Studies in Calcium, Parathyroid Hormone and Calcitonin Metabolism and Associated Skeletal Disorders.

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THESIS

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ABSTRACT. The thesis submitted to the University of Sydney for examination for the Doctorate of Medical Science comprises a compilation of 28 published manuscripts in the field of calcium metabolism,parathyroid hormone, calcitonin and vitamin D metabolism, and related skeletal disorders.These scientific presentations are deemed to have made a significant contribution to knowledge in the field.The papers are presented in the thesis in chronological order of publication but in the discussion herewith are grouped according to defined subjects.

Paget's Disease of Bone.

In our medical clinic we had a large number of patients with Paget's disease of bone which until the mid 1970s was untreatable. After the discovery of calcitonin, and the finding that calcitonin powerfully inhibited the activity of osteoclasts in bone, calcitonin became available as a therapy for Paget's disease, the first medication to be used for this purpose.

Paper 4 describes the effective use of calcitonin given by injection in the treatment of 100 patients with Paget's disease of bone, a large study by international standards and one of the pioneer studies, and which showed a marked reduction in the serum alkaline phosphatase and a reduction in bone pain. A raised alkaline phosphatase was a marker of the activity and the extent of bone involvement in Paget's disease, and a fall induced by calcitonin was a manifestation of reduced activity of the abnormal process in bone.

Paper 1 describes our unique finding of the frequent association of Paget's disease with hyperparathyroidism. Occasionally, the serum calcium is elevated in patients with Paget's disease thought previously to be due to the high rate of bone turnover due to excess activity of bone resorbing osteoclasts as part of the Pagetic pathophysiological process, but often in our study due to hyperparathyroidism treatable by parathyroidectomy.

Paper 11 describes the effective use of intravenous pamidronate in the treatment of Paget'disease, a bisphosphonate, which together with similar bisphosphonates, has now replaced calcitonin as the most effective treatment for Paget' disease.This is one of the early papers describing the effective use of pamidronate in the treatment of Paget's disease which can often be given as a single intravenous infusion.

Calcitonin.

Paper 8 describes changes in serum calcitonin in relation to a defined episode of exercise in men sufficient to cause an elevation in serum adrenaline and noradrenaline. No significant rise or fall in the serum calcitonin was observed following exercise. This paper contributed significantly to the understanding of factors which might influence the prevailing levels of calcitonin in serum.

Paper 9 describes the relationship between serum parathyroid hormone, serum calcitonin and serum inorganic phosphate and carefully measured parameters of osteoblast and osteoclast activity in patients having dialysis for end stage chronic renal failure. The dynamic and static aspects of bone metabolism were examined in bone biopsy samples. The major finding was the high correlation between the serum calcitonin and bone aluminium, a unique finding and worthy of further study.

Paper 12 described the responses of serum calcitonin to intravenous pentagastrin in patients with the MEN2A syndrome in which elevated serum calcitonin levels are a marker of medullary carcinoma of the thyroid gland. Previously it was thought that an exaggerated rise in the serum calcitonin after intravenous pentagastrin was diagnostic of medullary carcinoma of the thyroid gland not necessarily clinically apparent and often occult in siblings or children of patients with this autosomally dominant genetic disorder.This widely cited paper showed that pentagastrin infusions sometimes produced false positive results which could have lead to the un-neccesary surgical removal of the thyroid gland in patients so identified.

Hypoparathyroidism.

Paper 2 described the occurrence of calcification in brain structures associated with hypoparathyroidism. The mechanism of this phenomenon is unknown but this case report adds to the clinical perception of this association.

Paper 3 describes the association of diabetes insipidus with primary hypoparathyroidism in a patient in whom primary adrenal failure (Addison's Disease) was also present as part of an autoimmune polyglandular endocrine deficiency syndrome. This is the first description of diabetes insipidus in a patient with this disorder.

Hypercalcaemia.

Paper 5 describes the occurrence of elevated levels of serum vasopressin in patients with hypercalcaemia showing that polyuria, a poorly understood feature of hypercalcaemia was due to resistance to vasopressin action at the renal tubule level and not due to vasopressin deficiency.

Paper 15 described the elevated serum levels of 1,25-OH vitamin D in a patient with a T-cell lymphoma associated with hypercalcaemia and emphasised the importance of measuring serum 1,25-OH vitamin D in the investigation of hypercalcaemia. The sequence of investigation in this patient is described.

Hyperparathyroidism.

Paper 6. Our clinic saw large numbers of patients with primary hyperparathyroidism usually treated by surgical removal of a parathyroid tumour but sometimes not. There was controversy as the whether surgery could be withheld safely in some patients with primary hyperparathyroidism without the subsequent development of adverse events. In this study with significant longtitudinal follow up, many patients with hypercalcaemia who did not have surgery did not lose significant bone mass or lose significant renal function or develop renal calculi.

Paper 10 described a subsequent study of fore-arm bone mineral density in patients before and after parathyroidectomy in women. Following parathyroidectomy a significant rise was observed in patients who had had a parathyroidectomy whereas in women of similar age who did not have hypercalcaemia there was a significant fall in the bone mineral density suggesting benefit in terms of preservation of bone mineral density induced by parathyroidectomy.

Paper 14 published in collaboration with our unit's parathyroid surgeons,described surgical outcomes in a very large number of patients, 733, with primary hyperparathyroidism.By international standards this is a large study describing the experience of a single unit.

Paper 25 describes a cohort of patients with primary hyperparathyroidism studied between 1960 and 2011 and was designed specifically to study mortality in long term follow up. The mortality rate of community dwelling persons of the same age and sex was compared to mortality in the hyperparathyroid population. The time of diagnosis was the starting point and the number of years lived after this was recorded and compared with the life expectancy of the community living population beginning at the same time as the diagnosis of primary hyperparathyroidism was made in each individual person. There was a significant increase in mortality in the hyperparathyroid population with an average loss of 7.5 years in life expectancy.

Osteoporosis.

Paper 7 describes longtitudinal measurements of forearm bone mineral density highlighting the usefulness of this simple measure in the diagnosis and treatment of osteoporosis.

Paper 17 described for the first time bone mineral density in patients with chronic fatigue syndrome and the correlation of low bone mineral density with low mean exercise times (METs). The importance of this study was the finding of low bone mineral density in persons who had persistently low levels of exercise.

Paper 18 described the effect of high dose inhaled glucocorticoids on bone mineral density in pre-pubertal children with asthma and showed a significant inverse correlation between the daily dose of inhaled glucocorticoid and the accretion of bone mineral density.

Paper 19 studied the effect of the isoflavone, formononetin, on serum LDL cholesterol and forearm bone mineral density over a 6 month period. This study was subsequently complemented by a much larger study described in Paper 24 where formononetin was given in a randomised, placebo controlled double blind format for 2 years to post-menopausal women and showed that the use of formononetin was associated with a significant fall in serum LDL cholesterol levels but not with a significant rise in bone density.

Paper 28 described the significant relationship between a low bone mineral density measured by DEXA and by QCT in the radius and tibia and the mortality in patients receiving dialysis for end stage renal failure.

Paper 16 described the effect of venesection on bone mineral density in a pre-menopausal women with haemochromatosis and supplemented the sparse information on this subject.

Page 26 described a unique study of the relationship between the occurrence of heterozygosity in the HFE gene (C282Y) and bone mineral density in post-menopausal women and showed that the presence of this heterozygous mutation was associated with low bone mineral density compared to community dwelling women.

Paper 20 described a large study of post-menopausal women given supplemental vitamin D or placebo in a randomised double blind format followed for two years. Although the serum 25-OH vitamin D rose in those given vitamin D there was no significant impact on bone mineral density when compared to the control group, in this group of women who were already vitamin D replete.

Paper 22 described a large cohort of patients with osteoporosis given intravenous pamidronate every 3 months to prevent loss of bone mineral density. 84 patients were treated

and there was a significant increase in the bone mineral density in the lumbar spine and femoral neck. Many of these patients were receiving glucocorticoid therapy and responded in the same way as those not receiving glucocorticoid therapy. This was an important study demonstrating the efficacy of intravenous bisphosphonates before zolendronate became available.

Paper 23 describes a controlled clinical trial in progress in which a defined and supervised level of exercise is studied in relation to the fall in bone mineral density in post-menopausal women with breast cancer given an aromatase inhibitor likely to accelerate the rate of loss of bone mineral density. Patients are randomised to receive the exercise program or not.Follow up will continue for 12 months.

Miscellaneous.

Paper 13 described the effect of methotrexate on the proliferation of osteosarcoma cells in culture. Osteosarcoma cells have features of osteoblast cells found in bone.This study was carried out because of the widespread use of methotrexate in the treatment of rheumatoid arthritis and concern about the deleterious effects of methotrexate on bone.

Paper 21 described the relationship between circulating FGF-23 levels and the development of oncogenic osteomalacia. This was a comprehensive study examining the impact of FGF-23 on the renal tubular handling of inorganic phosphate and the synthesis of 1,25-OH vitamin D and showed that the fall in the serum FGF-23 after removal of the FGF-23 secreting bone tumour was associated with the healing the the osteomalacia and the restoration of normal levels of serum inorganic phosphate and serum 1,25-OH vitamin D.

Paper 27 describes the most comprehensive study to date of a patient with osteogenesis imperfecta ossium with long term follow up. The clinical, metabolic and radiologic findings are described.The abnormal function of osteoblasts taken from abnormal bone in tissue culture is described. A gene expression study was performed and compared to a gene expression study carried out using osteoblasts

in culture from a normal male of the same age.The expression of certain genes was markedly abnormal. The paper was the first to describe serum complement deficiency in osteogeneis imperfecta ossium.

The published work collated herein is submitted for examination for the degree of Doctorate of Medical Science, University of Sydney, in the belief that the quality and extent of the published work is deemed sufficient to merit the conferring of the degree of Doctorate of Medical Science. The papers submitted are those published between 1971 and 2017.The original work carried out in this time comprises 81 papers divided as follows:

1. Lipoprotein and triglyceride metabolism.15 papers
2. Disorders related to calcium, parathyroid hormone and calcitonin metabolism and associated skeletal disorders.36 papers
3. Thyroid gland disorders including thyroid carcinoma.9 papers
4. Research related to diabetes mellitus.4papers
5. Studies of vasopressin, prolactin and growth hormone.9 papers
6. Clinical and metabolic studies of chronic fatigue syndrome.4 papers
7. Studies relevant to clinical disorders of internal medicine.4 papers

On the advice of the Prima Facie committee 28 papers on calcium, parathyroid hormone and calcitonin metabolism and associated skeletal disorders have been selected for examination and are presented within the framework of a thesis.The papers considered as a whole are thought to represent a significant contribution to the discipline of endocrinology and metabolic bone disease, and are the product of original research. The papers are presented in chronological order and each paper is accompanied by a commentary highlighting the perceived scientific importance of the paper and the specific contribution made by Phillip Clifton-Bligh. The number of citations for each paper is given for both Google Scholar and Research Gate and the number of Reads for each paper is given for Research Gate as of 25th February 2017. The wider spectrum of research carried out by Phillip Clifton-Bligh is documented as a list of papers published between 1971 and 2017 from which the 28 for examination have been selected.

The title of the thesis is" **Studies in calcium,parathyroid hormone and calcitonin metabolism and associated skeletal disorders"**

Paper 1. Paget's disease in bone and hyperparathyroidism:coincidence or causal relationship? S Posen, P Clifton-Bligh, M Wilkinson. Calcified Tissue International 1978; 26:107-109.

P Clifton-Bligh contributed to the accumulation and assessment of the data. The large numbers of patients with Paget's disease and hyperparathyroidism seen in the Department of Endocrinology at Sydney Hospital enabled this study to be carried out. Of 173 patients studied with Paget's disease, 9 also had primary hyperparathyroidism. Based on published prevalence figures, the incidence of primary hyperparathyroidism in the population with Paget's disease is greatly increased.

This study contributes significantly to the assessment and management of patients with Paget's disease.

Citations.

Google Scholar 30

Research Gate 15

Reads.

Research Gate 2

Paget's Disease of Bone and Hyperparathyroidism: Coincidence or Causal Relationship?

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Summary. We studied 173 patients with Paget's disease and 105 patients with hyperparathyroidism. Nine patients were found to have both disorders.

Key words: Paget's Disease — Hyperparathryoidism

Introduction

Since Albright et al. (1) reported the coexistence of hyperparathyroidism and Paget's disease in a 44-year-old woman, there have been at least 16 similar reports in the English language (2–4). It is generally thought (1, 4–6) that the two disorders occur in the same individual no more frequently than would be expected by chance.

In this paper we report a further 9 patients with both these disorders and discuss the possible relationships between them.

Patients and Methods

A retrospective survey was made of all patients with Paget's disease seen in this unit between 1964 and 1977. A similar survey was made of all patients with proven primary hyperparathyroidism seen in this unit over the same period (7).

The diagnosis of Paget's disease was based on the radiological

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criteria of Brailsford (8) and Steinbach (9) as interpreted by two independent radiologists. At least one serum calcium determination (10, 11) was performed in each patient.

The diagnosis of primary hyperparathyroidism was based on the criteria of Pratley et al. (7): these consist of (a) abnormal parathyroid tissue removed at operation or seen at autopsy, (b) hypercalcemia ($\geq$ 10.8 mg/100 ml), and (c) absence of nitrogenous retention (BUN $\leq$ 50 mg/100 ml). Full radiological skeletal surveys were not made in patients with hyperparathyroidism. However, X-rays of the chest, lumbar spine, pelvis, and hands were taken in each case with skull X-rays in the majority.

Results

During the relevant period, 173 patients with Paget's disease were studied. The diagnosis of primary hyperparathyroidism was established surgically or at autopsy in 105 patients.

Five pagetic patients who were hypercalcemic were submitted to neck exploration. Each of these had a single parathroid adenoma and all became normocalcemic after parathyroidectomy.

A further 4 pagetic patients were observed to be hypercalcemic on two or more occasions over a period of at least 12 months. All of these had elevated serum immunoreactive parathyroid hormone values (12) (Table 1), although none of them showed any evidence of malignancy. However, these 4 patients have not been submitted to neck exploration because of their age and/or because of a lack of symptoms. None of the pagetic patients were immobilized and none suffered from fractures at the time of study.

Table 1. Clinical details of 9 patients with both osteitis deformans and primary hyperparathyroidism.

Initials	Sex	Age at diagnosis		Highest serum calcium (11) (mg/ml)	Highest serum PTH (12) (ng/ml)	Highest serum alk. phos. (24) (U/liter)	Operative findings
		Paget's	Hyperpara-thyroidism				
B.P.	F	49	49	12.2	0.6	188	Parathyroid adenoma (260 mg)
E.K.[a]	F	62	62	12.2	—	469[b]	Parathyroid adenoma (14.8 g)
J.B.K.	M	43	43	11.9	—	91	Parathyroid adenoma (700 mg)
I.McD.	M	51	63	11.7	0	2950	Parathyroid adenoma (260 mg)
M.B.	F	48	54	14.1	0.6	263	Parathyroid adenoma (1.4 g)
C.C.	F	55	50	11.6	0.8	639	—
D.L.	F	81	81	11.8	2.3	335	—
D.B.	F	48	48	12.1	0.7	326	—
G.R.	F	67	67	11.3	1.5	243	—
Normal values				9.2–10.0	<0.4	15–85	

[a] Previously reported (23) (Case 3)
[b] Fell to 42 after parathyroidectomy. Rose to 386 10 years later

Discussion

Paget's disease is estimated to occur in 2% to 4% of populations studied during the second half of life (13–15). The prevalence of Paget's disease among our hyperparathyroid patients is therefore not significantly different from that in the general population.

The prevalence of hyperparathyroidism in the population varies from 5/10,000 (16) to 10/10,000 (17) to 30/10,000 (18). Even if the highest figure is used and even if the 4 nonoperated patients are omitted from these calculations, 5/173 is still grossly elevated ($X^2 = 24.28$; $P < 0.001$).

The mechanism of the apparent association between Paget's disease and hyperparathyroidism is not clear. As far as we are aware, the pagetic patients were selected only on the basis of their skeletal symptoms, whereas most of the hyperparathyroid patients were referred because of renal calculi or symptomless hypercalcemia (7). None of the patients had received calcitonin therapy. An abnormal skeletal response to normal amounts of circulating parathyroid hormone (2) is excluded by the operative findings.

Hyperparathyroidism has been reported in isolated patients with other bone disorders such as polyostotic fibrous dysplasia (19) and neurofibromatosis (20), and it seems possible that some metabolite derived from skeletal tissue directly or indirectly stimulates parathyroid cell proliferation and hypersecretion. It appears likely that the hypercalcemia

described in some patients with Paget's disease (21, 22) is due to associated hyperparathyroidism.

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Paper 2. Computerized tomography of the brain in surgical hypoparathyroidism. S Posen, P Clifton-Bligh, T Cromer. Annals of Internal Medicine 1979; 91:415-417

P Clifton-Bligh made a significant contribution to the identification and diagnosis of patients with this disorder. The paper describes four patients with surgically induced hypoparathyroidism who developed intracranial calcification identified by computerized tomography of the brain. This may be an important sequel to unrecognised hypocalcaemia following thyroid surgery and emphasises the importance of follow up measures of serum calcium following thyroid surgery. Because intracerebral calcification can be associated with dementia the prevention and adequate treat ment of hypocalcaemia may be important to prevent this irreversible process.

This paper is considered to be an important contribution to the need to recognise the deleterious effects of intracerebral calcification associated with hypocalcaemia following thyroidectomy.

Citations.

Google Scholar 21

Research Gate 19

Reads.

Research Gate 2

In experimental animals and humans there are distinct differences in invasiveness between *Brucella* species, and these differences in virulence are reflected in the response of the host's tissues and the severity of illness (4). *Brucella melitensis* is more invasive than *B. abortus* and causes a more acute form of illness frequently leading to serious complications. It is tempting to postulate that the generally milder disease caused by *B. abortus* is a result of the host's ability to localize the infection in a granulomatous response. One possible explanation for the differences in tissue histology is sampling error. If larger amounts of tissue had been obtained, granulomas might have been found in serial sections. This seems unlikely, however, since the absence of granulomas was a consistent finding in disease due to *B. melitensis* and granulomas were present in both patients with disease due to *B. abortus*. Basic differences in the pathogenesis of infection between *B. abortus* and *B. melitensis* appears to be a more plausible explanation for the differences noted. Recent studies in our laboratory with experimental infection due to strains of *B. abortus* and *B. melitensis* in mice appear to confirm this concept. Animals infected with *B. abortus* formed hepatic granulomas and remained chronically infected for more than 60 d, while animals infected with *B. melitensis* did not form epithelioid granulomas and cleared their livers of bacteria in less than 20 d (abstract; *Clin Res.* 1978;26:772).

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Computerized Tomography of the Brain in Surgical Hypoparathyroidism

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PATIENTS with hypoparathyroidism develop radiologic opacities in the basal ganglia and other parts of the brain (1, 2). The condition is believed to be relatively rare in surgical hypoparathyroidism (3-5), presumably because patients with this disorder are treated more expeditiously than are patients with other forms of hypoparathyroidism. We have recently used computerized tomography to demonstrate intracerebral calcification in four patients with surgical hypoparathyroidism whose skull roentgenograms showed no abnormality.

Patient 1 is a housewife who developed tetany after thyroidectomy at age 34 years and was given calcium intravenously in the immediate postoperative period. No further bouts of tetany occurred, and no medication was given for 22 years. A left-sided cataract was diagnosed and a lens extraction done at the age of 53 years. At the age of 56 a second thyroidectomy was done because of the recurrence of a multinodular goiter, and again no treatment was given despite repeated bouts of tetany. At the age of 61 the patient presented to Sydney Hospital because of epileptiform seizures.

Physical examination showed a left iridectomy scar, a dense right cataract, two thyroidectomy scars, bilateral Dupuytren's contractures, and left-sided Parkinsonian tremor. The patient had no dementia. Serum calcium level was 1.55 mmol/L (6.2 mg/dL) and serum inorganic phosphate, 2.28 mmol/L (7.0 mg/dL). Immunoreactive parathyroid hormone, measured repeatedly between 1973 and 1979, has not been detectable on any occasion. Other biochemical tests were within normal limits. Between the ages of 61 and 73 years she received ergocalciferol and calcium supplements (6) and, with the exception of two episodes of hypercalcemia associated with high serum 25-hydroxyvitamin D concentrations (7), has remained biochemically normal. The Parkinsonian tremor has gradually worsened, and

Table 1. (Continued)

Liver Histology	Brucella Agglutinin Titers	Cultures	Patient
Resolving hepatitis, no granulomas	1:3200	*Brucella melitensis*	1
Nonspecific hepatitis, no granulomas	1:2460	*B. melitensis*	2
Nonspecific hepatitis, no granulomas	1:160	*B. melitensis*	3
Focal necrosis, no granulomas	>1:320	*B. melitensis*	4
Foci of inflammatory cells, no granulomas	>1:640	*B. melitensis*	5
Noncaseating granulomas	1:2560	*Brucella abortus*	6
Noncaseating granulomas	>1:320	*B. abortus*	7

Patient	Sex	Age at Time of CT Examination	Duration of Presumed Hypocalcemia (8)	Duration of Therapy	Areas of Calcification Seen on CT
		←————————————— yr ———————————→			
1	F	73	27	12	Thalami, caudate nuclei, dentate nuclei
2	F	68	15	13	Thalami, caudate nuclei, dentate nuclei (see Figure 1)
3	F	58	23	12	Thalami, caudate nuclei, dentate nuclei
4	F	56	28	6	Thalami, caudate nuclei
5	F	71	0.3	21	None
6	M	80	0	19	Choroid plexus

* Plain roentgenograms of the skull were normal in each case. Several of the patients have been reported in previous publications (4, 6-8).

the patient currently receives 75 mg of carbidopa and 750 mg of levodopa per day.

Skull roentgenograms have been normal on several occasions. Computerized tomography of the brain without contrast medium showed widespread calcification involving the heads of both caudate nuclei, the thalami, and both dentate nuclei (Figure 1).

Figure 1. Calcification of the dentate nuclei (*above*) and basal ganglia (*below*) in Patient 2 (*see* Table 1). Plain roentgenograms of the skull were normal.

We have examined five other patients with long-standing surgical hypoparathyroidism and normal skull roentgenograms by means of computerized tomography (Table 1). Three (all with presumed hypocalcemia of long duration [8]) showed changes similar to those observed in the first patient, although none had symptoms of basal ganglia dysfunction. The two patients whose computerized tomography scans were normal had received vitamin D and calcium supplements immediately after surgery.

It is known that computerized tomography is more sensitive in detecting cerebral calcification than are plain roentgenograms of the skull (9). Because the demonstration of basal ganglia calcification makes no difference to the current management of individual hypoparathyroid patients, we do not at present recommend computerized tomography for all patients with this condition (10).

Our findings indicate, however, that intracerebral calcification is more prevalent in surgical hypoparathyroidism than is generally believed. In a series previously published from this unit (4), only two of 41 patients with surgical hypoparathyroidism showed intracranial calcification on skull roentgenograms. Dimich and colleagues (3) found no intracerebral calcification in 16 patients with surgical hypoparathyroidism. Smith and associates (5) in 1973 found only 20 published case reports of basal ganglia calcification in surgical hypoparathyroidism. It appears likely, in view of our findings here, that these figures are underestimates.

The mechanisms of cerebral calcification in hypocalcemic states and the reasons for its localization to particular sites remain unknown. Each of the six patients described in Table 1 had brief episodes of documented iatrogenic hypercalcemia (6, 7). Only those with prolonged hypocalcemia, however, showed dense basal ganglia, and it appears likely that the calcification is "dystrophic" rather than "metastatic."

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Methemoglobinemia from Sniffing Butyl Nitrite

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BUTYL NITRITE has become popular in the drug culture as an aphrodisiac (1, 2). Amyl nitrite has been used for such purposes for several years (3), but its availability is legally restricted. The butyl congener, on the other hand, is sold over the counter. Although it is marketed as a "room odorizer," butyl nitrite enthusiasts achieve stimulation by sniffing the compound directly from the bottle, in the manner of amyl nitrite "poppers."

Although the physiologic effects of amyl nitrite are generally understood (4), little is known about the effects of butyl nitrite. However, we have recently studied this problem in a 25-year-old man partially deficient in nicotinamide adenine dinucleotide, reduced (NADH)-methemoglobin reductase and in a group of volunteers with normal reductase activity. We have documented that butyl nitrite inhalation can cause subclinical methemoglobinemia in normal subjects and higher levels of methemoglobin in reductase-deficient persons.

Our patient first presented to a local emergency room complaining of a grayish complexion, which developed after inhaling butyl nitrite. He was found to have a methemoglobin concentration of 18% (2.8 g/dL). Several months later he reappeared with a methemoglobin level of 7.7% (1.2 g/dL), again after butyl nitrite inhalation.

Although our patient gave no history suggesting familial methemoglobinemia, it was discovered that he and his father had a partial deficiency of NADH-methemoglobin reductase activity, as shown by the impaired ability of their erythrocyte
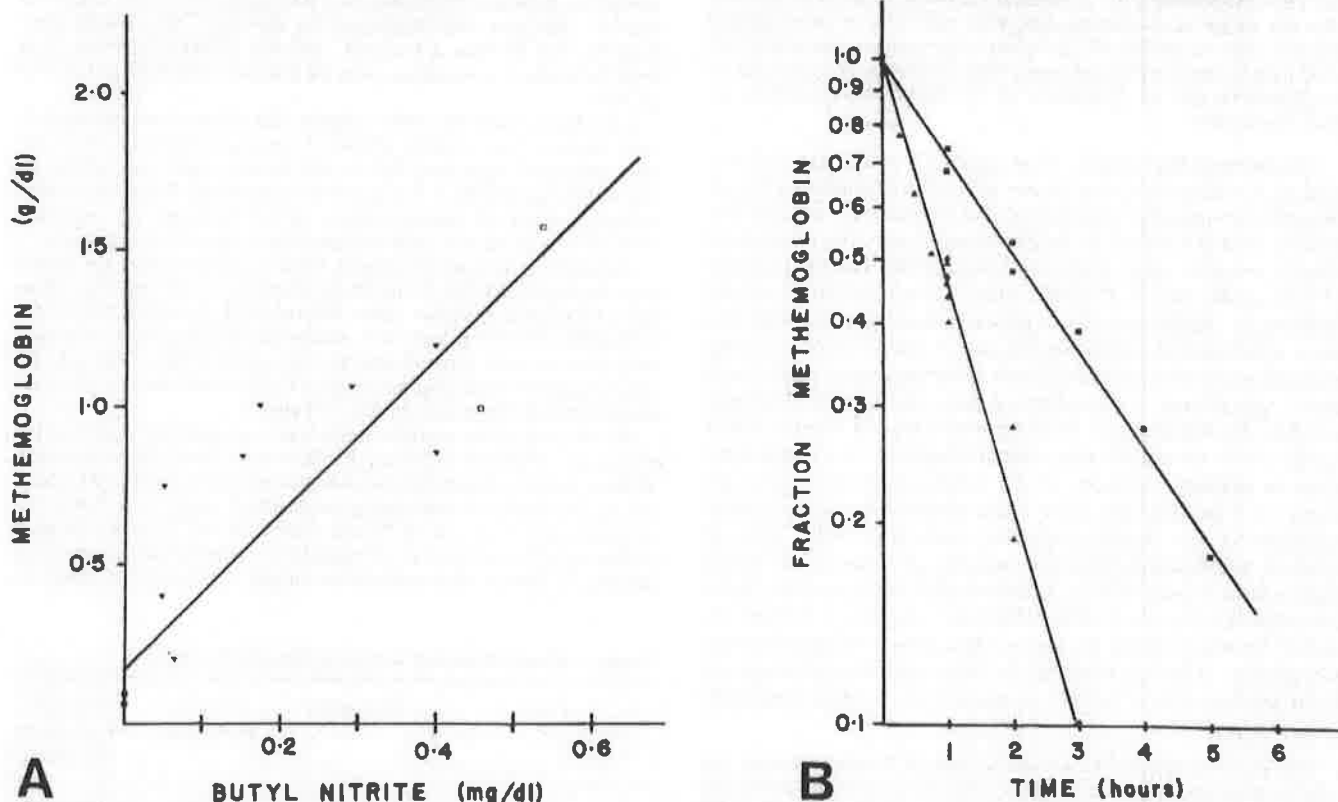

Figure 1A. Methemoglobin concentration versus simultaneously measured blood butyl nitrite level in six normal volunteers and the reductase-deficient patient. Points (●) indicate the six methemoglobin levels of 0.05 to 0.07 g/dL measured in the control subjects before butyl nitrite inhalation. Points (▼) represent levels of methemoglobin in the control subjects after 6- or 12-min nitrite inhalation. The control data have been fitted with a least-squares regression line (r = 0.85, SEM = 0.26 g/dL). Points marked by squares indicate values for the methemoglobin reductase deficient patient before nitrite exposure (■) and after 6- or 12-min inhalation (□). B. Methemoglobin clearance for six normal control subjects (▲) and the reductase-deficient patient (∗). The methemoglobin levels have been normalized and are expressed as fractions of the initial concentration. The zero time points indicate the six initial values for the control subjects and two for the patient. The data for the control subjects and for the patient have been fitted with least-squares regression lines. For the normal curve, half life = 55 min. For the curve for the reductase-deficient subject, half life = 126 min.

Paper 3. The association of diabetes insipidus with hypoparathyroidism, Addison's disease and mucocutaneous candidiasis. P Clifton-Bligh, C Lee, H Smith, S Posen. Australian and NZ Journal of Medicine 1980; 10:548-541

This paper is important as it is the first report of diabetes insipidus occurring in a patient with cortisol and parathyroid hormone deficiency. P C lifton-Bligh carried out all the investigations described in this paper. These included parathyroid hormone infusions, infusion of lysine vasopressin and infusion of synacthen. Blood and urine samples were meticulously collected by P Clifton-Bligh and the results obtained subject to scrutiny and interpretation. The paper was written by P Clifton-Bligh.

This paper is the first to describe an association of diabetes insipidus with Addison's disease and hypoparathyroidism.

Citations.

CASE REPORT

The Association of Diabetes Insipidus with Hypoparathyroidism, Addison's Disease and Mucocutaneous Candidiasis

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Summary: The association of diabetes insipidus with hypoparathyroidism, Addison's disease and mucocutaneous candidiasis. P. Clifton-Bligh, C. Lee, H. Smith and S. Posen, *Aust. N.Z. J. Med.*, 1980, **10**, pp. 548-551.

A case of central diabetes insipidus is reported in association with idiopathic hypoparathyroidism, adrenal failure and mucocutaneous candidiasis.

The diabetes insipidus was recognised at the same time as the discovery of aldosterone and cortisol deficiency, and occurred several years after the initial onset of mucocutaneous candidiasis and hypocalcaemia. Control of the diabetes insipidus was achieved initially with nasal aqueous lysine vasopressin and later with desmopressin.

The development of diabetes insipidus may be a further aspect of endocrine secretory failure associated with idiopathic hypoparathyroidism.

Following the first description of idiopathic hypoparathyroidism in 1926[1], and the subsequent definition of criteria for the diagnosis of hypoparathyroidism[2], this condition has been described in association with numerous disorders. The first occurrence with mucocutaneous lesions was noted in 1929[3] and later concurrence with Addison's disease[4-13], anterior hypopituitarism[14,15], pernicious anaemia[11,16], Hashimoto's thyroiditis[10], ovarian failure[13,17-19] and isolated aldosterone deficiency[20] were reported.

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The association of hypoparathyroidism with diabetes insipidus has not been described in detail. In 1941 Lichtwitz[21] mentioned a 51-year-old patient with diabetes insipidus who subsequently developed hypocalcaemia and tetany, but supporting data were not presented. Craig *et al.*[6] described a child of six years with a low serum calcium and a large fluid intake and output but the cause of the latter was not determined. Kunin and his coworkers[22] described a 20-year-old man with idiopathic hypoparathyroidism, Addison's disease and polyuria.

The present report deals with a patient in whom the combination of mucocutaneous candidiasis, idiopathic hypoparathyroidism, Addison's disease and central diabetes insipidus are documented.

Case Report

The patient (male, D.O.B. 21.7.52) presented at the age of four years with a history of oral monilia, recurrent since infancy. Monilial infection of the nails, dental caries and gum ulceration were also observed. There was no history of fits or tetany. Griseofulvin and diodoquin failed to improve the oral monilia. At the age of ten years, the serum calcium was found to be 6·2 mg/100 ml, and the serum inorganic phosphate 7·0 mg/100 ml. The serum albumin was 4·8 g/100 ml, serum globulin 3·6 g/100 ml, and serum alkaline phosphatase 22 King Armstrong units/100 ml. The 24-hour urinary calcium was 28 mg. X-rays of the skull, hands, and long bones, a faecal fat determination, jejunal biopsy, and sweat electrolytes were normal. An Ellsworth-Howard test was inconclusive. A slit lamp examination of the corneas and lenses was normal.

He was given 25,000 IU of ergocalciferol daily, later increased to 250,000 IU weekly.

He was reviewed in 1971 at the age of 19 years when he had no symptoms of tetany, diarrhoea or nocturia. He was of normal intelligence, height 170·2 cm. The haemoglobin was 17·1 g/100 ml, the serum creatinine 1·0 mg/100 ml, and the plasma cortisol 22·1 μg/100 ml, all values normal.

The serum IgG was 2000 mg/100 ml (normal 800-2000), serum IgA 110 mg/100 ml (normal 90-250) and serum IgM 100 mg/100 ml (normal 50-150).

The *in vitro* lymphocyte response to candida antigen showed no stimulation (control: 20 times stimulation). A skin

test to candida antigen was positive for the immediate response but negative for the delayed response.

In November 1974, at the age of 22 years, he presented with 10 kg weight loss which had occurred over a three-month period. He gave a history of increasing fatigue, poor appetite, and increasing thirst and polyuria sufficiently severe to interfere with sleep.

On examination the lying blood pressure was 80/70 and the standing blood pressure was unrecordable. Vitiligo was present on the left thigh. There was no buccal or excess skin pigmentation. Monilial infection of the nails, tongue and buccal mucosa was present. The visual fields were normal. The heart, thorax, abdomen and nervous system were clinically normal.

The serum sodium was 122 mmol/l, serum potassium 4·9 mmol/l, serum chloride 83 mmol/l, serum bicarbonate 26 mmol/l, serum creatinine 0·8 mg/100 ml, serum calcium 7·5 mg/100 ml, and serum inorganic phosphate 5·9 mg/100 ml. Thyroid function tests (serum thyroxine and ETR) were normal. The serum TSH was 4·0 mU/L (normal less than 5 mU/L). The plasma ACTH on 19 November, 1974 before investigation or treatment was 79 pg/ml (upper limit of normal is less than 100 pg/ml). The skull X-ray was normal.

On 19 November, 1974 an eight-hour infusion was carried out with 250 μg of Synacthen® in 500 ml of normal saline. At the start of infusion the plasma cortisol was 22·4 μg/100 ml and at the end, 26·0 μg/100 ml. The plasma renin was 183 ng/ml/hour (normal 5·2±4·4 SD). No aldosterone was detected in the urine during Synacthen® infusion or for 16 hours afterwards. Adrenal tomograms showed no adrenal calcification. Antiadrenal antibodies were detected in serum, but antibodies to thyroid and parathyroid tissue were not detected.

Cortisone acetate 37·5 mg daily and 9-alpha fluorohydrocortisone 0·1 mg daily, were commenced and ergocalciferol 250,000 IU/week, continued. His condition rapidly improved on this regime with weight increase, restoration of energy and normalisation of blood pressure, but thirstiness and polyuria continued. He passed 6050 ml of urine on 28 November, 1974. The serum sodium became normal.

On 11 January, 1975 a water deprivation test was carried out (Table 1). During the test the weight decreased by 2·0 kg (4·2% of body weight). The failure of urinary osmolarity to rise despite adequate dehydration, with a subsequent rise following pitressin indicated central diabetes insipidus. Following the use of aqueous lysine vasopressin therapy there was complete amelioration of thirstiness and polyuria. On 5 February, 1976 a further water deprivation test was carried out.

TABLE 1

Water deprivation test

Time (hours)	Weight (kg)	Urine volume (ml)	Urine osmolarity (mOsm/kg)
0	47·4		
1	46·6	680	49
2	45·9	600	58
3	45·4	640	66
5 units aqueous pitressin given subcutaneously			
5	45·0	280	230

TABLE 2

Infusion of synthetic ACTH*

Day	Plasma cortisol (μg/100 ml)	24-hour urine 17-ketosteroids (mg)	24-hour urine 17-ketogenic steroids (mg)
0	2·5	6·3	0·8
1	4·5		
2	5·0		
3	6·0		
4	7·5		
5	9·5	5·1	4·0

*250 μg of Synacthen® was infused over eight hours on each day in 500 ml of normal saline. The blood for plasma cortisol measurement was taken at the 8th hour on each infusion.

The normal range for 24-hour urine ketosteroids is 3·5 to 20·0 mg and for 17-ketogenic steroids 4·5 to 14·5 mg.

Carbamazepine which is believed to release ADH from the hypothalamus[23] was administered during the test, but did not cause any change in urine osmolarity.

On 28 March, 1975 after cortisone acetate had been replaced with dexamethasone 0·5 mg bd, a five-day infusion with Synacthen was performed (Table 2). The 24-hour urine 17-ketosteroids and 17-ketogenic steroids were measured before and on the last day of the infusions (Table 2). The failure of adrenal steroids to rise adequately in plasma or urine confirmed the diagnosis of primary adrenal failure.

On 2 May, 1975 an insulin infusion test was carried out with 0·075 u/kg of soluble insulin. The blood glucose fell to 24 mg/100 ml. The plasma ACTH showed some fluctuation in value but no tendency to rise (Table 3). The serum growth hormone rose from 8·1 ng/ml to 14·0 ng/ml.

On 2 February, 1978 he was admitted and cortisone acetate withdrawn. Twenty-eight hours later the plasma ACTH was 241 pg/ml and the plasma cortisol 3·6 μg/100 ml. In another patient with Addison's disease the plasma ACTH rose from 55 pg/ml to 348 pg/ml when cortisone acetate was withdrawn for 24 hours. On a further occasion when cortisone acetate was withdrawn the plasma ACTH was 81 pg/ml 24 hours later. One hour after an intramuscular injection of 5 U of aqueous lysine vasopressin the plasma ACTH was 128 pg/ml.

On 13 August, 1975 an infusion of bovine parathyroid hormone (Lilly-PTH) was commenced, with a plan to infuse 300 units over one hour. On the control day and on the infusion day aqueous lysine vasopressin was given intravenously at a rate of 0·25 U/hour throughout the collection periods to maintain water balance comparable on the two consecutive days, and to ensure that any urinary cyclic AMP attributable to vasopressin was constant on the two days. Ten minutes after the infusion of PTH was begun, redness, swelling and urticaria appeared in the infusion arm followed by erythema on the face and chest. An anaphylactic reaction was thought to have occurred. Urine collections for phosphate, creatinine and cyclic AMP were continued, however (Table 4). A rise in the urine phosphate/creatinine ratio and in the urine cyclic AMP/creatinine ratio was seen confirming that hypoparathyroidism was due to parathyroid hormone deficiency.

Other investigations were carried out as follows: plasma androgens were 0.95 μg/100 ml (normal $0.4–1.0$), karyotype: normal 46 XY pattern. LHRH infusion test: the serum LH rose from 16 ng/ml (LER 907 standard) to 180 ng/ml, and serum FSH from 205 ng/ml (LER 907 standard) to 350 ng/ml. TRH infusion test: the serum TSH rose from 4.8 mU/L to 13.6 mU/L and the serum prolactin from 6.0 ng/ml to 22.4 ng/ml. All these responses are considered normal.

At various times following his first admission to hospital, the serum immunoreactive PTH was measured[24] (Table 5). On several occasions when the serum calcium was below normal, immunoreactive PTH was detected in the serum.

Discussion

In this patient as in other similar cases[4–13], mucocutaneous candidiasis had its onset in early childhood and preceded the onset of hypocalcaemia. The combination of hypocalcaemia, an elevated serum inorganic phosphate, a normal serum creatinine and a rise in the urinary phosphate/creatinine and in the urine cyclic AMP/creatinine ratio after PTH infusion confirmed the diagnosis of hypoparathyroidism due to parathyroid gland secretory failure. A further patient with pseudohypoparathyroidism studied by us developed an anaphylactic reaction during a PTH infusion but no rise in urinary cyclic AMP was observed, so that a rise in urinary cyclic AMP excretion could not be attributed to the anaphylactic reaction itself. The

serum PTH was measured on numerous occasions over several years and at all times when the serum calcium was below normal PTH was detectable in the serum. On one occasion when the serum calcium was normal, the serum PTH was undetectable. The PTH secreted may be biologically ineffective and unable to maintain a normal serum calcium.[25] Immunoreactive PTH has been found in the serum of other patients with hypoparathyroidism.[26]

Twelve years after the appearance of hypocalcaemia, the symptoms of anorexia, weight loss and weakness occurred. The low serum sodium suggested the diagnosis of cortisol deficiency, but the first plasma cortisol measured was unusually high, 22.1 μg/100 ml, for symptomatic cortisol deficiency. The low urine aldosterone combined with a high plasma renin indicated aldosterone secretory failure, and the low serum sodium and postural hypotension may have been due to aldosterone deficiency at a time when cortisol secretion was maintained at a relatively high level. Subsequently, however, eight-hour infusions of synthetic ACTH on five consecutive days caused a subnormal rise in blood and urinary glucocorticoids indicating that primary adrenal insufficiency had developed.

TABLE 3

Plasma ACTH* during insulin-induced hypoglycaemia

Time (minutes)	0	15	30	45	60	90	120
Plasma ACTH (pg/ml)	123	79	48	102	125	45	45
Blood glucose (mg/100 ml)	76	41	24	35	47	60	57

*Plasma ACTH was measured by radioimmunoassay using the Radioamersham ACTH kit assay.

TABLE 4

The effect of PTH infusion on urinary phosphate and cyclic AMP*

	Phosphate/ creatinine	Cyclic AMP pM/gram creatinine
Control		
0–2 hours	0.34	10.6×10^5
PTH infusion		
0–2 hours	0.74	11.3×10^5
2–4 hours	1.02	204×10^5
4–6 hours	1.12	25×10^5

*50 units of bovine PTH (Lilly) was infused intravenously for ten minutes beginning at the end of the second hour on the infusion day.

TABLE 5

Serum immunoreactive parathyroid hormone at various times

Date	Serum PTH* (ng/ml)	Serum calcium (mg/100 ml)
18.11.74	1.20	7.1
19.12.74	0.30	6.8
3.2.75	0.38	8.4
13.8.75	not detected	9.3
14.8.75	0.45	8.6
28.11.75	0.68	8.4

*The upper limit of normal in the assay used is 0.40 ng/ml.[24]

Wiley et al.[27] reported that vasopressin deficient rats had diminished corticosterone response to ACTH in low dose. Chronic treatment with vasopressin restored responsiveness. In the present study, the five-day infusion with synthetic ACTH was performed after the patient had been receiving vasopressin for some weeks but cortisol responses to synthetic ACTH remained low.

Thirstiness and polyuria persisted after treatment with cortisone acetate and 9-alpha fluorohydrocortisone and two separate water deprivation tests indicated central diabetes insipidus. The symptoms of polyuria and thirstiness were abolished with aqueous lysine vasopressin and later desmopressin.

Pituitary ACTH secretion has been difficult to evaluate. The value for plasma ACTH on the day of admission was 79 pg/ml, well below the range seen for a patient with untreated Addison's disease. The plasma ACTH was measured again after discontinuing cortisone acetate for 24–28 hours and values of 81 pg/ml and 241 pg/ml were obtained.

The latter rise in plasma ACTH was similar to that seen in other patients with documented Addison's disease following discontinuation of cortisone replacement for 24 hours. However, the plasma ACTH following insulin induced hypoglycaemia and after vasopressin injection[28] did not show a convincing rise. The evidence favours impairment of pituitary ACTH release as well as primary adrenal failure.

Gillies and Lowry[29] suggested that vasopressin may have an important role in ACTH release from the anterior pituitary. It is possible that in the patient described here, defective vasopressin release may have limited normal pituitary ACTH release in response to various stimuli.

Diabetes insipidus has not been described as part of multiple endocrine deficiency syndromes and it is possible that its development here may have been fortuitous. It has not been possible to examine whether antibodies to any component of ADH secreting cells were present in the circulation. Bottazzo and Doniach[30] have described the presence of antibodies to pituitary cells in patients with multiple endocrine deficiency syndromes but specific antibodies to

posterior pituitary tissue have not been described. Despite its apparent rarity it is nevertheless conceivable that central diabetes insipidus as seen here is part of a general failure of peptide hormone synthesis and release from several endocrine glands.

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Paper 4. Paget's disease of bone. Experiences with 100 patients treated with salmon calcitonin. HS Grunstein, P Clifton-Bligh, S Posen. The Medical Journal of Australia September 15th 1981; 278-280

P Clifton-Bligh was involved in the diagnosis, treatment and assessment of many patients described in this study. The Endocrine Unit of Sydney Hospital had a very large clinical experience with the assessment and treatment of patients with Paget's disease especially after porcine and then salmon calcitonin became available for treatment. In the present study 53 men with Paget's disease were treated for a mean duration of 10.5 months with salmon calcitonin injections and 47 women were treated for a mean interval of 7.8 months. The most common indication for treatment was pain in affected bones. Side effects of nausea, flushing, diarrhoea and headache were reported by many patients. 18 patients discontinued therapy because of the severity of these side effects. Some of them were later able to tolerate porcine calcitonin. Significant improvement in terms of pain relief occurred in 58 patients. Pain in Paget's disease is often particularly troublesome at night. Three patients with extradural compression of the spinal cord at the thoracic level showed improvement after surgical decompression. Ten patients showed no improvement in defective hearing after calcitonin therapy. Aortic valve lesions were confirmed by echocardiography in 26 patients who had a systolic murmur defined on clinical examination. This finding was an important addition to the spectrum of abnormalities seen in Paget's disease. The mean serum alkaline phosphatase was reduced by 39.3% by calcitonin therapy.

This paper is considered to be an important addition to the world literature on the treatment of Paget's disease with calcitonin, especially as the large number of patients treated had a wide variation in the severity of the disorder.

Warfarin therapy was ceased in two patients. One boy, aged three years and nine months, with an aortic prosthesis received warfarin for one month prior to his return to Madagascar; he died suddenly at three months but no autopsy was performed. The other patient, a 52-year-old man, ceased his warfarin 12 months after his successful aortic valve replacement; 10 days later he developed a dense hemiplegia but made a good recovery. The other 144 patients were anticoagulated with warfarin therapy.

Late complications occurred in seven patients. One patient died of a low cardiac output state and one of alcoholic cardiomyopathy. Suspected or definite thromboembolic complications occurred in five patients of whom two died. The woman with the mesenteric embolus, the man who developed a hemiplegia, and the boy who returned to Madagascar have already been discussed. A 64-year-old woman with a mitral valve prosthesis, who was receiving warfarin, developed a transient visual defect and left arm weakness one month after operation; she made a good recovery. A 61-year-old lady, who was receiving warfarin for an aortic prosthesis, developed intermittent episodes of lights before her eyes one month after discharge; these were considered to be embolic in origin and she made a good recovery. No patient developed valve thrombosis.

Thus the late complications include one patient with definite, two with probable, and two with possible systemic emboli. Table one shows the frequency of thromboembolism in the hospital survivors. These complications occurred in three of the nine patients not receiving warfarin (33 per 100 patient years), but in only two of the 144 patients receiving warfarin therapy (1.4 per 100 patient years). In contrast, patients receiving anticoagulants with ball valves have an incidence of thromboembolism of 2.8 to 3.2 per 100 patient years, and with tilting disc valves the incidence is 2.1 to 3.5 per 100 patient years.

TABLE 1

Thromboembolic Complications in 153 Hospital Survivors With St Jude Medical Valves

| Cardiac Valve Replaced | Number of Valves | | | |
| | Patients Receiving Warfarin | | Patients Not Receiving Warfarin | |
	With Complications	Without Complications	With Complications	Without Complications
Mitral	1	59	1	0
Aortic	1	94	2	6
Total patients	2	142	3	6

Eleven survivors had combined aortic and mitral valve implants

The St Jude medical valve therefore has low frequency of thromboembolic complications when warfarin therapy is maintained, but a high frequency of such complications if warfarin is either not given or if it is ceased. The St Jude valve has been shown in previous studies[1] to have an excellent haemodynamic profile, and in this study an acceptable incidence of thromembolism, provided anticoagulant therapy is given.

REFERENCE

[1] Proceedings of the Second International Symposium on the St Jude Medical Valve, San Diego, U.S.A., March 12th to 14th, 1981

PAGET'S DISEASE OF BONE

EXPERIENCES WITH 100 PATIENTS TREATED WITH SALMON CALCITONIN [*]

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One hundred patients with Paget's disease of bone were treated with salmon calcitonin. Seventy per cent of patients who presented with pain reported improvement in symptoms. Side effects which occurred in 53 patients were more severe in women, necessitating the withdrawal of therapy in 30% of women as against 7.5% of men. Five of the patients with intolerable side effects were subsequently able to tolerate porcine calcitonin, while six were unable to tolerate salmon, porcine or human calcitonin. No audiological improvement occurred. Aortic valve lesions were detected in 11 patients. After a mean treatment period of 7.8 months, serum alkaline phosphatase level was reduced by 39.3%. Total 24-hour urinary hydroxyproline was reduced by 46.1% after 11.2 months of treatment.

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IN 1977, Plehwe *et alii* reported their experiences with porcine calcitonin in Paget's disease of bone.[1] Since then, over 300 patients with Paget's disease have been seen in this unit. The majority were not given any specific anti-Pagetic therapy, either because they were asymptomatic or because they did not consider their symptoms sufficiently severe to warrant injections. Salmon calcitonin was given to 100 patients; our experiences in this group are presented in this paper.

PATIENTS AND METHODS

The diagnosis of Paget's disease was made on the basis of the usual radiological criteria,[2] and most X-ray films were seen by two independent radiologists. Fifty-three men and 47 women (mean age, 64.6 years and 63.0 years respectively) were treated with salmon calcitonin. The total duration of treatment was 920 patient months, with a mean of 10.5 months for men and 7.8 months for women.

The indications for calcitonin therapy were pain (83 patients), neurological complications (seven patients), pathological fractures (four patients), impending hip surgery (five patients) and high output cardiac failure in one patient. Salmon calcitonin was given by subcutaneous injection (mostly by the patients themselves) in doses of 50 units (USV) a day in 43 patients, 100 units (Sandoz) three times a week in 47 patients and both of these regimens at some time in four patients. Other regimens (usually 100 units daily) were employed in six patients.

RESULTS

Side effects, which occurred in 53 patients, included nausea (37 patients), flushing of palms, soles, face or ears (17 patients), urinary frequency (10 patients), diarrhoea (four patients), headache (three patients), and wheals or erythema at injection sites (three patients). In 18 patients, the side effects were sufficiently severe to result in discontinuation of therapy. Eleven of these patients (one man, 10 women) had received calcitonin for six weeks or less, while seven patients (three men, four women) had been taking calcitonin for three months or longer before the side effects became intolerable. In eleven patients, non-salmon calcitonins were subsequently tried. Five tolerated porcine calcitonin, while six patients were unable to tolerate either salmon, porcine or human

calcitonin. Change from one type of salmon calcitonin to another was unsuccessful in two patients.

Intolerable side effects were more frequent in women (14 of 47 patients) than in men (four of 53 patients), and more frequent among patients taking 100 units three times a week (13 of 51 patients) than amongst patients receiving daily injections (seven of 47 patients).[1] The sex difference was statistically significant ($P < 0.005$), but the difference between the two dosage schedules was not.

The results of treatment of 100 patients with salmon calcitonin are set out as follows.

Significant improvement	58
Slight improvement	4
No improvement	11
Therapy for less than two months	10
Therapy for indications other than pain	17

Among 83 patients who were given calcitonin because of pain, 10 (12%) discontinued therapy within two months because of side effects, 58 (70%) reported significant improvement, four (5%) reported slight improvement, while 11 (13%) had no improvement. Of the patients who failed to note any improvement, two were believed to suffer from nerve-root compression, five had osteoarthritis, one suffered from intermittent claudication and two had severe non-organic illnesses. The proportion of "non-improvers" was similar for patients receiving 100 units three times a week and for those receiving 50 units a day.
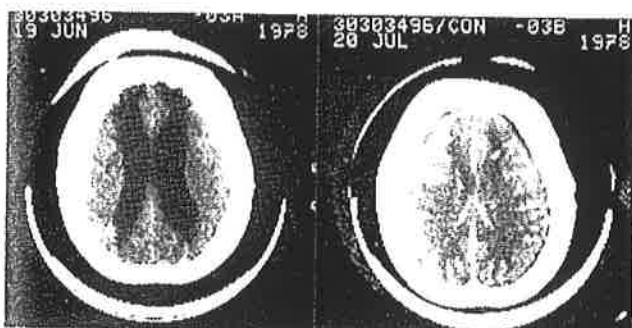

FIGURE 1: CT scan of the brain before and after the insertion of a ventriculoperitoneal shunt. Note the thick calvarium and the dilated ventricles (left) before surgery. The patient was demented before surgery, and improved considerably after insertion of the shunt.

FIGURE 2: Osteogenic sarcoma in a lumbar vertebra of a 67-year-old woman who became completely paraplegic after attempted decompression. The right X-ray film was taken two months after the left.

Six of seven patients who presented with neurological complications were also treated surgically; therapy with salmon calcitonin was commenced at the same time. The clinical condition of the three patients with extradural compression of the thoracic spinal cord improved after surgical decompression. Two patients with hydrocephalus showed an improvement after insertion of a ventriculoperitoneal shunt (Figure 1). One patient with severe lumbar nerve-root compression sustained a massive intraoperative haemorrhage, and became paraplegic. Her back pain persisted, and she was subsequently shown to have a sarcoma (Figure 2). The condition of one patient with severe cerebellar signs believed to be caused by posterior fossa compression remained unchanged after calcitonin therapy.

Audiograms were performed in 10 patients before and after calcitonin therapy, with a mean time interval of 2.8 years (range of one to five years) between the tests. No objective improvement was noted in any patient. Hearing was unchanged in five patients, slight deterioration had occurred in four patients, and major deterioration in one patient.

Cardiac murmurs (predominantly aortic systolic murmurs) were noted in 26 patients. Echocardiography was performed in 20 patients, 11 of whom showed aortic valve lesions. The results of echocardiography in 20 patients with "ejection" murmurs were as follows.

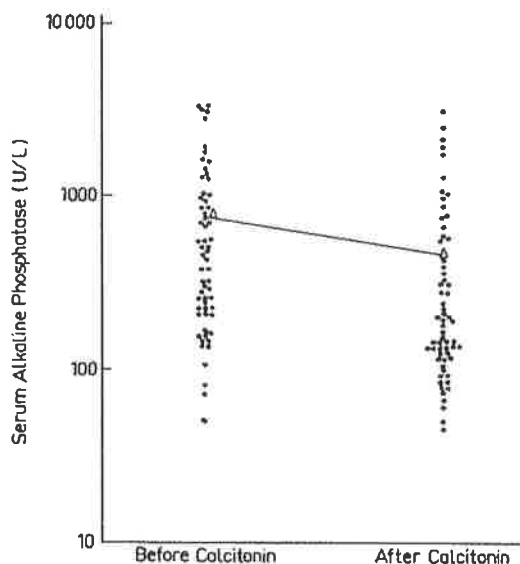|	
Aortic valvular lesion	11
Aortic valve not seen	1
Right ventricular dilatation or outflow obstruction	2
Congestive cardiomyopathy	1
Normal echocardiogram	5

FIGURE 3. Serum alkaline phosphatase levels (logarithmic scale) before and after calcitonin therapy. The mean duration of treatment was 7.8 months (range, one to 32 months). The mean serum alkaline phosphatase values are indicated by triangles joined by the solid line.

Mean serum alkaline phosphatase level was reduced by 39.3% after a mean period of treatment of 7.8 months (Figure 3). The rate of decline in serum alkaline phosphatase concentration was almost identical in patients receiving 50 units a day and in those receiving 100 units three times a week. In 11 patients, sequential measurements of serum alkaline phosphatase concentration were available after cessation of calcitonin therapy. Pretreatment values were reached after a mean period of 9.6 months (range, three to 20 months).

Mean urinary hydroxyproline was reduced by 46.1% after a mean period of treatment of 11.2 months (Figure 4).

DISCUSSION

Salmon calcitonin was effective in the relief of pain in 79% of patients treated for two months or more. The high "success rate" is attributed to the exclusion of patients who discontinued therapy early, and to the selection of patients whose symptoms were thought to arise from affected areas and who were sufficiently disabled to warrant injections.[1]

Two patients were treated by both schedules

Side effects were common, though usually mild, and shortlived. The number of patients demanding discontinuation of calcitonin because of side effects was larger than that reported in other series.[2] Discontinuation was more likely to occur in patients receiving 100 units three times a week than in those receiving 50 units a day, although neither dosage was tolerated by the two patients in whom both schedules were tested. The reason for the significantly larger number of women (30%) discontinuing treatment compared to men (7.5%) is not known. Almost 50% of patients who were unable to tolerate salmon calcitonin were able to tolerate porcine material.
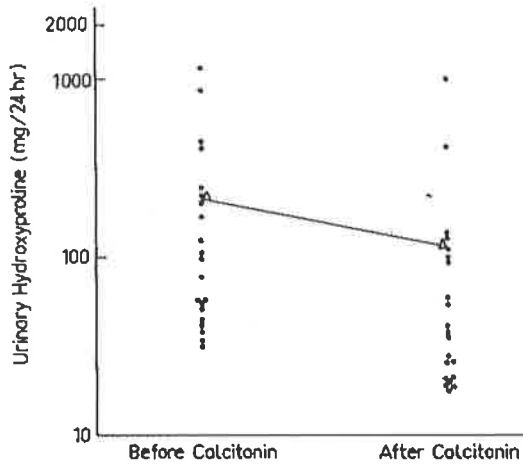

FIGURE 4: Total 24 hour urinary hydroxyproline (logarithmic scale) before and after calcitonin therapy. The mean duration of treatment was 11.2 months (range, two to 38 months). Symbols as in Figure 3

Six patients with neurological complications were treated surgically in the first instance. There were no patients in whom improvement in neurological signs could be attributed solely to calcitonin. As in previous reports,[4] there was no improvement in hearing.

The high prevalence of aortic valve disease is believed to be coincidental, as both Paget's disease and an abnormal aortic valve are commonly found in this age group.

The fall in alkaline phosphatase levels was not as great as has been previously reported.[5] However, in several patients the "post-treatment" measurements were made within two months of commencement of therapy when the serum alkaline phosphatase values may not yet have reached their nadir. The improvement in symptoms could not be correlated with biochemical changes.

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ESTIMATION OF P_{50} FROM A SINGLE VENOUS BLOOD SAMPLE

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The oxygen tension at 50% saturation of haemoglobin, or P_{50} was calculated from a single O_2 tension and saturation measurement on a venous blood sample by means of the proportional displacement assumption of Severinghaus and a parallel displacement assumption. These calculations were compared with a two-point technique, based on Hill's formula, which was assumed to be accurate. The results obtained from the simpler, one-point techniques correlated well with the more complex two-point estimation. This correlation remained for subjects with normal, high, and low P_{50}.

THE POSITION of the haemoglobin-oxygen dissociation curve can conveniently be expressed as the oxygen tension at which haemoglobin is 50% saturated, or P_{50}. An estimation of P_{50} is not only useful in the study of abnormal haemoglobin-oxygen affinity states such as carbon monoxide poisoning and haemoglobinopathies, and in the investigation of polycythaemia, but also in the analysis of oxygen delivery in many circulatory and respiratory disease states.

Traditional procedures to determine P_{50} require multiple-point analysis to allow construction of a curve, or at least a two-point determination of corresponding oxygen saturation and tension measurements, and a solution by means of Hill's equation.[2] These procedures are complex, time-consuming and, as a result, often not readily available. By assuming that any displacement of the curve is predictable in form, it is possible to obtain P_{50} from a single point estimation.

The purpose of this paper is to compare the two-point estimation using Hill's equation with single-point estimations based on one of two assumptions. The proportional displacement assumption is that, in the saturation range of 40% to 60%, the displacement of the curve being measured from the ideal curve is proportional to the oxygen tension (as proposed by Severinghaus[1]). The parallel displacement assumption is that, in the same saturation range, the measured curve is parallel to the ideal curve, the displacement being constant for all oxygen tensions (Figure 1).
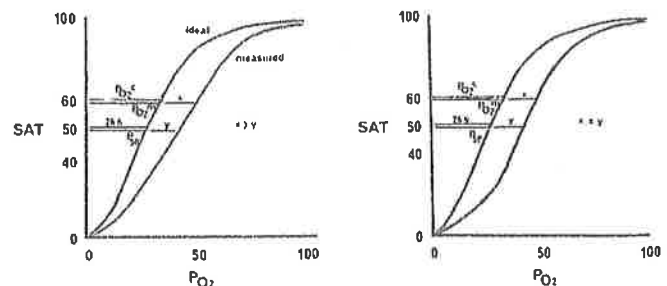

FIGURE 1: Displacement of the haemoglobin-oxygen dissociation curve in the proportional displacement assumption and in the parallel displacement assumption. Left, — Proportional displacement assumption. The distance between the curve being measured and the ideal curve at 50% saturation (x) increases to the distance y in proportion with the increase in PO_2 at higher saturation. Hence, the ratio $P_{50}/26.6$ is the same as the ratio PO_2''/PO_2'. Right, — Parallel displacement assumption. The distance between the measured and ideal curves remains constant in the saturation range being measured (x=y). Hence, $P_{50} - 26.6 = PO_2'' - PO_2'$.

Paper 5. Plasma vasopressin in hypercalcaemic states. BG Robinson, P Clifton-Bligh, S Posen, BJ Morris. The Australian and NZ Journal of Medicine 1983; 13:5-7

P Clifton-Bligh contributed significantly to this study with respect to the concept and design and in the provision of hypercalcaemic patients. Hypercalcaemia leads to a defect in the renal concentrating ability. It has not been clear whether this is due to interference with the action of vasopressin or due to suppression of vasopressin secretion. Six hypercalcaemic and eight control subjects participated in this study. The modification by BG Robinson of the Skowsky assay for measuring vasopressin was described in detail. The mean serum creatinine concentrations and the mean creatinine clearance values were not different between the hypercalcaemic and the eucalcaemic groups. Urinary osmolarity was significantly lower in the hypercalcaemic group. Plasma vasopressin was significantly higher in the hypercalcaemic group. It was assumed that the osmotic stimulus to vasopressin release was the same in the two groups as the plasma osmolarity was not significantly different between the two groups.

This paper provides significant additional evidence that hypercalcaemia interferes with the action of vasopressin on the renal tubule.

Citations.

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The work described in this paper was part of a submission by BG Robinson for a MSc degree at the University of Sydney.

PLASMA VASOPRESSIN IN HYPERCALCAEMIC STATES

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Abstract:
Plasma vasopressin was measured by radioimmunoassay in eight normal subjects and in six patients with hypercalcaemia. Vasopressin levels were significantly elevated in the hypercalcaemic patients, although urine osmolalities were lower than in controls. This finding is consistent with a renal resistance to the action of endogenous vasopressin in hypercalcaemia. (Aust NZ J Med 1983; 13: 5-7.)
Key words: Vasopressin, hypercalcaemia.

INTRODUCTION
Hypercalcaemia leads to defects in renal concentrating ability.[1] The mechanism of this defect is believed to be due to abnormalities relating to impaired sodium absorption in the ascending loop of Henle,[2] although the exact pathogenesis remains in doubt.[3,4]

This work was undertaken to study the relationship between hypercalcaemia and plasma vasopressin as it has not been clear whether the defect in renal concentrating ability is due to suppression of vasopressin secretion or due to resistance to the action of circulating endogenous vasopressin. The results of this work have been presented previously in abstract form.[5]

PATIENTS AND METHODS
Six consecutive hypercalcaemic patients, whose clinical data are shown in Table 1, fasted overnight from 10 p.m. Plasma samples were collected at 9 a.m. the next morning with the patients sitting. The patients were symptomatically well at the time of sampling and had not experienced nausea or vomiting. Eight normal subjects prepared in the same way acted as controls. None of the subjects were taking any medications known to influence renal water clearance.

Plasma and urine osmolality were measured by freezing point depression on a Knauer Semimikro osmometer (Knauer, Berlin, West Germany), serum calcium (corrected for specific gravity) by atomic absorptiometry, serum and urine creatinine by standard AutoAnalyzer methods and plasma vasopressin by the method of Skowsky et al.,[6] modified as follows: One ml of 2N HCl was added to 2 ml of plasma. The turbid mixture was centrifuged for 10 minutes at 1500 g and the supernate was transferred to another tube to which 2 ml of a suspension (5 mg/ml of distilled water) of bentonite (Sigma, St Louis, Mo. USA) was added. This tube was agitated for 30 minutes in an iced water bath and centrifuged at 2000 g for 10 minutes. The supernate was discarded and the pellet containing the vasopressin was resuspended in 2 ml of freshly made solution of cold acetone in 1N HCl (80:20, v:v). The suspension was agitated for 30 seconds on a vortex mixer and allowed to stand for 15 minutes by which time separation into three layers had occurred.

The top (lipid) layer was aspirated and discarded. After centrifugation for 10 minutes at 2000 g the supernatant was poured into another tube where it was snap frozen in liquid N_2, freeze dried and stored at $-20\,^{\circ}\mathrm{C}$. Assays were performed in this tube within one week of the extraction procedure.

Initials	Age	Sex	Serum calcium mm/L	Serum creatinine mm/L	Plasma vasopressin μU/ml	Plasma osmolality mOsm/kg	urine osmolality mOsm/kg
				Controls			
WC	33	M	2.38	0.10	3.7	288	1100
VM	28	F	2.37	0.07	2.9	284	580
PR	31	M	2.41	0.09	3.5	287	600
JY	32	F	2.41	0.07	2.7	285	668
BR	26	M	2.47	0.10	4.7	286	980
TL	26	F	2.28	0.06	3.1	286	1040
RR	29	M	2.20	0.08	3.4	288	746
MH	25	M	2.44	0.11	1.7	282	870
Mean ± 1 SD			2.37 ± 0.09	0.09 ± 0.02	3.2 ± 0.9	286 ± 2	823 ± 203
			Patients with Hypercalcaemia				
GH*	54	M	3.30	0.11	5.0	284	292
AP	73	F	3.32	0.13	5.7	292	510
JC	54	M	3.14	0.12	7.5	289	606
DA	52	F	2.82	0.11	8.4	290	620
SD	34	M	3.20	0.07	4.3	288	600
JW	48	M	2.60	0.09	2.1	286	770
Mean ± 1 SD			3.06 ± 0.29	0.11 ± 0.02	5.5 ± 2.3	288 ± 3	566 ± 158
P value: Controls vs patients			<0.001	NS	<0.05	NS	<0.025

* GH had untreated Addison's disease. All the other patients had primary hyperparathyroidism.

The lyophilised synthetic vasopressin standards (Calbiochem, La Jolla, California) were reconstituted in phosphate buffer* and serially diluted on the extraction day. Two hundred ul of each dilution of the standards were added to 1800 ul of low vasopressin plasma obtained from water loaded individuals. These standard mixtures were extracted simultaneously with test sera, freeze-dried and stored in the same manner. The recovery of cold vasopressin added to low vasopressin plasma was $70 \pm 5\%$ and did not differ from the recovery of labelled vasopressin.

The freeze dried samples and standards were reconstituted with 50 ul of 0.1 N NaOH, 100 ul of buffer* containing antivasopressin antibody (Ferring, Malmo, Sweden, Rabbit #19), and 750 ul of buffer.* The mixture was allowed to stand for 24 hours at 4 °C at which time I^{125} vasopressin (New England, Nuclear, specific activity 900-2,200 uCi/ug) 3,500 cpm/tube was added in 100 ul of buffer.*

A fresh shipment of I^{125}-labelled vasopressin was obtained each month and was not repurified before use. Incubation was allowed to proceed for three days at 4 °C after the addition of labelled material. Dextran T70-Norit A charcoal was used to separate the antibody-bound vasopressin from "free" vasopressin. Both antibody-bound and "free"

fractions were counted for four minutes in a Packard gamma counter (Model 5220). Assay blanks consisted of extracted low vasopressin plasma made up in the same manner as samples and standards but without antibody. Each standard was thawed only once before use.

The assay routinely detected 0.5 uU (1.4 pg or 1.4 fmol) of vasopressin. The intra-assay co-efficient of variation at 50% displacement was 8% while the inter-assay variation was 14%. Oxytocin and 1-desamino-8-D-arginine vasopressin did not cause any displacement of labelled vasopressin from the antibody at concentrations of 400,000 pg and 1000 pg/tube respectively. Lysine vasopressin caused 50% displacement of labelled vasopressin from the antibody at concentrations of 12 pg/tube. Samples from control subjects and from hyper-calcaemic patients were measured in the same assay.

RESULTS

Plasma vasopressin values are shown in Table 1. The mean serum creatinine concentrations and creatinine clearance rates (not shown) were not significantly different in the two groups. The mean urine volume was significantly higher in hyper-calcaemic patients than in normal subjects whereas urinary osmolality was significantly lower in the hypercalcaemic patients. Plasma vasopressin concentrations were significantly higher in patients with hypercalcaemia than in normal subjects $(p < 0.05)$.

* Containing 8.7 gram NaCl/L, 1.42 gram Na₂HPO₄/L, 1 gram sodium azide/L and 10 gram bovine serum albumin/L. The pH was adjusted to 7.4 with 2N HCl.

DISCUSSION

This work shows that the renal concentrating defect of hypercalcaemic patients is associated with an increase in vasopressin, presumably due to an increase in vasopressin release. While we did not examine the effects of small changes in calcium concentrations on the vasopressin assay, we consider it unlikely that such changes were responsible for the differences shown in Table 1. We also believe that while the patients and controls were not age-matched it is unlikely that an increase in age would result in high plasma vasopressin values. It has been shown in older age persons that an increased osmotic stimulus brought about by an infusion of hypertonic saline will increase the plasma vasopressin concentration more than in younger persons.[7] However, upright posture producing an orthostatic stimulus to vasopressin release leads to increased plasma vasopressin concentrations in younger persons compared with those of older age.[8] In our study group, the plasma osmolality under the conditions of study was not different in the older hypercalcaemic patients from the younger eucalcaemic controls and therefore the osmotic stimulus to vasopressin secretion was presumed to be the same in both groups. Also, after the overnight fast, subjects were studied while sitting, so that any orthostatic stimulus to vasopressin secretion should have been expressed more markedly in the younger eucalcaemic subjects. The significance of the difference found in the plasma vasopressin levels between the two groups acquires greater force in the light of the above.

Increased serum calcium concentrations[9] and dehydration[10] can stimulate vasopressin release and either stimulus (or both) may have been operative in our hypercalcaemic patients.

Hammer and coworkers[9] stated that experimental hypercalcaemia raised plasma vasopressin only in the presence of parathyroid hormone excess. One of our patients had Addison's disease and the dehydration associated with sodium wasting may have contributed to the elevation of plasma vasopressin in his case. However, reduced free water clearance due to cortisol lack should have raised urine osmolality whereas in this patient the urine osmolality was low. This patient's data is included in the analysis.

It has been known for many years that high concentrations of calcium ions interfere with the renal effects of exogenous vasopressin in vitro[11,12] and in vivo.[16] This work provides additional evidence that hypercalcaemic states in man are associated with a renal resistance to endogenous vasopressin and confirms the recent work of Baylis et al.[13]

Increased serum calcium concentrations, in addition to promoting increased water loss through defective action of vasopressin at the renal level, and thereby stimulating vasopressin secretion by plasma volume contraction, may also facilitate the secretion of vasopressin from the posterior pituitary gland. Calcium may directly activate the pituitary actin-myosin system and exocytosis of vasopressin as has been proposed for insulin[14] and for the secretion of other pituitary hormones.[15] The finding in our patients that plasma osmolality was not significantly raised compared with controls lends support to the suggestion that raised serum calcium concentrations may directly stimulate vasopressin secretion.

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Paper 6. Is parathyroidectomy of benefit in primary hyperparathyroidism? S Posen, P Clifton-Bligh, TS Reeve, C Wagstaffe, M Wilkinson. Quarterly Journal of Medicine 1985; 54:241-251

P Clifton-Bligh assessed, treated and followed up many of the patients described in this study. The authors have a very large experience in the diagnosis, treatment and long term follow up of patients with primary hyperparathyroidism. At the time of writing there was considerable controversy as to whether there were some patients with primary hyperparathyroidism who show no discernible long term benefit after successful parathyroidectomy. Even now in 2017 there has been no randomised long term study evaluating patients who had or who did not have parathyroid surgery. The study reported in this paper involved 265 patients, 90 of whom did not have parathyroid surgery. Of these 37 had died. At the time of decision for or against surgery the serum calcium, serum parathyroid hormone, 24 hour urine calcium were significantly higher in patients submitted for neck exploration than in those who did not have surgery. The serum creatinine was not significantly different between the two groups.Twenty one deaths (15.1%) occurred in those who had had successful parathyroid surgery (average age at death 66.3 years), four deaths (12.5%) in those who had unsuccessfrul surgery (average age at death 65.1 years) and twelve deaths (15.6%) in those who did not have parathyroid surgery (average age at death 72.2 years). One patient had progressive respiratory failure from multiple thoracic fractures. There were more cancers in those with persistent hypercalcaemia.Patients without renal calculi at the time of presentation were very unlikely to form stones subsequently regardless of whether neck exploration was carried out or not. At the time of follow up there was no difference in the prevalence of hypertension between the three groups.There was no progressive rise in the serum calcium or the serum parathyroid hormone in the "unsuccessful" group or in the non-operated group. There was a significantly lower bone mineral content in the forearm in those who did not have surgery together with those in whom surgery was unsuccessful, when the groups were matched for age and duration of follow up.

This study is considered important in that it includes a large number of patients followed up for several years who did not have parathyroid surgery or in whom neck exploration was unsuccessful. There was little evidence of progressive harm in these patients except in those who had pre-existing renal calculi.

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Is Parathyroidectomy of Benefit in Primary Hyperparathyroidism?

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SUMMARY

A retrospective survey was performed on 265 patients with primary hyperparathyroidism who had received three forms of treatment on a non-randomised basis. 'Successful' surgery (normalisation of serum calcium) was carried out in 142 patients, 'unsuccessful' surgery (persistence of hypercalcaemia after neck exploration) in 33 and no surgery in 90. Patients subjected to surgery were significantly younger than patients in the unoperated group and their serum calcium values at the time of decision were approximately 10 per cent higher. The mean follow-up period was significantly longer in the operated groups.

The percentages of patients who had died were similar in each group. Clinical events relating to renal stones depended on the presence or absence of calculi at the time of decision rather than on the method of treatment. At the time of follow-up the prevalence of hypertension, renal impairment and vertebral crush fractures were similar in all three groups. Forearm osteo-densitometry showed a higher bone mineral content in the 'successful' group than in the other two groups.

In spite of the selection bias inherent in a study of this kind, it is clear that untreated hyperparathyroidism is compatible with long survival and a lack of demonstrable deleterious effects on kidney and bone.

INTRODUCTION

'...Still, she did wish that George Edzel's ears weren't quite so big (perhaps he'd been given just a spot too much parathyroid at Metre 328?).'

<div align="right">A. Huxley, Brave New World (1932)</div>

It is generally believed that the hyperparathyroid state is harmful and that patients with primary hyperparathyroidism benefit from parathyroidectomy (1). In order to verify this belief we performed a retrospective survey on 265 patients with primary hyperparathyroidism who had been referred over a 22-year period.

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PATIENTS AND METHODS

The patients had received three different types of treatment on a non-randomised basis:

A. *'Successful' operation.* There were 142 hypercalcaemic individuals who became and remained normocalcaemic after the surgical removal of histologically verified parathyroid tissue.

B. *'Unsuccessful' operation.* There were 33 hypercalcaemic individuals with elevated serum immunoreactive parathyroid hormone (iPTH) values who remained hypercalcaemic and whose serum iPTH remained elevated after neck exploration. Included in this group were nine patients whose original operation had been performed in other units and 10 individuals who became normocalcaemic for varying periods after surgery but who subsequently became hypercalcaemic again.

C. *No operation.* In 90 hypercalcaemic individuals with elevated serum iPTH values neck exploration was either not recommended or refused.

In each group, women outnumbered men approximately 2:1 (172 women, 93 men) and in each group women were older than men (mean age of all women = 54.83 years vs mean age of all men = 48.52 years).

Patients with serum creatinine values of 0.3 mmol/l (3.3 mg/dl) or more at presentation were excluded. Patients with malignancies were excluded unless *either* removal of parathyroid tissue led to normocalcaemia *or* a parathyroid adenoma was found at autopsy *or* there was a time interval of at least five years between the first documented serum calcium elevation and the diagnosis of a malignant disorder.

All patients were sent a circular letter inviting them to attend. A total of 154 patients were examined by one of us while 45 patients were examined by their own physicians who supplied relevant information, radiographs and sera. Twenty-two patients were contactable and allegedly well but unwilling or unable to see either one of us or their physicians. Thirty-seven patients had died and seven could not be traced. The proportions of patients who attended were similar in the three groups. Follow-up biochemical results were available for 15 of the patients who had subsequently died or disappeared.

In 13 cases the original decision not to operate had been changed during the period of observation. In three patients (all males) there had been spectacular rises in serum calcium with the development of symptoms attributed to hypercalcaemia, while in three patients the decision had been changed because of minor rises in serum calcium. In one patient, surgery was recommended on account of recurrent calculi. In six patients the decision to operate was based on pressure from the patients or from colleagues. Each of these 13 patients was classified only with the group in which he/she spent most of the period of observation.

During the interview the patients were asked specific questions relating to symptoms of cerebrovascular disease, major abdominal episodes and urinary calculi (2) dating from the time when the decision on operation had been taken. No attempt was made to distinguish between 'old' and 'new' stones. A blood pressure reading was taken after the patient had been seated for 10 min and details of antihypertensive and other drug therapy were recorded.

Blood was taken for the measurement of serum calcium, serum creatinine, and serum immunoreactive parathyroid hormone (iPTH) (3). Twenty-four-hour urine calcium was measured and five standard radiographs were taken (lateral lumbar spine, lateral thoracic spine, hands, chest and pelvis). These were subsequently assessed for the presence or absence of osteoporosis on a semiquantitative basis and the results recorded on a 0−2 scale for each film, with 0 indicating no osteoporosis and 2 indicating 'definite' osteoporosis.

Bone mineral content was measured in a Model GT35 Novo Osteodensitometer (Novo Diag-
nostic Systems, Bagsvaerd, Denmark) in 13 women who had undergone 'successful' parathy-
roidectomy and in 15 women with persistent hypercalcaemia. These patients were matched for
age, handedness and duration of follow-up.

All values were expressed in terms of 'means and standard deviations' even when they showed
a non-Gaussian distribution. The statistical analyses for non-Gaussian values were performed
with the Mann—Whitney test for unpaired data. Prevalence data were calculated by means of
the chi-square test.

RESULTS

Table 1 shows that patients not submitted to surgery were significantly older at the time of
decision than patients in the two other groups, that they had a lower prevalence of renal calculi
and that they were followed up for a significantly shorter period.

Table 2 shows that at the time of decision serum calcium, serum iPTH and urine calcium
values were significantly higher in the patients submitted to operation whereas serum creatinine
values were similar in the operated and unoperated patients. There were 14 patients with urine
calcium values of less than 2 mmol/24 h. Five of these were in the 'successful' group, two in the
'unsuccessful' group and seven in the unoperated group.

Table 3 shows that 37 patients had died before follow-up. Twenty-one deaths had occurred
in the individuals who had undergone 'successful' parathyroidectomy (15.1 per cent), four
amongst patients who had had unsuccessful operations (12.5 per cent) and 12 in the unoperated
group (15.6 per cent). Mean ages at the time of death were 66.3 years in the 'successful' group,
65.1 years in the 'unsuccessful' group and 72.2 years in the unoperated group. Except for one
78-year-old woman in the 'successful' group who died from intractable cardiac failure 72 h after
parathyroidectomy and one 73-year-old woman in the 'unsuccessful' group who died of respira-
tory failure due to progressive osteoporosis with multiple spontaneous fractures, there were no
deaths directly attributable to hyperparathyroidism. In each group the majority of deaths were
attributed to cardiac or cerebrovascular causes.

Six patients presented with a well-documented history of pancreatitis. Two of these had
successful parathyroidectomies, while four were treated conservatively. Only one patient had a

TABLE 1. *Differences between operated and unoperated patients*

	N	Mean age (years)* ±1 SD	Prevalence of renal stones (%)*	Mean duration of follow-up (years) ±1 SD
Successful operation	142	50.2 ±13.7	69.7	7.8 ±4.8
Unsuccessful operation	33	49.0 ±12.5	48.5	6.6 ±5.3
No operation	90	56.0** ±14.8	27.8***	5.2** ±3.4

*At time of decision; **p value for difference between all
operated patients and unoperated patients < 0.005 (t-test for
normally distributed values); ***p value for difference between
all operated and unoperated patients < 0.001 (χ^2-test).

TABLE 2. *Selected biochemical parameters at presentation in three groups of patients with primary hyperparathyroidism (means ±1 SD)*

	Serum calcium (mmol/l)	Serum iPTH (ng/ml)	Serum creatinine (mmol/l)	Urine calcium (mmol/24 h)
Successful operation (n = 142)	3.10 ± 0.39	1.41 ± 1.67 (n = 93)	0.11 ± 0.05	8.32 ± 3.98 (n = 89)
Unsuccessful operation (n = 33)	3.10 ± 0.43	1.14 ± 1.16 (n = 24)	0.13 ± 0.06	7.19 ± 4.78 (n = 16)
No operation (n = 90)	2.82* ± 0.16	0.79** ± 0.60 (n = 76)	0.11 ± 0.05	6.30*** ± 3.68 (n = 40)
Normal range	2.30 −2.50	< 0.4	0.06−0.11	2.5−7.5

*Difference from operated groups $p < 0.0001$; **difference from operated groups $p < 0.005$; ***difference from operated groups $p < 0.05$; (Mann—Whitney test for unpaired data).

further attack of documented pancreatitis during the period of observation. He was an alcoholic who had undergone a 'successful' parathyroidectomy five years earlier.

Table 4 shows the prevalence of various malignancies in the three groups of patients. There were more cancers amongst the patients with persistent hypercalcaemia than amonst the nor-mocalcaemic patients. Even amongst the 'unsuccessful' patients who were matched for age and sex with the 'successful' group, the prevalence of malignancies was significantly greater ($p < 0.01$ for difference between 'successful' and 'unsuccessful' groups). When the patients with inciden-tally discovered thyroid carcinomas were excluded from the calculations the difference was still significant at the 1 per cent level. No correlation could be established between the duration of follow-up and the development of malignancies.

Fig. 1 shows the prevalence of renal calculi (2) at the time of follow-up or death in 206 patients with adequate data. It is seen that in each category, the prevalence of renal stones *after*

TABLE 3. *Status of three groups of hyper-parathyroid patients at the time of follow-up*

	Alive	Dead*	Unknown
Successful operation	118	21 (15.1)	3
Unsuccessful operation	28	4 (12.5)	1
No operation	75	12 (15.6)	3

*Percentages (in parentheses) refer to those patients whose status was known.

TABLE 4. *The prevalence of malignant disorders (excluding skin cancers) amongst three groups of patients with primary hyperparathyroidism* *

	Large bowel	Hyper- nephroma	Thyroid	Other
Successful operation (8/139)	1	1	2	4
Unsuccessful operation (7/32)	2	2	1	2
No operation (9/87)	3	1	0	5
Total	6	4	3	11

*Numbers in parentheses indicate total number of patients with cancer and number of patients in each group other than those lost to follow-up.

the decision was made depended largely on whether or not stones were present at the time of decision. Patients without calculi at the time of presentation were very unlikely to form stones afterwards, regardless of whether a neck exploration was performed or not. Patients with stones at the time of presentation had an approximately 50 per cent chance of further stone formation or clinical 'events' relating to pre-existing stones.
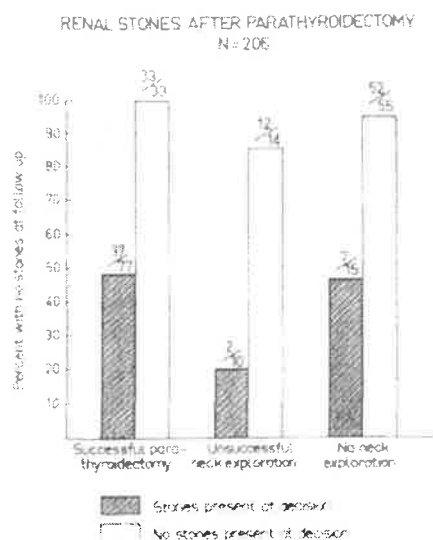

FIG. 1. The relationship between parathyroidectomy and the formation or recurrence of renal calculi during the period of observation. Amongst patients without evidence of renal stones at presentation (clear bars) very few went on to form stones or suffer from stone symptoms subsequently. Patients with a history of stones at presentation (hatched bars) had a 50 per cent chance of further 'events' during the period of observation regardless of whether or not a parathyroidectomy was performed. The fractions above the bars refer to patients *without* stones at follow-up.

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TABLE 5. *The prevalence of hypertension* at the time of follow-up in three groups of patients with primary hyperparathyroidism*

	No. with adequate data	No. with hypertension	Percentage with hypertension
Successful operation	96	45	46.9
Unsuccessful operation	26	13	50.0
No operation	67	36	53.7
Total	189	94	46.7

*Defined as a diastolic blood pressure of 100 or more and/or the regular consumption of antihypertensive medication.

Table 5 shows the prevalence of hypertension at the time of follow-up in each of the three groups. There was no significant difference between the hypercalcaemic and the normocalcaemic patients.

Table 6 shows the biochemical status of the three groups of patients at the time of follow-up Serum calcium, serum iPTH and urine calcium had fallen significantly in the 'successful' group whereas these values remained relatively unchanged in the other two groups. However, even in the 'unsuccessful' and the unoperated groups, serum calcium concentrations were lower at follow-up than at the time of decision.

Eleven of the 'successful' patients had serum immunoreactive PTH values above the upper limit of our reference range even though they were all normocalcaemic. Three of these patients

TABLE 6. *Mean biochemical values at the time of follow-up in three groups of hyperparathyroid patients (means ± 1 SD)*

	Serum calcium (mmol/l)	Serum iPTH (ng/ml)	Serum creatinine (mmol/l)	Urine calcium (mmol/24 h)
Successful operation	2.38* ± 0.13 ($n = 107$)	0.30* ± 0.20 ($n = 92$)	0.12 ± 0.12 ($n = 111$)	3.38* ± 2.26 ($n = 66$)
Unsuccessful operation	2.82*,*** ± 0.21 ($n = 28$)	1.29** ± 1.49 ($n = 28$)	0.13 ± 0.08 ($n = 27$)	5.97** ± 4.20 ($n = 10$)
No operation	2.71*, *** ± 0.30 ($n = 77$)	0.91** ± 0.67 ($n = 70$)	0.11 ± 0.05 ($n = 76$)	5.54** ± 3.36 ($n = 47$)

* = Significantly different ($p < 0.001$) from value at time of decision (see Table 2); ** = significantly different ($p < 0.001$) from successful group, but not from other group with persistent hypercalcaemia; *** = significantly different ($p < 0.001$) from successful group but also significantly different from other group with persistent hypercalcaemia ($p < 0.01$) (Mann—Whitney test for unpaired data).

TABLE 7. *The prevalence of Paget's disease amongst patients with primary hyperparathyroidism*

	No. with adequate data	No. with Paget's disease	Percentage with Paget's disease
Successful operation	81	4	4.9
Unsuccessful operation	19	2	10.5
No operation	61	11	18.0
Total	161	17	10.6*

*6.4 per cent of all patients.

TABLE 8. *The prevalence of vertebral crush fractures amongst patients with primary hyperparathyroidism*

	No. with adequate data	No. with vertebral crush fractures	No. with bone scores of 5 or greater
Successful operation	81	9 (11.1)	12 (14.8)
Unsuccessful operation	19	2 (10.5)	3 (15.8)
No operation	61	11 (18.0)	8 (13.1)

Percentages in parentheses.

TABLE 9. *Forearm osteodensitometry in 28 patients with primary hyperparathyroidism (all female, all right-handed)*

	Successful operation	Unsuccessful operation or no operation
Number	13	15
Age (years)	57.1 ± 7.6	56.0 ± 7.2
Duration of follow-up (years)	7.2 ± 4.0	7.6 ± 5.0
Mineral content — right (arbitrary units†)	35.7 ± 6.6	29.0 ± 4.1*
Mineral content — left (arbitrary units†)	34.5 ± 6.7	28.9 ± 4.6**

*t = 2.98; $p < 0.01$; **t = 2.14; $p < 0.05$.
†One unit corresponds to approximately 0.03 g/cm of bone.

had mild degrees of renal impairment while in eight there was no obvious cause for elevated serum PTH concentrations.

Table 7 shows the prevalence of Paget's disease amongst the three groups of hyperparathyroid patients. Most of these patients had been referred because of Paget's disease, with hypercalcaemia being detected during the initial work-up (4).

Table 8 shows that vertebral crush fractures and 'high' osteoporotic scores were no more common at the time of follow-up in one group than in others. While each group of patients contained several individuals with crush fractures of vertebrae and high osteoporotic scores the majority showed no skeletal abnormalities on standard radiological films.

Table 9 shows that the bone mineral content of the forearms of normocalcaemic patients was significantly greater than that of hypercalcaemic patients.

DISCUSSION

We recognise that the patients in the unoperated group were not matched with the other two groups and that the decisions for surgery reflected, to some extent, judgements concerning the desirability of surgery. It is therefore not possible, with the data presented in this study, to construct life tables or to produce other statistical assessments concerning the benefits of parathyroidectomy. Such statistical data can only be derived from a randomised prospective study and as the Mayo Clinic experience (5) has shown such a study is unlikely to be forthcoming in a democratic society.

Nevertheless, it is clear from our experience and from several other published and unpublished series (Table 10) that untreated hyperparathyroidism is compatible with long survival and a lack of demonstrable deleterious effects on kidney or bone. If chronic hypercalcaemia and/or chronic parathyroid hormone excess aggravate the atheromatous process, such aggravation was not apparent in our studies. The reputed toxicity of parathyroid hormone to the function of peripheral nerves (9), the heart (10) and bone marrow (11) did not give rise to clinical problems in the 'unsuccessful' and unoperated groups. Renal impairment and evidence of cerebrovascular disease were no more prevalent in the unoperated and the 'unsuccessful' groups than in patients who had undergone a successful parathyroidectomy.

Osteodensitometric measurements performed during this survey and in other studies (12) suggest that bone mineral density is reduced in the hyperparathyroid state. However there is

TABLE 10. *Other published and unpublished series describing patients with 'untreated' hyperparathyroidism* *

Authors	N	Mean follow-up period (years)	Deaths (N)
Scholz and Purnell (5)	86	10 (approx.)	35
Adams (6)	31	4.0	4
Van't Hoff *et al.* (7)	32	4.2	3
Harrop *et al.* (8)	51	3.5	4
This paper	119†	5.7	16

*Total: 319 patients observed for 1975 patient-years.
†Comprises 'unsuccessful' and unoperated groups except for four patients who could not be traced.

other evidence (13) that hyperparathyroidism enhances bone accretion and intermittent parathyroid hormone administration has actually been advocated as treatment for osteoporosis (14). Dauphine *et al.* (15) claimed that radiological osteoporosis was more common at the time of presentation in patients with hyperparathyroidism than in controls. We were unable at the time of follow-up to show any radiological differences between operated and unoperated patients and suspect that there may have been bias in the selection of controls studied by Dauphine *et al.* (15).

It is known (16) that hyperparathyroidism is associated with a high prevalence of hypertension. Our data support the findings of Jones *et al.* (17) who were unable to demonstrate any beneficial effects of parathyroidectomy on hypertension or renal function and who concluded that hypertension does not constitute an indication for parathyroidectomy.

It is not possible, in a study of this kind, to determine whether there is an association between primary hyperparathyroidism and pancreatitis (18). However, there was no evidence from this group of patients or from other series (Table 10) that a conservative approach predisposed the patients to further attacks of pancreatitis.

It is generally believed that hyperparathyroidism is a risk factor for urinary stone formation (19) and there is *in vitro* evidence (20) that parathyroidectomy diminishes crystal formation in urine. However, in answer to a patient's question, 'Will I form kidney stones if I do not have this operation?' one has to reply that this depends on whether or not there was a previous history of urinary calculi (Fig. 1). Patients with a history of renal stones have a 50 per cent chance of suffering from further episodes regardless of whether they undergo a parathyroidectomy. Patients without a previous history of calculi are unlikely to form stones even if they remain hypercalcaemic and hypercalciuric.

We do not wish to give the impression that we regard parathyroidectomy as a useless operation. The procedure is relatively free of morbidity (21, 22) even in elderly patients (23), it renders the patients normocalcaemic (Table 6) and relieves them of the symptoms of hypercalcaemia. Parathyroid osteopathy (24) and hypercalcaemic myopathy (25) have been documented to disappear after parathyroidectomy. The bone mineral content of the forearms was clearly reduced in patients with peristent hyperparathyroidism.

Moreover, the chronic hypercalcaemic state is associated with characteristic cardiac lesions (26), severe hypercalcaemia may lead to renal damage and death (27) and it is impossible, in unoperated patients, to exclude parathyroid carcinoma with certainty (28). It is known (29) that high calcium concentrations in growth media may act as a mitogen for some malignant cells and the prevalence of malignant disorders was apparently higher amongst the hypercalcaemic patients than amongst the normocalcaemics (Table 4) though this finding was not confirmed by Ljunghall *et al.* (30). The follow-up period of the unoperated patients was relatively short and it is possible that in another five or 10 years more skeletal complications may become apparent.

For these reasons we generally recommend surgery in young patients, in patients with symptoms attributable to the hyperparathyroid state and in patients with serum calcium values in excess of 3.0 mmol/l (12 mg/dl). However, there is no evidence that parathyroidectomy prolongs life or that it cures the multiplicity of non-specific symptoms that have been described with hyperparathyroidism. The theoretical dangers of not operating on hyperparathyroid patients ('they might require thiazides and/or digoxin' or 'if you do not do it now they might have to have an operation when they are old and unfit') do not appear to have impaired the health of our unoperated patients.

We have therefore adopted the attitude that parathyroidectomy is to hyperparathyroidism what cholecystectomy is to cholelithiasis. We have become increasingly conservative in older, asymptomatic patients (31) and in patients who have previously had unsuccessful neck ex-

ploration even if these patients are young and even if serum calcium values are in excess of 3.0 mmol/l. To date we have not had cause to regret this tolerance.

ACKNOWLEDGEMENTS

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Paper 7. Is there a place for forearm osteodensitometry in clinical screening studies. B Robinson, C Wagstaffe, J Roche, P Clifton-Bligh, S Posen. The Medical Journal of Australia 1987; 146:297-299

P Clifton-Bligh made a significant contribution in the recruitment and assessment of the patients included in this study, and in the interpretation of the results. The present study compares the bone mineral density measured in the forearm by osteodensitometry with the volumetric bone mineral density measured in the lumbar spine by quantitative computed tomography in 125 subjects. 64 of the 125 were deemed to be normal after review for intercurrent and past illness and of current medications. 27 subjects with one or more vertebral crush fractures were studied. 21 subjects with back pain but without crush fractures were studied. The bone mineral density of the forearm was measured distally beginning at a point where the radius and ulna are 8mm apart and thence for 1 cm proximally. The volumetric bone mineral density in the lumbar vertebrae was measured in trabecular bone by quantitative computed tomography.Women older than 60 years had significantly lower bone mineral content than younger women. Women at all age groups had significantly lower forearm bone mineral content than age matched men. The volumetric bone densi5ty of trabecular bone in the lumbar spine showed no significant difference between male and female but there was a significant age related decline in both sexes. 21 of the 27 patients with vertebral crush fractures had a volumetric spinal trabecular bone mineral content of $75mg/cm^3$ or less. Both forearm bone mineral content and spinal trabecular bone mineral density were lower in male and female patients with vertebral crush fractures than among control subjects. The measurement of forearm bone mineral content is considered to be a powerful tool to identify subjects at risk of fracture. A forearm bone mineral content of less than 35 units had a 93% sensitivity in predicting a spinal trabecular bone mineral density of less than $75mg/cm^3$.

This study highlights the value of measuring forearm bone mineral content as a screening tool to identify those at risk for osteoporosis. It should be emphasised that this is a cross sectional study.

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Is there a place for forearm osteodensitometry in clinical screening studies?

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ABSTRACT In order to evaluate forearm osteodensitometry for its potential to detect subjects with a low spinal mineral content and/or vetebral fractures, single-photon absorptiometry of the forearm and estimations of spinal mineral content by computed tomography were performed in 124 normal and abnormal subjects. Eighty-one per cent (22/27) of patients with vertebral crush fractures had a low spinal mineral content. In contrast, among 64 apparently normal individuals, six patients (four women, two men; 9.4%) had a low spinal mineral content. Forearm osteoden-sitometry showed a significant positive correlation with spinal mineral content. A forearm value in women in excess of 35 arbitrary units was associated with a spinal value of 75.1-mg equivalent dipotassium phosphate (K_2HPO_4) or above in 29/31 cases. A forearm value in women of 28.5 arbitrary units or lower was associated with a spinal value of 75-mg equivalent K_2HPO_4 or less in 20/24 subjects. While of no predictive value for the spine in patients with intermediate readings, forearm osteodensitometry is nevertheless considered a useful screening procedure for spinal osteoporosis.

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T he estimation of bone mineral in the appendicular skeleton by single-photon absorptiometry was described more than 20 years ago.[1] In recent years, newer techniques have been described for the measurement of spinal mineral content,[2,3] and much statistical information has been

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gathered in relation to the axial and peripheral skeletons in cross-sectional and longitudinal studies.[4,5]

The question now arises as to whether there is any longer a place in clinical medicine for the measurement of mineral content in the forearm. Under what circumstances should a practising clinician say to a patient, "You need a forearm mineral estimation"? This question has aroused considerable controversy. Some investigators consider forearm measurement virtually useless while others regard it as a valuable screening test.[6,7]

We recently performed a cross-sectional evaluation of 125 normal and abnormal subjects by clinical examination, forearm osteodensitometry and spinal computed tomography, the results and conclusions of which are reported in this communication.

Subjects and methods

Sixty-four apparently normal subjects (31 men, 33 women) aged 23 to 73 years were recruited from hospital staff members and from local golf, bowling and service clubs. These subjects were questioned regarding handedness, previous illnesses and medications. Those who were suffering from illnesses or taking medications that were believed to influence skeletal mass were excluded. Premenopausal women were included regardless of whether or not they were or had been taking oestrogen-containing compounds. An additional 139 normal subjects (63 men, 76 women) were similarly recruited but in these individuals only forearm osteodensitometry was performed.

Sixty-one subjects with pathological symptoms (15 men, 46 women) aged 21 to 79 years were studied. Twenty-seven of these had been referred because of recurrent or persistent back pain and had been found, on radiological examination, to have sustained one or more vertebral crush fractures. Twenty-one patients with vertebral crush fractures were compared with age- and sex-matched control subjects.

Twenty-one patients with back pain did not have a crush fracture. There were seven patients who had sustained multiple peripheral fractures and six patients who had received corticosteroid agents for

12 months or longer.

Forearm osteodensitometry was performed with a Novo Osteodensitometer, model GT35 (Novo Instruments, Copenhagen, Denmark). This instrument measures mineral content in the bones of the distal forearm; commences at a point where the distance between radius and ulna is 8 mm; and moves proximally from this point. Results are given in arbitrary units, each unit being the equivalent of 0.033 g/cm. The radiation dose is approximately 30 μSv and the coefficient of variation is 1.0% (same operator, same day). The bone density was measured in both arms in each subject but only the results from the dominant arm were used for statistical purposes.

Spinal mineral content was estimated by computed tomography with a Siemens DR2 instrument according to the method of Genant et al.[8] Representative volumes (approximately 4 cm^3) of trabecular bone in the bodies of three non-collapsed lumbar vertebrae were measured, averaged and expressed as mineral equivalents of dipotassium phosphate (K_2HPO_4) in mg/cm^3. The radiation exposure for each examination is approximately 1.70 mSv while the coefficient of variation is 4.6% (same operator, same day). No consistent differences were noted between different vertebrae from T_{12} to L_5.

Results

Forearm osteodensitometry in these 203 normal individuals gave results that were very similar to those that had been obtained by other investigators. A profound sex difference was observed in all age groups (Figure 1). Women who were aged 60 years or over showed significantly lower values than did younger women (two sample t-test, $P<0.01$) but there were no other significant age-related differences. Right-handed persons had a mean of 5.8% more mineral in their right arms than in their left arms, whereas individuals who described themselves as left-handed had a mean of 2.3% more mineral in the left forearm than in the right forearm.

Figure 2 shows that, in contrast to the forearm, the spinal mineral content of normal subjects showed no significant sex
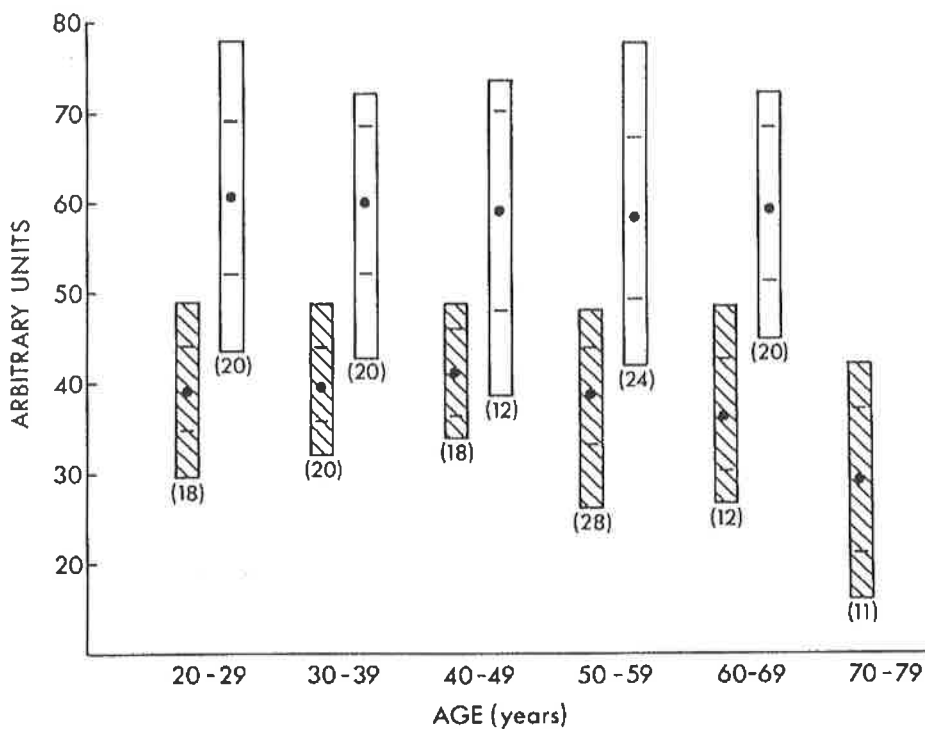
FIGURE 1: Forearm osteodensitometry values in 96 normal men (clear bars) and 107 normal women (hatched bars) aged 20–79 years. Note the profound sex difference at all ages but the relative lack of age-related changes except for women aged 60 years and over. The boxes represent observed ranges, the points are the means and the lines indicate one standard deviation from the mean. One arbitrary unit is equivalent to 0.033-g mineral/cm.
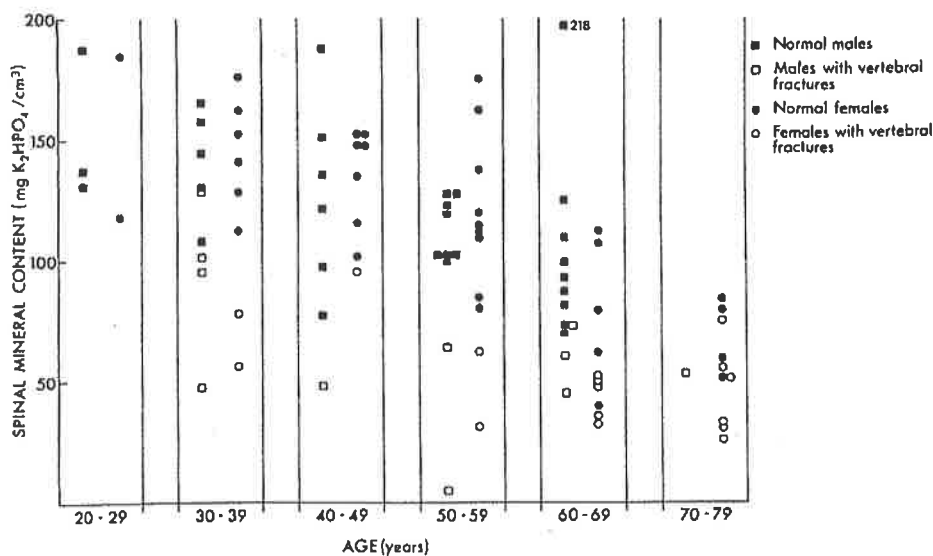

FIGURE 2: Spinal mineral content as measured by computed tomography in 64 normal subjects and in 27 patients with vertebral crush fractures. Note that 81% of patients with vertebral crush fractures have a spinal mineral content of 75-mg equivalent K_2HPO_4/cm^3 or less.

differences before the age of 59 years. An age-related decline was observed over the entire age range in both sexes ($r = -0.81$ in men, $P < 0.001$; $r = -0.78$ in women, $P < 0.001$).

Figure 2 shows the spinal mineral content of 27 patients with crush fractures. Twenty-two (81%) of 27 patients with vertebral crush fractures had a spinal mineral content of 75-mg equivalent K_2HPO_4/cm^3 or less. In contrast only 6/64 (9.4%) normal subjects

had values of less than 75-mg equivalent K_2HPO_4. Thus, for detecting the presence of vertebral crush fractures, a low spinal mineral content has a sensitivity of 81% and a specificity of 91%.

The Table shows vertebral and forearm mineral content values in patients with vertebral crush fractures and in control subjects who were matched for sex and age. Both forearm and spinal mineral contents were lower among male and female patients

with crush fractures than among control subjects.

Figure 3 shows the correlation between forearm and spinal measurements in 78 women with normal and abnormal findings. In this small group of subjects there was no significant difference between the regression equations that were calculated for both groups. The equation was $y = 4.07x - 43$ for all subjects, where y = spinal mineral content in mg equivalent K_2HPO_4/cm^3 and x = forearm osteodensitometry results in arbitrary units.

There was a significant positive correlation between spinal and forearm measurements ($r = 0.711$ in women; $P < 0.001$). In women, a good discrimination was obtained between spinal values above and below 75-mg equivalent K_2HPO_4/cm^3 when forearm cut-off points were established at 35 units and 28.5 units arbitrarily. Ninety-three per cent of subjects with forearm values of 35 units or above had a spinal mineral content of 75.1-mg equivalent K_2HPO_4/cm^3 or more. Eighty-three per cent of subjects with forearm values of 28.5 units or less had a spinal mineral content of 75-mg equivalent K_2HPO_4/cm^3 or less. Among subjects with forearm values between these two readings (28.6–34.9 units), 50% had a spinal mineral content of 75-mg equivalent K_2HPO_4/cm^3 or less.

Thus, a forearm bone mineral content of less than 28.5 units had a sensitivity of 83% in the prediction of a low spinal mineral content. A forearm bone mineral content of less than 35 arbitrary units had a sensitivity of 93% in predicting a low spinal mineral content.

Discussion

Forearm osteodensitometry has much to recommend it as a screening test for osteoporosis. The test involves mobile instruments, it is relatively cheap, radiation exposure is low and precision is high.[9] High forearm readings (in excess of 35 arbitrary units in women) make the presence of spinal osteoporosis highly unlikely (2/32 patients; 6.3%), while low forearm values (28.5 arbitrary units or less) were associated with spinal osteoporosis in 83% of cases in this series (Figure 3).

We realize that forearm measurements involve predominantly cortical bone and that changes in cancellous bone are not necessarily reflected in the forearm. Indeed, as Figure 3 indicates, it is possible to obtain readings close to zero for cancellous bone in the spine, whereas the cortical bone values of the forearm never fall below 15 arbitrary units. Therefore, forearm osteodensitometry should be regarded as a screening test similar to serum lipid estimations. Screening tests, by definition, are not "reliable" from a diagnostic point of view. Forearm osteodensitometry will not identify all subjects with a low spinal mineral content and, depending

TABLE: Forearm and spinal mineral content in patients with vertebral fractures and in matched control subjects

|	n	Mean age (years)	Mean forearm values (arbitrary units)	Mean spinal mineral content (mg equivalent K_2HPO_4/cm^3)
Control subjects (men)	9	46.55	66.28 ± 7.74	134.19 ± 38.87
Patients with vertebral fractures (men)	9	45.67	50.69 ± 10.48 $P < 0.02*$	69.53 ± 35.63 $P < 0.0025*$
Control subjects (women)	12	59.16	32.91 ± 5.83	90.85 ± 35.19
Patients with crush fractures (women)	12	59.83	29.11 ± 5.64 $P < 0.001*$	51.87 ± 20.76 $P < 0.001*$

*Paired t-test.
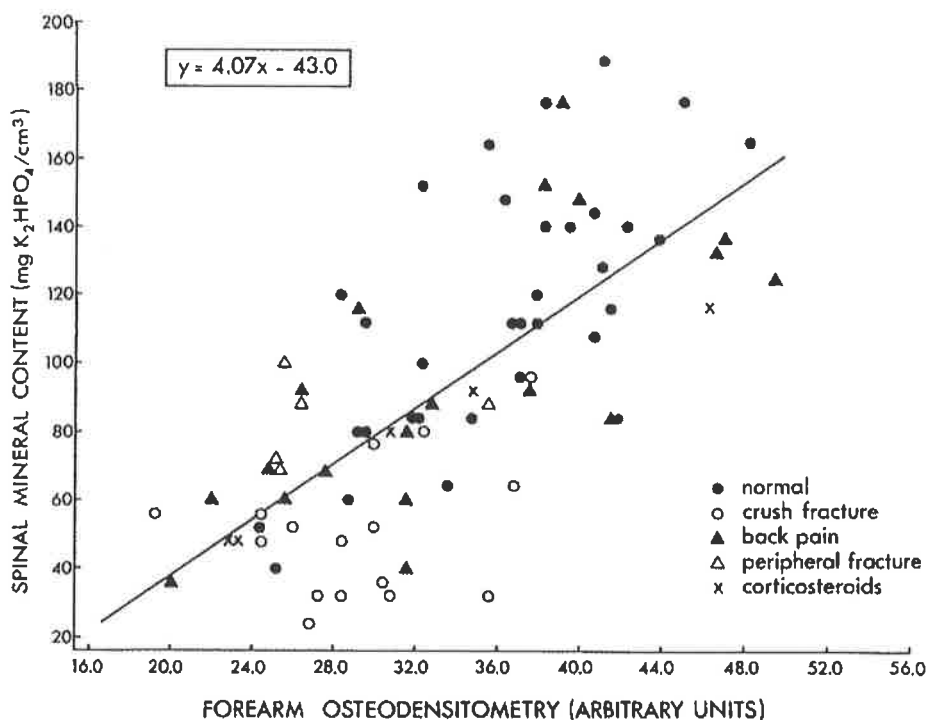
FIGURE 3: The correlation between forearm densitometry and spinal mineral content in 78 women. Thirty-two women had no disease or symptoms, 16 women had crush fractures, 20 women had back pain but no vertebral fractures, five women had peripheral fractures and five women were taking corticosteroid agents.

on the cut-off points that are selected, there are bound to be false-positive and false-negative results.[10] The value of all bone mineral measurements in the prediction of spinal or peripheral fractures remains to be determined.[11-13]

It would be unreasonable to expect any bone density measurement to predict 100% of fractures. Fractures are caused by multiple factors of which a reduction in bone mass is only one and, possibly, not the most important.[12] However, forearm osteodensitometry is a powerful tool to identify subjects at risk of a fracture. A menopausal woman who wishes to know whether she requires prophylactic therapy and whose forearm mineral content is 36 arbitrary units needs to be given different advice from a woman whose forearm value is 26 units. Similarly, a patient with back pain and a relatively normal back x-ray film reasonably can be reassured on the basis of a high forearm mineral value that she is not osteoporotic at this moment.

This work confirms the data of Cann et al., who showed that a "low" spinal mineral content is associated with vertebral fractures,[14] although it is recognized that single-energy computed tomography underestimates spinal mineral content.[15] It also confirms the data of Grubb et al.[16] and Nordin et al.[17] who showed that a low forearm mineral content is associated with a low spinal mineral content.

Therefore, we recommend that in spite of its limitations forearm osteodensitometry be employed as a screening procedure in all patients who are considered to be at risk of developing spinal osteoporosis. This includes menopausal individuals, individuals who are receiving corticosteroid therapy and patients whose spinal x-ray films suggest a diminished bone mineral density. If values are low, patients should be treated or at least investigated further for the presence of osteoporosis. If the values are high, patients can be reassured that they almost certainly do not have osteoporosis at this time. Patients with intermediate forearm mineral values may require further tests such as spinal computed tomography or dual-photon absorptiometry. As other single-photon absorptiometers are developed the "grey area" may well diminish.[7]

The value of forearm (or any other) osteodensitometry in the prediction of hip fractures remains problematical.[9]

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Paper 8. The effect of exercise on circulating immunoreactive calcitonin in men. ME O'Neill, M Wilkinson, BG Robinson, DB McDowall, KA Cooper, MS Mihailidou, DB Frewin, P Clifton-Bligh, SN Hunyor. Hormone and Metabolic Research 1990; 22:546-550

P Clifton-Bligh made an important contribution to this study in the design and execution of the study, in the analysis of the results and in the development of the calcitonin assay. The major clinical usefulness of the serum calcitonin assay is in the diagnosis of medullary carcinoma of the thyroid gland. It is important to study variables which might affect the reproducibility of the serum calcitonin assay. Exercise has been shown to increase the serum ionised calcium and acute rises in serum ionised calcium had been shown previously to increase the serum calcitonin. These findings underpinned the rationale for this study.Previous studies had involved exercise at variable durations and intensity. The present study examined whether moderate duration/moderate to high intensity exercise affected serum calcitonin levels specifically in males and whether any change in serum calcitonin was linked to changes in plasma catecholamines. 13 healthy males were exercised to a symptom limited maximal level using the Bruce protocol. The subjects had been fasted for at least four hours. The values for serum calcitonin, serum calcium and serum parathyroid hormone were corrected for plasma volume changes during exercise. Pre-, peak, and post exercise serum calcitonin concentrations were measured. Uncorrected and corrected serum calcitonin did not change with exercise. The serum calcium was increased at peak exercise. Plasma noradrenaline, adrenaline and dopamine were significantly increased at peak exercise. The increase in the total plasma calcium during exercise may have been too small or too brief to stimulate increased calcitonin secretion.

The main relevance of this study comes from the finding that serum calcitonin did not increase during moderate exercise in males.

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The Effect of Exercise on Circulating Immunoreactive Calcitonin in Men

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Summary

Moderate-duration exercise increases serum catecholamine and serum calcium levels and might as a result be also expected to increase the levels of circulating serum immunoreactive human calcitonin (HCT). To explore this possibility, HCT was studied during and after moderate duration symptom-limited dynamic exercise in 13 healthy males, mean age 28 ± 6.9 (SD) years. The mean duration of exercise using the Bruce treadmill protocol was 14.1 ± 2.2 (SD) minutes. The mean heart rate (HR) peaked at 185 ± 6 (SD) bpm which was 96.1% of the predicted maximal HR for age. Values for HCT, uncorrected for changes in plasma volume, showed a minimal decrease in the recovery phase, whilst HCT corrected for changes in plasma volume did not alter during exercise or recovery. The serum parathyroid hormone (PTH) also did not change. At peak exercise, uncorrected but not corrected values for plasma noradrenaline, adrenaline and dopamine had increased significantly. Corrected plasma total calcium increased during recovery. In summary, dynamic weight-bearing moderate-duration exercise did not elevate HCT in healthy males.

Key words

Calcitonin – Exercise – Medullary Thyroid Carcinoma – Catecholamines – Parathyroid Hormone – Males

Introduction

In order to use the measurement of circulating serum immunoreactive human calcitonin (HCT) as a reliable screening investigation for medullary carcinoma of the thyroid (MTC), it is important to have an understanding of the variables which might influence it. For example, eating (*Wilson* and *Foster* 1985) and alcohol ingestion (*Weatherall, Ledingham* and *Warell* 1984) are known to increase HCT.

HCT secretion also increases in response to an acute increase in plasma ionized calcium (*Hamburger, Crosnier* and *Grunfield* 1979) and several studies have reported that moderate to high intensity exercise may increase plasma ionized calcium (*Nielson, Christiansen, Hartling* and *Trap-Jansen* 1977; *Vora, Kukreja, York, Bowser, Hargis* and *Williams* 1983; *Ljunghall, Joborn, Benson, Fellstrom, Wide* and *Akerstrom* 1984; *Aloia, Rasulo, Deftos, Vaswani* and *Yeh* 1985; *Cunningham, Segre, Slatopolsky* and *Avioli* 1985). Therefore, we hypothesized that exercise-related increases in plasma ionized calcium might increase HCT.

Vora, Williams, Hargis, Bowser, Kawahara, Jackson, Henderson and *Kukreja* (1978) found that beta-adrenergic stimulation with isoprenaline in man increased HCT but *Epstein, Heath* III and *Bell* (1983) did not, so that the question as to whether exercise-induced increases in circulating catecholamines might influence HCT was unresolved.

The conflicting results found in previous studies that have explored the effect of exercise on HCT may be due to differences in the exercise intensities and durations tested, in the associated exercise-induced changes in calcium and in the sex of the subjects tested. *Aloia* et al. (1985) found that in males, there was an increase in HCT after 10 minutes of moderate intensity exercise at 40%–50% of maximal oxygen consumption, which was out of proportion to hemoconcentration changes and was associated with an increase in plasma total and ionized calcium levels. In contrast, *Nishiyama, Tomoeda, Ohta, Higuchi* and *Matsuda* (1988) also examined the HCT response in males to moderate intensity exercise (50% of maximum capacity), but found no change in HCT. They exercised their subjects for a more prolonged period of 30 minutes and it was associated with a decrease in ionized calcium levels. *Cunningham* et al. (1985) found that HCT was not influenced by maximal exercise of very short duration (60–130 seconds). Despite increasing plasma ionized calcium, this exercise duration may have been too brief to see an effect on HCT. Also, the inclusion of four females in their sample of 12 subjects may have minimized any HCT changes because females have lower calcium-stimulated HCT rises than males (*Deftos, Weisman, Williams, Karpf, Frumar, Davidson, Parthemore* and *Judd* 1980).

Table 1 Pre-, peak and post-exercise values for hormonal and biochemical variables for Group 1. Mean and pooled standard error (SE) determined from repeated measures analysis of variance (RMAV) are given.

	N	Pre	Peak	Post-Ex	RMAV	SE
Calcitonin (pg/ml)	11	36.4	39.7	31.3*	*	1.84
Corrected	10	35.6	35.1	31.4	NS	1.65
Total calcium (mmol/l)	12	2.40	2.56*	2.37	*	0.022
Corrected	10	2.40	2.35	2.52*	**	0.033
Bicarbonate (mmol/l)	11	27.0	20.4[+]	23.5	**	1.2
Corrected	10	27.0	18.8[+]	25.4	**	1.3
Phosphate (mmol/l)	11	1.14	1.43[+]	1.13	**	0.053
Corrected	10	1.14	1.31	1.23	NS	0.056
PTH (ng/ml)	12	0.21	0.22	0.23	NS	0.031
Corrected	10	0.21	0.20	0.27	NS	0.034

Values corrected for changes in plasma volume with exercise and recovery are indicated by "Corrected", NS/not significant, *$P < 0.05$,
[+] $P < 0.01$ and ** $P < 0.005$.

Hence, with regard to interpreting HCT results, recent high-intensity short-duration exercise (*Cunningham* et al. 1985) or moderate-intensity prolonged exercise *Nishiyama* et al. 1988) should not influence HCT. However, the study by *Aloia* et al. (1985) raises the possibility that moderate duration, moderate intensity exercise may influence HCT and the interpretation of HCT screening results. Their findings have not yet been confirmed by other studies. Therefore, the present study sought to confirm whether moderate-duration, moderate to high intensity exercise in healthy males was associated with changes in HCT and whether exercise-related changes in catecholamines and/or plasma calcium occurred and could be linked with changes in HCT.

Methods and Design

Group 1 consisted of 13 healthy males who exercised to a symptom-limited maximal level using the standard Bruce treadmill protocol. Venous blood samples (25 to 30 mls) were taken in the standing position prior to exercise, at peak exercise and 30 minutes into recovery for the following estimations: HCT, parathyroid hormone (PTH), biochemical profile (for plasma total calcium, bicarbonate and phosphate), hematocrit and hemoglobin. Group 2 comprised a subset of 7 of these males who had extra blood withdrawn at 3 and 9 minutes of exercise for the above variables, in addition to venous samples before exercise, and at 3 and 9 minutes and at peak exercise for noradrenaline (NA), adrenaline (AD) and dopamine (DA). Hemolyzed samples were excluded. During exercise, heart rate (HR) was monitored continuously and systolic blood pressure (SBP) was measured every three minutes.

A wide range of fitness levels was represented among the subjects, all of whom were free of any medications. The study was approved by the institutional Ethics Review Committee and all subjects gave informed consent. They abstained from food, alcohol and smoking, for at least 4 hours prior to their test.

HCT was measured by a modification of the method *Heynen* and *Franchimont* (1974). Anti-human calcitonin antibody raised in a goat, which bound selectively to the 17–32 amino acid sequence of the calcitonin molecule was used. Synthetic human calcitonin (Organon) was used to prepare standards in a buffer described by *Heynen* and *Franchimont* (1974) and for labelling with I^{125} by the procedure of *Marx, Woodward* and *Aurbach* (1972), which is known to preserve the biological activity of the labelled calcitonin. Nondescript

serum was not used to dilute calcitonin standards because of the difficulty in obtaining calcitonin-free serum. When calcitonin standards were prepared with sera from patients with severe untreated hypocalcaemia who had very low HCT values not significantly different from zero, and compared with standards prepared in buffer, identical curves were obtained for standards ranging between 0 and 50 pg per assay tube. Hence, we used standards prepared in buffer in the present study. Each serum sample was assayed in aliquots of 100 uL and 200 uL in triplicate. A non-specific binding blank was included in each sample. If proportional increases in the measured calcitonin value with increased aliquot volume did not occur, the values were discarded. Quality control sera at multiple dilutions were included with each assay. The interassay coefficient of variation for values of 200 pg/ml was 12%. The non-specific binding was 3%. The sensitivity of the assay was 20 pg/ml. The upper limit of the reference range was 200 pg/ml.

The radio-immunoassay method previously described by *Kleerekoper, Ingham, McCarthy* and *Posen* (1974) was used to measure PTH (1–84). It has inter- and intra-assay variabilities of 9.2% and 8.1% respectively at the upper limit of the normal range, which was 0.4 ng/ml.

Venous samples for NA, AD and DA were collected on ice in heparinized tubes (lithium heparin 125 U/10 ml) containing glutathione (5 mmol/l), spun immediately in a refrigerated centrifuge, separated and kept at −20 °C until assayed. A modification of the radioenzymatic technique of *Da Prada* and *Zurcher* (1976), as described by *Cummings, Russell, Frewin* and *Miller* (1984) was used. In our laboratory, the recovery rate was 60–62% and the coefficient of variation of the assay was 5% at the concentrations found in this study. The lower limit of detection was 0.05 pmol/ml.

Corrected results, which take into account plasma volume changes with exercise and recovery, were calculated using equations developed by *van Beaumont, Underkofler* and *van Beaumont* (1981). Due to clotting of some of the blood samples for hemoglobin and hematocrit, the number of subjects included in the corrected data is less than for the uncorrected data. This accounts for the slight difference in the group 1 uncorrected and corrected baseline values.

Data analysis was performed using the Minitab Statistics Package. The values given in the text are means (± standard deviation [SD]). Repeated measures analysis of variance (RMAV) was used as the test of statistical significance. The significance level was set at $P < 0.05$. The number of subjects (N) with complete sets of data

Table 2 Pre-exercise (Pre) and exercise (3 minute, 9 minute and peak) values for hormonal and biochemical variables for group 2 (N = 7)

	Pre	3 min	9 min	Peak	RMAV	SE
Calcitonin (pg/ml)	39.6	41.3	36.8	36.7	NS	2.22
Corrected	39.6	41.0	22.5	33.6	NS	1.90
Total calcium (mmol/l)	2.42	2.43	2.44	2.61^+	**	0.022
Corrected	2.42	2.38	2.40	2.39	NS	0.028
Bicarbonate (mmol/l)	28.3	28.9	27.6	20.9^+	**	0.47
Corrected	28.3	28.8	27.5	19.1^+	**	0.51
Phosphate (mmol/l)	1.13	1.11	1.21^*	1.49^+	**	0.039
Corrected	1.13	1.04	1.20^+	1.36^+	**	0.038
Noradrenaline (pmol/ml)	1.40	1.60	2.48	14.21^+	**	1.38
Corrected	1.40	1.62	2.48	12.90^+	**	1.28
Adrenaline (pmol/ml)	0.14	0.20	0.34	2.34^+	**	0.22
Corrected	0.14	0.20	0.34	2.15^+	**	0.21
Dopamine (pmol/ml)	0.24	0.22	0.23	0.53^+	**	0.041
Corrected	0.24	0.23	0.26	0.48^+	**	0.039
PTH (ng/ml)	0.24	0.24	0.22	0.23	NS	0.041
Corrected	0.24	0.25	0.18	0.21	NS	0.042

Abbreviations as for Table 1.

used in RMAV is given in Table 1. The pooled standard error (SE) was calculated from RMAV (Tables 1 and 2). The student Newman-Keul's test was used to determine which exercise levels were statistically different from baseline values. Pre, peak and post-exercise results are given for group 1 (in Table 1), the purpose of analysing the group 2 results (see Table 2) was to determine whether exercise-induced changes commenced at 3 minutes, 9 minutes or peak exercise. To see if the group 2 results were likely to be representative of the whole group, unpaired t-tests were used to compare characteristics between the 7 males in group 2 and the remaining 6 males.

Results (Tables 1 and 2)

Group 1 had a mean age of 28.3 (± 6.9) years and exercised for 14.1 (± 2.2) minutes, reaching 96.1 % (± 6.4 %) of the predicted maximal HR for age. At peak exercise, HR was 185 (± 15) beats per minute and SBP was 160 (± 23) mmHg. There was no significant difference between the subset of 7 males in group 2 and the remaining 6 males when considering their ages or any of the above exercise-related variables.

Uncorrected and corrected values for HCT did not change with exercise. Corrected HCT did not change during recovery, indicating that the post-exercise decline in uncorrected HCT was due to hemodilution.

The increase in uncorrected total plasma calcium that occurred with peak exercise but not by 9 minutes of exercise, was due to hemoconcentration because the corrected data did not change during exercise. However, corrected plasma total calcium increased post-exercise.

Uncorrected and corrected bicarbonate values decreased with peak exercise, but not by 9 minutes of exercise. Uncorrected plasma phosphate values increased by 9 minutes

of exercise and then continued to rise until peak exercise. Corrected phosphate results for group 1 showed similar trends but did not reach statistical significance.

With peak exercise, values for NA, AD and DA had increased significantly, but such changes were not evident by 9 minutes of exercise, when 79 % (± 6 %) of the predicted maximal HR for age had been achieved.

Uncorrected and corrected PTH values did not change with exercise or recovery.

During recovery, uncorrected values for plasma total calcium and uncorrected and corrected values for bicarbonate and phosphate returned to baseline levels.

Discussion

Although vigorous symptom-limited, moderate-duration dynamic treadmill exercise was associated with increases in plasma catecholamines and uncorrected total plasma calcium in healthy males, no significant changes in HCT were observed at peak exercise. The findings of the present study therefore differ to those of *Aloia* et al. (1985) but support the conclusions of *Nishiyama* et al. (1988) and *Cunningham* et al. (1985) that HCT does not change with dynamic exercise. *Cunningham* et al. (1985) postulated that exercise may modify the influence of plasma ionized calcium on serum HCT secretion and/or alter its peripheral metabolism. The increase in uncorrected total plasma calcium levels during exercise in our study may have been too small or too brief to stimulate increased HCT secretion. Although we may have missed a change in HCT in early recovery because of sampling 30 minutes into recovery, *Cunningham* et al. (1985) did not detect this when they sampled at 5 and 15 minutes into recovery.

The present study showed that a potential adrenergic stimulus to HCT secretion was applied during vigorous exercise with the demonstration of increases in serum catecholamine levels. The lack of an exercise-related HCT response may relate to the short duration of increased catecholamine stimulation which was less than 5 minutes duration.

The exercise intensities tested by *Aloia* et al. (1985) were unlikely to result in a markedly increased adrenergic stimulus to HCT release because, in our study, catecholamines did not increase until more than 79% of predicted maximal HR for age was achieved. Therefore, the increase in HCT with exercise that they found may have occurred independently of adrenergic stimulation.

The findings of *Cunningham* et al. (1985), *Nishiyama* et al. (1988) and the present study indicate that dynamic weight-bearing aerobic exertion prior to venesection for screening basal HCT tests for MTC would not significantly influence the HCT results in healthy subjects. If an "at risk" subject exercised dynamically before being tested and had an elevated HCT level, we would interpret this as being a true positive result and not due to exercise. However, this cannot necessarily be extrapolated to other activities, such as isometric or isotonic resistance exercise. *Dalsky, Stocke, Ehsani, Slatopolsky, Lee* and *Birge* (1988) postulated that skeletal loading stress is the critical factor in the response of bone to weight-bearing exercise rather than the exercise per se, because there was no correlation between improvements in aerobic capacity and bone mineral content in post-menopausal females after training. The possibility that HCT secretion may be promoted by the greater skeletal loading and very high adrenergic response associated with resistance activities (*Shepherd* 1987), as not yet been studied. Another area that has not been explored is the effect of exercise on human calcitonin concentration and action at a cellular level in bone and other tissues.

The statistically significant decrease in uncorrected HCT values at 30 minutes post-exercise was not large enough to be of clinical importance in normal males.

The uncorrected values for plasma total calcium increased with exercise. Several studies have detected exercise-induced increases in uncorrected values for plasma ionized calcium (*Cunningham* et al. 1985; *Nielson* et al. 1977; *Vora* et al. 1983; *Ljunghall* et al. 1984; *Aloia* et al. 1985), which *Vora* et al. (1983) have found to change in parallel with total plasma calcium. Our finding that corrected plasma total calcium values do not change during exercise is consistent with the results of *Greenleaf, Convertino, Stremel, Bernauer, Adams, Vignau* and *Brock* (1977). In contrast, *Aloia* et al. (1985) and *Cunningham* et al. (1985) found that corrected total calcium and ionized calcium values also increased during exercise. The significant post-exercise increase in corrected plasma total calcium in our study may have been due to redistribution of calcium from other compartments into the plasma space.

Despite the observation that the beta-adrenergic agonist isoprenaline increases PTH at rest in man (*Kukreja, Gurgis, Bowser, Henderson, Fisherman* and *Williams* 1975), we found that increases in plasma catecholamine levels due to moderate duration exercise were not accompanied by changes in PTH levels. This confirms the results of previous studies (*Cunningham* et al. 1985; *Vora* et al. 1983; *Ljunghall* et al. 1984) for which several hypotheses have been advanced. The catecholamine-induced stimulus may be insufficient to stimulate PTH secretion (*Vora* et al. 1983), may be opposed by the increase in uncorrected plasma calcium and/or may be modified by metabolic acidosis (*Cunningham* et al. 1985; *Vora* et al. 1983). However, the effect of exercise on PTH is controversial and it is important to consider the associated ionized plasma calcium response to exercise. *Aloia* et al. (1985) found that PTH decreased with moderate duration exercise and this may have been secondary to the increase in ionized plasma calcium. In contrast, *Ljunghall, Joborn, Roxin, Rastad, Wide* and *Akerstrom* (1986) and *Nishiyama* et al. (1988) found that PTH increased with prolonged exercise which was associated with a decrease in ionized calcium levels.

The importance of correcting hormonal and biochemical values for plasma volume changes resulting from exercise is highlighted by the findings in this study and that of *Cunningham* et al. (1985). Conclusions concerning the effects of exercise are markedly different when considering plasma volume corrected data as opposed to uncorrected data. A case could be made for using the uncorrected values of the variables studied because receptors are exposed to such concentrations. Also, clinicians use uncorrected data when interpreting screening HCT results. However, true release into or removal from the plasma compartment is more likely to be mirrored by the values corrected for hemodilution or hemoconcentration.

Conclusions

Moderate duration, moderate to high intensity, aerobic weight-bearing exercise did not change corrected HCT levels in healthy males but did cause a small but statistically significant decrease in uncorrected HCT levels during recovery. Moderately prolonged vigorous dynamic exercise prior to performing screening basal HCT levels for MTC would not cause false positive results in males.

Acknowledgements

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Paper 9. Immunoreactive serum calcitonin and skeletal histology in chronic renal failure. T Furlong, YL Chan, C Cornish, D McDowall, J Mahony, P Clifton-Bligh. Nephrology 1991; 58:138-143

P Clifton-Bligh collated the data,arranged the statistical analysis and wrote the paper. The bone biopsies had been carried out by T Furlong. The serum calcitonin assay was developed by P Clifton-Bligh. The patients were recruited by YL Chan. The study was carried out to further evaluate relationships between serum calcitonin and parameters of bone formation and resorption as acquired from the quantitative analysis of bone biopsies. 32 females and 18 males having regular haemodialysis were studied. The calcium content of the dialysis fluid was 1.8mmol/L. Calcium, phosphate, parathyroid hormone, calcitonin was measured in serum obtained immediately after dialysis. Aluminium was measured in a small piece of bone core obtained at biopsy. The mean values for serum calcitonin in both men and women were above the upper limit of the referenc e range. The serum parathyroid hormone was highly correlated with the osteoclast resorption surface (P=0.004). The serum parathyroid hormone was also highly correlated with the active osteoblast surface (P=0.0024). The serum inorganic phosphate was negatively correlated with the active osteoblast surface (P=0.0082). Bone aluminium was not significantly correlated with either the osteoclast resorption surface or the active osteoblast surface. In the multivariate analysis there was a strong positive correlation between bone aluminium and serum calcitonin (P=0.0078). Serum calcitonin in the multivariate analysis did not correlate with the osteoclast resorption surface or the active osteoblast surface. Interestingly, the high levels of serum calcitonin did not overcome the stimulating effect of circulating parathyroid hormone on osteoclast activity or the active osteoblast surface.

This paper is considered to be important because of the high level of correlation in the multivariate analysis between the serum calcitonin and bone aluminium, a new finding. More work is required to study the effect of aluminium intake on the serum calcitonin and between the serum calcitonin and the absorption of aluminium from the GIT.

Some of the bone biopsies for the assessment of histomorphometric parameters were carried out by Y.Chan as part of his PhD project. The re-analysis of the data, the development and application of the calcitonin assay and the writing of the paper was carried out by P Clifton-Bligh. The development of the theoretical concept underlying this project was by P Clifton-Bligh.

Immunoreactive Serum Calcitonin and Skeletal Histology in Chronic Renal Failure

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Abstract. Serum calcitonin and serum parathyroid hormone (PTH) were measured in 50 patients undergoing regular haemodialysis for end-stage chronic renal failure, and an analysis of osteoclast and osteoblast activities was made in bone biopsies obtained by iliac crest trephine. Osteoclast and osteoblast activities were studied in a multivariate analysis in relation to factors which might reasonably be thought to influence activity, namely serum calcitonin, serum PTH, serum calcium, serum inorganic phosphate, and bone aluminium. Only serum PTH correlated strongly with osteoclast activity (p = 0.0047). Serum PTH correlated also with osteoblast activity (p = 0.0024). Serum inorganic phosphate correlated negatively with osteoblast activity (p = 0.0082). Serum calcitonin did not correlate with osteoclast or osteoblast activities but did correlate strongly with bone aluminium in a multivariate analysis (p = 0.0078). Bone aluminium did not correlate independently with osteoclast or osteoblast activities. This study affirms the implied powerful role of PTH in influencing osteoclast and osteoblast activities in end-stage chronic renal failure.

Introduction

Patients with chronic renal failure receiving haemodialysis almost invariably exhibit osteodystrophy marked by varying degrees of osteoclast overactivity and/or defects in the mineralisation of osteoid. The increase in osteoclast activity is thought to be mediated by increased levels of circulating parathyroid hormone (PTH) [1–4]. Serum calcitonin concentrations are also raised above normal in the majority of patients with chronic renal failure [4–9]. Calcitonin has been shown to reduce osteoclast activity in experimental animals [10–12], and in bone culture systems, the PTH-stimulated release of calcium assumed to be osteoclast mediated was inhibited by calcitonin [13, 14]. The effects of sustained elevations in serum calcitonin on cellular activity in bone have been studied in patients with medullary carcinoma of the thyroid [15] in whom the appositional rate of newly mineralising bone was reduced suggesting a reduction in osteoblast activity.

The question arises as to whether the elevation in serum calcitonin frequently observed in patients with chronic renal failure can also inhibit osteoblast activity and restrain the stimulatory effects of PTH on osteoclast activity. Kanis et al. [16] found that after bilateral nephrectomy, serum calcitonin rose, and osteoclast activity was reduced suggesting a link between circulating calcitonin and osteoclast activity. High levels of serum calcitonin were found to be associated with lower levels of serum alkaline phosphatase in patients with chronic renal failure [17] raising the possibility that osteoblast activity might be inhibited by calcitonin. On the other hand, Malluche et al. [4] found no relationship between osteoclast activity and serum calcitonin in patients with chronic renal failure.

The present investigation was undertaken to further evaluate the relationship between circulating calcitonin and bone cell activity in patients with chronic renal failure. This study is an extension of the one carried out

by Chan et al. [18] modified to include only patients who also had measures of serum calcitonin.

Patients and Methods

Fifty patients (18 males, 32 females) aged 23–65 years, and having regular haemodialysis for end-stage chronic renal failure were studied. The mean age of the males was 46.7±14.0 years, and that of the females 49.8±11.1 years. The calcium content of the dialysis fluid was 1.8 mmol/l.

Blood was taken immediately before dialysis for the measurement of serum calcium, specific gravity, calcitonin, PTH, and inorganic phosphate. The serum calcium value was corrected if the value for the specific gravity of serum was greater or less than 1.027 [19]. At the same time, a bone biopsy was taken from the anterior iliac crest, and sections stained to demonstrate osteoclasts [20] and active osteoblasts [21] in separate sections from undecalcified bone. The percentage of trabecular bone surface undergoing active resorption by osteoclasts, and the percentage of trabecular surface occupied by active osteoblasts was calculated. This latter parameter has been shown to correlate with mineralisation surface as assessed by tetracycline labelling [3]. Double tetracycline labelling of bone surfaces was attempted, but only a small fraction of patients took up the label [18], and then only those with 'pure hyperparathyroidism'. Therefore, during subsequent analysis, a meaningful correlation between serum calcitonin and tetracycline labelling in the whole group could not be undertaken. The fraction of trabecular surface occupied by active osteoblasts was used as an indirect marker of osteoblast function.

Serum Calcitonin

Serum calcitonin was measured by a modification of the method of Heynen and Franchimont [22]. Anti-human calcitonin antibody raised in a goat, bound selectively to the 17–32 amino acid sequence of the calcitonin molecule. Synthetic human calcitonin (Organon) was used to prepare standards in the buffer described by Heynen and Franchimont [22], and for labelling with ^{125}I by the procedure of Marx et al. [23], which is known to preserve the biological activity of labelled calcitonin. Non-descript serum was not used to dilute calcitonin standards because of the difficulty in obtaining calcitonin-free serum. Serum from thyroidectomised persons frequently contained substantial amounts of immunoassayable calcitonin, as found by others [24]. Occasional patients with severe untreated hypocalcaemia had very low serum calcitonin values not significantly different from zero. When calcitonin standards were prepared with these sera and compared with standards prepared in buffer, identical curves were obtained for standards ranging between 0 and 50 pg per assay tube. Therefore, standards in buffer were used throughout the study. Each serum sample was assayed in aliquots of 100 and 200 µl in triplicate, and a non-specific binding blank was included with each sample. If proportional increases in the measured calcitonin value with increased aliquot volume did not occur, the values were discarded. Quality control sera at multiple dilutions were included with each assay. The interassay coefficient of variation for values of 200 pg/ml was 12%. The non-specific binding was 3%. The sensitivity of the assay was 20 pg/ml; the upper limit of the reference range was 200 pg/ml.

Serum PTH

Serum PTH was assayed by a method previously described [25]. The upper limit of normal was 0.4 ng/ml. A high correlation between values obtained with this assay in patients with chronic renal failure and values obtained by a method measuring bioactive PTH has previously been demonstrated [26].

Bone Aluminium

A small piece (1–2 mm) of the skeletal biopsy core was rinsed with deionised water and dried at 110°C for 16 h. Bone aluminium concentrations were measured in this material by the method of LeGendre and Alfrey [27].

Statistics

Each parameter was checked to ensure that the data were normally distributed in the population studied. Multivariate analysis was carried out with osteoclast resorption surface and active osteoblast surface as the dependent variables [28].

Results

The biochemical data are shown in table 1, and the histological data and bone aluminium in table 2. The mean serum calcitonin value for male patients was 285±90 pg/ml, and for female patients 237±93 pg/ml. The difference between male and female patients was not significant.

There was no correlation between age and serum calcitonin. In the multivariate analysis, with either osteoclast resorption surface or active osteoblast surface as the dependent variable, analysis was undertaken against factors which might have influenced the function of bone cells at the tissue level and included serum calcitonin, serum PTH, serum calcium, serum inorganic phosphate, and bone aluminium. The results are shown in table 3 in which p values for each correlation are given. With respect to osteoclast resorption surface, only serum PTH showed a significant positive correlation (p = 0.0047). With respect to active osteoblast surface, serum PTH showed a positive correlation (p = 0.0024), and serum inorganic phosphate showed a negative correlation (p = 0.0082). Bone aluminium was not significantly correlated with either osteoclast resorption surface or active osteoblast surface.

Bone aluminium was also studied in relation to the biochemical parameters serum calcitonin, serum PTH, serum calcium, and serum inorganic phosphate in multivariate analysis (table 3). The relationship between bone aluminium and serum PTH was not significant, but surprisingly, there was a strong positive correlation between bone aluminium and serum calcitonin (p = 0.0078).

Table 1. Parameters in patients undergoing chronic haemodialysis

Patient No.	Sex	Age years	Serum calcitonin pg/ml	Serum PTH ng/ml	Serum phosphate mm/l	Serum calcium mm/l
1	F	31	165	4.25	1.85	2.99
2	F	43	196	2.35	1.41	2.88
3	F	57	256	4.80	2.11	2.49
4	M	45	473	3.00	1.42	2.62
5	F	45	453	1.80	1.21	2.76
6	M	50	210	4.90	1.94	2.44
7	M	57	375	0.92	1.44	2.71
8	M	61	95	5.00	1.31	2.47
9	F	52	134	5.40	1.37	2.74
10	F	60	366	4.30	1.81	2.88
11	F	53	169	11.60	1.50	2.67
12	M	60	271	2.75	1.55	2.82
13	F	56	148	10.10	2.27	2.53
14	F	44	199	3.10	1.86	2.31
15	M	46	376	5.00	–	2.42
16	M	56	258	1.60	1.37	2.41
17	M	23	234	1.48	1.50	2.53
18	M	65	307	4.30	1.42	2.85
19	F	63	156	4.30	2.03	2.17
20	F	57	221	0.75	1.10	2.53
21	F	29	183	5.30	2.35	2.30
22	M	33	285	6.00	1.12	3.11
23	F	53	147	3.00	1.44	2.49
24	M	21	253	0.78	1.66	2.76
25	M	64	215	5.50	1.87	2.67
26	F	61	266	0.30	1.24	2.72
27	F	53	285	0.50	1.85	2.71
28	F	28	197	1.85	2.11	2.60
29	F	59	170	1.55	1.76	2.35
30	M	43	271	2.70	1.50	2.61
31	F	53	177	1.35	1.30	2.99
32	F	55	162	3.25	1.42	2.41
33	F	59	364	1.54	1.93	2.66
34	F	34	220	2.10	1.20	2.55
35	F	53	198	3.25	1.27	2.70
36	F	61	249	2.00	1.63	2.39
37	F	62	398	6.70	1.33	–
38	M	61	422	1.16	1.13	2.75
39	M	36	210	2.05	1.42	2.22
40	F	35	418	2.95	1.56	2.47
41	F	38	388	3.60	1.15	2.74
42	M	51	358	1.73	1.25	2.61
43	F	28	152	7.00	1.64	2.58
44	F	60	254	0.82	1.12	2.58
45	F	49	172	0.55	2.41	2.93
46	F	45	114	6.45	1.63	2.40
47	F	60	284	1.18	1.75	2.89
48	M	48	218	2.20	1.79	2.40
49	F	59	330	1.62	0.79	2.89
50	M	32	305	5.20	2.63	2.63

Table 2. Bone tissue parameters in patients undergoing chronic haemodialysis

Patient	Sex	Osteoclast resorption[a]	Active osteoblast surface[b]	Bone aluminium mg/kg/dry weight
1	F	6.02	2.15	46
2	F	3.68	1.38	76
3	F	5.86	5.89	108
4	M	1.92	2.31	84
5	F	0.70	2.23	49
6	M	14.01	17.49	71
7	M	2.01	0.69	101
8	M	7.70	18.66	63
9	F	18.29	25.86	12
10	F	9.69	–	130
11	F	18.23	16.12	–
12	M	0.93	6.08	98
13	F	7.25	7.28	66
14	F	0.42	–	38
15	M	7.28	15.13	146
16	M	1.88	–	136
17	M	6.51	11.00	33
18	M	5.46	–	48
19	F	9.96	20.95	45
20	F	9.62	7.80	–
21	F	12.49	6.58	54
22	M	7.60	32.51	57
23	F	6.57	6.84	42
24	M	2.59	5.64	58
25	M	5.54	6.84	31
26	F	0.25	–	23
27	F	5.25	–	23
28	F	5.70	–	73
29	F	5.11	–	27
30	M	11.02	3.50	52
31	F	2.20	6.39	77
32	F	0.17	–	21
33	F	1.75	–	160
34	F	2.04	–	61
35	F	4.24	10.61	38
36	F	1.06	2.37	6
37	F	15.57	3.46	27
38	M	3.34	3.54	104
39	M	5.77	11.07	62
40	F	1.06	–	37
41	F	7.32	12.79	67
42	M	0.91	–	41
43	F	3.84	–	4
44	F	2.91	7.97	53
45	F	0.05	–	12
46	F	9.79	15.59	116
47	F	1.25	1.56	52
48	M	9.87	5.60	74
49	F	1.53	4.77	–
50	M	1.45	6.51	143

Histomorphometric parameters obtained from 8 normal adults (mean ± 1 SD): osteoclast resorption: 1.7 ± 0.5%; active osteoblastic surface 5.9 ± 1.3%.

[a] Percent of trabecular surface involved by osteoclast resorption.
[b] Percent of trabecular bone surface occupied by active osteoblasts.

Table 3. Multivariate analysis

Dependent variable	Significance
Osteoclast resorption surface	
versus serum calcitonin	p = 0.1538
versus serum PTH	p = 0.0047
versus serum inorganic phosphate	p = 0.6341
versus serum calcium	p = 0.5332
versus bone aluminium	p = 0.7713
Active osteoblast surface	
versus serum calcitonin	p = 0.2150
versus serum PTH	p = 0.0024
versus serum inorganic phosphate[a]	p = 0.0082
versus serum calcium	p = 0.5978
versus bone aluminium	p = 0.7876
Bone aluminium	
versus serum calcitonin	p = 0.0078
versus serum PTH	p = 0.4218
versus serum inorganic phosphatase	p = 0.1735
versus serum calcium	p = 0.7742

[a] The correlation coefficient between active osteoblast surface and serum phosphate was negative.

Discussion

The major purpose of the present study was to examine osteoclast activity in the presence of the sustained elevations in serum calcitonin which occur in chronic renal failure. A recognised target cell for calcitonin is the osteoclast [10, 29]. Osteoclast activity was assessed as the percentage of trabecular surface undergoing osteoclast resorption. In the present study, a correlation between serum calcitonin and osteoclast activity could not be demonstrated, confirming the findings of Malluche et al. [4]. It is possible that under circumstances in which serum calcitonin remains continuously elevated, a down-regulation of its effect on the cell level may occur [30]. Alternatively, the assay of serum immunoreactive calcitonin by the present method may not accurately reflect the biological activity of circulating calcitonin which is known to circulate in multiple molecular forms in patients with chronic renal failure [31]. No attempt was made to measure monomeric calcitonin in the present study. However, in a study by Mulder et al. [8], the ratio of low-molecular weight (presumed monomeric) to high-molecular weight calcitonin in the serum of patients receiving haemodialysis was similar to normal controls implying that biologically active calcitonin was proportional to total circulating calcitonin. Although calcitonin in chronic renal failure circulates in multiple molecular forms it is

not known to what extent each form contributes to the biological activity at the bone cell level.

With respect to osteoblast activity as assessed by active osteoblast surface, there was no correlation between this parameter and serum calcitonin in multivariate analysis. This contrasts with the finding of Kanis et al. [16] who studied the relationship in the changing circumstances after nephrectomy. A strong positive correlation between osteoblast activity and serum PTH was observed in the present study, which supports the findings of de Vernejoul et al. [1] and Nilsson et al. [2]. In our study, this relationship was maintained even when possible influences of bone aluminium or serum calcitonin on osteoblast activity were accounted for. In addition, with multivariate analysis, serum inorganic phosphate was negatively correlated with osteoblast activity implying that high levels of serum inorganic phosphate may inhibit osteoblast activity independently of the stimulatory effect of serum PTH. This observation was not apparent in the univarate analysis of Chan et al. [18].

An interesting finding was the strong positive correlation in the multivariate analysis between serum calcitonin and bone aluminium, an observation which has not been previously made. This could be interpreted in a number of ways, for example, that high serum calcitonin levels in some way facilitated aluminium deposition in bone. A study of the effect of calcitonin on the tissue deposition of aluminium or blood levels of aluminium has not been made. Alternatively, if bone aluminium reflected the aluminium content of other tissues, e.g. parafollicular C cells, then the high tissue aluminium level might stimulate calcitonin release. In this respect, it is of interest that the aluminium content of parathyroid cells is increased in haemodialysed patients with chronic renal failure [32], and that aluminium may inhibit PTH release [33]. In addition, Hodsman et al. [34] found an inverse relationship between serum PTH and bone aluminium, a relationship not seen in the present study.

The effect of administered aluminium hydroxide on serum calcitonin was studied in non-dialysed patients with chronic renal failure by Takamoto et al. [35]. Serum calcitonin did not change, whereas serum inorganic phosphate, and serum PTH fell.

In summary, the high correlation between PTH and both osteoclast resorption surface and active osteoblast surface implies a powerful role of PTH in influencing osteoclast and osteoblast functions in patients with chronic renal failure receiving dialysis. Osteoclasts do not have receptors for PTH [36], and the simultaneous increase in osteoblast and osteoclast activities under the

influence of PTH may occur by primary activation of osteoblasts and secondary activation of osteoclasts by cell contact [37], which cannot be overcome by the high levels of calcitonin found in patients with chronic renal failure. The presumed failure of calcitonin to sustain its inhibiting effect on osteoclasts in patients with end-stage chronic renal failure is in contrast to the ability of calcitonin to inhibit PTH-mediated bone resorption in in vitro bone culture systems.

Acknowledgments

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This paper was presented by P Clifton-Bligh at a meeting on hyperparathyroidism at the National Institutes of Health, Bethesda, USA. P Clifton-Bligh carried out the data analysis and the paper was written by P Clifton-Bligh.The paper presented further evidence of the relationship between bone mineral content and hyperparathyroidism. Bone mineral content was measured in the distal forearm in patients before and after parathyroidectomy and compared with forearm bone mineral content in patients with continuing hyperparathyroidism and in normal controls. The bone mineral content of the distal forearm was measured at a point where the radius and ulna are 8 mm apart and for 1 cm proximal to this point. In a study of 28 patients, the forearm bone mineral content rose by 2.25% over a 12 month period after successful parathyroidectomy. In 10 women over the age of 40 years there was a significant rise in the forearm bone mineral content after successful parathyroidectomy studied over 2 years. The greatest rise was in the first 12 months. In a study of 12 normal female controls over the age of 40 years, the forearm bone mineral content fell significantly over a 2 year period ($P<0.001$). The mean fall in the forearm bone mineral content in the women with continuing hyperparathyroidism was significantly different from the mean rise in the women who had had a successful parathyroidectomy ($P<0.05$). The change in the bone mineral content in the ongoing hyperparathyroid group did not correlate with the serum parathyroid hormone. The forearm bone mineral content at time 0 in the control group was significantly higher than the forearm bone mineral content at time 0 in the hyperparathyroid groups. The gain in forearm bone mineral content after successful parathyroidectomy was greatest in those who had the lowest initial forearm bone mineral content. An important aspect of the present study was that many of the initial 244 patients reviewed with primary hyperparathyroidism had confounding factors with respect to the ongoing use of medication or the presence of intercurrent illness which may have influenced the changes in forearm bone mineral content over time. Some other reported studies have not controlled for these additional factors.

The present study is considered to be a significant contribution to the evaluation of bone mineral content in persons with hyperparathyroidism.

Longitudinal Changes in Forearm Bone Mineral Content in Primary Hyperparathyroidism

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ABSTRACT

Forearm bone mineral content was measured in 28 patients with primary hyperparathyroidism before and 1 year after successful parathyroidectomy. The forearm bone mineral content rose from a mean value of 1.068 to 1.092 g/cm ($P < 0.05$, paired t-test). Those patients with the lower initial values had the largest rise. In an additional study, the forearm bone mineral content was measured in 10 women over the age of 40 years (mean age 58.6 ± 7.9SD years) with hyperparathyroidism before and for 2 years after successful parathyroidectomy and compared with the forearm bone mineral content measured over 2 years in 12 women (mean age 56.3 ± 5.5SD years) with continuing hyperparathyroidism and with the forearm bone mineral content of 12 eucalcemic control women (mean age 58.8 ± 8.2SD years), also measured over 2 years. The parathyroidectomized group gained bone, whereas the ongoing hyperparathyroid group and the eucalcemic control group lost bone. The difference between the parathyroidectomized group and the ongoing hyperparathyroid group was significant after 2 years ($P < 0.05$). The percentage loss of forearm bone mineral in the eucalcemic control subjects was not significantly different from the percentage loss of forearm bone mineral in the ongoing hyperparathyroid group, although the initial mean bone mineral content in the eucalcemic group was significantly higher than in the ongoing hyperparathyroid group, suggesting that a possible determinant of bone mineral loss in women in this age group is the initial bone mineral content.

INTRODUCTION

PRIMARY HYPERPARATHYROIDISM has been associated with excessive loss of bone mineral, which is believed to result from hyperstimulation of osteoclastic bone resorption by excessive amounts of circulating parathyroid hormone.[1-5] Cross-sectional studies of patients with primary hyperparathyroidism have shown diminished bone mineral content measured by photon absorptiometry[6-8] and by quantitative computed tomography.[9,10] In severe disease, conventional radiography may reveal subperiosteal erosions, lytic lesions, and diffuse osteopenia.[1,5,10-12] An increased prevalence of vertebral crush fractures has also been reported,[12,13] although there is some uncertainty about the suitability of the control groups in these studies. Parathyroidectomy in patients without overt complications of the disease is controversial.[14-22] Increasing

availability of bone mineral densitometry has allowed postoperative changes in specific bones to be monitored. Some investigators found no change after parathyroidectomy; others reported significant increases.[6,12,23-26] In many of these studies, however, other factors that may influence bone mass were not rigorously sought and simultaneous studies of appropriate control populations were not undertaken. When an increase in the bone mineral density after parathyroidectomy has been observed, it has been short term, with no further increase seen by the end of the second postoperative year.[23,24,30] On the other hand, untreated hyperparathyroidism has been shown in some studies to be associated with a decline in bone mineral content.[6,13]

In the present study, the bone mineral content of the distal forearm was measured in patients before and after parathyroidectomy and compared with changes in the

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forearm bone mineral content measured in patients with continuing hyperparathyroidism and in normal control subjects.

PATIENTS AND METHODS

Between 1983 and 1986, 244 patients with primary hyperparathyroidism were evaluated. The diagnosis was based on the finding of an elevated serum calcium concentration, an elevated serum parathyroid hormone, and an absence of other causes of hypercalcemia. A number of patients with primary hyperparathyroidism did not undergo parathyroidectomy because they lacked symptoms and signs associated with hyperparathyroidism and were regularly followed. Of the 244 patients, 136 had parathyroidectomy and 92 did not; 16 patients had advanced renal failure and were excluded from the present analysis. The indications for parathyroidectomy generally were a serum calcium of more than 3.00 mmol/liter, a history of peptic ulceration, and the occurrence of renal calculi, pancreatitis, renal impairment, or muscle weakness. Patients who had measurements of forearm bone mineral content at the time of diagnosis and for up to 2 years subsequently were considered further and were compared with eucalcemic control patients who were studied over the same interval of time. Patients or control subjects who were taking glucocorticoids, thyroxine, phenytoin, thiazide diuretics, estrogens, progestins, anabolic steroids, calcitonin, vitamin D, or excess alcohol were excluded from analysis. Patients or control subjects who had celiac disease, osteomalacia, chronic hepatitis, rheumatoid arthritis, or diabetes mellitus were excluded. Those with a serum creatinine >0.20 mmol/liter were excluded. One patient in the parathyroidectomized group had a gastrectomy 18 years before parathyroidectomy. She was not taking vitamin D. The forearm bone mineral content was measured in the dominant arm in each patient or control subject with a Novo osteodensitometer. The values are given as g/cm. Measurements of the bone mineral content were commenced at a point where the distal radius and ulna were 8 mm apart, and six sweeps were made proximally. The precision of measurement was 1%.

After the exclusions mentioned, the remaining patients were divided into four study groups. In group A, 28 patients with primary hyperparathyroidism (21 women and 7 men, aged between 20 and 77 years) underwent parathyroidectomy and were rendered eucalcemic. Measurements of bone mineral content were made either before or within a few days of surgery and at least once again during 12 months after surgery. Groups B, C, and D, described here, represent three age-matched groups of females over the age of 40.

Group B, 10 women over the age of 40 with primary hyperparathyroidism, were treated successfully with parathyroidectomy and rendered eucalcemic. The mean age was 58.6 ± 7.9 years, mean ± standard deviation (SD). Initial measurements of bone mineral content were made either before surgery or within a few days of surgery. The 10 patients in this group are also included in group A.

Group C, 12 women over the age of 40 with hypercalcemia due to hyperparathyroidism, were not subjected to parathyroidectomy (9) or remained hypercalcemic after unsuccessful surgery (3). The mean age was 56.3 ± 5.5 (SD).

Control group D consisted of 12 eucalcemic women over the age of 40 years (mean age 58.8 ● 8.2 SD years). In groups B, C, and D estimates of forearm bone mineral content were made at 0, 12, and 24 months. Serum calcium and creatinine concentrations were measured by standard autoanalyzer methods. Serum parathyroid hormone was measured by a method previously described.[27] The normal range for this assay is 0–0.4 ng/ml. The polyclonal antiparathyroid hormone antibody used in the radioimmunoassay "sees" the whole 1–84 human parathyroid hormone molecule but not the C-terminal fragments. The assay is a one-site radioimmunoassay.

RESULTS

In group A, 28 patients aged between 20 and 77 years were studied over a 12 month period following successful parathyroidectomy. The mean forearm bone mineral content at time zero was 1.068 g/cm ± 0.412 (SD) and rose over a 12 month period by 0.024 g/cm, or 2.25% ($P <$ 0.05 paired t-test). The 28 patients were ranked from 1 to 28 in terms of initial forearm bone mineral content. The 14 patients with higher initial bone mineral content (above 0.927 g/cm), whose mean age was 41.9 ± 16.7 years (SD), showed a mean change of +0.004 g/cm over the 12 month period from 1.354 to 1.358 g/cm (SD), whereas the 14 patients with the lower initial bone mineral content (below or equal to 0.927 g/cm), whose mean age was 59.6 ± 9.7
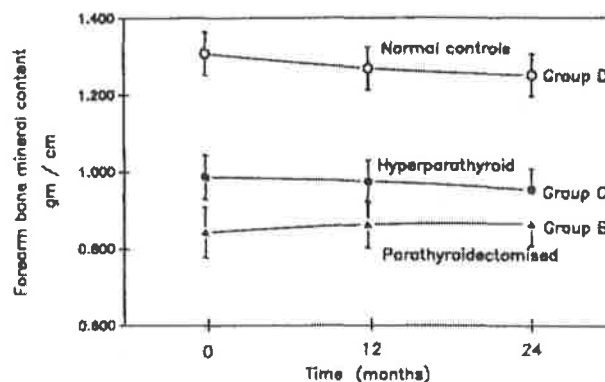

FIG. 1. Mean changes in the forearm bone mineral content over a 2 year period in women over the age of 40 who underwent successful parathyroidectomy (group B), who remained hyperparathyroid (group C), compared with normal control subjects (group D). Each data point shows the mean ± SEM (standard error of the mean). The significance of the changes in each group and comparisons between groups are given in the text.

(SD) years, showed a mean change of 0.045 g/cm from 0.781 to 0.826 g/cm ($P < 0.025$). The difference between the mean ages of the two groups was significant ($P < 0.005$).

In group B, 10 women over the age of 40 years underwent successful parathyroidectomy for hyperparathyroidism and were followed for 2 years. The mean serum calcium before parathyroidectomy was 3.02 ± 0.26 (SD) mmol/liter (range 2.65–3.40). The mean PTH before surgery was 1.51 ± 0.60 (SD) ng/ml. The mean forearm bone mineral content at the time of surgery was 0.843 ± 0.208 (SD) g/cm and rose by a mean of 0.021 g/cm, or 2.49%, over 2 years to 0.864 ± 0.183 (SD) g/cm. The greatest part of the rise, 2.25%, occurred in the first 12 months, and 0.24% in the second 12 months. The rise in the bone mineral content over the 2 year period was not significant.

In group C, 12 women over the age of 40 years were studied with ongoing hyperparathyroidism. The mean serum calcium was 2.83 ± 0.14 (SD) mmol/liter (range 2.61–3.04). The mean serum PTH was 0.75 ± 0.28 (SD) ng/ml. The mean forearm bone mineral content at the time of initial assessment was 0.987 ± 0.201 (SD) g/cm and over a 2 year period fell by a mean of 0.035 g/cm, or 3.55%, to 0.952 ± 0.190 (SD) g/cm. The fall in the bone mineral content over the 2 year period was not significant. In the 9 patients who had not had a neck operation the initial mean bone mineral content was 0.967 g/cm and the mean fall in bone mineral content was 0.040 g/cm. In the 3 patients who had an unsuccessful neck exploration the initial mean bone mineral content was 1.049 g/cm, falling by a mean of 0.026 g/cm.

In group D, 12 normal women over the age of 40 years were studied. They were not on any medications known to influence bone mass and had no intercurrent previous illness known to influence bone mass. The mean forearm bone mineral content at the time of initial assessment was 1.308 ± 0.193 (SD) g/cm and over a 2 year period fell by a mean of 0.057 g/cm, or 4.36%, to 1.251 ± 0.193 (SD) g/cm. The fall was significant ($P < 0.001$, paired t-test). The mean fall in forearm bone mineral content of the eucalcemic control subjects in group D was not significantly different from the fall in the ongoing hyperparathyroid group C. The mean fall in the forearm bone mineral content of the control subjects was significantly different compared to the mean rise in the forearm bone mineral content in the parathyroidectomized patients in group B ($P < 0.005$). Also the mean fall in the forearm bone mineral content for the ongoing hyperparathyroid group was significantly different from the mean rise in the forearm bone mineral content of the parathyroidectomized group ($P < 0.05$). The changes in the bone mineral content in groups B, C, and D are shown in Figure 1.

The percentage change over a 2 year period between the control group and the untreated hyperparathyroid group was not significantly different. If the percentage losses of bone in the control group and the untreated hyperparathyroid group were combined and then compared with the percentage gain in bone in the parathyroidectomized group B, there was a significant difference ($P < 0.005$), suggesting that parathyroidectomy and restoration of eucal-

cemia did in some way prevent bone loss during the 2 years of observation.

The initial bone mineral content in the ongoing hyperparathyroidism group and in the parathyroidectomized group before surgery did not correlate with the serum calcium or with the serum PTH. The change in bone mineral content in the ongoing hyperparathyroid group over a 2 year period did not correlate with the serum PTH.

The forearm bone mineral content of the control group at time zero, 1.308 g/cm, was significantly different from both the forearm bone mineral content at time zero in the group with ongoing hyperparathyroidism, 0.987 g/cm ($P < 0.001$), and from the forearm bone mineral content at time zero in the parathyroidectomized group B, 0.843 g/cm ($P < 0.001$). The forearm bone mineral content at time zero in the group with ongoing hyperparathyroidism (group C) was not significantly different from the forearm bone mineral content at time zero in the group who had successful parathyroidectomy (group B).

DISCUSSION

In a group of 28 patients with primary hyperparathyroidism, both men and women of diverse age, who were studied for 1 year after successful parathyroidectomy, the forearm bone mineral content rose significantly by 2.25%. Furthermore, those patients who had the lower initial bone mineral content and who were also older showed the largest increase in the 12 month period. This is similar to the data of Mautalen et al.,[24] except that age was not taken into account in this latter study. Mautalen et al.[24] also showed a high negative correlation between the initial forearm bone mineral content and the subsequent rise in bone mass. A separate aspect of the present study was the evaluation of three groups of women of similar mean age (over the age of 40 years) for a 2 year period. Many of this group of 34 women had a past hysterectomy or could not remember exactly when menopause occurred, but because the mean ages of the three groups was not significantly different, it was assumed that any influence of menopause on bone mineral loss in the three groups would be comparable. In addition, any patient who had an intercurrent disease or was receiving ongoing medication that might influence bone mineral content was excluded from the study. An important finding was that, whereas the normal eucalcemic control subjects and ongoing hyperparathyroid patients lost bone, those having a successful parathyroidectomy did not. The difference between the eucalcemic control subjects and the parathyroidectomized group was significant. In the female parathyroidectomized group B there was a small nonsignificant rise in the forearm bone mineral content over 2 years, and it seems unlikely that the forearm bone mineral content would ultimately be restored to control values. Martin et al.[30] also found that the increment in forearm bone mineral content that occurred after parathyroidectomy was less with each succeeding year. However, Alhava et al.[26] found that forearm bone

mineral content increased toward normal control values for 4-5 years after parathyroidectomy, but thereafter increased rates of loss were again observed.

In the present study, patients with ongoing hyperparathyroidism lost bone, compared to the initial bone mineral content, at the same percentage rate as normal control subjects, notwithstanding that their baseline bone mineral content was significantly lower than the forearm bone mineral content of control subjects. The question arises as to why patients with hyperparathyroidism have lower forearm bone mineral content compared to age- and sex-matched control subjects. It has usually been assumed that low bone mineral content is due to excess resorption of bone by osteoclasts under the influence of increased circulating parathyroid hormone over a prolonged period. If this process is proportional to the initial bone mineral content, then bone loss follows an exponential function. If patients with hyperparathyroidism originally had normal bone mass before the onset of hyperparathyroidism, then taking into consideration the rate at which bone loss was observed in this study, hyperparathyroidism must have been present for many years before diagnosis, or, during an earlier time before the commencement of the present observation, there may have been an accelerated loss of bone mineral. The concept that excess parathyroid hormone is an important factor responsible for bone loss is supported by the finding that bone mineral content increased after the serum parathyroid hormone was rendered normal by parathyroidectomy (group A). An alternative possibility is that intrinsically low bone mineral content may predispose an individual to an increase in parathyroid function and subsequent hypercalcemia, rather than increased parathyroid activity causing a low bone mineral content. If the former is true, then the rate of loss of bone mineral would not correlate with the serum PTH, and this was the case in the present study. Also, in another study,[8] the serum PTH did not correlate with the forearm bone mineral content although relationships between serum PTH and a single measurement of the bone mineral content do not take into account the influence of the time that the excess parathyroid hormone has been operating at the bone cell level. In the present study, the group with ongoing hyperparathyroidism, with a mean serum calcium of 2.82 mm/liter, had a percentage loss of bone mineral over a 2 year period that was similar to the percentage loss of bone mineral in normal control subjects, and this finding is similar to that observed by Rao et al.[28] in patients with comparable degrees of hyperparathyroidism. Also in the latter group, an increased risk of minimal trauma fracture was not observed,[29] in contrast to the findings of other groups.[12,23] We do not have any data on the incidence of fracture in our group of patients.

The decision to carry out a neck exploration for hyperparathyroidism is based upon an assumed benefit from parathyroidectomy. If patients with mild hyperparathyroidism do not have an increased risk of minimal trauma fracture, then restoration of eucalcemia by surgery does not confer benefit in skeletal terms. In the present study,

however, those patients with the lowest forearm bone mineral content, 0.927 g/cm or lower, had the largest rise in bone mineral content after successful parathyroidectomy (5.76% in 1 year), and this may allow some reduction in the risk of minimal trauma fracture, although this is not proven.

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Paper 11. Single-day intravenous pamidronate in patients with Paget's disease. M Hooper, P Clifton-Bligh, GM Marel, FV Lang J Tancred, D McDowall, L Forman. Journal of Arthritis and Rheumatism 1994; 23:276-277

This was a collaborative study between MJ Hooper and P Clifton-Bligh who each made equal contributions to the recruitment and treatment of the patients with Paget's disease, and who had extensive experience over several years studying patients with Paget's disease. The first effective treatment for Paget's disease was calcitonin, subsequently replaced by bisphosphonates. The present study used the bisphosphonate, pamidronate in varying doses infused intravenously and patients were studied at 2,4, 8,12 and 24 weeks subsequently. The doses of pamidronate were 20mg ,30mg, 45mg,and 60mg. The pamidronate was infused over a 2 hour period. The single day infusion was followed by a rapid and sustained fall in the serum alkaline phosphatase (a marker of the activity of the Paget's disease) of 35-60%. The serum alkaline phosphatase was normalised in 56% of those with mild to moderate disease and in only 5% of those with severe disease. The nadir of the serum alkaline phosphatase was seen after 8 weeks and was greatest, a fall of 60%, when the dose of 60mg of pamidronate was used.

The study which involved a large number of patients with Paget's disease is considered to be a significant contribution in the study of the recently introduced therapy for Paget's disease and its application in clinical practice.

Citations.

Single-Day Intravenous Pamidronate in Paget's Disease

By Michael J. Hooper, Philip Clifton-Bligh, Geoffrey M. Marel, Fay V. Lang,

Janet Tancred, Deborah McDowall, and Lindee Forman

Although the efficacy of pamidronate (APD) in Paget's disease is established, the optimal dose and regimen are not known. In this article, further findings using a single-day intravenous infusion are reported, comparing the responses of 114 subjects treated with doses of 20 mg (n = 35), 30 mg (n = 26), 45 mg (n = 29), and 60 mg (n = 24). Assessments of clinical and biochemical response were made at 2, 4, 8, 12, and 24 weeks. Patients with persistent disease activity were retreated after 24 weeks. The single-day infusion of APD was followed by a rapid and sustained biochemical response, but in only 24% of patients did alkaline phosphatase (AP) levels normalize. Of patients in whom the serum AP level normalized, 93% had initial values less than three times the upper limit of normal. Although there was no significant difference in response between the lower dosage groups, there was a greater response in patients treated with a higher dose of APD. The percentage decrease in AP from baseline was similar after the first and second infusions. These findings show that a single-day infusion of APD is effective in the treatment of Paget's disease and that a dose-response relationship exists.
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INDEX WORDS: Paget's disease of bone; pamidronate; serum alkaline phosphatase.

THE INHIBITORY effect of pamidronate (APD) on osteoclastic bone resorption and its efficacy in the treatment of Paget's disease are established. However, the optimum dosage regimen remains unclear. We report here our findings using single-day intravenous (IV) infusions.

SUBJECTS AND METHODS

We treated 114 patients with Paget's disease (Table 1) with IV APD as part of a dose-finding study to establish the efficacy of a single daily infusion. These data compare the responses of subjects treated with APD in doses of 20 mg (n = 35), 30 mg (n = 26), 45 mg (n = 29), and 60 mg (n = 24). APD was infused at a rate of 7.5 or 15 mg/h in normal saline in an ambulatory setting. Assessments of clinical and biochemical response were made at 2, 4, 8, 12, and 24 weeks. Patients with persistent disease activity were retreated after 24 weeks. Severity of the disease was determined by initial alkaline phosphatase (AP) levels with patients having initial AP less than three times the upper limit of normal defined as having mild to moderate disease and those with initial AP greater than this defined as having severe disease. Three categories of response to treatment were defined as follows: normalization of AP level; moderate response, decrease in AP level > 30% of the initial value; and minimal response, decrease in AP < 30% of the initial value.
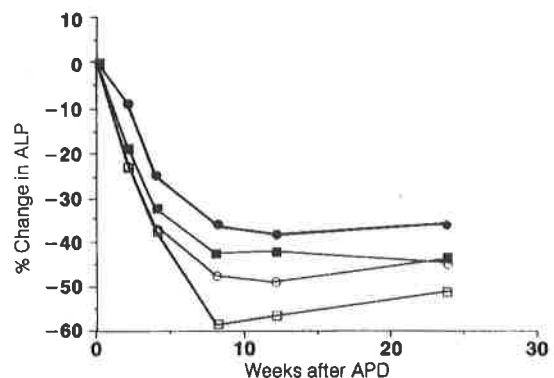

Fig 1: Percentage decrease in serum AP level with differing dosage levels of APD. In patients treated with 60 mg, data are presented only for the 13 followed for 24 weeks after infusion. ●, 20-mg dose; ■, 30-mg dose; ○, 45-mg dose; ☐, 60-mg dose.

Table 1: Demographic Data for Each Dosage Group of Patients Treated
With Intravenous Pamidronate

Dosage	n	M:F Ratio	Age (yr) Mean (Range)	Polystotic- Monostotic Ratio	AP (mU/L) Mean (Range)
20 mg	35	1.9	62 (31-82)	6.0	693 (85-2,930)
30 mg	26	1.9	73 (49-90)	2.7	585 (65-2,515)
45 mg	29	1.2	69 (38-87)	2.2	542 (95-2,515)
60 mg	13	0.9	64 (44-80)	3.3	395 (65-1,135)
Total	114	1.5	67 (31-90)	3.4	554 (65-2,930)

RESULTS

The biochemical responses reflected by changes in AP are shown diagramatically in Fig 1. The single-day infusion of APD was followed by a rapid and sustained biochemical response, but AP level normalized in only 24% of patients. Of patients in whom the AP level normalized, 93% had mild to moderate disease activity. A greater proportion of patients with mild to moderate disease and those who received higher doses of APD experienced moderate responses or normalization of AP levels. Although there was no significant difference in response between the lower dose groups in our study, there was a significantly greater response in patients treated with a higher dose of APD (Fig 1). The percentage decreases in AP level from baseline were similar after the first and second infusions.

CONCLUSIONS

These findings show that a single-day IV infusion of APD is effective in the treatment of Paget's disease. Normalization of AP occurs in 50% of those with mild to moderate disease activity but in few (5%) with severe disease. And that a dose-response relationship exists with APD.

Paper 12. The identification of false positive responses to the pentagastrin stimulation test in RET mutation negative members of MEN-2A families. DJ Marsh, D McDowell, VJ Hyland, SD Andrew, M Schnitzler, EL Gaskin, DF Nevell, T Diamond, L Delbridge, P Clifton-Bligh, BG Robibson. Clinical Endocrinology 1996; 44:213-220

P Clifton-Bligh developed the calcitonin assay, performed many of the pentagastrin tests and was involved in the analysis of the data. The pentagastrin test was used as a stimulatory test for calcitonin release and and exaggerated response compared to normal was thought to indicate C-Cell hyperplasia as a precursor to the development of medullary thyroid carcinoma. However, responses in normal persons vary widely. The present study examined pentagastrin responses in RET mutation negative family members of patients with the MEN-2A syndrome. Pentagastrin tests were performed as routine screening proceedures on individuals who were members of MEN-2A or familial medullary thyroid carcinoma families. 39 persons were subject to pentagastrin stimulation. 32 were RET mutation negative and 7 were RET mutation positive. The normal range for the test was a peak serum calcitonin of less than 200pg/ml. The highest peak serum calcitonin in a RET mutation positive male was 15,965pg/ml. The mean of peak values for the serum calcitonin in the RET mutation positive carriers was 4884pg/ml. 4 patients who were RET mutation negative, had repeat pentagastrin tests over periods of 1.2 to 8.9 years and the calcitonin responses showed a stepwise increase.Two of these patients with abnormal calcitonin responses to pentagastrin subsequently were subject to thyroidectomy and excess numbers of C cells on the histological tissue were not seen. The highest peak serum calcitonin after pentagastrin in RET mutation negative males was 687pg/ml. 4 of the 6 RET mutation positive males had peak serum calcitonins above this. Pentagastrin responsiveness is not mediated exclusively via a mutation in RET. One of the male patients, RET mutation negative, with an exaggerated calcitonin response to pentagastrin had C cell hyperplasia on histology. It is not known whether these mutation negative patients progress to malignancy. However, pentagastrin testing is not recommended in RET mutation negative patients. The 6 male patients who were RET mutation positive did have exaggerated calcitonin responses to pentagastrin.

This paper is considered to be an important contribution to the evaluation of families with a history of MEN-2A or familial medullary thyroid carcinoma.

Citations.

The identification of false positive responses to the pentagastrin stimulation test in RET mutation negative members of MEN 2A families

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Summary

OBJECTIVE The pentagastrin stimulation test is the traditional test used for the identification of asymptomatic individuals in multiple endocrine neoplasia type 2A (MEN 2A) and familial medullary thyroid carcinoma (FMTC). The identification of mutations in the RET proto-oncogene segregating with the disease phenotype in MEN 2A and FMTC families has made it possible to re-examine the validity of using this test for the identification of affected family members.
DESIGN Sequential and single pentagastrin stimulation test data were collected following the identification of RET mutation positive and RET mutation negative members of families with MEN 2A or FMTC.
PATIENTS RET mutations were identified in 16 Australian and New Zealand MEN 2A or FMTC families. An analysis of 39 individuals from these families was included in this study. Thirty-two individuals (14 males, 18 females) had previously been determined as RET mutation negative. Seven individuals (6 males, 1 female) had previously been determined as RET mutation positive. Two RET mutation negative males had thyroidectomy based on prior pentagastrin test results.

MEASUREMENTS Serum calcitonin levels in response to stimulation with pentagastrin were measured at 0, 1, 2, 5 and 10 minutes post injection. Mutation analysis of the RET proto-oncogene was performed in all individuals. In two RET mutation negative individuals from two MEN 2A families, thyroidectomy was performed and C-cells were quantitated in order to determine the diagnosis of C-cell hyperplasia.
RESULTS There was a statistically significant difference ($P < 0.013$) between RET mutation negative male and female mean peak calcitonin responses of 282 ± 236 and 96 ± 62 (mean $\pm$SD) ng/l respectively. False positive responses to pentagastrin stimulation were identified in seven individuals who were RET mutation negative in two of the 16 families. Histologic examination of the thyroid glands in the two RET mutation negative individuals who had thyroidectomy demonstrated C-cell hyperplasia in one but not in the other.
CONCLUSIONS There is considerable overlap between pentagastrin test results in individuals who are RET mutation positive and those who are RET mutation negative. These results indicate a need for routine performance of RET proto-oncogene analysis on all individuals at risk of developing MEN 2A or FMTC and a coupling of pentagastrin test results and RET proto-oncogene analysis in the decision to proceed with thyroidectomy.

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Multiple endocrine neoplasia type 2A (MEN 2A) is an inherited cancer syndrome showing autosomal dominance and incomplete penetrance. Medullary thyroid carcinoma (MTC) occurs in all presentations of this syndrome. It may occur alone in familial medullary thyroid carcinoma (FMTC) or in conjunction with phaeochromocytomas and in some instances parathyroid hyperplasia (MEN 2A), and with phaeochromocytomas, mucosal neuromas, Marfanoid habitus and ganglioneuromatosis of the gastrointestinal tract in multiple endocrine neoplasia type 2B (MEN 2B).

The traditional method for the identification of asymptomatic individuals at risk of inheriting one of these syndromes is the pentagastrin stimulation test that measures a patient's serum

calcitonin levels over set time intervals in response to stimulation with pentagastrin or calcium. A positive test is thought to be an indication of the presence of C-cell hyperplasia or MTC. It is well established that C-cell hyperplasia is a precursor to the development of MTC in MEN 2A and FMTC (Wolfe *et al.*, 1973; Graze *et al.*, 1978). However, both C-cell numbers and an individual's response to pentagastrin stimulation can vary widely in the normal population (Gibson *et al.*, 1982; Landsvater *et al.*, 1993).

Point mutations in the extracellular domain of the RET proto-oncogene segregating with the disease phenotype in families with MEN 2A and FMTC have been identified (Mulligan *et al.*, 1993; Donis-Keller *et al.*, 1993) . Mutations have been found in 97% of unrelated MEN 2A patients (Mulligan *et al.*, 1994). A single mutation consistent with the MEN 2B phenotype has been found in the tyrosine kinase domain of RET (Carlson *et al.*, 1994; Eng *et al.*, 1994; Hofstra *et al.*, 1994). Screening programmes established to identify these mutations have enabled the determination of mutant gene carrier status in the majority of individuals at risk of developing these syndromes (Chi *et al.*, 1994; Lips *et al.*, 1994; Marsh *et al.*, 1994; Wells *et al.*, 1994; Xue *et al.*, 1994).

Prior to the identification of these mutations, several groups had reported individuals believed to be displaying a false positive response to the pentagastrin stimulation test (Lips *et al.*, 1987; Gagel *et al.*, 1988; Landsvater *et al.*, 1993; Barbot *et al.*, 1994). In the present study, the calcitonin responses to pentagastrin stimulation in individuals in MEN 2A and FMTC families found to be RET mutation negative were retrospectively assessed and false positives were identified.

Materials and methods

Subjects

Thirty-nine members of 16 Australian and New Zealand MEN 2A and FMTC families at risk of inheriting one of these syndromes underwent biochemical and genetic analyses. Informed written consent was obtained from all individuals participating in this study. Ethical approval for this research was granted by the Human Ethics Committee of the Royal North Shore Hospital.

Biochemical screening: pentagastrin stimulation testing and calcitonin assay

Pentagastrin stimulation tests were performed as a routine screening procedure on individuals who were members of MEN 2A and FMTC families.

The pentagastrin stimulation test was performed supine after an overnight fast. Pentagastrin (0·5 µg/kg, ICI Pharmaceuticals

Melbourne, Australia) was injected intravenously over 5 s and blood was taken for calcitonin determination at 0, 1, 2, 5 and 10 minutes. The method for determination of calcitonin by radioimmunoassay has previously been reported (Furlong *et al.*, 1991) and is a modification of the method used by Heynen and Franchimont (1974). In brief, anti-human calcitonin antibody was raised in a goat injected with synthetic human calcitonin (Organon Tiknika, Boxtel, NL) and Freund's adjuvant and was found to bind selectively to the 17–32 amino acid sequence of the calcitonin molecule. Synthetic human calcitonin (Peninsular Laboratories Inc., Belmont, CA) was used to prepare standards in the buffer described by Heynen and Franchimont (1974) and for labelling with ^{125}I by the procedure of Marx *et al.* (1972), which is known to preserve the biological activity of labelled calcitonin. Each serum sample was assayed in aliquots of 100 and 200 µl in triplicate within 2 weeks of collection, and a non-specific binding blank was included with each sample. If proportional increases in the measured calcitonin value with increased aliquot volume did not occur, the values were discarded. Quality control sera at multiple dilutions were included in each assay and no difference in measured values was observed over a 2-year period. The interassay coefficient of variation at a concentration of 200 ng/l serum was 12%. The normal range for this test has been quoted as less than 200 ng/l peak stimulated calcitonin response. All calcitonin radioimmunoassays were performed at Royal North Shore Hospital.

Histopathological analysis of the thyroid gland

Tissue sections were hybridized with a rabbit anti-human calcitonin antibody (Dako Corporation, CA, USA) and the antibody was detected using the immunoperoxidase method via an avidin biotin complex (Hsu *et al.*, 1981). C-cells were counted in 3·1mm^2 field areas at ×100 magnification. C-cell hyperplasia was diagnosed if greater than 50 C-cells per field were present (Albores-Saavedra *et al.*, 1988).

Direct mutation analysis

Mutations were detected by a method that involved restriction endonuclease digestion of polymerase chain reaction products. This technique involved the gain or loss of a restriction endonuclease site generated by either a point mutation with or without a primer mediated nucleotide substitution (Marsh *et al.*, 1994).

Taq cycle sequencing was performed on DNA from exons 10 and 11 of the RET proto-oncogene in a known RET mutation positive individual from each family studied using the Cyclist DNA sequencing kit (Stratagene, La Jolla, CA) to confirm the presence of the mutation.

Statistical analysis

Calcitonin values are expressed as mean ± standard deviation. A two-tailed Student's *t*-test was performed to determine whether there was a significant difference ($P < 0.05$) between male and female RET mutation negative individuals. All statistical analyses were performed on Microsoft Excel version 5.0.

Results

Peak calcitonin values after pentagastrin stimulation for 32 asymptomatic members of MEN 2A and FMTC families subsequently identified as RET mutation negative and seven members of MEN 2A and FMTC families identified as RET mutation positive are shown in Fig. 1. The highest peak calcitonin value in the serum of males and females shown to be RET mutation negative were 687 and 275 ng/l respectively. The mean and standard deviation of the peak calcitonin value in the serum of RET mutation negative males and females was 282 ± 236 and 96 ± 62 ng/l respectively. In general, RET mutation negative males had a higher peak calcitonin value than RET mutation negative females ($P < 0.013$). The means and standard deviations of the basal
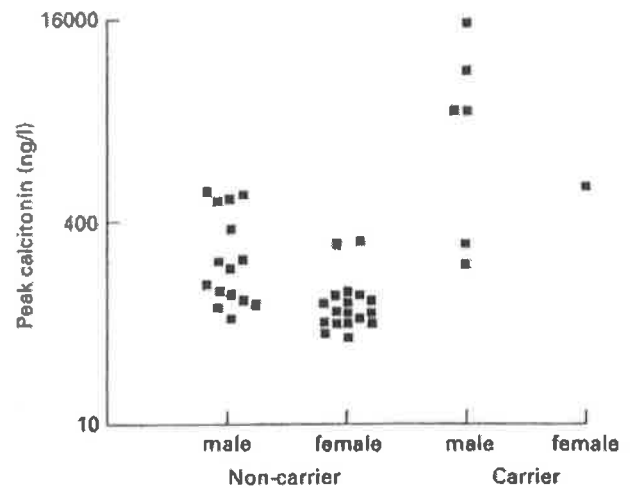

Fig. 1 Log peak calcitonin levels following pentagastrin stimulation for RET mutation positive and negative males and females who are members of MEN 2A and FMTC families.

calcitonin level for RET mutation negative males and females were 80 ± 22 and 70 ± 32 ng/l respectively. The highest peak pentagastrin stimulated calcitonin value in the serum of a RET mutation positive male was 15 965 ng/l. The mean and standard

Table 1 Calcitonin levels after pentagastrin stimulation in non-carriers of the RET mutation identified in two MEN2A families

Subject	Sex	Age (years)	0	1	2	5	10
031/II-3	female	39·4	152	177	188	180	155
		43·8	121	151	184	171	153
		46·3	159	256	245	QNS	192
031/II-4	male	28·9	78	55	99	90	69
		31	53	216	225	N/A	138
		37·8	63	560	426	N/A	360
031/III-1	male	12·8	97	187	198	162	126
		15·2	99	258	239	187	148
		16·7	134	317	319	235	199
		19·7	119	646	508	351	285
031/III-4	male	15·2	97	687	501	382	249
031/III-5	female	12·8	109	253	233	177	142
126/III-4	male	25·3	72	204	212	190	164
		26·5	99	349	309	287	231
126/III-6	male	27·6	65	600	480	398	278

(Column group header: *Serum calcitonin (ng/l)* → *Time (min)*)

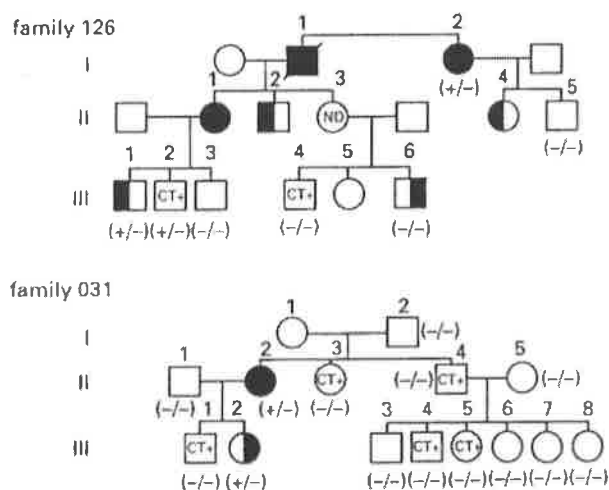QNS, Quantity not sufficient for calcitonin assay; N/A, sample not taken at this time interval.

Fig. 2 MEN 2A families containing individuals who are RET
mutation negative and display high calcitonin measurements.
Family members are identified as follows: ●, MTC and
phaeochromocytoma; ◐, MTC only; ◑, C-cell hyperplasia only; CT+
abnormal peak calcitonin response; ND, calcitonin not determined.
(+/−) indicates that the individual is RET mutation positive; (−/−)
indicates that the individual is RET mutation negative.

deviation of the peak calcitonin value in the serum of RET
mutation positive males was 4884 ± 5929 ng/l. The mean and
standard deviation of the basal calcitonin level for these males
was 393 ± 339 ng/l. The single peak calcitonin result available
for a RET mutation positive female was 801 ng/l with a basal
calcitonin level of 82 ng/l.

Sequential pentagastrin stimulation data from individuals in
two families is presented for three males and one female who
were shown by direct mutation analysis to be RET mutation
negative (Table 1). Peak calcitonin levels in these individuals
show a rise over a range of periods from 1·2 to 8·9 years. A
single pentagastrin test result is available for three other RET
mutation negative individuals (Table 1). Patient consent was
not given for additional pentagastrin stimulation testing in
individuals from family 031.

The RET mutation identified in affected members of family
031 occurred in exon 11 at codon 634 causing the substitution
of a cysteine (TGC) by an arginine (CGC). The RET mutation
identified in affected members of family 126 also occurred in
exon 11 at codon 634 causing the substitution of a cysteine
(TGC) by a glycine (GGC) (Marsh *et al.*, 1994).

Individuals in families 031 and 126 found subsequently to be
RET mutation negative showed marked increases in calcitonin

levels after stimulation with pentagastrin (Fig. 2 and Table 1).
As a consequence of the pentagastrin stimulation test data and
prior to the analysis of their RET locus, thyroidectomy was
performed on individuals 126/III-6 and 031/III-1. Histology in
individual 126/III-6 revealed variation in the number of C-cells
per × 100 field with a maximum of 150 C-cells in any one field
at this magnification. In some fields, small groups of C-cells and
clusters of up to 30 C-cells were observed (Fig. 3a). In contrast,
individual 031/III-1 had a maximum of only 39 C-cells in any
one field at × 100 magnification and clustering was not present
(Fig. 3b).

Discussion

The identification of mutations in the RET proto-oncogene in
association with MEN 2A and FMTC has enabled re-evaluation
of the interpretation of pentagastrin stimulation test results in
the management of these families. The recent identification of
mutations associated with the MEN 2B phenotype would allow
a similar evaluation of the pentagastrin stimulation test in MEN
2B families.

Pentagastrin stimulation tests are normally performed in
members of multiple endocrine neoplasia type 2 and FMTC
families as well as in relatives of apparent sporadic cases of
MTC. We have collected data from this test in both RET
mutation positive and RET mutation negative individuals who
are members of families in which RET mutations have been
identified and shown to segregate with MTC (Fig. 1). The
analysis of pentagastrin stimulation data in a large number of
individuals subsequently shown to be RET mutation positive
was not possible as it was not uncommon for these individuals
to present with clinical symptoms and only basal calcitonin
measurements were performed.

Barbot *et al.* (1994) examined pentagastrin stimulation test
results in members of families in whom genetic status at the
RET locus had been predicted by linkage analysis. Earlier
analyses of the pentagastrin test using either an immunoradio-
metric assay (Guilloteau *et al.*, 1990) or an extraction calcitonin
assay (Kao & Gharib, 1993) did not discriminate between tests
performed in relatives of patients with sporadic MTC and
members of families with MEN 2A or FMTC. An accurate
estimation of false-positive responses to pentagastrin in these
studies was difficult to obtain since direct mutation analysis at
the RET locus was not possible at the time these studies were
performed.

A number of studies have found peak calcitonin levels after
pentagastrin stimulation to be higher in males than in females

Fig. 3 Histopathology of individuals a, 126/III-6 and b, 031/III-1 showing a section of the thyroid gland stained for calcitonin to identify the
presence of C-cells. × 100.
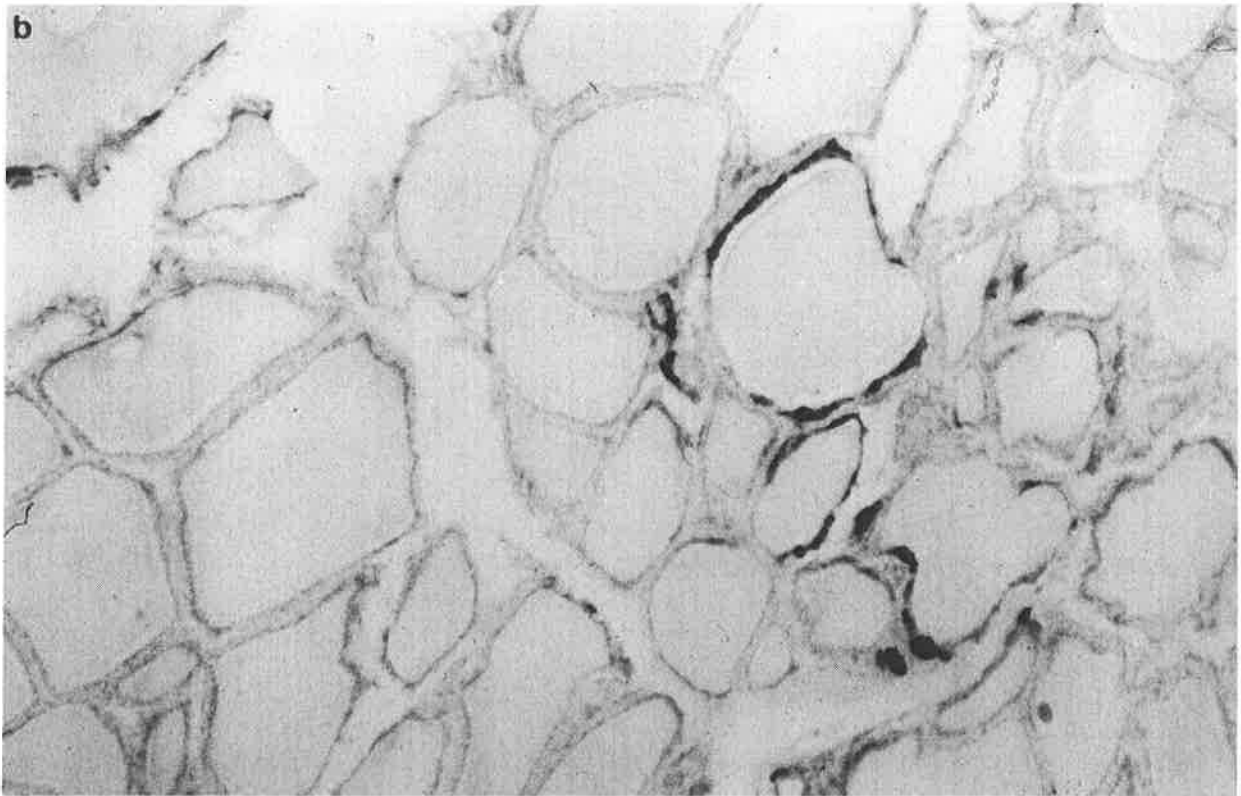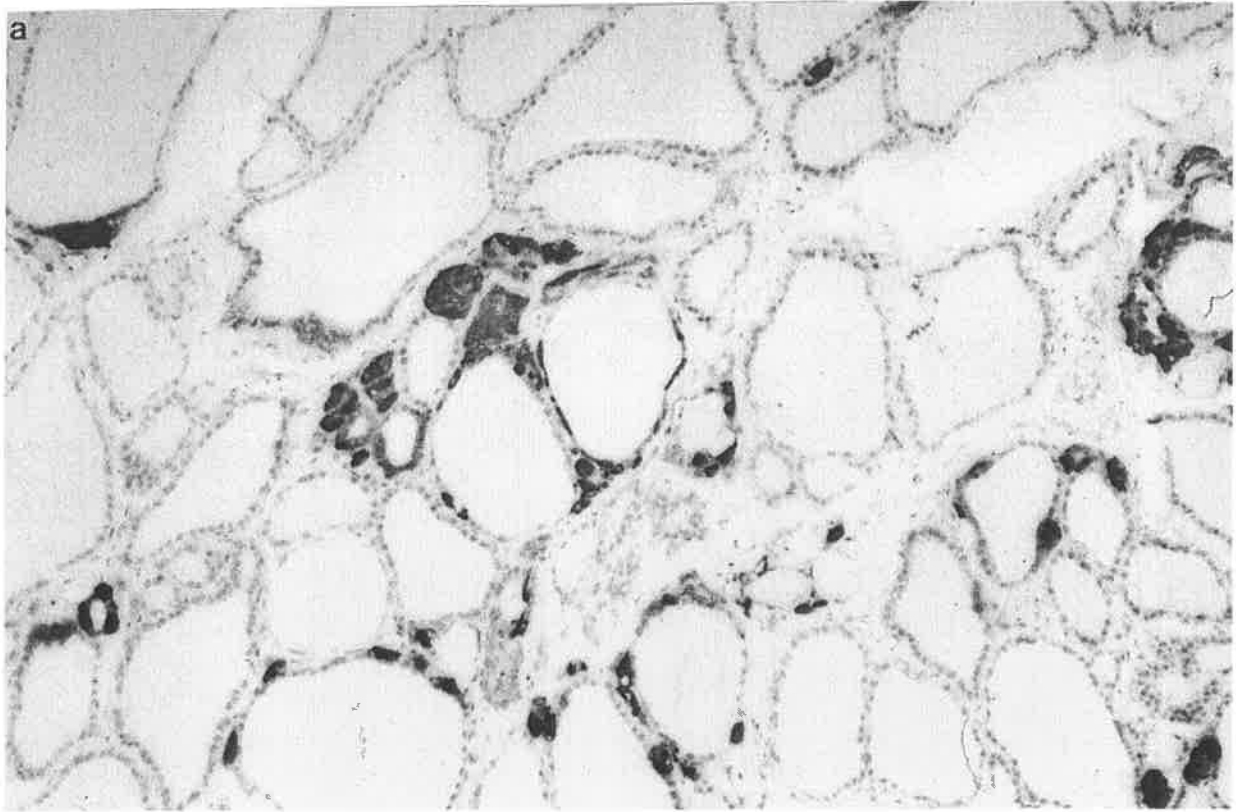
(Deftos *et al.*, 1980; Lips *et al.*, 1987). Our analysis of individuals shown to be RET mutation negative also shows a large variation between males and females with the peak mean and standard deviation values for males and females in this group being 282 ± 236 and 96 ± 62 ng/l respectively. There is no difference in the penetrance of MEN 2A or FMTC despite the sex differences in pentagastrin responsiveness. This implies that penetrance and pentagastrin responsiveness are not both mediated exclusively via a mutation in RET.

Two males, 126/III-6 and 031/III-1, with peak calcitonin values of 600 and 646 ng/l respectively had thyroidectomy performed. These males were subsequently shown to be RET mutation negative. The histology of 126/III-6 satisfied the criterion set for the diagnosis of C-cell hyperplasia that at least 50 cells be present at ×100 magnification (Fig. 3a) (Albores-Saavedra *et al.*, 1988). The histology of 031/III-1 did not satisfy this criterion (Fig. 3b). These individuals highlight the fact that significant increases in calcitonin levels can occur after pentagastrin stimulation in RET mutation negative individuals and that this may or may not be associated with C-cell hyperplasia.

Anatomical and inter-individual variation in C-cell number is a documented phenomenon (Wolfe *et al.*, 1973; 1974; Gibson *et al.*, 1982). Within a normal human adult thyroid gland C-cells are predominantly solitary and are more prolific about the mid to upper thirds of the lateral lobes and are at lower frequencies in the isthmus (Wolfe *et al.*, 1973; 1974). Thus, for comparative purposes it is important that thyroid sections taken for the diagnosis of C-cell hyperplasia be from this lateral lobe area.

One autopsy study had shown a correlation between increasing age and C-cell numbers in males, postulating that this observation may be due to a normal maturation process (Gibson *et al.*, 1982). This study was unable to show the same correlation in females; however, this may have been due to a smaller population size and narrower age distribution of females available for study. Landsvater *et al.* (1993) state that as much as 5–10% of the random population have an abnormal pentagastrin stimulation test result. This variation may be normal, as inferred in the studies of Gibson *et al.* (1982), or some of the higher values may be caused by renal insufficiency (Silva *et al.*, 1977; Mulder *et al.*, 1982; Garancini *et al.*, 1983; Escalada *et al.*, 1993) or other malignancies (Milhaud *et al.*, 1974; Hein *et al.*, 1989; Price *et al.*, 1992).

While progression of C-cell hyperplasia to MTC is recognized in affected members of MEN 2A and FMTC families (Grauer *et al.*, 1990) the relationship, if any, between C-cell hyperplasia and the possible progression to malignancy in family members shown to be RET mutation negative is yet to be elucidated. The two thyroidectomies reported in this study on individuals shown retrospectively to be RET mutation negative showed no histological evidence of malignancy, even though C-cell hyperplasia was clearly evident in 126/III-6. Thus progressive increases in calcitonin response in RET mutation negative individuals may be due either to increased C-cell numbers unrelated to MEN 2A or FMTC, or to increasing responsiveness to pentagastrin, or to both.

In the present study it would appear that false positive results from the pentagastrin stimulation test can occur in members of MEN 2A and FMTC families who are RET mutation negative. This has also been observed by Lips *et al.* (1994) and McMahon *et al.* (1994) using techniques of direct mutation analysis, by Barbot *et al.* (1994) and Landsvater *et al.* (1993) using linkage analysis and by Lips *et al.* (1987) and Gagel *et al.* (1988) on the basis of clinical and histopathological data.

We have performed direct mutation analysis in 16 families and have observed false-positive calcitonin responses to pentagastrin stimulation in seven individuals from two of these families. The presence of an additional gene in these two families, not linked to RET, which is involved in mediating these false positive responses to pentagastrin stimulation is possible. There are no clinical differences between these two families and those in which false-positive responses did not occur. We have not observed any case of MTC in family members who are RET mutation negative. We and others (Lips *et al.*, 1994; Wells *et al.*, 1994) recommend that RET mutation negative individuals do not require repeat pentagastrin testing.

We suggest that results obtained from the pentagastrin stimulation test should in future be coupled with the results of direct mutation analysis in MEN 2A and FMTC families where possible before thyroidectomy is considered in an individual found to be RET mutation positive. RET mutation negative individuals who are members of MEN 2A and FMTC families and who have apparent false positive pentagastrin stimulation test results should be investigated further for signs of other disease. We suggest that if no other evidence of disease is identified, these individuals should be classified as having high normal responses for the pentagastrin stimulation test and thyroidectomy should not be performed.

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Paper 13. Effect of methotrexate and sulphasalazine in UMR 106 rat osteosarcoma cells. SJ Preston, P Clifton-Bligh, MR Laurent, C Jackson, RS Mason. British Journal of Rheumatology 1997; 36:178-184

P Clifton-Bligh made a significant contribution in the conception, planning and execution of this study. Methotrexate is a drug used commonly to treat rheumatoid arthritis but has been associated with the development of osteoporosis and fractures. The present paper describes studies in vitro of the effect of methotrexate on the function of UMR rat osteosarcoma cells as a surrogate for osteoblasts. Short term administration of high dose methotrexate to Wistar rats had previously been shown to profoundly inhibit osteoblast function.UMR 106 cells have some characteristics of osteoblasts in that they produce ground substance which will mineralize. The cells also produce alkaline phosphatase. The cells respond to 1,25-OH vitamin D. The effect of methotrexate was studied at concentrations usually seen in the blood of patients treated with methotrexate. At concentrations of greater than 10nm methotrexate inhibited proliferation of UMR 106 cells.Mehtotrexate did not inhibit alkaline phosphatase production. Incubation of UMR-106 cells with 1,25-oh vitamin D did not protect UMR 106 cells from the toxic effects of methotrexate. The effect of methotrexate in concentrations of 10,50,100nm had no effect on umbilical endothelial cell proliferation. Serum methotrexate levels of 0.1 to 1.0micromol/L are seen within 4 hours of receiving a dose of 7.5 to 15mg of methotrexate. UMR 106 cells showed toxic effects at relatively lower doses of methotrexate compared the effects in other cell lines. Addition of folinic acid reversed the toxic effect of methotrexate.

This paper is considered to be an important contribution to the understanding of the impact of methotrexate on bone cell metabolism which is relevant to its application as therapy in rheumatoid arthritis.

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EFFECT OF METHOTREXATE AND SULPHASALAZINE ON UMR 106 RAT OSTEOSARCOMA CELLS

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SUMMARY

Methotrexate is commonly used in the treatment of rheumatoid arthritis. An osteopathy has been described in children treated with methotrexate for leukaemia, consisting of bone pain, osteoporosis and fractures. Animals given short-term high-dose and long-term low-dose methotrexate have both reduced bone formation and increased resorption on histomorphometry. As patients with rheumatic diseases have numerous risk factors for osteoporosis, possible additional risk from low-dose methotrexate is of relevance to the rheumatologist. To investigate further the mechanism of osteoporosis in animals and man, *in vitro* studies were carried out on an osteoblast cell line, using concentrations found in patients with rheumatic disease. UMR 106 rat osteoblast-like osteosarcoma cells were incubated with methotrexate, and also with sulphasalazine, an anti-rheumatic drug with no known effect on bone, for comparison. A dose-dependent toxic effect of methotrexate on the cell line was observed using concentrations found in patients with rheumatic disease. This was not observed with sulphasalazine. The reduced bone formation observed in animals and man may be due to a direct effect of methotrexate on the osteoblast.

KEY WORDS: Osteoporosis, Methotrexate, Sulphasalazine, Osteoblast, Cell culture.

METHOTREXATE and sulphasalazine are agents frequently used in the treatment of inflammatory rheumatic diseases, particularly rheumatoid arthritis. Methotrexate is a potent competitive antagonist of folic acid and inhibits the enzyme dihydrofolate reductase [1], although in patients with rheumatoid arthritis, other mechanisms including an anti-inflammatory action may be important [2]. Sulphasalazine is a weak inhibitor of folic acid metabolism [3], although the mechanism of action of the drug in rheumatoid arthritis has not been defined. Sulphasalazine is metabolized *in vivo* to sulphapyridine and 5-aminosalicylic acid, with serum sulphapyridine concentrations having a better correlation with both efficacy and toxicity than sulphasalazine concentrations [4].

Methotrexate osteopathy has been described in children treated with maintenance methotrexate for acute lymphocytic leukaemia [5]. A triad of bone pain, fractures and osteoporosis was seen in these children that did not resolve until methotrexate was stopped. We have published observations in patients with rheumatic disease treated with methotrexate who developed a similar clinical syndrome [6], raising the possibility that methotrexate is an additional risk factor for osteoporosis in these patients.

An osteopenic effect of methotrexate on rat bone has been demonstrated with both chronic low-dose [7] and short-term high-dose administration [8]. After short-term high-dose administration of methotrexate to Wistar rats, osteoblast function was observed to be profoundly inhibited, with a significant reduction in

absolute osteoid volume and osteoid thickness, in the presence of normal osteoblast numbers and a 60% reduction in bone formation rates (BFR) [8]. Rats given methotrexate i.p. for 16 weeks in doses that yielded similar serum methotrexate levels in humans $(0.6 \pm 0.1\ \mu\text{M})$ were found to have both decreased bone formation and increased resorption on histomorphometry [7]. Methotrexate-induced osteoporosis has also been demonstrated in mice, although only after high doses for prolonged periods [9]. Sulphasalazine has no known effect on bone, although to our knowledge this has never been studied.

UMR 106 is a clonal osteosarcoma cell line originally induced in the rat by injections of radioactive phosphorus. The tumours formed by these cells have the ability of differentiated osteoblasts to form a bone-like ground substance which is then mineralized [10]. Osteoblasts differ in their phenotypic expression according to their stage of maturation. Alkaline phosphatase is a marker of differentiation [11] and is produced by UMR 106, representing a later stage in osteoblast maturation [12]. It does not, however, have measurable mRNA for bone Gla protein [13], which is a marker of the most differentiated osteoblast [14, 15].

UMR 106-01 and 106-06 are subclones of the UMR 106 cells. The only substantial known difference between them in phenotype is the expression of calcitonin receptors, more typical for osteoclasts, in UMR 106-06 cells [16]. UMR 106 has receptors for growth hormone [17], leukaemia inhibitory factor [18] and 1,25-dihydroxyvitamin D [19]. The number of 1,25-dihydroxyvitamin D receptors in UMR 106 increases in the presence of 1,25-dihydroxyvitamin D [20], suggesting induction of a more mature cell phenotype as seen in other cell lines [21, 22]. Recently, transforming growth factor-β (TGF-β) has also been

proposed as an inducer of differentiation in this cell line [23], based on increases in parathyroid hormone (PTH) and vitamin D receptors after TGF-β exposure.

Short-term exposure, of <72 h, of human bone cells to 1.25-dihydroxyvitamin D produces stimulation of proliferation, possibly due to recruitment of previously non-dividing cells [24]. Inhibitory effects on cell proliferation are seen with longer duration of exposure in association with increased alkaline phosphatase activity [24]. Osteosarcoma cell lines differ from primary osteoblast cultures, however, in that expression of genes indicative of the mature cell is concomitant with proliferation, rather than occurring exclusively after proliferation [14].

The aim of the present study was to assess the effect of methotrexate, sulphasalazine and sulphapyridine on UMR 106 proliferation and function using concentrations found in patients treated with these agents for rheumatic disease. The effect of the addition of 1,25-dihydroxyvitamin D and folinic acid (leucovorin) with methotrexate was also determined.

MATERIALS AND METHODS

Cell culture
UMR 106 cells were a generous gift from Dr T. J. Martin, St Vincents Institute, Melbourne. Cells were cultured in DMEM (Sigma Chemical Co., St Louis, MO, USA) plus 5% fetal calf serum (FCS) containing 30 μg/ml transferrin, 5 μg/ml insulin, 3.5 mg/ml glucose, 3.7 mg/ml sodium bicarbonate and 0.11 mg/ml sodium pyruvate (pH 7.2). Cells were incubated in 5% CO_2 in humidified air at 37°C. Medium was changed every 2 days and at confluence the cells were split 1:3 using trypsin/ethylenediaminetetra-acetic acid (EDTA).

Human umbilical vein endothelial tissue (HUVE) was isolated from placental tissue according to the method of Jaffe *et al.* [25]. Cells were cultured in medium 199 (ICN Biomedicals, Cleveland, USA) containing 20% FCS, 50 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, MA, USA) and 50 μg/ml heparin (Sigma Chemical Co., St Louis, MO, USA).

All experiments were repeated at least three times and results are given as either a percentage for pooled data or as a typical example. All data are presented as means ± S.D.

Statistical analysis
Statistical analysis was performed using ANOVA and the Wilcoxon ranked sum test for non-parametric data (SPIDA, Macquarie University, Sydney).

Proliferation assay
Cell proliferation was measured using a modification of the methylene blue colorimetric assay of Oliver *et al.* [26]. Cells were added to a 96 well plate and incubated for up to 168 h. Test agents were added at the time of plating into 96 wells. At the end of the incubation period, culture medium was removed and cells were fixed by adding 100 μl of 10% formol saline to each well for at least 30 min. The fixative was then removed and 100 μl of filtered 1% (w/v) methylene blue in 0.01 M borate buffer (pH 8.5) was added to each well. After 30 min, excess dye was removed and plates were washed serially in three tanks of borate buffer (pH 8.5). Excess buffer was removed and 100 μl/well of 1:1 (v/v) ethanol and 0.1 M HCl added. The plates were gently shaken and the absorbance read at 620 nm, using a microplate photometer.

Cell viability
Cell viability was assessed using trypan blue exclusion. After incubation, medium was removed and 200 μl/well trypsin/EDTA added. After the cells had lifted off the plate, 200 μl DMEM/FCS was added to each well and cells were collected in individual tubes. An equal volume of trypan blue (0.1%) was mixed with the cells for each tube and viable and total cells counted manually. Cell viability is expressed as a percentage of total cell count.

Alkaline phosphatase activity
Alkaline phosphatase activity was measured by a modification of the method of Bessey *et al.* [27]. This assay is performed in 24 well plates and based on the formation of a yellow complex when p-nitrophenol is made alkaline. After incubation with test agents, the cells were washed three times in warm phosphate-buffered saline (PBS), followed by the addition of 1 ml of reagent [4 ml 1 N HCl, 9.6% (v/v) 2-amino-2-methyl-1-propanol (Sigma Chemical Co., St Louis, MO, USA), 595 mg% (w/v) p-nitrophenyl phosphate (Sigma Chemical Co., St Louis, MO, USA)] to each well. After 10–15 min, 800 μl alkaline phosphatase reagent was added to test tubes containing 1 ml of 1 M NaOH and immediately vortexed. Absorbance was read at 410 nm, results converted using a standard curve and expressed as Sigma units/ml. Results were then corrected for protein content using the Bradford [28] protein assay (Biorad, Hercules, CA, USA) and expressed per milligram of protein.

Vitamin D, methotrexate, leucovorin, sulphasalazine and sulphapyridine
1,25-Dihydroxyvitamin D (vitamin D) was kindly donated by Roche Products (Dee Why, Sydney, Australia). Vitamin D in ethanol was diluted in a minimum of 0.1% to obtain a final concentration per well from 10 nM to 10 pM.

Purified methotrexate was a generous gift from Lederle (New York, USA) and dissolved in PBS. Leucovorin (folinic acid) was in solution as the calcium salt (Sigma Chemical Co., St Louis, MO, USA). Sulphasalazine was a generous gift from Pharmacia (Uppsala, Sweden) and sulphapyridine (4-amino-*N*-2-pyridinyl-benzenesulphonamide) was obtained from Sigma Chemical Co. (St Louis, MO, USA). Sulphasalazine was in suspension with PBS and sulphapyridine was dissolved in PBS/NaOH with a final pH of stock solution of 9.8. Cells were incubated with sulphasalazine and sulphapyridine from 24 to 96 h.

Vehicle was added to all control wells to exclude non-specific effects.

RESULTS

Cell proliferation

Initial cell proliferation studies determined the validity of using the methylene blue assay of Oliver et al. [26] as a method of cell quantification. Cells were plated into 96 well plates from 1000 to 30 000 cells/well and incubated from 12 to 48 h. The doubling times of the UMR 106 cells in culture were observed to be between 18 and 20 h.

There was a linear relationship between viable cell number and absorbance between 0.5 and 2.5 at 620 nm (Fig. 1). The number of cells in subsequent experiments was 2000–20 000.

Effect of methotrexate on UMR 106 proliferation and function

Cell proliferation and viability. Methotrexate was added to UMR 106 cells in concentrations ranging from 0.1 pM to 10 nM for up to 7 days. Using the methylene blue assay, methotrexate at concentrations > 10 nM significantly inhibited cell proliferation ($P < 0.01$; Fig. 2a). This effect was seen on the cells within 24 h. No effect of methotrexate was seen on cell adhesion, which occurred within a few hours of plating out. There was also no change in effect at each concentration of methotrexate from 24 to 168 h of incubation (data not shown).

Trypan blue studies demonstrated that the effect of methotrexate was largely due to direct toxicity on the cell line rather than inhibition of proliferation, as cell viability at 100 nM methotrexate was $<25\%$ compared with $>95\%$ at or below 10 nM (Fig. 2b).

UMR 106 cells were incubated for 48 h with methotrexate between the concentrations of 10 and 100 nM (Figs 3 and 4). Concentrations of methotrexate at 40, 60, 80 and 100 nM significantly inhibited proliferation ($P < 0.01$) and viability, with trypan blue

FIG. 2.—(a) The effect of methotrexate on UMR 106 proliferation. Cells were plated at 2000 cells/well and incubated for 72 h. A 100 nM concentration of methotrexate significantly inhibited proliferation ($P < 0.01$)**. (b) UMR 106 viability after methotrexate exposure. Cells were plated into 24 well plates at 20 000 cells/well and incubated with methotrexate for 72 h. At 100 nM methotrexate, there was a significant reduction in cell viability ($P < 0.01$)**.

studies confirming a dose-dependent toxicity of methotrexate on the UMR 106 cell line (Fig. 4). No significant effect of methotrexate on cell viability when compared to control was seen at concentrations of 10 and 20 nM. At 40 nM ($P = 0.004$), 60 nM ($P < 0.001$) and 80 nM ($P < 0.001$) methotrexate concentrations,
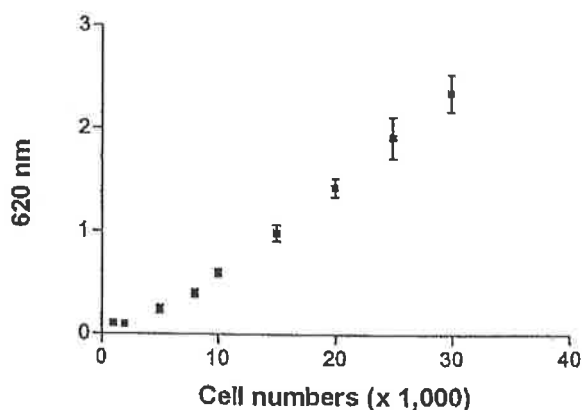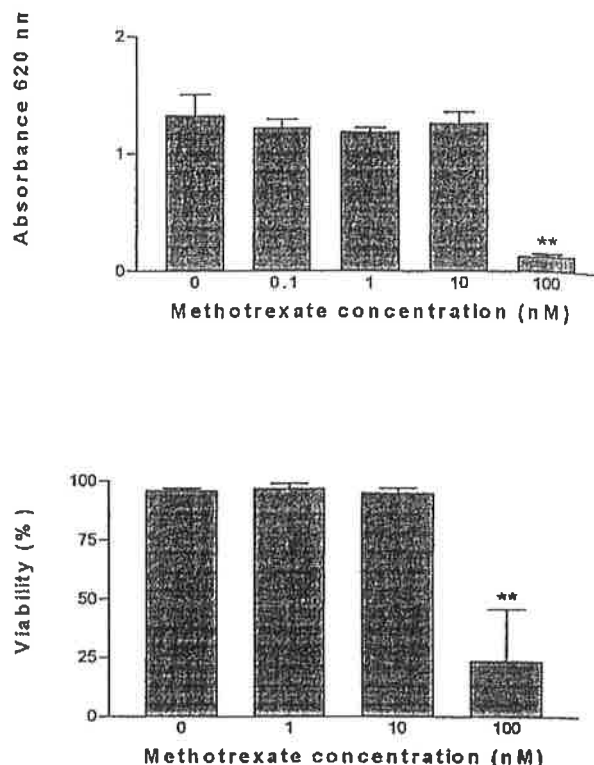

FIG. 1.—Relationship between cell counts and absorbance at 620 nm using the methylene blue proliferation assay. After 24 h incubation, there was a strong correlation between cell counts in the range of 1000–30 000 cells/well and absorbance at 620 nm ($r = 0.99$; $P < 0.001$).
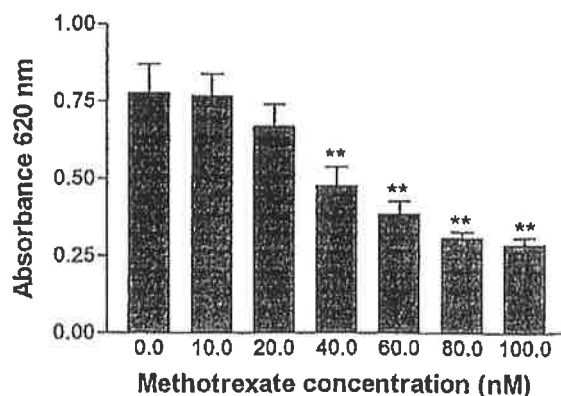

FIG. 3.—Effect of methotrexate on UMR 106 proliferation at concentrations between 100 and 10 nM. Cells were plated at 5000 cells/well and incubated with methotrexate for 48 h. P values are <0.01 for 100, 80, 60 and 40 nM methotrexate**. There was no significant difference between control and 20 and 10 nM methotrexate.
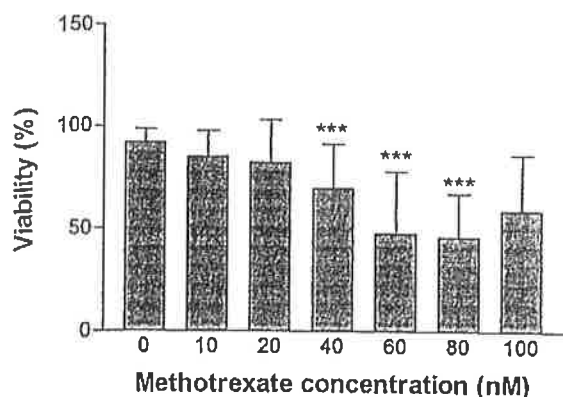
FIG. 4.—Effect of methotrexate on cell viability expressed as a percentage of viable cells in total cell number. Cells were plated at 20 000 cells/well and incubated with methotrexate for 48 h. Methotrexate at concentrations of >20 nM significantly reduced cell viability when compared to control ($P < 0.001$)***.
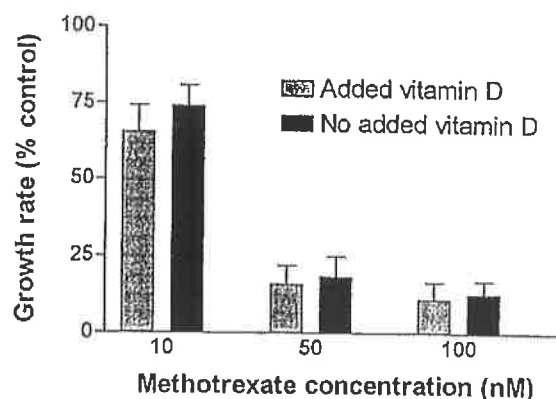

FIG. 5.—Effect of pre-incubation for 48 h with 10 nM 1,25-dihydroxy-vitamin D on UMR 106 proliferation. Cells were plated at 2000 cells/well and incubated with 1,25-dihydroxyvitamin D or control for 48 h. Methotrexate at 10, 50 and 100 nM was then added and incubation continued for a further 48 h. The methylene blue assay was used to assess proliferation. Results are expressed as a percentage of control values for cell growth. There was no significant difference between 1,25-dihydroxyvitamin D and control wells at any concentration of methotrexate.

however, there was a significant reduction in cell viability. The unexpected finding of higher cell viability at 100 nM methotrexate when compared to 60 and 80 nM is likely to be due to the greater S.D. found at 100 nM due to extremely low cell numbers.

Alkaline phosphatase activity. Alkaline phosphatase activity was measured after incubation of UMR 106 cells with methotrexate from 48 to 72 h. No significant inhibition of alkaline phosphatase activity was seen at any concentration of methotrexate (data not shown).

Effect of 1,25-dihydroxyvitamin D. One potential reason for the susceptibility of the UMR 106 cell line to methotrexate is that it is a maturation-related phenomenon. UMR 106, although relatively mature in phenotype, does not produce osteocalcin, the marker of the most differentiated osteoblast [15]. 1,25-Dihydroxy-vitamin D is known to produce cell maturation and cells were incubated for 48 h with 10 nM 1,25-dihydroxy-vitamin D, a concentration known to stimulate maximally 1,25-dihydroxyvitamin D receptor upregu-lation in UMR 106 cells [20]. This was to determine whether vitamin D had a protective effect against subsequent addition of methotrexate. In these experi-ments, cells were incubated for 48 h with 10 nM 1,25-dihydroxyvitamin D and then incubated for a further 48 h with 10, 50 and 100 nM methotrexate (Fig. 5). No protective effect against methotrexate toxicity was seen after pre-incubation of the cells for 48 h with 1,25-dihydroxyvitamin D.

Effect of folinic acid (leucovorin). The effect of methotrexate on cell viability was prevented by the addition of folinic acid at the time of addition of methotrexate and 4 h after the addition of methotrex-ate. Concentrations of leucovorin effective in prevent-ing methotrexate toxicity were 1 and 10 μM (Fig. 6). There was no effect on cell proliferation from 10 μM folinic acid alone.

Human umbilical vein endothelial cells. The effect of methotrexate on a non-osseous cell line was assessed by the addition of methotrexate to human umbilical vein cells in culture. Concentrations of methotrexate at

10, 50 and 100 nM had no effect on human umbilical vein endothelial cell proliferation as assessed by the methylene blue assay (data not shown).

Effect of sulphasalazine and sulphapyridine on UMR 106 proliferation and function

Cell proliferation and viability. Sulphasalazine and sulphapyridine were incubated with UMR 106 cells from 24 to 96 h at concentrations ranging from 0.1 to 100 μg/ml. Sulphasalazine had no inhibitory effect on UMR 106 after 24 h proliferation at any concentration (data not shown). At 48 h or longer, however, sulphasalazine at 100 μg/ml significantly
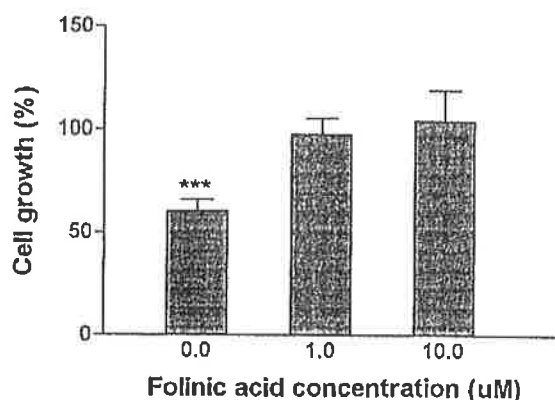
FIG. 6.—Effect of folinic acid and methotrexate on UMR 106 proliferation. Cells were plated at 2000 cells/well and incubated for 48 h. Folinic acid at 1 and 10 μM prevented methotrexate toxicity. Results are expressed as a percentage of control. Results show 100 nM methotrexate with 0, 1 and 10 μM concentrations of folinic acid. Proliferation with 100 nM methotrexate without folinic acid was significantly reduced when compared to control ($P < 0.001$). When methotrexate was combined with folinic acid in the concentrations shown, however, there was no significant difference in proliferation when compared to control.
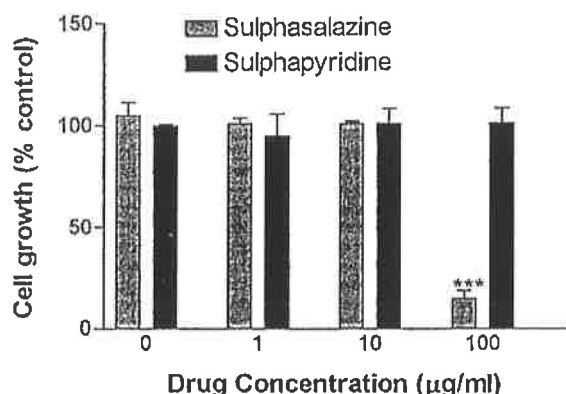
FIG. 7.—Effect of sulphasalazine and sulphapyridine on UMR 106 proliferation. Cells were plated at 2000 cells/well and incubated for 72 h. Sulphasalazine significantly inhibited UMR 106 proliferation at 100 μg/ml ($P < 0.001$)***. There was no significant effect of sulphasalazine on UMR 106 proliferation at other concentrations. There was no significant effect of sulphapyridine on UMR 106 proliferation at any concentration.

inhibited proliferation ($P < 0.001$) (Fig. 7). Trypan blue studies revealed evidence of cell toxicity at this concentration, with viability at 100 μg/ml sulphasalazine being 33% (S.D. 11.6%) compared with 94% (S.D. 2.4%) for the control. There was no significant effect of sulphasalazine on proliferation at other concentrations. Sulphapyridine had no effect on UMR 106 proliferation at any concentration (Fig. 7) in the time periods studied.

Alkaline phosphatase activity. For alkaline phosphatase activity assays, UMR 106 cells were incubated with sulphasalazine or sulphapyridine from 48 to 72 h. No inhibition of UMR 106 alkaline phosphatase activity was seen at concentrations of sulphasalazine that did not have a direct inhibitory effect on cell viability and no inhibitory effect of sulphapyridine was seen at any concentration (data not shown).

DISCUSSION

The methylene blue colorimetric assay measures cell proliferation. This method has been successfully used in a variety of cell types, including the rat osteosarcoma cell ROS 17/2.8, and has been found to be reliable for quantitative evaluation, despite the highly basophilic cytoplasm in such cells [29]. A dye-based assay to assess cell proliferation has major advantages when studying the effect of methotrexate on a cell line. Traditional assays using radioactive thymidine uptake must be interpreted with care because of the enzyme-blocking actions of the drug on thymidylate synthetase [2]. We found a linear relationship between absorbance and cell number using the methylene blue assay, and found it to be a useful method for cell quantification of the UMR 106 line.

Methotrexate was toxic to the UMR 106 cell line at concentrations > 10 nM with the effect evident at 24 h. This is well within the serum concentrations of methotrexate achieved with dosages used in rheumatic disease. Serum concentrations of methotrexate in

patients within the first 4 h of having taken their single weekly dose are usually in the range of 0.1–1.0 μM for doses between 7.5 and 15 mg [31]. In contrast, we found no significant effect on human umbilical vein endothelial cell viability after exposure to 100 nM methotrexate for 48 h. Human umbilical vein endothelial cells have previously been shown to exhibit maximal inhibition of tritiated uridine incorporation at 100 nM methotrexate, without any evident effect on cell viability [32]. Concentrations of FCS were different in the media of the two cell lines, but as methotrexate is such a potent folate antagonist, differences in culture media were not thought to explain the variable cell sensitivity. Methotrexate at 1 μM was found to be toxic to peripheral blood mononuclear cells in culture, with viable cell yields 34% that of the control after 4 days in culture with methotrexate [33]. In comparison with other cell lines, therefore, UMR 106 cells showed toxic effects at relatively lower doses of methotrexate, suggesting an increased sensitivity of osteoblast-like cells to the drug.

The mechanism of methotrexate toxicity in lymphocytes has been shown to be mediated through inhibition of folic acid metabolism, as demonstrated by 'rescue' of the cells with high concentrations of folinic acid [33]. In UMR 106 cells, toxicity of methotrexate was similarly prevented by the addition of folinic acid at both 1 and 10 μM concentrations. This demonstrates that the mechanism of methotrexate toxicity in UMR 106 cells is also through inhibition of folic acid metabolism and that there are no unique aspects of the folic acid pathway in osteoblasts. Sensitivity may be determined by differences in cell origin and morphology. Osteoblasts may be more sensitive to the anti-folate actions of methotrexate due to the importance of protein synthesis and secretion in the cell. Toxicity to cells of methotrexate is related to both dose and duration of exposure [34]. Methotrexate is taken into cells and undergoes polyglutamation, and in this form remains a potent inhibitor of folic acid [35]. High methotrexate concentrations have been found in synovial membrane and cortical and trabecular bone of patients with rheumatoid arthritis [36], and tissues remain exposed for prolonged periods to the anti-folate actions of the drug. It is likely, therefore, that a potential toxic effect of methotrexate on osteoblasts is not limited to the time of peak serum levels shortly after drug ingestion. Continuous exposure of cells to methotrexate in tissue culture, however, may not entirely simulate the situation *in vivo* of weekly oral pulse therapy.

There is little published work on the effect of methotrexate on osteoblasts. Methotrexate has been studied in a mouse calvarial cell line in concentrations from 0.5 nM to 0.6 μM [37]. No effect on cells counts or alkaline phosphatase activity was found at these concentrations, although matrix calcification was diminished at concentrations in the upper range. It was concluded that osteoblast function is inhibited by low mean concentration of methotrexate in a dose-responsive manner. UMR 106 demonstrates

greater sensitivity to methotrexate than the mouse calvarial cell line. Possible explanations are that the sensitivity is either species specific or maturation related. Rats are known to be more sensitive to the toxic effects of methotrexate than mice, based on studies comparing dose–toxicity responses in different animals [38]. Although UMR 106 represents a later stage in osteoblast differentiation, it may still be more progenitor cell in type when compared to the fetal calvarial cell line and this may also account for increased susceptibility to methotrexate toxicity. Of the two possibilities, however, species-specific sensitivity is supported by our finding of a lack of protective effect following pre-incubation with 1,25-dihydroxyvitamin D, a hormone capable of inducing cellular maturity [20], on subsequent methotrexate toxicity. Differential toxicity studies between species based on the development of side-effects and LD50 [38] would predict that human cell lines would be at least as, if not more, susceptible than the rat.

Sulphasalazine was found to be toxic to the UMR 106 cell line after 48 h at concentrations of 100 μg/ml. However, neither sulphasalazine nor sulphapyridine were found to have any significant effect on the cell line in the concentrations found in the serum of patients with arthritis. Expected concentrations are 5.0 μg/ml for sulphasalazine and 18.8 μg/ml for free sulphapyridine when sulphasalazine is taken for more than 4 weeks at a daily 2 g dose [30].

In summary, toxicity on an osteoblast cell line was demonstrated using concentrations of methotrexate found in patients with rheumatic disease. Although extrapolation from transformed cell lines to the *in vivo* situation requires caution, this finding lends support to the observations that the osteopathy demonstrated in animals and man is potentially mediated through the osteoblast. The increased osteoclastic action observed in animal bone histomorphometry after administration of methotrexate [7] may be a secondary phenomenon. According to the theory of Rodan and Martin [39], osteoblasts in the anabolic state form a protective inhibitory layer over bone matrix which, after exposure to bone-resorbing hormones, change shape, allowing osteoclasts access to the matrix. If there is perturbation in osteoblast form due to toxicity from an external agent such as methotrexate, then potentially access of osteoclasts to the matrix is also increased.

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Paper 14. Surgery for primary hyperparathyroidism 1962-1996: indications and outcomes. LW Delbridge, NA Younes, AI Guinea, TS Reeve, P Clifton-Bligh, BG Robinson. The Medical Journal of Australia 1998; 168:153-156

By international standards this is a very large study of primary hyperparathyroidism. P Clifton-Bligh made a significant contribution with respect to the assessment, diagnosis, treatment of a large number of the patients described in this report. In the 1960's and the 1970's the most common indication for surgery was the presence of renal calculi but from the 1980's onwards there was a marked increase in patients diagnosed by incidental biochemical screening. In the 1990's low bone mineral density was the most common indication for surgery (31%). Of the 733 patients diagnosed with hyperparathyroidism, all except 6 were rendered eucalcaemic by parathyroidectomy. 76% had one gland disease. 4 gland disease was seen in 17%. Two had parathyroid carcinoma. Two patients had permanent hypocalcaemia after surgery. 6 had a permanent vocal cord paralysis. 6 patients who had unsuccessful neck exploration were subsequently shown to have hypercalcaemia not due to hyperparathyroidism. One patient with recurrent hypercalcaemia was shown to have the MEN-1 syndrome. General muscle weakness and fatigue were common symptoms in hyperparathyroidism.

This paper is considered to be an important contribution to the world experience of this disorder describing changes in presentation in a large number of patients over 34 years and most importantly describing outcomes.

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Surgery for primary hyperparathyroidism 1962–1996: indications and outcomes

Leigh W Delbridge, Nidal A Younes, Ana I Guinea,
Thomas S Reeve, Phillip Clifton-Bligh and Bruce G Robinson

The past few decades have seen dramatic changes in the apparent incidence, presentation and management of primary hyperparathyroidism. With advances such as ready access to serum calcium and parathyroid hormone measurements, the condition is being increasingly recognised and treated, and new patterns of presentation are being seen. The classic bone disease osteitis fibrosa cystica, the principal manifestation early this century, is now rare.[1] A clinical picture has emerged characterised either by an absence of symptoms or by subtle and vague symptoms,[2-4] such as fatigue, weakness, and variable aches and pains, with the condition often suspected only because of an incidental finding of an elevated serum calcium level on biochemical testing. The recent introduction of bone mineral density screening by osteodensitometry seems also to have increased detection of cases of primary hyperparathyroidism.[5]

Our aim was to examine the changes over the past three decades in presentation and management of primary hyperparathyroidism at a single large referral centre, and to analyse the indications for, and outcomes of, surgery for this condition.

Methods

Subjects were all patients who underwent surgery for primary hyperparathyroidism in the Endocrine Surgical Unit at Royal

Abstract

Objective: To examine changes over the past three decades in the indications for, and outcomes of, surgery for primary hyperparathyroidism.

Design: Survey of a prospective hospital database.

Setting: Royal North Shore Hospital (a tertiary referral and university teaching hospital), Sydney, New South Wales, January 1962 to December 1996.

Patients: All 733 patients who underwent neck exploration for primary hyperparathyroidism.

Results: The annual number of parathyroidectomies increased virtually exponentially, from a mean of two in 1962–1969 to 73 in 1996. In the 1960s and 1970s, the most common indication for surgery was the presence of renal calculi (58% and 43%, respectively), but in the 1980s there was a marked increase in presentation of asymptomatic disease after biochemical screening (19%). In the 1990s, low bone mineral density detected by osteodensitometry has become the most common indication for surgery (31%). After initial operation, 11 patients (2%) had persistent hypercalcaemia, with five of these cured by reoperation — an overall failure rate of 1%.

Conclusions: Surgery for primary hyperparathyroidism has become increasingly common, with low bone mineral density replacing renal calculi as the most common indication for surgery. Neck exploration in experienced hands results in an overall cure rate of 99%.

North Shore Hospital, Sydney, New South Wales, from January 1962 (date of the first parathyroidectomy at the unit) until December 1996. Information was obtained from the prospective database of all endocrine surgical procedures maintained at that hospital since January 1957. The database was searched for any follow-up to December 1997.

The diagnosis of primary hyperparathyroidism was based on the finding of an elevated serum calcium concentration and, when available, an inappropriately normal or elevated parathyroid hormone concentration. Before 1972, parathyroid hormone assays were not available, and the diagnosis was based on a combination of biochemical, radiological and clinical changes (eg, renal stones, urinary tract infection, abdominal pain and neuropsychological disturbances). Patients with secondary or tertiary hyperparathyroidism were excluded from the study.

Information was obtained on the presentation, indications for surgery, operative details, postoperative complications, histopathological results and surgical outcomes. Persistent hyperparathyroidism was defined as hypercalcaemia continuing after surgery. Recurrent hyperparathyroidism was defined as hypercalcaemia returning after a minimum of six months of postoperative normocalcaemia.

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1: Indications for parathyroidectomies performed at Royal North Shore Hospital, Sydney, New South Wales, 1962–1996

Indication	Number of parathyroidectomies (% of total for time period)				
	1962–1969	1970–1979	1980–1989	1990–1996	Total
Renal calculi	11 (58%)	31 (43%)	63 (24%)	57 (15%)	162 (22%)
Osteoporosis*	0	2 (3%)	18 (7%)	117 (31%)	137 (19%)
Asymptomatic	0	5 (7%)	48 (19%)	52 (14%)	105 (14%)
Neuromuscular†	0	7 (10%)	36 (14%)	40 (11%)	83 (11%)
Neuropsychiatric‡	1 (5%)	5 (7%)	21 (8%)	36 (9%)	63 (9%)
Incidental§	5 (26%)	11 (15%)	21 (8%)	30 (8%)	67 (9%)
Parathyroid crisis¶	1 (5%)	1 (1%)	10 (4%)	27 (7%)	39 (5%)
Abdominal**	1 (5%)	4 (6%)	20 (8%)	13 (3%)	38 (5%)
Other	0	7 (10%)	23 (9%)	9 (2%)	39 (5%)
Total	19	73	260	381	733

* Osteoporosis = low bone mineral density or presence of osteoporotic fractures.
† Neuromuscular = muscle weakness, fatigue, lethargy or similar symptoms.
‡ Neuropsychiatric = depression, insomnia, memory loss or similar symptoms
§ Incidental = parathyroid tumour found during a thyroid operation.
¶ Parathyroid crisis = acute admission with marked hypercalcaemia, vomiting and dehydration.
** Abdominal = abdominal pain, constipation, peptic ulceration, pancreatitis or similar symptoms.

Results

Between January 1962 and December 1996, 733 patients underwent neck exploration for primary hyperparathyroidism at Royal North Shore Hospital. They comprised 161 males (22%) and 572 females (78%), with an age range of 9–96 years (median age, 53 years for males and 60 years for females).

Annual numbers of operations for primary hyperparathyroidism are shown in Figure 1. The number has risen virtually exponentially, from one to four annually in the 1960s, to 73 in 1996.

Indications for surgery: These are shown in Box 1. The presence of renal calculi was the principal indication for surgery between 1962 and 1969, accounting for 58% of operations, but has progressively decreased in importance, accounting for only 15% of operations between 1990 and 1996.

Numbers of asymptomatic patients (in whom none of the recognised symptoms of primary hyperparathyroidism could be identified preoperatively) increased in the 1970s and 1980s, but remained low overall (105 patients, 14%).

Between 1990 and 1996, the most marked change was the increase in patients with low bone mineral density detected on screening for osteoporosis (by osteodensitometry or quantitative computed tomography) as the principal indication for surgery. In the 1990s, it was the most common indication (31% of cases), followed by neuromuscular or neuropsychiatric symptoms (20%) and renal calculi (15%), with only 14% of cases considered truly asymptomatic.

Surgical and pathological findings: At surgery, single-gland disease (presence of only one enlarged gland) was found in 556 patients (76%), two-gland disease in 40 (5%), three-gland disease in two (0.3%), four-or-more-gland disease in 121 (17%), carcinoma in two (0.3%), cyst in seven (1%), and no parathyroid abnormality in five (0.7%).

Complications of surgery: Postoperative hypocalcaemia requiring calcium supplementation was seen in 81 patients (11%), but only two of these had permanent hypoparathyroidism. Eight patients developed a wound infection (1%), eight required reoperation for haemorrhage (1%), six had a permanent vocal cord palsy (1%), and one required a temporary tracheostomy because of intraoral haemorrhage caused by injury to the tongue. One patient with preexisting ischaemic heart disease died in the immediate postoperative period.

Outcomes of surgery: Hypercalcaemia was cured immediately in 716 of the 733 patients. In six of the remaining 17, initial neck exploration showed no abnormalities, and they were subsequently shown to have conditions other than hyperparathyroidism, for which surgery was not indicated: sarcoidosis (three), familial hypocalciuric hypercalcaemia (two), and persistent hypercalcaemia with no apparent cause (one).

Eleven patients had persistent hypercalcaemia caused by primary hyperparathyroidism after initial surgery, giving an initial cure rate of 98%. Details of initial and subsequent surgery for these 11 are shown in Box 2. Five were cured by re-exploration of the neck, two remained hypercalcaemic after a further unsuccessful re-exploration, and four had not undergone further surgery (two refused and two were awaiting further assessment). Thus, the overall cure rate for surgery was 99%.

Hypercalcaemia was known to have recurred in two patients, at a mean of 10.5 years after successful surgery. Recurrence was associated with multiple endocrine neoplasia Type 1 syndrome (MEN1) in one of these patients and was cured by removal of the remnant and autotransplantation. The second patient had mild asymptomatic hyperparathyroidism for which reoperation was not indicated.

Discussion

We found that, as expected, surgery for primary hyperparathyroidism became increasingly common at Royal North Shore Hospital over the past three decades. In the 1990s, low bone mineral density replaced renal calculi as the most common indication for surgery. Initial neck exploration resulted in cure in 98% of cases.

Figure 1: Number of parathyroidectomies performed annually at Royal North Shore Hospital, Sydney, NSW, 1962–1996
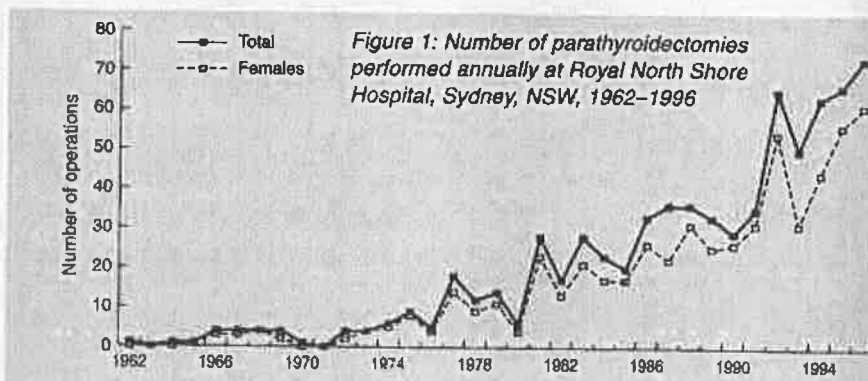
2: Details of 11 patients with an initial failed parathyroidectomy and persistent hyperparathyroidism at Royal North Shore Hospital, Sydney, New South Wales, 1962–1996

Age, sex	Indication	Initial operation			Subsequent operations		Outcome
		Year	Abnormalities found and procedure		Year	Abnormalities found and procedure	
46, F	Renal calculi	1977	2 normal glands found		1977	No abnormality found at reoperation	Persistent
35, F	Renal calculi	1981	Single adenoma removed		1981 1981 1982	Spilled parathyroid remnants found at each of three reoperations	Persistent
44, F	Renal calculi	1984	4 hyperplastic glands seen, subtotal parathyroidectomy		None	Diagnosed as MEN1; patient declined reoperation	Persistent
38, M	Renal calculi	1986	4 hyperplastic glands seen, subtotal parathyroidectomy		1986	Total parathyroidectomy	Cured
73, F	Neuromuscular symptoms	1989	2 normal superior glands seen		1989	Adenoma found in mediastinum and removed via median sternotomy	Cured
49, F	Asymptomatic	1990	3 hyperplastic glands seen, subtotal parathyroidectomy		1990	No abnormality found, but hypercalcaemia resolved	Cured
69, F	Neuromuscular symptoms	1991	2 hyperplastic glands seen, both removed		1991	Adenoma found in mediastinum and removed via cervical incision	Cured
52, F	Osteoporosis	1993	2 hyperplastic glands and 1 normal gland seen, both enlarged glands removed		1993	4th superior hyperplastic gland removed	Cured
67, F	Osteoporosis	1994	3 normal glands seen		None	5th mediastinal tumour found by computed tomography; patient refused reoperation	Persistent
44, F	Osteoporosis	1995	2 hyperplastic glands, both removed		None	Awaiting further assessment	Persistent
64, F	Osteoporosis	1996	3 normal glands seen		None	Awaiting further assessment	Persistent

MEN1 = multiple endocrine neoplasia Type 1 syndrome.

Primary hyperparathyroidism occurs relatively frequently in the community, with an incidence of at least 1 in 1000 individuals,[6] and may be as frequent as 1 in every 500 women over the age of 50 years.[7] The very small numbers of patients being diagnosed and treated as recently as two decades ago related to lack of ready access to serum calcium and parathyroid hormone measurements, as well as lack of awareness of the disease.

Surgery is indicated in patients with symptoms or a high serum calcium level, and in asymptomatic patients who are not suitable for conservative management.[8] The commonest indication for surgery now is the presence of low bone mineral density (36% of operations in 1996). Recovery of bone mass has been documented after successful parathyroid surgery in many series, even in patients with mild or asymptomatic hyperparathyroidism.[5]

The presence of renal calculi remains a major indication for surgery. Although the percentage of patients undergoing neck exploration for this indication has declined significantly each decade, the actual numbers have, in fact, steadily increased. Renal calculus formation is reduced after successful parathyroid surgery,[9] although preformed stones or those associated with idiopathic hypercalciuria may continue to be passed.[10]

Marked muscular atrophy is rarely

Figure 2: Neck explorations, showing: (a) parathyroid glands; (b) lobe of thyroid gland; (c) trachea.

seen nowadays, but muscular weakness contributing to a general feeling of tiredness and malaise is noticed in most patients with primary hyperparathyroidism.[11] We found neuromuscular disease was the primary indication in 11% of our patients. Abnormalities included muscular atrophy, generalised weakness and fatigue, which are thought to be related to low plasma phosphate level and possibly hypokalaemia. Many patients with these symptoms have reported improvement after parathyroidectomy.[12,13]

Neuropsychiatric symptoms were the primary indication for surgery in only 9% of patients in this series, but may be found (if sought) in significant numbers of patients with primary hyperparathyroidism (reported incidence, 30%–100%).[14] Symptoms include depression, anxiety, fatigue, lassitude, concentration difficulties, and failing memory. They have been reported to improve or disappear in most patients after surgery.[12,15] A recent study showed that the most dramatic changes are reductions in body pain and improvements in vitality and emotional function.[16]

Similarly, although abdominal symptoms (which may be related to peptic ulcer disease, pancreatitis or constipation) were the primary indication for surgery in only 5% of our patients, they may be seen in up to 20% of patients with primary hyperparathyroidism.[17]

In our series, 14% of patients appeared asymptomatic. The proportion of patients with primary hyperparathyroidism reported to be asymptomatic varies greatly, from 2% to 80%,[3,17-19] possibly depending on the care with which they are evaluated. Indeed, vague psychiatric and neuromuscular symptoms and generalised weakness may be fully appreciated only in retrospect, once normocalcaemia has been achieved by surgery.[20]

It is important to consider surgery even in the asymptomatic, as there is increasing evidence that primary hyperparathyroidism affects longevity. Several studies have shown that untreated individuals with mild hypercalcaemia have a reduced survival rate.[21] A study of 441 patients followed up for a mean of eight years showed that successful parathyroid surgery reduced the risk of dying,[21] while a more recent study of 896 patients confirmed this result and showed that the duration of hyperparathyroidism is also

a factor, with early surgery reducing the risk of dying.[22] A National Institutes of Health consensus statement from 1990 addressing the management of asymptomatic primary hyperparathyroidism recommends that "all patients with primary hyperparathyroidism should be considered to be candidates for surgery".[8]

The aim of surgery in primary hyperparathyroidism is to identify and remove all abnormal parathyroid tissue. As multiple-gland disease is common (22% of patients in this series), the mainstay of good surgical technique is to identify all (four or more) parathyroid glands in order to differentiate normal from abnormal glands (see Figure 2). We believe that current passing interest in "minimal access" parathyroid surgery (endoscopic or unilateral minimal incisions based on preoperative localisation) is misguided. Such techniques should be avoided as they will inevitably increase failure rates from unsuspected multiple-gland disease for, at best, a very marginal cosmetic advantage.

Localisation techniques such as ultrasonography, computed tomography and scintigraphy with sestamibi have not shown sufficient sensitivity and specificity to justify routine use before initial operation and are certainly not cost-effective.[23,24] False positive and false negative results from preoperative localisation tests may add confusion, especially for the inexperienced surgeon. Indeed, surgery undertaken by those not experienced in the procedure has been shown to be associated with a high failure rate and need for reoperation, as well as increased complications.[25] For example, a Scandinavian study showed that surgery performed in units doing fewer than 10 parathyroidectomies per year resulted in only 70% of patients achieving long-term normocalcaemia,[25] whereas in experienced units a success rate of 98% should be achieved.

Acknowledgements

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Paper 15. Calcitriol mediated hypercalcaemia in a T cell rich B cell lymphoma. JJ Moore, JP Isbister, P Clifton-Bligh, RP Eckstein. Australian and NZ Journal of Medicine 1998; 28:479-480

P Clifton-Bligh made a significant contribution in the investigation and treatment of hypercalcaemia in this patient. Hypercalcaemia due to an elevated serum1,25-OH vitamin D had not been previously reported in T cell rich B lymphoma. The hypercalcaemia was of late onset in the evolution of the disease and occurred after already significant deterioration in renal function. A renal biopsy showed nephrocalcinosis. This may have been due to undocumented hypercalciuria before the development of hypercalcaemia related to high levels of serum1,25-OH vitamin D.

This paper is considered to be an important contribution to the understanding of causes of hypercalcaemia in persons with lymphoma and describes the sequence of investigation.

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Calcitriol mediated hypercalcaemia in a T cell rich B cell lymphoma

Hypercalcaemia complicates 15% of cases of non-Hodgkin's lymphoma (NHL) and in approximately 30% of these cases the hypercalcaemia is mediated by calcitriol (1,25 OH vitamin D).[1] The syndrome is characterised by intestinal hyperabsorption of calcium, normal serum phosphate, increased renal excretion of calcium, normal or suppressed serum para-thyroid hormone (PTH) and normal para-thyroid hormone-related peptide (PTHrP) concentrations. We report a case of calcitriol mediated hypercalcaemia in a patient with a T cell rich B cell lymphoma, a recently described entity characterised by a minor population of clonal B cells (<10%) distributed in a background of polyclonal T cells.[2]

A previously well 48-year-old man presented to our unit with a 12 month history of low back pain and 5 kg of weight loss. A recent computerised tomography (CT) scan of the lower back revealed a para-vertebral mass at the level of L4 with no evidence of cord compression. A biopsy of the para-vertebral mass showed a heavy lymphoid infiltrate of bone by small lymphocytes with a predominant T cell immunophenotype (CD3 positive) with a smaller population of larger atypical B cells. A provisional diagnosis of T cell rich B cell lymphoma was made (Figure 1). Further staging revealed splenomegaly with no marrow or node involvement. HTLV-1 and HIV serology were negative. The serum calcium was normal at 2.26 mmol/L (2.05-2.55 mmol/L). Initial therapy entailed corticosteroids and radiotherapy to the para-vertebral mass with significant clinical and radiological improvement.

The patient represented seven months later with increasing splenomegaly and worsening renal function (creatinine 0.63 mmol/L). A renal biopsy was performed which revealed acute tubular degeneration, nephrocalcinosis and a lymphoid infiltrate of CD3 positive cells. At this time the serum calcium remained normal (2.28 mmol/L). After rehydration he was commenced on monthly cycles of cyclosphosphamide and prednisone with improvement of his renal function (creatinine 0.16 mmol/L). There was no progression of disease until he represented eight months later with weight loss, increasing hepatosplenomegaly and hypercalcaemia (serum calcium 3.8 mmol/L). Hormone assays at the time showed: parathyroid hormone <0.10 ng/mL (0-0.4), parathyroid hormone related peptide 0.5 pmol/L (0-2.6), 25 OH vitamin D 14 nmol/L (18-128) and 1,25 OH vitamin D 206 pmol/L (45-175) confirming calcitriol as the main mediator of the
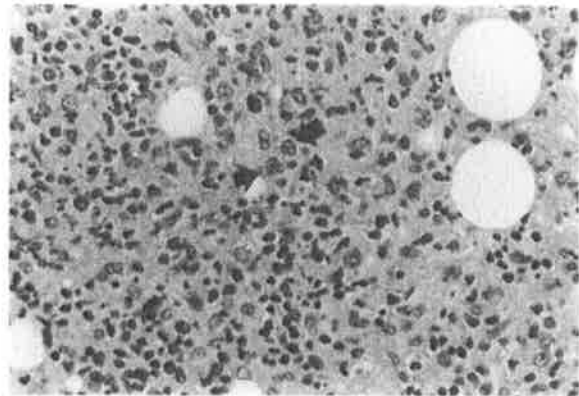

Figure 1: Occasional large cleaved follicle-centre lymphoma cells (arrows) are seen in a background of small lymphocytes and histiocytes (Haematoxylin and eosin).

hypercalcaemia. This responded to corticosteroid and bisphosphonate therapy followed by CHOP chemotherapy. In view of the patient's rapidly progressive disease he proceeded to syngeneic bone marrow transplant, however, this was complicated by necrotising jejunitis and adult respiratory distress syndrome and he died ten days post transplant.

Calcitriol mediated hypercalcaemia, although well described in B cell lymphomas, has not previously been reported in T cell rich B cell lymphoma. The significant renal impairment and nephrocalcinosis preceded the hypercalcaemia by 15 months in this patient. It has been suggested that hypercalcuria and elevated calcitriol are the forerunner of calcitriol mediated hypercalcaemia[3] and this may account for the renal failure in our patient. We conclude that calcitriol mediated hypercalcaemia can occur in T cell rich B cell lymphoma and that the concurrence of renal impairment in lymphoma should alert the physician to the possibility of hypercalcuria and nephrocalcinosis, even in the presence of normocalcaemia. ∎

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Rehabilitation length of stay after hip fracture

The current trend in organisation of rehabilitation services is to move rehabilitation programmes off acute hospital sites along with other sub-acute medical care (psychiatric care is an exception). We wondered whether this move may influence the efficiency of care provided. Certainly it has been shown in Scandinavia that rehabilitation following proximal femoral fracture provided away from acute hospital campuses appeared to increase total cost of the episode of care.[1]

We have audited treatment which was provided to patients after proximal femoral fracture at five acute care hospitals in Northern Sydney in 1993-94. Rehabilitation for these patients was provided in 12 facilities of which four were located on acute hospital sites and eight were 'off site' in both public and private hospitals.

The median length of stay for patients in units providing 'on site' rehabilitation (n=143) was 19 days (interquartile range 13-30 days) and for patients in 'off site' facilities (n=166) the median was 29 days (interquartile range 20-42), indicating a highly significant increase in length of stay and thus total costs in the 'off site' facilities (z=-5.09, p=0.0001, Wilcoxon's sum rank test). Age and other background characteristics of patients were similar across the hospitals. The median acute hospital length of stay was 11 days with no major variation across the five hospitals.

We conclude that it is likely that there are factors relating to the care culture in sub-acute hospitals which may account for this significant difference in median length of stay. Other structural issues, such as the availability of support staff and other facilities, may impede the efficiency of delivery of rehabilitation programmes in these facilities.

Policies that encourage a move of sub-acute care away from acute hospital sites are likely to be counter-productive to the overall provision of efficient rehabilitation services. A higher total cost for treatment is associated with the increased length of stay in 'off site' facilities. This should be considered before such policies are implemented. ∎

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Paper 16. Haemochromatosis and osteopenia. Case report: Effect of venesection on bone mineral density in an eugonadal woman with haemochromatosis. EJ Hibbert, GR Fulcher, L Coyle, F Gates, P Clifton-Bligh, D Stiel. Journal of Gastroenterology and Hepatology 1999; 14:176-178

P Clifton-Bligh made a significant contribution with respect to the assessment of this patient and in the selection of therapeutic options. Osteopenia is well described in association with haemochromatosis. There are multiple possible mechanisms including a direct toxic effect of iron on bone. Often patients with haemochromatosis are hypogonadal and this may be a significant factor in the development of osteoporosis. This paper reports the effect of venesection designed to reduce blood levels of iron and ferritin in an eugonadal woman with haemochromatosis. This case is thought to be the first in which venesection led to an increase in bone mass in a person who was not hypogonadal. The patient was a 41 year old premenopausal woman with a serum ferritin of 513 microgram/L, markedly raised. A liver biopsy showed grade three iron staining. Regular venesection was undertaken with a plan to lower the serum ferritin to normal. Bone mineral density was measured before venesection was commenced and at 12 and 24 months after the commencement of venesection. Her menstrual cycle remained regular throughout this time. The serum FSH was 7.5mU/L, a premenopausal value. The bone mineral density of the lumbar spine, femoral neck, and greater trochanter rose between 0 and 12 months, the lumber spine by 7.2%, the femoral neck by 7.0%. A smaller rise in bone mineral density occurred in the femoral neck and greater trochanter between 12 and 24 months. The rise in the bone mineral density was not thought to be due to the effect of the concomitant use of 1000mg of calcium day. She was not deficient in 25-OH vitamin D. This report is in contrast to a study in eugonadal men with haemochromatosis in whom venesection did not prevent loss of bone mineral density over a 24 month interval. It was not possible in the present study to evaluate osteoblast function using bone cell markers or by histomorphometric examination of bone.

This paper is considered to be an important contribution to the study of the effect of iron excess on bone metabolism.

HAEMOCHROMATOSIS AND OSTEOPENIA

CASE REPORT: Effect of venesection on bone mineral density in an eugonadal woman with haemochromatosis

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Abstract
Background: A 41-year-old premenopausal woman with newly diagnosed haemochromatosis was found to have osteopenia on screening bone mineral densitometry.
Methods and Results: Liver biopsy showed grade 3 haemochromatosis with an hepatic iron index of 4. Investigation for secondary factors for osteopenia revealed no cause. The patient was clinically and biochemically eugonadal. Following venesection of 8 L blood (4 g iron) over 17 months and calcium supplementation, her bone density rose significantly. Neck of femur bone density increased by 6.0% over 13 months and lumbar vertebral bone density increased by 7.2%. There are no previous reports of response of bone density to venesection in eugonadal patients or in women with haemochromatosis.

Key words: bone mineral density, haemochromatosis, osteoporosis, osteopenia, venesection.

INTRODUCTION

Osteopenia is a well-described association with haemochromatosis, occurring in 15–65% of cases.[1] The aetiology of bone loss in haemochromatosis is unclear. Hypogonadism,[2,3] vitamin D deficiency,[4] abnormalities of parathyroid function,[5,6] chronic liver disease[7] and a direct toxic effect of iron on bone[8,9] are putative mechanisms. If iron directly alters the balance between bone formation and resorption, it is possible that venesection might result in a measurable increase in bone mineral density (BMD). To the best of our knowledge, there are no published studies showing a positive effect of venesection on BMD in patients with haemochromatosis uncomplicated by hypogonadism. We, therefore, report the case of an eugonadal woman with newly diagnosed haemochromatosis with sequential measures of BMD.

CASE REPORT

The patient is a 41-year-old premenopausal woman who presented with fatigue, lethargy and decreased libido. She was found to be hypothyroid 4 years previously but was clinically and biochemically euthyroid at the time of study (free thyroxine (FT_4) 18.4, normal range 8–24 pmol/L; serum thyroid-stimulating hormone (TSH) 0.15, normal range 0.1–6.3 mU/L) while taking thyroxine 125 µg/day. She had normal menses, no overt arthropathy or arthralgia and no symptoms of cardiovascular disease. She had a history of five pregnancies resulting in two living children. She drank alcohol 10 g/day, did not smoke, weighed 63 kg and had a body mass index (BMI) of 22.5 kg/m^2. The remainder of her physical examination was normal. Specifically, there were no stigmata of haemochromatosis. Her initial biochemistry is presented in Table 1. Of note, her serum ferritin was 513 µg/L at presentation (approximately three times the upper limit of normal) and transferrin saturation was 79.5%.

A liver biopsy revealed grade 3 iron staining with a mild increase in portal fibrosis. The hepatic iron index was 4. She was commenced on regular venesection, initially weekly, thence every 2 weeks and then intermittently to maintain her serum ferritin level in the normal range. Blood (5.5 L) was venesected between May 1995 and October 1995 and a further 2.5 L between October 1995 and October 1996 (a total of 4 g iron). She was given calcium supplementation as calcium carbonate 600 mg once daily. Her serum ferritin at the last visit was 206 µg/L. The initial BMD and repeat studies after 12 and 24 months treatment are presented in Table 2. The patient remained ambulant throughout the time of the study without any significant change in her level of physical activity or weight. Her menstrual cycle also

remained regular throughout. Serum TSH remained low, but within normal limits, throughout the time of venesection with normal levels of free T_3 and free T_4. Serum 25-hydroxy-vitamin D level was normal.

DISCUSSION

We believe this to be the first report of improvement in BMD in an eugonadal patient with haemochromatosis after venesection. It also appears to be the first report describing the effects of venesection on BMD in a woman with haemochromatosis.

There was a dramatic increase in both femoral neck and spine BMD during the 17 months venesection. Bone mineral density of the neck of the femur rose by

6.0% (from 0.760 to 0.805 g/cm^2) over 13 months. Lumbar vertebral BMD (L2–L4) rose 7.2% from 1.262 to 1.352 g/cm^2 over the same time period. The improvement in BMD is unlikely to be due to calcium alone, as there are no reports showing this degree of improvement with calcium supplementation in premenopausal women. It is unlikely that factors relating to physical activity, gonadal status, thyroid function, vitamin D or BMI account for the improvement in BMD as they did not change significantly over the period of observation.

Osteopenia in haemochromatosis has been strongly associated with gonadal status in some,[1-3] but not all reports.[7] In a study comparing eugonadal and hypogonadal men with haemochromatosis who had undergone venesection for between 2.3 and 10 years, Diamond *et al.* showed that bone histomorphometry findings differed between the two groups.[2] Hypogonadal men had increased parameters of resorption, primarily with inappropriately low parameters of bone formation and lower BMD than eugonadal men. Eugonadal venesected men showed evidence of greater osteoblastic activity than eugonadal non-venesected men. Radial BMD was unexpectedly lower in the venesected men while BMD at other sites was similar. In a further report, a young male whose presentation of haemochromatosis was an osteoporotic fracture, is described.[9] This patient was eugonadal with no causes for osteoporosis other than haemochromatosis. The response to venesection was not reported.

In a 2 year prospective study, Diamond *et al.* examined changes of BMD in response to venesection in eugonadal men with haemochromatosis and in response to venesection and testosterone replacement in hypogonadal men with haemochromatosis.[3] Improvements in BMD appeared to occur only in hypogonadal patients treated with venesection and testosterone

Table 1 Initial biochemistry results of the patient

Parameter	Patient (normal range)
Serum iron (μmol/L)	33 (8–30)
Total iron binding capacity (μmol/L)	37 (45–74)
Transferrin (g/L)	1.66 (1.82–3.57)
Transferrin saturation (%)	79.5
Ferritin (μg/L)	513 (15–150)
Follicle stimulating hormone (mIU/L)	7.5
Luteinizing hormone (mIU/L)	7.8
Aspartate aminotransferase (U/L)	19 (5–35)
Alanine aminotransferase (U/L)	23 (7–50)
Gamma-glutamyltranspeptidase (U/L)	19 (<65)
25-hydroxy-vitamin D (nmol/L)	69 (42–169)

Table 2 Bone mineral density (BMD) following venesection

Year	BMD (g/cm^2)	BMC (g)	Z score	T score	% change
Lumbar 2–4					
1995	1.262	64.04	+1.51	+0.95	
1996	1.352	67.12	+2.11	+1.51	+7.2
1997	1.336	67.52	+2.03	+1.41	−1.3
Femoral neck					
1995	0.760	3.934	−1.51	−2.14	
1996	0.805	4.096	−1.10	−1.76	+6.0
1997	0.808	4.07	−1.04	−1.73	+0.4
Wards triangle					
1995	0.601	NA			
1996	0.634				
1997	0.659				
Greater trochanter					
1995	0.735	9.741			
1996	0.766	10.38			
1997	0.769	10.55			

External and internal calibrations were performed daily using a hydroxyapatite phantom in perspex. The coefficient of variation (CV) at each site is as follows: L-S spine 1.0%, neck of femur 1.2%, greater trochanter 1.8%, Ward's triangle 5.3%. BMC, bone mineral content; Z score, standard deviations from the mean of an age-matched population; T score, standard deviations from the mean of a young reference population; NA, not available.

replacement. Specifically, after treatment with weekly venesection and testosterone (250 mg intramuscularly every 3 weeks), there was a 13.1% increase in lumbar spine BMD. In contrast, in eugonadal men treated with venesection alone, BMD of the lumbar spine decreased by $3.5 \pm 2.8\%$ over 24 months while forearm BMD did not change. The authors, thus concluded that the mechanism for the osteopenia of haemochromatosis was different in hypogonadal and eugonadal men.

Although hepatic cirrhosis *per se* is associated with diminished bone formation and lower osteoblast surfaces,[7] none of the eugonadal men in retrospective[2] or prospective[3] studies of response to venesection had cirrhosis. Similarly, the current patient did not have cirrhosis on liver biopsy. Thus, while the development of cirrhosis may contribute to osteopenia in some patients, neither it, nor hypogonadism, provide a complete explanation.

The BMD increase in the present patient indicates that osteopenia in haemochromatosis has important determinants other than gonadal and hepatic status. One possible explanation is that iron deposition directly impairs osteoblast function. Direct iron deposition in bone has been demonstrated by magnetic resonance imaging studies in patients with haemochromatosis.[10] Furthermore, Ebina *et al.* demonstrated iron deposition both in osteoblasts and osteoclasts using a rat model of renal insufficiency.[8] The authors proposed that iron has a cellular effect on osteoblast activity by altering lipid peroxidation.[8] Unfortunately, in the present patient, we do not have direct evidence that osteoblast activity was initially impaired, nor that it increased following venesection, as bone biopsy was not justified on clinical grounds.

The clinical presentation and progress of the current patient differed from patients studied by Diamond *et al.*[2,3] in several important respects. First, at the commencement of venesection, she showed more significant osteopenia at the femoral neck than at the spine. In contrast, Diamond *et al.* reported a greater decrease of BMD at trabecular rather than cortical sites.[3] Second, she showed an increase in BMD in response to venesection both at the spine and femoral neck. Third, Diamond *et al.* reported the findings of a male population. Finally, it is likely that the current patient was studied relatively early in the course of her disease. These differences raise a number of interesting questions. Are there significant gender differences in the effects of iron on cortical and trabecular bone? Does

response to venesection differ between the sexes, or is the response of bone to venesection dependent on the duration of exposure of bone to excess iron? Further studies into these issues are indicated.

In conclusion, we described a patient with idiopathic haemochromatosis who was screened for osteopenia because of the association between haemochromatosis and osteoporosis. The significant increase in BMD following venesection in the absence of any other demonstrable interventions provides supportive clinical evidence for a direct osteopenic effect of iron.

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Paper 17. Bone density and body composition in young women with chronic fatigue syndrome. L Hoskin, P Clifton-Bligh, R Hansen, G Fulcher, F Gates. Annals of the NY Academy of Sciences 2000; 904:625-627

P Clifton-Bligh made a significant contribution to this paper with respect to the concept and design of the study, the recruitment and assessment of the patients and the development of the methodology. This is a unique study of 37 young women with chronic fatigue syndrome(CFS), mean age 25 years, who were compared with 20 healthy controls mean age 24 years. The CFS and control participants were not significantly different in height and weight or in terms of exogenous estrogen exposure. Fat free soft tissue mass was significantly lower in the CFS group. Bone mineral density was significantly lower in the trochanteric region of the hip ($P<0.002$) but not in the spine, forearm or femoral neck. Activity levels were assessed using mean exercise time (MET). The activity levels of CFS patients were significantly lower than those of controls ($P<0.0001$). The serum 25-OH vitamin D was not significantly different between the two groups.

This paper is considered to be a significant contribution to the study of body composition in patients with CFS.

Citations.

 Google Scholar 10

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Reads.

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The work described in this paper was submitted as part of a thesis for the award of a MSc at the University of Sydney.

Bone Density and Body Composition in Young Women with Chronic Fatigue Syndrome

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INTRODUCTION

Chronic fatigue syndrome (CFS) is a clinically defined syndrome with a cluster of symptoms, including persistent fatigue, nausea, and weakness and a marked inability to undertake physical activity or to exercise.[1] The incidence of CFS is estimated to be 20,000 cases per year.[2] The aim of this case-control study was to determine whether young women with CFS had differences in bone mineral density or body composition, compared to a group of healthy controls of comparable age, height, and weight.

METHODS

Thirty-seven nulliparous women (mean age, 25 years), who met the Oxford Criteria for diagnosis of CFS,[3] and 20 healthy controls (mean age, 24 years), who were not taking oral estrogen, were screened by a physician; their bone density at the hip, spine, and forearm and their whole body composition were measured by DXA, as well as serum levels of 25-OH vitamin D. Habitual daily activity was measured using a three-day questionnaire to establish an MET (mean exercise time) score.[4] All participants completed a questionnaire on calcium intake[5] and were queried about their previous fracture history and estrogen therapy.

RESULTS

The CFS and control groups were not significantly different in height, weight, past fracture history, or estrogen exposure (TABLE 1). Fat-free soft tissue mass (FFSTM) was significantly lower in the CFS group, as was fat-free mass (FFM) (TABLE 2). Bone mineral density was significantly lower at the trochanteric region of the hip ($p < 0.002$) in the CFS group; however, no significant differences were found at the other sites measured (TABLE 3).

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TABLE 1. Comparison of selected characteristics between CFS and control participants

	CFS ($n = 37$)		Control ($n = 20$)		
	Mean ± SD	Range	Mean ± SD	Range	p value
Age (years)	25	16–38	24	16–36	
Weight (kg)	60.2 ± 11.36	43.60–87.70	62.1 ± 6.71	50.0–72.20	0.48
Height (cm)	166.1 ± 5.99	153.0–175.80	165.2 ± 6.58	153.0–177.0	0.61
Menarche (years)	13.2 ± 1.38	10.0–16.0	13.13 ± 1.86	11.0–16.00	0.69
		CFS	Control	χ^2	
Family history osteoporosis		12 (32%)	5 (25%)	$p > 0.05$	
History of previous fracture		17 (45%)	7 (35%)	$p > 0.97$	
History of >1 previous fracture		5 (13%)	2 (10%)	$p > 0.97$	
Previous estrogen therapy		16 (44%)	9 (45%)	$p = 0.90$	

TABLE 2. Comparison of body composition between CFS and control participants using nonparametric (Mann-Whitney U) analysis

	CFS ($n = 37$)		Control ($n = 20$)		
	Median	Interquartile range	Median	Interquartile range	p value
TFM	21,730	17,103–28,151	20,925	17,274–24,700	0.68
FFSTM	33,442	29,452–39,523	36,827	34,733–42,523	**0.01**
FFM	36,008	31,749–42,245	39,388	37,382–45,277	**0.02**

The activity levels of the CFS subjects were significantly lower than those of the control subjects ($p < 0.0001$), as was calcium intake of the CFS group ($p < 0.006$).

Serum levels of 25-OH vitamin D were not significantly different between the groups ($p < 0.19$). FFSTM was the best predictor of bone mineral density at all sites using univariate analysis, followed by weight. In multivariate analysis, having the condition of CFS was not an independent predictor of bone mineral density. Multivariate analysis identified FFSTM and vitamin D levels as the best predictors of bone mineral density. Activity and vitamin D levels correlated with bone mineral density at the trochanter in all subjects. Activity levels correlated with spinal bone mineral density, but not with FFSTM or FFM.

CONCLUSIONS

This study identifies differences in bone mineral density and body composition between young women with CFS and healthy controls. The findings of a relationship between habitual daily activity and hip-spine bone density suggest that there may be

TABLE 3. Comparison of bone mineral density by DXA between CFS and control participants using Mann-Whitney U analysis

	CFS ($n = 37$)		Control ($n = 20$)		
	Median	Interquartile range	Median	Interquartile range	p value
TBMD[a]	0.950	0.910–0.995	0.975	0.920–1.010	0.23
BMC	2667.0	2362.0–2835.0	2639.5	2367.0–2728.0	0.78
Fem. neck	0.928	0.825–0.984	0.942	0.898–1.060	0.15
Trochanter	0.717	0.653–0.751	0.768	0.716–0.821	**0.002**
Wards	0.780	0.701–0.862	0.810	0.781–0.891	0.07
L2–L4	1.083	0.980–1.173	1.036	0.966–1.136	0.55
Distal rad	0.315	0.295–0.337	0.323	0.302–0.379	0.22
Proximal radius	0.694	0.645–0.742	0.667	0.633–0.708	0.35
8 mm region	0.419	0.381–0.449	0.407	0.386–0.462	0.92

[a]Total bone mineral density.

some metabolic disturbance involved in the syndrome. Women with CFS should be encouraged to maintain mobility and to exercise in order to minimize the impact of reduced activity and immobilization on the integrity of muscle mass and bone.

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Paper 18. Effects of high-dose inhaled corticosteroids on bone metabolism in prepubertal children with asthma. HDW Allen, IG Thong, P Clifton-Bligh, S Holmes, L Nery, K Byth Wilson. Pediatric Pulmonology 2000; 29:188-193

P Clifton-Bligh made a significant contribution to this paper with respect to the development of the protocol, the collection and analysis of the data, and the writing of the paper. This study was undertaken because only a few studies have examined the effects of inhaled corticosteroids on bone metabolism in children and in particular whether inhaled corticosteroids could impede the acquisition of bone mineral during growth.The present study evaluated bone metabolism in pre-pubertal children over a period of 9-20 months who were receiving inhaled steroids in doses ranging from 400-2000 microgram per day in the form of either beclomethasone or budesonide. Some children received short courses of oral prednisone for acute exacerbations of asthma. Serum testosterone in boys and serum estradiol in girls was used to define the pre-pubertal status. There were 30 boys and 18 girls. Nine children not using inhaled steroids were studied in parallel as controls. Compliance in the asthmatic children was assessed every 2 months. The average daily dose of inhaled steroid was expressed as mg/m^2 surface area per day. Total body bone mineral content was measured at baseline and again at 9-20 months. Changes between these times was assumed to be linear and a derived 12 month value obtained. Bone mineral content was measured in the lumbar spine L2, L3, L4 and the average areas of these vertebrae were calculated. Again, derived 12 month values were used during analysis. The mean age of the boys was 7.8 years and of the girls 7.7 years. The average daily dose of inhaled steroids in the beclomethasone treated group was $0.59mg/m^2$/day and in the budesonide group $0.81mg/m^2$/day, during the period of observation. Serum inorganic phosphate concentrations were lower in the treatment group compared to the control group. The mean baseline total bone mineral content was not significantly different between boys and girls in the steroid treated group and the mean change in total bone mineral content was not significantly different between boys and girls after 12 months of treatment. Also, there were no significant differences in the changes in total bone mineral content between those treated with beclomethasone versus those treated with budesonide. The increase in total bone mineral content over 12 months was greater in control children compared to children treated with inhaled corticosteroids ($P<0.025$). The lean body mass was significantly greater at baseline in the controls compared to the children treated with inhaled steroids ($P<0.001$). In multiple regression analysis, the change in total bone mineral content was inversely related to the inhaled steroid dose ($P=0.016$). The change in the mean bone mineral density of L2,L3,L4 in the steroid treated children was $0.033g/cm^2$ and was $0.061g/cm^2$ in the controls ($P<0.025$).

This study is considered to be an important contribution to the understanding of accretion of bone mineral in pre-pubertal children receiving high dose inhaled corticosteroids, and contrasts with previous studies in which lower doses were used.

Citations.

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Effects of High-Dose Inhaled Corticosteroids on Bone Metabolism in Prepubertal Children With Asthma

Hugh D.W. Allen, FRACP,[1]* Ian G. Thong, MB, BS, PhD,[1] Phillip Clifton-Bligh, FRACP,[2] Susan Holmes, RN,[1] Liza Nery, RN,[2] and Karen Byth Wilson, PhD[2]

Summary. We studied the effect of inhaled corticosteroids on the increase in bone mineral content in prepubertal children with asthma. Forty-eight asthmatic, prepubertal children receiving either inhaled beclomethasone dipropionate or budesonide were evaluated. Nine children of similar age not receiving inhaled steroids served as controls. The average age of corticosteroid-treated children was 7.8 ± 2.4 years, and of control children, 8.4 ± 2.1 years (NS). The average dose of inhaled corticosteroids in the treated children was 0.67 ± 0.48 mg/m²/day, and they were followed over a 9–20-month period.

Total bone mineral content (TBMC) was measured at baseline and after 9–20 months. A derived value for 12 months' TBMC was calculated, assuming that changes in TBMC were linear with the passage of time. The change in TBMC over a 12-month period was 264 ± 68 mg for the corticosteroid-treated children and 330 ± 84 mg for control children ($P < 0.025$). In a multiple regression analysis in which adjustments were made for the effects of age, height, and weight, the change in TBMC in corticosteroid-treated children was inversely related to the inhaled steroid dose/m²/day ($P = 0.016$). The increase in the lumbar vertebral bone mineral density in control children was also significantly greater than in the corticosteroid-treated children ($P < 0.025$).

We conclude that inhaled steroids, at an average dose of 0.67mg/m²/day, when used in the treatment of asthma reduce the acquisition of bone mineral in prepubertal children. **Pediatr Pulmonol. 2000; 29:188–193.** © 2000 Wiley-Liss, Inc.

Key words: corticosteroid; asthma; beclomethasone; budesonide; bone mineral content; bone mineral density; controlled clinical trial; children.

INTRODUCTION

Oral corticosteroids are used to treat severe asthma, and are associated with adverse reactions such as growth retardation, adrenal suppression, and osteoporosis when excessive doses are given in daily or alternate-day regimes.[1-3] The increasing use of inhaled corticosteroids to treat asthma is based on their dose-dependent effectiveness in decreasing bronchial reactivity and airway inflammation, leading to improved asthma control with fewer side effects than with oral steroids.[4-6] Although side effects are relatively uncommon at doses up to 600 μg/day, there is uncertainty about the safety of doses greater than this when used over a period of years. One particular area of concern relates to the possible deleterious effects on bone metabolism. In adults, inhaled corticosteroids have reportedly reduced bone mineral density,[7] reduced total body calcium,[8] and lowered serum alkaline phosphatase[9] and osteocalcin.[10-13]

In children, only a few studies have examined the effects of inhaled corticosteroids on bone metabolism. Using dual energy x-ray absorptiometry (DEXA), König et al.[14] found that 300–800 μg/day of beclomethasone did not significantly alter the bone mineral density of the distal radius. Baraldi et al.[15] also found in asthmatic children receiving beclomethasone that the increase in bone mineral density (BMD) was not affected over a period of 6 months. Agertoft and Pederson[16] in a cross-sectional

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study of 157 budesonide-treated asthmatic children found that the dose of budesonide and the BMD were not related. On the other hand, dose-related changes in serum concentrations of osteocalcin and carboxypropeptide procollagen after the use of budesonide have been observed.[17] However, a more rapid lowering of serum osteocalcin in beclomethasone-treated children compared to control children was not found over a 6-month interval.[18] Wolthers et al.[19] found that in a period of several weeks, prednisone reduced the serum osteocalcin, but inhaled budesonide did not.

The present study examined prepubertal asthmatic children over a period of 9–20 months to assess the longitudinal effects of the inhaled corticosteroids beclomethasone dipropionate (BDP) or budesonide (BUD) on bone metabolism.

METHODS

Forty-eight prepubertal asthmatic children (30 boys and 18 girls), aged 5–14 years, were studied over 9–20 months. Therapeutic doses of inhaled steroids ranged from 400–2,000 μg/day. All patients used a bronchodilator as required. Some children required short courses of oral prednisone for acute exacerbations of asthma. Children were excluded if they required more than three short courses of oral corticosteroids within the study period. Serum testosterone in boys and serum estradiol in girls were used to determine pubertal status. In the former, a serum testosterone of 3.2 nmol/L or greater, and in the latter, a serum estradiol of 40 pmol/L or greater, were taken to indicate the onset of puberty. Pubertal children identified in this manner were excluded from the study. All patients were instructed in the use of a spacer device if using BDP. BUD was administered via a turbuhaler. Mouth rinsing was encouraged after each dose of inhaled corticosteroid, although this may affect the bioavailability of the corticosteroids used.

The 48 children receiving inhaled corticosteroids were compared with 9 nonsteroid-using control children (7

boys and 2 girls), whose ages ranged from 6–13 years. Of the controls, 2 boys were asthmatics who did not require and had not received oral or inhaled corticosteroids, while the remaining seven (5 boys and 2 girls) were healthy nonasthmatic children.

In each child, height, weight, and surface area were measured at baseline and again after 9–20 months. Height was always measured by the same observer, using a stadiometer. The type and dose of inhaled steroids used over the study period were recorded. This included aerosol corticosteroids used for allergic rhinitis. Oral corticosteroids used during the year were documented. Compliance was assessed every 2 months, at which time patients were telephoned to record hospitalizations, school missed, and the amount of inhaled and oral corticosteroids used. The canisters containing the aerosol corticosteroids were not weighed sequentially. The canisters were not fitted with a device designed to accurately count the number of actuations.

The average daily dose of inhaled steroid was expressed as mg/m^2 of body surface area per day. The total oral corticosteroids used in 12 months were expressed as mg/m^2/year. A nonfasting blood sample was taken to measure the serum testosterone or estradiol, calcium, phosphate, osteocalcin, and insulin growth factor-1 (IGF-1). Osteocalcin was measured by radioimmunoassay (RIA), using a commercially available kit.[20]

The total body bone mineral content (TBMC) was measured using dual energy x-ray absorptiometry (Norland Corporation, Fort Atkinson, WI). Measurements were made in the anterior-posterior direction with the patient supine. Values at baseline and again 9–20 months later were compared. It was assumed that linear changes occurred between baseline and 9–20 months of therapy, and a value for a 12-month time point was calculated. The lean body mass (LBM) and total fat mass (TFM) were calculated from a whole-body dual energy x-ray scan. Measurements were made at baseline and again at 9–20 months. A 12-month value was calculated as for the TBMC.

Bone mineral content (BMC) was also measured in the lumbar spine (L$_2$, L$_3$, and L$_4$), using the same osteodensitometer. Measurements were made at baseline and after 9–20 months of treatment with inhaled corticosteroids. A derived 12-month value was calculated as for TBMC. The average value of the three vertebrae was used. The average area of L$_2$, L$_3$, and L$_4$ was calculated by the osteodensitometer. A derived 12-month value for area was calculated as for TBMC, assuming that in an individual the change in vertebral area was linear between baseline and 20 months. The average vertebral density (BMD) in g/cm^2 was calculated by dividing the BMC by the area. Measurements of vertebral BMC and BMD were made in 47 children. In one child the measurements were inadvertently omitted.

Abbreviations	
BDP	Beclomethasone dipropionate
BMC	Bone mineral content
BMD	Bone mineral density
BUD	Budesonide
DEXA	Dual energy X-ray absorptiometry
IGF	Insulin growth factor
LBM	Lean body mass
m^2	Surface area in square meters
RIA	Radioimmunoassay
SA	Surface area
SD	Standard deviation
TBMC	Total bone mineral content
TFM	Total fat mass

TABLE 1—Data at Time 0 (Baseline) for Controls and Children Treated With Inhaled Corticosteroids[1]

	Corticosteroid-treated	Controls	P
Age in years	7.8 ± 2.4	8.4 ± 1.7	NS
Insulin growth factor-1 (U/mL)	0.72 ± 0.29	0.87 ± 0.28	NS
Osteocalcin (ng/mL)	15.65 ± 6.54	16.09 ± 3.34	NS
Lean body mass (g)	18,327 ± 4,289	22,975 ± 3,231	<0.001
Total fat mass (g)	7,161 ± 4,475	5,662 ± 3,872	NS
Total bone mineral content (g)	975 ± 333	1,133 ± 297	NS

[1]Mean ± SD. NS, no significance.

The study protocol was approved by the Medical Research Ethics Committee of Royal North Shore Hospital, and informed consent was obtained from the parent(s)/guardian(s) of each patient and from the patients themselves.

Statistical Methods

Unpaired *t*-tests were used to test for differences between groups. Multiple linear regression analysis was used to examine the joint effects of age, height, weight, and the dose of inhaled corticosteroid/m², on the outcome of interest, i.e., the change in total bone mineral content. Rank correlation coefficients were used to quantify the degree of association between variables.

RESULTS

There were 48 children in the corticosteroid-dependent group, 30 boys and 18 girls. Anthropometric data relating to this group are shown in Table 1. The mean age of the boys was 7.8 ± 2.5 (SD) years, and of the girls, 7.7 ± 2.0 (SD) years; the ages were not significantly different (NS).

The average daily use of inhaled steroid (nasal plus bronchial) in the 12 months before commencement of the study in the corticosteroid-treated group was 0.77 ± 0.54 (SD) mg/m²/day. The average daily use of inhaled steroid in the BDP-treated group was 0.74 ± 0.47 (SD) mg/m²/day, and in the BUD-treated group, 0.86 ± 0.70 (SD) mg/m²/day in the 12 months before commencement of the study.

The average daily use of inhaled steroid during the 9–20 months of the study in the corticosteroid-treated group was 0.67 ± 0.48 (SD) mg/m²/day. The average daily use of inhaled steroid in the BDP-treated group was 0.59 ± 0.48 (SD) mg/m²/day, and in the BUD-treated group, 0.81 ± 0.46 (SD) mg/m²/day. The difference between the BDP and the BUD group was not significant.

The serum calcium between the two groups was not significantly different at baseline. Serum inorganic phos-

TABLE 2—Change in Mean Values Over a 12-Month Period in Controls and in Children Treated With Inhaled Corticosteroids[1]

	Corticosteroid-treated	Controls	P
Total bone mineral content (g)	264 ± 68	330 ± 84	<0.025
Lean body mass (g)	1,463 ± 1,889	1,874 ± 2,399	NS
Total fat mass (g)	1,742 ± 2,151	2,007 ± 1,696	NS

[1]Mean ± SD. NS, no significance.

phate concentrations were significantly lower in the treatment group (P < 0.025) at baseline compared to the normal group. The serum osteocalcin and IGF-1 concentrations were not significantly different between the two groups at baseline.

The mean baseline TBMC of the boys treated with inhaled corticosteroids was 982.7 ± 374.6 (SD) g, and of the girls, 961.7 ± 260.2 (SD) g (NS). The mean change in TBMC over a 12-month period in the boys was 267.6 ± 74.0 (SD) g, and in the girls, 257.9 ± 58.3 (SD) g (NS). The data for TBMC in boys and girls were therefore combined in subsequent analysis. Thus the mean TBMC at baseline in corticosteroid-treated children was 974.9 ± 333.4 (SD) g, and after 12 months it was 1,239.3 ± 362.9 (SD) g. The mean change over 12 months was 264.0 ± 68.0 (SD) g (Table 2). The mean change in TBMC between BDP- and BUD-treated children was not significantly different.

The mean age of the children in the control group was 8.4 ± 2.1 (SD) years, not significantly different from the children receiving inhaled steroids. Data relating to the controls and corticosteroid-treated children are shown in Table 1. The TBMC at baseline in control children was 1,133.4 ± 297.2 (SD) g, and after 12 months was 1,463.0 ± 321.9 (SD) g. The mean change in TBMC over 12 months was 329.6 ± 84.0 (SD) g. This change was greater than in the children receiving inhaled corticosteroids (P < 0.025). There was no significant relationship between the change in TBMC in corticosteroid-treated children and the dose of oral corticosteroids given over the 12-month treatment period. There was no significant relationship between the dose of inhaled corticosteroids given in a 12-month period and the dose of oral corticosteroids given during the same period (r = 0.30, P = 0.2).

The lean body mass (LBM) in the control patients was significantly greater than that for corticosteroid-treated children at baseline (P < 0.001). The total fat mass (TFM) was not significantly different between controls and corticosteroid-treated patients at baseline. There was no significant difference between the change in LBM and in TFM over the 12-month period for controls and corticosteroid-treated children.

A multiple regression analysis was performed in

TABLE 3—Bone Measurements in the Spine of Lumbar Vertebrae 2 to 4[1]

	Time in months	Corticosteroid-treated	n	Controls	n	P
Bone mineral content (g)	0	15.04 ± 3.94	47	18.34 ± 3.84	9	<0.025
Bone mineral content (g)	12	16.88 ± 4.86	47	21.12 ± 5.41	9	<0.025
Area (cm^2)	0	26.93 ± 4.89	47	29.42 ± 3.59	9	NS
Area (cm^2)	12	28.42 ± 5.25	47	30.66 ± 3.54	9	NS
Bone mineral density (g/cm^2)	0	0.55 ± 0.07	47	0.62 ± 0.09	9	<0.01
Bone mineral density (g/cm^2)	12	0.59 ± 0.08	47	0.68 ± 0.11	9	<0.005
Change in bone mineral content		1.83 ± 1.51	47	2.78 ± 2.14	9	NS
Change in area		1.49 ± 1.38	47	1.24 ± 1.81	9	NS
Change in bone mineral density		0.03 ± 0.03	47	0.06 ± 0.04	9	<0.025

[1]NS, no significance.

which adjustments were made for the effects of age, height, and weight measured at the time of entry into the study. The change in TBMC in corticosteroid-treated children, adjusted for these variables, was inversely related to the inhaled steroid dose/m^2/day ($P = 0.016$). The R^2 for the model was 0.36, which means that 36% of the observed variability in the change in TBMC was accounted for by the joint effects of age, height, weight, and inhaled steroid dose.

Changes in BMC and BMD were also studied in the lumbar spine (L_2–L_4) for the corticosteroid-treated children and for the control children. The data are shown in Table 3. One child in the corticosteroid-treated group did not have osteodensitometry of the spine performed, and for this reason there are 47 subjects in the corticosteroid-treated group analysis. The mean BMC at baseline in the corticosteroid-treated children was 15.04 ± 3.94 (SD) g, and in the control children, 18.34 ± 3.84 (SD) g ($P <$ 0.025). The mean area of vertebral bodies (L_2–L_4) measured by the osteodensitometer was 26.93 ± 4.89 (SD) cm^2 in the corticosteroid-treated children and $29.42 \pm$ 3.58 (SD) cm^2 in the control children (NS). The mean BMD of the vertebral bodies at baseline in the corticosteroid-treated children was 0.553 ± 0.066 (SD) g/cm^2, and 0.622 ± 0.086 (SD) g/cm^2 in the control children (P < 0.01). The change in BMC of the vertebral bodies over a 12-month period in the corticosteroid-treated children was 1.83 ± 1.51 (SD) g, and in the control children, 2.78 ± 2.14 (SD) g (NS). The change in mean area of the vertebral bodies over a 12-month period was 1.49 ± 1.38 (SD) cm^2 in the corticosteroid-treated children and 1.24 ± 1.81 (SD) cm^2 in the control children (NS). The change in mean BMD of the vertebral bodies over a 12-month period in the corticosteroid-treated children was $0.033 \pm$ 0.031 (SD) g/cm^2, and 0.061 ± 0.042 (SD) g/cm^2 in the control children (P < 0.025).

Thirty-one of the 48 children treated with inhaled corticosteroids had a synacthen test at the beginning of the observation period. There was no statistically significant correlation between the serum cortisol increment after synacthen between 0–30 min and the change in TBMC over the subsequent 12 months.

DISCUSSION

In this study, we found that inhaled corticosteroids used to treat prepubertal asthmatic children were significantly associated with a reduction in the rate of accretion of bone mass. The increase in total body bone mineral content over a 12-month interval was less in children using inhaled corticosteroids compared with age-matched noncorticosteroid-treated controls. There was also a significant inverse correlation between the dose of inhaled corticosteroid measured in mg/m^2/day and the rate of increase in total body bone mineral content.

In the lumbar vertebrae (L_2–L_4), the change in BMC in the corticosteroid-treated children over a 12-month period was not significantly different from that of control children. However, the increase in BMD of the lumbar vertebrae in the control children was significantly greater than that of the corticosteroid-treated children. It is very difficult to assess the change in true BMD over time in children, because the bones increase in volume in three dimensions, whereas osteodensitometry measures the change in projected area. Interestingly, the change in area over a 12-month period in the corticosteroid-treated children was greater than that of control children, although not significantly so. This makes it very likely that the change in true BMD in g/cm^3 in the corticosteroid-treated children will have been significantly less than that of control children. Further studies of bone mineral content and bone mineral density are being carried out in these patients to determine whether peak bone mass ultimately is significantly less than that achieved in control noncorticosteroid-treated subjects.

These results agree with adult studies which describe bone loss associated with supraphysiological doses of exogenous steroids. However, an important difference between adults and children is that our children receiving inhaled corticosteroids continued to deposit bone, albeit at a slower rate. In the study of Martinati et al.[21] in which asthmatic children received an average of 0.32 mg of inhaled corticosteroid/day, bone mineral density was not different from that in a cromoglycate-treated control group. In a study of 35 asthmatic children by Baraldi et

al.,[15] 0.3–0.4 mg of inhaled beclomethasone/day did not influence the increase in BMD over a 6-month period compared with controls. In a study of 157 asthmatic children of mean age 10.3 years receiving budesonide in an average dose of 0.504 mg/day, the correlation between BMD and budesonide dose was not significant.[16] This latter study was cross-sectional and not longitudinal. However, the average dose of inhaled corticosteroid in our children was 0.67 mg/m^2/day, or 0.66 mg/day. In our study, the daily dose of inhaled corticosteroids was higher than in the three studies quoted above, and this may account for the finding that spinal BMD and TBMC increased at a significantly slower rate in corticosteroid-treated children than in controls.

In our group of 48 corticosteroid-treated children, 24 required at least one short course of oral corticosteroids. Therefore, it could be argued that the oral corticosteroids contributed to the observed slower rate of bone formation. However, there was no significant relationship between the dose of oral corticosteroids given over a 12-month period and the rate of bone formation. In contrast to methods which collect data retrospectively,[8] our data were collected longitudinally with regular assessment of oral steroid use.

Osteocalcin is a serum marker whose concentrations reflect osteoblast activity. In adults and children, short-term administration of high doses of inhaled corticosteroids have lowered osteocalcin.[11,17,22] In contrast, we found no significant differences in serum osteocalcin levels between the group of children using inhaled corticosteroids and the noncorticosteroid-using controls at the time of entry into the study, although the corticosteroid-using children had been receiving inhaled corticosteroids for at least 12 months before entry into the study. In a two-part study, it has been shown that serum osteocalcin levels in children using inhaled steroids over a period of years were similar to concentrations seen in children who had never used corticosteroids, whereas serum osteocalcin levels fell significantly soon after a child was started on inhaled corticosteroid.[17] In other studies, however, the use of inhaled beclomethasone in asthmatic children did not affect the rate of decline in serum osteocalcin compared to control children.[18]

In children using inhaled corticosteroids at the time of entry into the study, the serum inorganic phosphate concentrations were significantly lower than the values in control children. This could be explained by phosphaturia as a consequence of secondary hyperparathyroidism, or decreased tubular reabsorption of phosphate caused by the corticosteroid used.[23] However, renal excretion of inorganic phosphate and serum parathyroid hormone levels were not measured in the present study.

We found that there was a large individual variation in both the dietary intake of calcium and exercise on direct questioning, but quantification of this variation was not undertaken. It is possible that differences in physical activity levels between the control group and the corticosteroid-treated group may have affected the accretion of bone mineral. However, the rate of accumulation of lean body mass over the study period was not significantly different between the control group and the corticosteroid-treated group.

As with any study examining the effects of inhaled corticosteroids in asthma, it was difficult to exclude the effects of asthma per se from those specifically due to corticosteroids. The study of a control population of untreated children with asthma of comparable severity to corticosteroid-treated children is not ethically possible.

A study looking at serum osteocalcin levels in children with chronic rheumatic disease showed that children with active disease had reduced levels of serum osteocalcin, but those with inactive disease had normal osteocalcin levels.[24] Although different disease processes were involved, this might suggest that the normal serum osteocalcin levels, seen in the corticosteroid-treated asthmatic children, indicated well-controlled asthma.

In summary, the present study has shown that treatment with inhaled corticosteroid of prepubertal children with asthma, the increase in total bone mineral content and in vertebral bone mineral density was less over a 9–20-month period than that which occurred in an age-matched group of control children. The increase in BMC in lumbar vertebrae L_2 to L_4, however, was not significantly different between the two groups. There was no significant difference in the two groups in the change occurring in total fat mass and total lean body mass. The dose of inhaled steroids expressed as mg/m^2/day was inversely correlated with the change in total bone mineral content, when the effects of age, height, and weight were included in the analysis.

We plan to carry this study forward until peak bone mass is reached, to determine whether asthmatic children treated with inhaled corticosteroids have less bone mineral content than untreated controls. An attempt will also be made to assess differences in physical activity between control subjects and those treated with corticosteroids.

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Paper 19. The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism. P Clifton-Bligh, RJ Baber, GR Fulcher, M-L Nery, T Moreton.Menopause 2001; 8:259-265.

P Clifton-Bligh had a major role in the formulation, initiation and completion of the trial and also in the analysis of the data and preparation of the manuscript. This study was carried out to assess the effect of isoflavones of red clover origin on lipid and bone metabolism. The dominant isoflavone in Rimostil is formononetin and this isoflavone had not been previously studied in this context. The red clover isoflavones bind to the estrogen receptor ER-beta with greater affinity than to ER-beta. The purpose of the study was ultimately to determine whether beneficial effects could be achieved with formononetin and ultimately whether undesirable effects such as seen with estrogen therapy would be diminished. The study is considered to be exploratory. Doses of 28.5mg/day, 57mg/day, 85.5mg/day were given for six months. There were significant increases in serum HDL-cholesterol and significant reductions in serum apolipoprotein B. The bone mineral density in the proximal radius and ulna was significantly increased but there was no significant change in the bone mineral density of the distal radius and ulna. Importantly there was no increase in endometrial thickness at the end of six months of treatment with any dose of Rimostil. There was no significant change in the urinary excretion of deoxypyridinoline (a marker of osteoclastic activity) over the six month period observed with any of the doses of Rimostil used. This study has several flaws namely the lack of a placebo group and the short duration of the study. A larger study was subsequently carried out over a two year period with a placebo control.

This study carried out in which patients were their own controls showed a significant rise in the serum HDL-cholesterol and a significant fall in the serum apolipoprotein B over a six month period when treated with Rimostil, predominantly formononetin.

The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism

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ABSTRACT

Objective: This study was undertaken to evaluate the effects of varying doses of phytoestrogens on lipid and bone metabolism in postmenopausal women.

Design: A novel red clover isoflavone preparation (Rimostil) containing genistein, daidzein, formononetin, and biochanin was administered to 46 postmenopausal women in a double-blind protocol after a single-blind placebo phase and followed by a single-blind washout phase. Patients were randomized to receive either 28.5 mg, 57 mg, or 85.5 mg of phytoestrogens daily for a 6-month period.

Results: At 6 months, the serum high-density lipoprotein cholesterol had risen significantly by 15.7–28.6% with different doses ($p = 0.007$, $p = 0.002$, $p = 0.027$), although the magnitude of the response was independent of the dose used. The serum apolipoprotein B fell significantly by 11.5–17.0% with different doses ($p = 0.005$, $p = 0.043$, $p = 0.007$) and the magnitude of the response was independent of the dose used. The bone mineral density of the proximal radius and ulna rose significantly by 4.1% over 6 months with 57 mg/day ($p = 0.002$) and by 3.0% with 85.5 mg/day ($p = 0.023$) of isoflavones. The response with 28.5 mg/day of isoflavones was not significant. There was no significant increase in endometrial thickness with any of the doses of isoflavone used.

Conclusion: These results show that the administration of an isoflavone combination extracted from red clover was associated with a significant increase in high-density lipoprotein cholesterol, a significant fall in apolipoprotein B, and a significant increase in the predominantly cortical bone of the proximal radius and ulna after 6 months of treatment. Interpretation of the results is undertaken cautiously because of the absence of a simultaneously studied control group.

Key Words: Menopause – Phytoestrogens – HDL cholesterol – Apolipoprotein B – Bone mineral density – 25OH-vitamin D.

Isoflavone phytoestrogens are not steroids but mimic some actions of steroidal estrogens. The phenolic ring in the isoflavone class of phytoestrogens enables the binding of these molecules to the intracellular estrogen receptors.[1] It is now well documented that at least two forms of estrogen receptor exist, ER-α and ER-β. The more recently discovered second form of estrogen receptor, ER-β, binds isoflavones with greater affinity than ER-α.[2] Since steroidal estrogens are known to modify cholesterol metabolism and bone density, foodstuffs that contain isoflavones, such as soy protein and extracts of red clover, have been studied for their ability to influence cholesterol and bone metabolism. For example when 45 mg of isoflavone-containing soy protein was administered daily to premenopausal women, the serum cholesterol was significantly reduced,[3] but the high-density lipoprotein (HDL) cholesterol concentrations did not change. In a different study, postmenopausal women given 40 g/day of isoflavone-containing protein showed a reduction in serum non-HDL cholesterol and an increase in serum HDL cholesterol.[4] Baum et al.[5] also showed an increase in the serum HDL cholesterol in postmeno-

pausal women given isoflavone-containing soy flour for 24 weeks. However, in another study, 58 postmenopausal women given isoflavone-containing soy flour for 14 weeks experienced no change in serum total cholesterol, HDL cholesterol, and triglycerides.[6] Anderson et al.,[7] in a meta-analysis of published studies, showed that 31–47 g/day of soy protein produced a fall in serum cholesterol and in serum low-density lipoprotein (LDL) cholesterol especially in those with high baseline serum cholesterol levels.

Both genistein and daidzein, isoflavones found in soy and in red clover, stimulated an increase in calcium content of rat femoral diaphyseal bone in culture.[8] In 2-month old rats subjected to ovariectomy, injections of genistein ameliorated bone loss, while histomorphometry showed increased rates of bone formation. Interestingly, genistein did not affect markers of bone resorption.[9] Genistein enhanced the proliferation and differentiation of human bone cells in culture.[10] Furthermore, female mice subjected to ovariectomy had markedly reduced trabecular bone in the distal femoral metaphysis that was prevented by genistein.[11] Daily administration for 6 months of soy protein containing 90 mg of isoflavones increased the bone mineral content and bone mineral density (BMD) of the lumbar spine in postmenopausal women, but no significant changes were seen at other skeletal sites. By contrast, daily administration of soy protein containing 55.6 mg of isoflavones for 6 months did not preserve bone mass.[4]

Rimostil is a recently developed red clover isoflavone mix containing daidzein and genistein. In addition, and in contrast to soy protein, Rimostil contains the isoflavones formononetin and biochanin. The effects of this extract on bone density and serum lipids have not been previously investigated. For this reason, the present study was undertaken to evaluate the effects of varying doses of isoflavones extracted from red clover on lipid and bone metabolism in postmenopausal women. The study consisted of a single-blind placebo baseline, a double-blind treatment phase, followed by a single-blind placebo washout phase.

PATIENTS AND METHODS

Fifty women, at least 1 year past their last menstrual period, aged less than 65 years and who had a serum follicle-stimulating hormone level of at least 40 mIU/mL, were recruited into the study. Women were excluded from the study if they had received hormonal treatment within 3 months of study entry, had a history of estrogen-dependent neoplasm, including breast can-

cer, acute or chronic liver disease, diabetes mellitus, hypertension (blood pressure > 160/90), or a body mass index (BMI) of >33 kg/m^2. All subjects were randomly assigned to one of three groups designated to receive 28.5 mg/day, 57 mg/day, or 85.5 mg/day of total isoflavones. Each patient also received 1000 mg of calcium daily. At the point of recruitment into the study and at every visit thereafter, patients were instructed to minimize the intake of isoflavone-containing food. The diet of participants was not otherwise modified. At time −1 month, all patients were administered three 500-mg placebo tablets. One month later, the double-blind treatment phase commenced in which subjects were randomized to receive one of the three isoflavone doses. Active tablets each contained 28.5 mg total isoflavone content and were presented in identical 500-mg tablet form. At this time, patients in the 28.5 mg/day group received one active and two placebo tablets per day, those in the 57 mg/day group received two active and one placebo tablet per day, and those in the 85.5 mg/day group received three active tablets per day. These combinations of tablets were administered daily for 6 months. This was followed by a 2-month washout period during which all patients were switched back to three placebo tablets per day. Two participants from the 28.5 mg/day group, one from the 57 mg/day group, and one from the 85.5 mg/day group withdrew from the trial after initial assessment, leaving 46 women who completed the trial. The isoflavones contained in the active tablets were obtained from red clover by a standardized extraction process and contained daidzein, genistein, formononetin, and biochanin in a novel proprietary ratio (Rimostil; Novogen Ltd., North Ryde, Australia). Twenty-four hour urine isoflavone analysis was undertaken at −1, 0, 3, 6, and 8 months. Daidzein, genistein, equol, biochanin, formononetin, and O-desmethylangolensin were analyzed in the urine using a modification of previously published methods.[12,13] Aliquots (10 ml) of urine were mixed with 100 ml of glucuronidase. The mixture was incubated for 24 h at 37°C after which it was extracted on a C-18 solid phase extraction column (Waters Pty. Ltd., Sydney, Australia). Isoflavones were eluted with 3 ml of methanol, and 10 ml of the extract was injected into the high-performance liquid chromatography system. The high-performance liquid chromatography system consisted of a 25-cm, 5 nM, C-18 stationary phase column (Symmetry, Waters Pty. Ltd.) and a gradient acetonitrile/water mobile phase. The limit of detection of the assay for each of the isoflavones measured was 5 ng/mL. The interassay coefficient of variation (CV) was <15%.

At 0, 3, and 6 months, the urine was assayed for deoxypyridinoline as a marker for osteoclast activity and corrected for creatinine excretion. Deoxypyridinoline was assayed by the Immulite Pyrilinks-D method (Diagnostic Products Corporation, Los Angeles, CA, USA). The interassay CV was 13.3% at 25 nmol/L and 3.3% at 100 nmol/L.

At −1, 0, 3, 6, and 8 months, nonfasting blood was drawn between 8:00 a.m. and 10:00 a.m. for the analysis of serum cholesterol, HDL cholesterol, LDL cholesterol, apolipoprotein B, and triglycerides. Measurement of total cholesterol and HDL cholesterol was performed on a Hitachi 747 analyser using a commercially available enzymatic colorimetric assay, and measurement of apolipoprotein B was performed on a Hitachi 902 analyser using an immunoturbidimetric test (Roche Diagnostics, Mannheim, Germany). LDL cholesterol was calculated using the Friedewald equation. The CV for total cholesterol was 1.92% at 6.87 mmol/L, the CV for HDL cholesterol was 2.97% at 2.31 mmol/L, and the CV for triglycerides was 3.55% at 1.55 mmol/L. The CV for apolipoprotein B was 3.80% at 1.83 mmol/L.

At 0, 3, and 6 months, the BMD of the right forearm was measured at three sites: proximal radius and ulna at a point one third of the distance between the ulnar stylus and the olecranon; radius and ulna at a point in the distal forearm where the radius and ulna are 8 mm apart and where scanning proceeds from this point 1 cm proximally; and distal radius and ulna. BMD was quantified using a Norland pDEXA bone densitometer (Norland Corporation, Fort Atkinson, WI, USA). The precision of the method was 0.65% in the proximal radius and ulna and 0.66% for the distal radius and ulna.

At 0 and 6 months, the endometrial thickness was measured by transvaginal ultrasound (Aloka Echo Camera, model SSD-500; Aloka Co. Ltd, Tokyo, Japan). At 0, 3, 6, and 8 months, the serum 25OH-vitamin D concentrations were measured. The 25OH-vitamin D assay used in the study was a commercially available ^{125}I RIA kit (DiaSorin, Stillwater, MN, USA).

The protocol for the study was approved by the Royal North Shore Hospital Human Research Ethics Committee before individual patients entered the study.

Statistical analysis

The statistical software package SPSS for Windows was used to analyze the data. One-way analysis of variance was used to test for homogeneity of continuous variables such as age, weight, height, and years since menopause across treatment groups. When heterogene-

TABLE 1. *Characteristics of the study group*

	Age (ys)	BMI Wt/Ht^2 kg/m^2	Years since LMP[a]
Group 1 28.5 mg/d isoflavones	55 ± 3.3	26.1 ± 2.8	5 ± 2.3
Group 2 57 mg/d isoflavones	59 ± 4.1	25.3 ± 6.2	9 ± 4.2
Group 3 85.5 mg/d isoflavones	56 ± 6.4	25.1 ± 4.5	6 ± 4.0

Values given are mean ± 1 SD.
[a]LMP, last menstrual period.

ity was detected, the least significant difference method of multiple comparisons was used to test for pairwise differences between groups.

The effect of treatment was assessed by the within patient change over time using the Wilcoxon signed rank test. Nonparametric two-way analysis of variance (Kruskal-Wallis) with repeated measures was used to test for effects of treatment, time, and their interaction on the continuous outcome variables such as blood lipid levels and BMDs.

Spearman rank correlation coefficients were used to quantify the degree of linear association between continuous variables. A significance level of 5% was considered to be statistically significant throughout.

RESULTS

The age, BMI, and years since menopause for the study groups are shown in Table 1. The mean ages of the three groups were not significantly different. There were no significant differences between groups for BMI. The number of years since menopause was significantly greater for group 2 when compared with groups 1 and 3 ($p = 0.035$).

The changes in the serum lipids are shown in Table 2. The total serum cholesterol did not change significantly in any treatment group. The serum HDL cholesterol rose significantly after 6 months of treatment with each of the doses given. With 28.5 mg of isoflavone daily, the serum HDL rose by 15.8% ($p = 0.007$), with 57 mg by 28.6% ($p = 0.002$), and with 85.5 mg by 15.7% ($p = 0.027$). There was no significant difference in the HDL response between different doses of isoflavones.

There was no significant change in the calculated serum LDL cholesterol concentrations over the 6-month treatment interval in any treatment group ($p = 0.874$), and the serum triglyceride concentrations did not change significantly in any treatment group ($p = 0.879$).

TABLE 2. *Serum lipids (mmol/L)*

	Time (mo)			
	0	3	6	8
28.5 mg				
n = 15				
TC[a]	5.92 ± 0.52	5.76 ± 1.11	6.22 ± 0.93	5.91 ± 0.44
HDL	1.75 ± 0.37	1.77 ± 0.37	2.01 ± 0.41	1.85 ± 0.41
LDL	3.69 ± 0.61	3.39 ± 1.07	3.76 ± 0.84	3.48 ± 0.49
ApoB	1.30 ± 0.27	1.20 ± 0.25	1.15 ± 0.23	1.04 ± 0.12
Tg	1.05 ± 0.38	1.20 ± 0.54	1.12 ± 0.41	1.37 ± 0.76
57 mg				
n = 16				
TC	5.94 ± 1.04	5.57 ± 1.01	6.15 ± 1.05	6.17 ± 1.15
HDL	1.60 ± 0.48	1.70 ± 0.53	1.95 ± 0.64	1.85 ± 0.41
LDL	3.51 ± 1.06	3.10 ± 1.06	3.41 ± 1.01	3.62 ± 1.18
ApoB	1.35 ± 0.28	1.25 ± 0.29	1.12 ± 0.28	1.17 ± 0.34
Tg	1.63 ± 0.68	1.34 ± 0.61	1.56 ± 0.70	1.61 ± 0.74
85.5 mg				
n = 15				
TC	5.50 ± 0.79	5.58 ± 0.97	5.83 ± 1.12	5.77 ± 0.98
HDL	1.61 ± 0.41	1.73 ± 0.42	1.82 ± 0.39	1.79 ± 0.45
LDL	3.38 ± 0.93	3.27 ± 0.93	3.35 ± 0.89	3.46 ± 1.02
ApoB	1.26 ± 0.30	1.23 ± 0.33	1.09 ± 0.29	1.12 ± 0.29
Tg	1.14 ± 0.52	1.06 ± 0.56	1.05 ± 0.51	1.13 ± 0.61

Values given are mean ± 1 SD.
[a]TC, total cholesterol; HDL, HDL cholesterol; LDL, LDL cholesterol; ApoB, apolipoprotein B; Tg, triglyceride.

The serum apolipoprotein B concentration fell significantly after 6 months of treatment with each of the doses given. With 28.5 mg of isoflavone daily, the serum apolipoprotein B fell by 11.5% ($p = 0.005$), with 57 mg by 17.0% ($p = 0.043$), and with 85.5 mg by 11.5% ($p = 0.007$). There was no significant difference in the apolipoprotein B response between different doses of isoflavones.

By 8 months, 2 months after cessation of the isoflavone active treatment phase, the serum HDL cholesterol had fallen by 9.4% in the 28.5 mg/day isoflavone treatment group, by 7.1% in the 57 mg/day treatment group, and by 1% in the 85.5 mg/day isoflavone treatment group.

The data for the changes in BMD in the forearm are shown in Table 3. The BMD in the proximal radius and ulna rose by 2.9% over the 6-month period with 28.5 mg of isoflavones daily ($p = 0.118$ NS), by 4.1% with 57 mg of isoflavones daily ($p = 0.002$), and by 3.0% with 85.5 mg of isoflavones daily ($p = 0.023$) (Wilcoxon signed rank test). The average increase in BMD for the whole group was 3.3%.

The BMD of the distal radius and ulna showed no significant change in relation to the doses of isoflavones used. The BMD of the radius and ulna at the 8-mm gap between radius and ulna showed a significant decline with time ($p = 0.037$, paired t test; $p = 0.057$, Wilcoxon signed rank test) independent of the

dose of isoflavones given. In the combined group of 45 patients, this fall was 1.5% over 6 months.

The data for the changes in urine deoxypyridinoline are shown in Table 4. There were no significant changes in urine deoxypyridinoline over time between 0 and 6 months and no differences between the doses of isoflavones used adjusted for times.

The total urine isoflavones increased from a baseline value of 1.820 ± 2.473 mg/24 h to 5.786 ± 5.555 mg/24 h after 6 months in the group treated with 28.5 mg of isoflavones ($p = 0.018$), from 0.271 ± 0.423 mg/24 h to 9.497 ± 5.687 mg/24 h in the group treated with 57 mg isoflavones ($p = 0.002$), and from 1.529 ± 3.508 mg/24 h to 18.767 ± 12.054 mg/24 h in the group treated with 85.5 mg isoflavones ($p = 0.0001$). There was a significant difference between doses ($p = 0.001$) in that 85.5 mg/day of isoflavones produced a larger urinary output than either 28.5 mg or 57 mg daily, which were comparable.

There was no correlation between the change in either the serum HDL cholesterol or the serum apolipoprotein B between 0 and 6 months of treatment and the change in total urine isoflavones over the same time interval. However, the change in the BMD of distal radius and ulna between 0 and 6 months was directly correlated with the increase in daidzein ($p = 0.017$). There was no correlation between the changes in BMD at other sites and the changes in individual urine isoflavones.

A puzzling and unexpected finding was that the serum 25OH-vitamin D declined significantly between 0 and 6 months of treatment and this decline was independent of the dose of isoflavones. The mean serum 25OH-vitamin D was 94.5 ± 28.2 nmol/L at time 0, and 75.3 ± 24.5 nmol/L after 6 months of treatment with isoflavones ($p < 0.0001$). At the eighth month, 2 months after treatment with isoflavones was ceased, the serum 25OH-vitamin D had risen to 79.6 ± 24.0 nmol/L. In comparison to the 6 month value, this rise was significant ($p < 0.001$).

The endometrial thickness, as assessed by transvaginal ultrasound, did not change significantly during the 6 months of treatment with isoflavones. Endometrial thickness at time 0 was 2.9 ± 1.1 mm, and after 6 months of isoflavones it was 3.1 ± 1.1 mm (NS). The endometrial thickness did not exceed 5 mm after 6 months of treatment with isoflavones in any of the study patients.

DISCUSSION

This study showed that the administration of an isoflavone combination extracted from red clover, Rimos-

TABLE 3. *BMD-forearm (g/cm²)*

Dose[a]		Time (mo)		
		0	3	6
28.5 mg	Distal radius and ulna	0.313 ± 0.062	0.312 ± 0.071	0.311 ± 0.061[b]
n = 15	Radius and ulna, 8 mm	0.418 ± 0.060	0.425 ± 0.071	0.424 ± 0.059
	Proximal radius and ulna	0.723 ± 0.071	0.739 ± 0.066	0.743 ± 0.066
57 mg	Distal radius and ulna	0.326 ± 0.044	0.323 ± 0.041	0.317 ± 0.039[b]
n = 15	Radius and ulna, 8 mm	0.438 ± 0.049	0.434 ± 0.049	0.428 ± 0.048
	Proximal radius and ulna	0.746 ± 0.059	0.768 ± 0.060	0.779 ± 0.062[c]
85.5 mg	Distal radius and ulna	0.318 ± 0.061	0.309 ± 0.053	0.316 ± 0.054[b]
n = 15	Radius and ulna, 8 mm	0.439 ± 0.066	0.426 ± 0.073	0.427 ± 0.074
	Proximal radius and ulna	0.732 ± 0.070	0.752 ± 0.084	0.754 ± 0.076[c]

Values given are mean ± 1 SD.
[a]Column one shows the daily dose of isoflavones given to each group.
[b]The change in BMD in the distal radius and ulna between 0 and 6 months is not significant.
[c]The change in the bone mineral of the proximal radius and ulna between 0 and 6 months is significant for the 57 mg and 85.5 mg groups but not for the 28.5 mg group.

TABLE 4. *Urinary deoxypyridinoline (nmol/mmol Cr)*

Dose	Time (mo)		
	0	3	6
28.5 mg	6.576 ± 2.671	6.831 ± 1.771	6.186 ± 1.934
57 mg	5.369 ± 1.711	7.056 ± 1.687	7.191 ± 2.800
85.5 mg	7.079 ± 3.415	7.233 ± 2.481	7.131 ± 2.676

Values are mean ± 1 SD.

til, was associated with a marked and significant increase in serum HDL cholesterol. This effect was seen at each dose level of isoflavones (28.5 mg/day, 57 mg/day, and 85.5 mg/day) and the magnitude of the response was not correlated with the dose of isoflavones. This study was designed to assess the possible effects of different doses of isoflavones on lipid parameters described and did not include a placebo arm. This is a major weakness of the study because it is therefore not possible to ascertain whether the observed changes in serum HDL cholesterol and serum apolipoprotein B are due to the effects of phytoestrogens administered or whether they are due to factors operating independent of any phytoestrogen effect. However, the fall in serum HDL cholesterol in the 2 months after isoflavones were ceased was partial evidence that the effects observed were due to the administration of isoflavones. It is also possible that a maximum effect on serum HDL cholesterol and serum apolipoprotein B had already been exerted by the lowest dose of phytoestrogen used, 28.5 mg/day, and that the use of higher doses did not exert any additional effects.

There is strong evidence in women that higher levels of serum HDL cholesterol are associated with a reduced risk of coronary heart disease. It has been calculated that a hypothetical 0.026 mmol/L increase in serum HDL cholesterol could translate into a 3.2% reduction in cardiovascular mortality in women.[14] The results reported here were equivalent to a 0.34 mmol/L increase in serum HDL cholesterol, which could imply, if the effect is linear, that a proportionately greater clinical benefit could be achieved. A sustained increase in serum HDL cholesterol brought about by the consumption of red clover-derived isoflavones might favor an environment in which deposition of cholesterol into the walls of arteries is reduced. However, in another study in which postmenopausal women with preexisting coronary atherosclerosis, whose average age was 66 years, were given Premarin 0.625 mg/day, the mean serum HDL cholesterol rose by 18.8%; but the rate of progression of coronary atherosclerosis, as assessed by angiography, was not different from placebo-treated women.[15]

An interesting aspect of the changes in serum HDL cholesterol observed in the present study was that since the 6-month response was greater than the 3-month response, the full expression of the response may not have been reached even at 6 months. The finding in the present study of no significant dose response effect with the different doses of isoflavones was similar to the finding of Baum et al.[5] who used isoflavone-containing soy protein at different doses. In that study, the increase in the serum HDL cholesterol was 5.2% for 50 mg of isoflavone and 3.6% for 90 mg of isoflavone. In our study, the response was substantially greater. One possible explanation for this is that extracts of red clover contain the additional isoflavones formononetin and biochanin, which may have more pronounced effects on the HDL metabolism than soy isoflavones. The HDL cholesterol response between individuals showed wide variability even with the same dose of isoflavones

and this response was independent of the amount of isoflavones secreted in the urine.

The significant fall in the plasma apolipoprotein B at each dose of isoflavone given is also an interesting finding. Previous studies have shown a direct relationship between plasma apolipoprotein B levels and the risk of premature coronary artery disease particularly in women,[16,17] so that a fall in the plasma apolipoprotein B levels might be associated with a reduced risk of premature coronary artery disease. The use of isoflavones in this study did not bring about a significant fall in plasma LDL cholesterol. Because the plasma apolipoprotein B did fall significantly, this would lead to a rise in the LDL cholesterol/apolipoprotein B ratio. A lower LDL cholesterol/apolipoprotein B ratio has been associated with the formation of smaller, dense LDL particles and increased numbers of these particles has been linked with an increased risk of premature coronary heart disease.[18,19] A rise in the LDL cholesterol/apolipoprotein B ratio would be expected to lead to an increased LDL particle size, which may be less atherogenic. It should be noted, however, that in the present study, apolipoprotein B was measured in whole plasma and not in the LDL fraction of plasma, so that true LDL cholesterol/apolipoprotein B ratios were not obtained. Alternatively, since LDL cholesterol is a calculated value based on HDL and triglyceride levels, the fact that triglyceride levels were measured nonfasting may have influenced the calculation of LDL cholesterol levels. However, there was no significant change in serum triglycerides with time or between different doses of phytoestrogens such that it might affect the derived LDL and HDL cholesterol calculations. Since apolipoprotein B is not affected by food, apolipoprotein B may be a more robust indicator of LDL changes in this study.

BMD was measured at three separate sites in the forearm. The forearm was chosen in this study because of the previously demonstrated high precision of measurements in the forearm and the expectation that any changes in the BMD brought about by increased consumption of isoflavones would be small over a 6-month period. In fact, the average increase with all doses in BMD in the proximal radius and ulna in a 6-month period was 3.3%. In the 57 mg/day group, a 4.1% increase was observed. Significant increases in BMD in the proximal radius and ulna were seen with 57 mg/day or 85.5 mg/day of isoflavones but not with 28.5 mg/day. Therefore, in the proximal radius and ulna, a threshold dose effect was seen with which a significant increase in bone mass occurred. The bone structure in the proximal radius and ulna is predominantly cortical bone, and

increases in cortical bone have not been seen in other studies with isoflavones. The distal radius and ulna contain predominantly trabecular bone, and the BMD at this site did not change significantly in the present study. In other studies, the BMD of the lumbar spine, predominantly trabecular bone, did increase after ingestion of isoflavones[4] and in ovariectomized mice, trabecular bone was preserved by genistein. In the present study, there was no correlation between changes in BMD at any site and changes in urinary genistein. In the distal radius and ulna, there was a positive correlation between changes in BMD and changes in urinary daidzein. The potential for isoflavones to preserve or increase bone mass in postmenopausal women is being further explored in a double-blind, placebo-controlled trial to be continued over 2 years.

The finding in the present study of an increase in the BMD of predominantly cortical bone is of particular interest because studies using estrogen in postmenopausal women, also having at least 1000 mg of calcium/day, have shown only a very small increase in the BMD of the proximal radius of less than 1%.[20] It should be noted that our patients received 1000–1200 mg/day of supplemental calcium throughout the study, and it is possible that the increase in BMD could have been due to the effect of calcium supplementation. However, in a separate study conducted by us[21] in which 80 postmenopausal women, average age 56.1 years, were given 1000 mg calcium/day, the BMD of the proximal radius and ulna increased by 0.94% in 12 months, somewhat less than the 3.3% increase observed in the present study in a 6-month period.

It is not known whether the effects of phytoestrogens in Rimostil on bone are mediated by ERα or ERβ, or both, or whether the action is one of stimulation of ER-mediated events or inhibition of the ability of the receptor to bind estradiol. Interestingly, in adult ERβ knockout mice, the cortical bone density of the tibia and femur is increased, whereas the trabecular bone density of the tibia and femur is unchanged,[22] a similar pattern of response seen in the present study. It cannot, however, be implied that any of the phytoestrogens used in the present study inhibit the function of the ERβ in bone.

A further interesting and unexpected finding was that the administration of isoflavones was associated with a significant fall in the serum 25OH-vitamin D concentrations. The mean serum 25OH-vitamin D concentrations after 6 months of isoflavone administration were still above the lower limit of normal and it is not certain what effect a fall of this magnitude would have on the maintenance of bone mass, or whether giving

supplemental vitamin D would enhance the response to isoflavones. The mechanism of the fall in the serum 25OH-vitamin D has not been determined. Twenty-nine of the 50 subjects entered during the summer and therefore completed the 6 months of isoflavone treatment during winter. This may have explained the fall in 25OH-vitamin D observed. The fall in serum 25OH-vitamin D could also have been due to an increase in the rate of inactivation of 25OH-vitamin D in the liver.[23] However, an increase in the BMD of the proximal radius and ulna occurred despite this fall in serum 25OH-vitamin D.

Finally, the isoflavones in the form administered did not cause an increase in endometrial thickness over the 6-month period of observation. This is consistent with previous data indicating the lack of endometrial effects by isoflavones,[24] supporting the safety of this type of intervention. The data reported in this study provide some evidence for a beneficial effect on cholesterol profile and bone density in postmenopausal women receiving a unique red clover isoflavone supplement. The magnitude of the effects here are likely to be clinically relevant, and although the absence of a simultaneously studied placebo group prevents a more robust link between phytoestrogens and the parameters studied, based on previously published risk factor analysis they may be associated with reduced risk of cardiovascular disease and bone fracture rates.

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Paper 20.Vitamin D supplementation and bone mineral density in early postmenopausal women. L Cooper, PB Clifton-Bligh, ML Nery, G Figtree, E Hibbert, BG Robinson. American Journal of Clinical Nutrition 2003; 77:1324-1329

P Clifton-Bligh had a major role in the design and supervision of this clinical trial, in the reading of the supporting bibliography and in the writing of the manuscript. This paper describes a major randomised double blind placebo controlled trial involving the use of supplementary vitamin D in early post-menopausal women over 2 years. The study was conducted on the assumption that supplemental vitamin D might prevent the loss of bone mineral density which occurs in early post-menopausal women. All study participants were given 1000mg of calcium per day and half were randomly assigned to receive vitamin D2, 10,000 units once weekly. The average age of the women was 56 years. 187 women were enrolled. All patients were selected on the basis that their bone mineral density t-score was above -2. The mean baseline value for the serum 25-OH vitamin D was 82.6nmol/L in the calcium only group and 81.6 nmol/L in the vitamin D treated group. Bone mineral density was measured in the lumbar spine, neck of femur, trochanter and proximal radius at baseline and thereafter at 6 month intervals for 2 years . The serum 25-OH vitamin D fell by 13.4nmol/L in the calcium only group and by 1.1nmol/L in the calcium plus vitamin D group over the two years (P=0.02). The serum 25-OH vitamin D rose by 5.3nmol/L in the first year and fell thereafter. The bone mineral density of the lumbar spine and femoral neck remained stable over the two year period but there was no significant difference between the calcium only group versus the calcium plus vitamin D group. The bone mineral density of the proximal radius fell significantly in both groups and the fall was not significantly different between the calcium only group and the calcium plus vitamin D group.

This paper is considered to be a significant contribution to the discussion as to whether supplemental vitamin D is effective in preserving bone mineral density in early postmenopausal women. The women had normal baseline serum 25-OH vitamin D levels and bone mineral densities above a t score of -2 .The women were studied in a randomised, placebo controlled format and the huge diligence required in the recruitment and the collection of data in this large group of women, 187 in number, should be emphasised.

Citations.

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The data in this paper is incorporated in the MSc thesis of L Cooper. This study was planned and supervised by P Clifton-Bligh. The analysis of some of the data, the interpretation of the data, the literature search, the writing of the paper and liaison with the publishing editors was carried out by P Clifton-Bligh.

Vitamin D supplementation and bone mineral density in early postmenopausal women[1-3]

Lucy Cooper, Phillip B Clifton-Bligh, M Liza Nery, Gemma Figtree, Stephen Twigg, Emily Hibbert, and Bruce G Robinson

ABSTRACT

Background: Increased vitamin D intake may preserve or increase bone mineral density (BMD) in older persons.

Objective: A 2-y double-blind study was undertaken to determine whether weekly administration of 10 000 units of vitamin D_2 maintained or increased BMD in younger postmenopausal women more efficiently than did calcium supplements alone.

Design: One hundred eighty-seven women who were ≥ 1 y postmenopausal were randomly assigned to take either 1000 mg Ca/d after the evening meal or 1000 mg Ca/d plus 10 000 U vitamin D_2/wk in a double-blind, placebo-controlled format. The BMD of the proximal forearm, lumbar spine, femoral neck, Ward's triangle, and femoral trochanter was measured at 6-mo intervals by osteodensitometry.

Results: During the 2-y period, there was no significant difference in the change in BMD at any site between the subjects taking calcium supplements and those taking calcium plus vitamin D_2. Both groups significantly ($P < 0.005$) gained BMD in Ward's triangle and the femoral trochanter but significantly ($P < 0.005$) lost bone in the proximal radius. There was no significant change in the lumbar spine or femoral neck BMD.

Conclusion: In younger postmenopausal women ($\bar{x}$ age: 56 y) whose average baseline serum 25-hydroxyvitamin D concentration was well within the normal range, the addition of 10 000 U vitamin D_2/wk to calcium supplementation at 1000 mg/d did not confer benefits on BMD beyond those achieved with calcium supplementation alone.   *Am J Clin Nutr* 2003;77:1324–9.

KEY WORDS   Menopause, vitamin D, bone density, postmenopausal women

INTRODUCTION

Bone mineral density (BMD) declines in women with the onset of menopause. There is both a reduction in the efficiency of absorption of calcium from the diet and an increased rate of bone resorption attributed to a decrease in serum estrogen, and the associated decrease in BMD may be accompanied by an increased risk of fracture due to minimal trauma. Studies have been reported in which additional calcium has been given by mouth in an attempt to overcome the negative calcium balance and reduce bone calcium loss (1–7). Variable results have been achieved with calcium supplementation, depending on concurrent dietary calcium intake, the number of years after menopause, the type of calcium used, and the bone site studied.

Serum parathyroid hormone (PTH) increases with age (8) and serum 25-hydroxyvitamin D [25(OH)D] declines with age (9, 10), and there is an inverse correlation between serum 25-hydroxyvitamin D_3 [25(OH)D_3] and serum PTH concentrations in older patients (11–15). Vitamin D deficiency is thought to contribute to bone loss in women (16, 17). In several studies, vitamin D has been given to postmenopausal women on the assumption that increases in concentrations of serum PTH may be suppressed and the rate of BMD loss slowed (9, 15, 18–20). Peacock et al (21) gave women ($\bar{x}$ age: 73.7 y) supplements containing 750 mg Ca/d, 15 µg 25(OH)D_3/d, or placebo. They found that supplemental calcium was more powerful than was 25(OH)D_3 in reducing the rate of BMD loss from the total hip, although the effect of calcium was greater when the serum 25(OH)D concentrations were lower. Hunter et al (22) gave 800 U of vitamin D_3/d for 2 y to postmenopausal monozygotic twins whose average age was 59 y. The change in the BMD of the spine and the neck of femur did not differ significantly between the placebo-treated group and the vitamin D_3–treated group. Vitamin D supplementation may increase BMD in older patients when the initial serum 25(OH)D concentration is low.

The present study examined the effects of vitamin D_2 supplementation on changes in BMD in younger ($\bar{x}$ age: 56 y) postmenopausal women who were also given 1000 mg Ca/d and compared those changes with the changes in BMD in women given 1000 mg Ca/d only. This study was undertaken and completed before the work of Hunter et al (22) and confirms that study's published findings.

SUBJECTS AND METHODS

Subjects

Healthy white women who were postmenopausal by 1–10 y and who were not receiving hormone replacement therapy were recruited through media advertisements. All study participants

were assessed by means of a medical history questionnaire. Subjects with malignant disease and those with a renal, hepatic, endocrine, or gastrointestinal disorder associated with abnormal calcium metabolism were excluded. Subjects who had used estrogen, progesterone, glucocorticoids, anticonvulsants, thiazide diuretics, vitamin D supplements, or other medications known to affect calcium or bone metabolism in the previous 12 mo were also excluded. Subjects with laboratory evidence of renal, hepatic, or endocrine disorder; a serum follicle-stimulating hormone concentration <40 mIU/mL, or BMD at any site ±2 SD from the mean for subjects matched for age were also excluded. One hundred eighty-seven women met all entry criteria and were enrolled in the study.

Study protocol

In this 2-y double blind, placebo-controlled study, all subjects received 1000 mg Ca/d and were randomly assigned to receive either placebo or 10 000 IU vitamin D_2 once a week. At the beginning of the study, subjects were assessed by physical examination. Medical, social, dietary, and exercise histories were recorded, and subjects were advised to report any significant variations to lifestyle during the study. Blood and urine were collected for the measurement of variables to assess bone metabolism, and BMD was measured at 6 sites to assess appendicular as well as axial changes to the skeleton. Subjects were seen 1 mo later to obtain blood for serum calcium and follicle-stimulating hormone measurement and then every 6 mo for the duration of the study. At each of the 6-mo visits, relevant medical problems were recorded and investigated as necessary, blood and urine were collected, BMD measurements were performed, and treatment compliance was assessed by tablet counts and diary review.

The Royal North Shore Hospital Ethics Committee approved the study protocol. All subjects provided written informed consent.

Supplements

Two calcium carbonate tablets (Cal-Sup; 3M Pharmaceutical, Sydney, Australia) each containing the equivalent of 500 mg elemental calcium were taken at bedtime. Vitamin D_2 (Ostelin; Boots Healthcare Pharmaceuticals, Sydney, Australia) was prepared in 2 batches; one was supplied at the beginning of the study and the other at the halfway point. The tablets were stored in lightproof containers at room temperature and underwent stability testing by the manufacturer immediately before supply. A certificate of analysis was issued with each batch. Placebo tablets consisted of lactose, microcrystalline cellulose, magnesium stearate, and coloring agents. The vitamin D_2 or placebo was taken every Sunday evening at bedtime.

Compliance and completion rate

Of the 187 women enrolled [94 receiving calcium (Ca group) and 93 receiving calcium and vitamin D (Ca+D group)], 153 completed the study—80 (85%) in the Ca group and 73 (78.5%) in the Ca+D group. Of those who withdrew, 12 did so for personal reasons (eg, family crisis, moving away, and lack of commitment), 6 developed unrelated intercurrent illness (eg, systemic lupus erythematosus or malignancy) and were thus disqualified on the basis of the study protocol, 2 had BMD measurements that fell >2 SD below the mean for age during the course of the study, and 6 developed symptoms necessitating hormone replacement therapy. Nine subjects withdrew as a result of treatment-related side effects: 5 had constipation or

abdominal discomfort, 2 were intolerant of the taste of the calcium supplement, 1 had a clinical diagnosis of renal calculus, and 1 developed hyperparathyroidism. Eight of these subjects were in the Ca+D group, which thus had significantly ($P = 0.02$) more treatment-related withdrawals. The mean (± SD) rate of compliance with treatment was 98.2 ± 6.1% for the Ca+D group and 97.7 ± 5.4% for the Ca group.

Bone density measurements

BMD was measured at 6-mo intervals at the lumbar spine, neck of femur, trochanter, Ward's triangle, proximal radius and ulna, and proximal radius with the use of a dual-energy X-ray absorptiometer (XR26; Norland Corp, Fort Atkinson, WI). External and internal calibrations were performed daily with the use of a hydroxyapatite phantom embedded in perspex (Norland Corp). The CVs were 1.0% for lumbar spine, 1.2% for neck of femur, 1.8% for trochanter, 5.3% for Ward's triangle, 0.8% for proximal radius and ulna, and 0.9% for proximal radius. There was no long-term drift in the phantom measurements.

Biochemistry

Blood and urine were collected at baseline and then at 6-mo intervals throughout the study, with an additional blood collection at 1 mo, as mentioned previously. Serum 25(OH)D, 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], and osteocalcin concentrations were measured by radioimmunoassay (Incstar Corp, Stillwater, MN). 25(OH)D$_2$ is measured with the same efficiency as 25(OH)D$_3$, and therefore, the value obtained will be the sum of the circulating 25(OH)D$_2$ and 25(OH)D$_3$ concentrations. Serum PTH was measured by in-house radioimmunoassay using polyclonal antibodies against the intact molecule (23). Urinary deoxypyridinoline crosslinks were performed with the use of a competitive enzyme-linked immunoassay (Metra Biosystems Inc, Mountain View, CA) on fasting second-morning voids. The intraassay and interassay CVs were <15.0%. At each 6-mo visit, 24-h urinary samples were collected for calcium and creatinine measurements. Serum analytes were measured on a biochemical analyzer (Boehringer Mannheim 747; Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's recommended methods. Urinary analytes were measured on a Beckman CX-7 Biochemical Analyzer (Beckman Instruments, Fullerton, CA) in accordance with the manufacturer's protocol. Serum and urine analyses were carried out in the Department of Biochemistry, Royal North Shore Hospital.

The height and weight of each participant were measured at each 6-mo visit. Dietary calcium intake was assessed at the outset of the study and again at 1 y by means of a food-frequency questionnaire (24) and subsequent calculation of daily intake. To ensure adequate randomization, sun exposure was calculated with the use of a questionnaire designed to assess time outdoors, activity undertaken, usage of sunscreen and frequency of application, and cloud cover.

Statistical analysis

A power analysis was undertaken before the study so that a change of 2% in BMD over 2 y could be detected with 80% power, with the use of a $P < 0.05$ significance level (two-sided test), provided that 74 persons were included in each arm of the study. Each patient's 6-mo data were used to construct a regression coefficient measuring the annual rate of change for that

TABLE 1

Baseline characteristics of the postmenopausal women supplemented with either calcium (Ca group) or calcium and vitamin D (Ca+D group) for 2 y[1]

	Ca group ($n = 94$)	Ca+D group ($n = 93$)
Age (y)	56.1 ± 4.7^2	56.5 ± 4.2
Years after menopause	5.4 ± 3.0	6.1 ± 2.8
Weight (kg)	67 ± 12	67 ± 11.9
Height (cm)	162.4 ± 5.8	162.4 ± 5.8
Dietary calcium (mg/d)[3]	811.2 ± 324.8	754.4 ± 288.3
Ethanol (g/d)	5.3 ± 9.6	6.4 ± 10
Sun exposure (min/d)	115.8 ± 80	113.7 ± 91.7
Smokers (n)	6	7

[1] There were no significant differences between the groups.

[2] $\overline{x} \pm$ SD.

[3] Dietary calcium was reassessed at 12 mo. The mean ($\pm$SD) dietary calcium in the Ca group was 825.7 ± 358.5 mg/d and that in the Ca+D group was 836.2 ± 393.9 mg/d.

person. The within-patient changes were compared between groups with the use of two-factor repeated-measures analysis of variance with interaction. The P values of the main effects of time and treatment and time-and-treatment interaction were obtained. Bonferroni's correction was used where multiple comparisons were made.

RESULTS

The baseline characteristics are shown in **Tables 1** and **2**. There were no significant differences between the 2 groups. The mean BMD at each site examined in the 2 treatment groups at 5 time points is shown in **Table 3**.

Bone mineral density

When studied through the 2-y studied period, the change in BMD at any of the sites studied did not differ significantly between subjects taking calcium supplements (Ca group) and subjects taking calcium and vitamin D_2 supplements (Ca+D group) (**Table 4**). There were no statistically significant interactions between the effects of time and treatment on the annual percent-

TABLE 2

Baseline values for biochemical variables in the postmenopausal women supplemented with either calcium (Ca group) or calcium and vitamin D (Ca+D group) for 2 y[1]

	Ca group ($n = 94$)	Ca+D group ($n = 93$)
Serum		
Calcium (mmol/L)	2.40 ± 0.10	2.40 ± 0.10
25(OH)D (nmol/L)	82.6 ± 27.0	81.6 ± 24.4
1,25(OH)$_2$D (pmol/L)	93.4 ± 29.7	93.5 ± 31.1
Phosphate (mmol/L)	1.17 ± 0.12	1.17 ± 0.11
PTH (ng/mL)	0.2 ± 0.1	0.2 ± 0.2
ALP (U/L)	89.1 ± 26.3	85.4 ± 18.8
Osteocalcin (ng/mL)	4.3 ± 1.7	4.6 ± 2.4
Urine		
DPYR (nmol/mmol creatinine)	4.7 ± 3.4	4.4 ± 1.7
Ca (mmol/d)	4.4 ± 2.0	4.0 ± 2.0

[1] $\overline{x} \pm$ SD. 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; PTH, parathyroid hormone; ALP, alkaline phosphatase; DPYR, deoxypyridinoline.

age changes from baseline over 2 y. The lumbar spine exhibited no significant percentage changes from baseline over 2 y. The trochanter and Ward's triangle exhibited significantly ($P < 0.005$) positive percentage changes from baseline. The proximal radius and the proximal radius and ulna showed significantly ($P < 0.005$) negative percentage changes over 2 y. The neck of the femur and the proximal radius had significantly ($P < 0.001$ and $P = 0.007$, respectively) different percentage changes from baseline in each year of the study (**Table 5**).

Biochemistry

The baseline indexes did not differ significantly between the 2 groups (Table 2). The analysis of the changes in the biochemical variables is shown in **Table 6**. Only 25(OH)D showed a significant ($P < 0.001$) interaction between the effects of time and treatment on the annual change. The concentrations of 25(OH)D changed significantly ($P < 0.05$, Bonferroni's adjustment for multiple comparisons) in the Ca+D group in both years of the study: in the first year, the mean ($\pm$ SD) concentrations increased by

TABLE 3

Bone mineral density measurements at 5 time points in the postmenopausal women supplemented with either calcium (Ca group) or calcium and vitamin D (Ca+D group) for 2 y[1]

	Baseline	6 mo	12 mo	18 mo	24 mo
			g/cm^2		
Ca group					
Lumbar spine (L2–L4)	0.965 ± 0.160	0.979 ± 0.156	0.974 ± 0.155	0.976 ± 0.163	0.981 ± 0.153
Neck of femur	0.813 ± 0.128	0.819 ± 0.113	0.823 ± 0.114	0.818 ± 0.109	0.840 ± 0.122
Trochanter	0.666 ± 0.099	0.676 ± 0.099	0.683 ± 0.101	0.694 ± 0.100	0.682 ± 0.101
Proximal radius and ulna	0.669 ± 0.078	0.675 ± 0.080	0.670 ± 0.083	0.683 ± 0.080	0.673 ± 0.080
Proximal radius	0.668 ± 0.075	0.669 ± 0.078	0.662 ± 0.081	0.674 ± 0.075	0.662 ± 0.078
Ca+D group					
Lumbar spine (L2–L4)	0.953 ± 0.148	0.966 ± 0.158	0.952 ± 0.148	0.962 ± 0.144	0.955 ± 0.146
Neck of femur	0.808 ± 0.123	0.810 ± 0.118	0.793 ± 0.110	0.810 ± 0.114	0.815 ± 0.108
Trochanter	0.647 ± 0.106	0.659 ± 0.103	0.654 ± 0.105	0.673 ± 0.100	0.658 ± 0.100
Proximal radius and ulna	0.672 ± 0.081	0.676 ± 0.087	0.670 ± 0.087	0.663 ± 0.096	0.670 ± 0.080
Proximal radius	0.671 ± 0.081	0.668 ± 0.085	0.664 ± 0.086	0.661 ± 0.083	0.663 ± 0.080

[1] $\overline{x} \pm$ SD. The number of subjects in the Ca and Ca+D groups, respectively, at the 5 time points were as follows: baseline, 94 and 93; 6 mo, 89 and 80; 12 mo, 84 and 74; 18 mo, 81 and 73; and 24 mo, 80 and 73.

TABLE 4
Annual rate of change in bone mineral density in the postmenopausal women supplemented with either calcium (Ca group) or calcium and vitamin D (Ca+D group) for 2 y[1]

	Ca group (n = 80)	Ca+D group (n = 73)
	%	
Lumbar spine (L2–L4)	0.24 ± 2.35	0.15 ± 2.49
Neck of femur	0.59 ± 2.06	0.26 ± 2.14
Trochanter	1.11 ± 2.41[2]	1.26 ± 2.99[2]
Ward's triangle	1.40 ± 4.18[2]	1.40 ± 4.20[2]
Proximal radius and ulna	−0.13 ± 4.06[3]	−0.67 ± 2.64[3]
Proximal radius	−0.73 ± 1.81[3]	−0.86 ± 1.60[3]

[1] $\bar{x} \pm$ SD. There were no statistically significant differences between annual percentage changes from baseline between the Ca and Ca+D groups in the 2-y study.

[2] Significant changes from baseline, $P < 0.005$.

[3] Significantly negative changes from baseline over 2 y, $P < 0.005$.

5.3 ± 18.1 nmol/L, and in the second year, they decreased by 6.4 ± 5.6 nmol/L. In contrast, the Ca group showed a steady decrease over the 2 y at a significant ($P < 0.05$, Bonferroni's adjustment for multiple comparisons) average annual rate of −6.7 ± 0.7 nmol/L. These data are shown graphically in **Figure 1**. Of the remaining variables, PTH, alkaline phosphatase (ALP), osteocalcin, urinary calcium (UCa), and serum calcium showed no statistically significant difference between the changes observed in year 1 and year 2. ALP, UCa, and serum calcium decreased significantly with time. There was no significant difference in the concentrations of PTH, ALP, osteocalcin, UCa, or serum calcium between the treatment groups.

There was no significant correlation between the starting serum 25(OH)D concentration and the subsequent change in BMD at any site in either group or any significant correlation between the percentage change in serum 25(OH)D or PTH and the change in BMD at any site in either group.

DISCUSSION

This study was designed to examine the efficacy of vitamin D supplementation, given as vitamin D_2 in a single dose of 10 000 U/wk, in maintaining BMD in the early postmenopausal period. The study found that there was no significant additional benefit to BMD in either the axial or appendicular skeleton of early postmenopausal women when vitamin D_2 in addition to calcium

was given, rather than calcium alone. A trial by Komulainen et al (25) examined healthy, early postmenopausal ($\bar{x}$ 1.2 y after menopause) women and failed to detect any significant benefit to BMD of daily supplementation with small amounts (300 IU) of vitamin D. In contrast, studies in elderly populations have shown supplements of vitamin D to be beneficial to BMD. Chapuy et al (18) gave 800 IU vitamin D/d and 1000 mg Ca/d to elderly women whose average serum 25(OH)D concentration before treatment was 40 nmol/L, and they found a significant increase in femoral neck BMD and a reduction in the rate of fracture. It is not clear in this study whether the beneficial effect is from the calcium or from the vitamin D supplementation. Ooms et al (19) gave 400 IU vitamin D_3/d to women with an average age of 80.1 y. The serum 25(OH)D concentration increased from 27 nmol/L to 62 nmol/L, and the femoral neck BMD increased significantly (1.8%) in the first year and by an additional 0.2% in the second year. The effects were independent of the serum 25(OH)D concentration at baseline. Dawson-Hughes et al (20) gave 70-y-old women 700 U vitamin D and calcium for 3 y. After one year of treatment, there was significantly less loss of total BMD and of spinal BMD but no change in femoral neck BMD. The baseline serum 25(OH)D concentration in the women in that study was 70.3 nmol/L, which is not very different from the baseline serum 25(OH)D concentration in the present study, but it increased to 109.7 nmol/L with 700 U vitamin D_3/d supplementation. As in the study by Ooms et al (19), most of the effects in the study by Dawson-Hughes et al (20) were seen within the first year of treatment. Adams et al (15) studied 12 women ($\bar{x}$ age: 60 y) with a baseline serum 25(OH)D concentration of 25.1 nmol/L who received 500 000 U vitamin D_2 over a 5-wk period, which is the same dose that our patients received over a 12-mo period. In that study, the serum PTH concentration was reduced by 32.9 pg/mL, and the spine and femoral neck BMD increased by 4–5% per year; these findings support the concept that vitamin D administration will increase BMD in persons whose initial serum 25(OH)D concentrations are much lower than those found in our patient group. In a randomized double-blind protocol, Peacock et al (21) gave women ($\bar{x}$ age: 73.7 y) either 750 mg Ca/d, 15 μg 25(OH)D_3/d, or placebo. The mean baseline serum 25(OH)D concentration was 65 nmol/L, and the concentration increased to 118 nmol/L with the 25(OH)D treatment. The effect of calcium supplements on reducing the loss of BMD from the total hip was greater than that seen when 25(OH)D_3 was given, and the effect of supplemental calcium was greater when the initial serum 25(OH)D_3

TABLE 5
Annual rate of change in bone mineral density in the postmenopausal women during years 1 and 2 of supplementation with either calcium (Ca group) or calcium and vitamin D (Ca+D group)[1]

	Year 1		Year 2		
	Ca group (n = 84)	Ca+D group (n = 74)	Ca group (n = 80)	Ca+D group (n = 73)	P[2]
	%		%		
Lumbar spine (L2–L4)	0.44 ± 4.20	−0.19 ± 4.13	0.59 ± 3.58	0.75 ± 3.73	0.6
Neck of femur	−0.40 ± 3.72	−1.81 ± 3.90[3]	2.15 ± 4.47	3.09 ± 4.90	<0.001
Trochanter	1.53 ± 4.09	1.31 ± 5.03	-0.02 ± 4.15	0.92 ± 5.17	0.5
Ward's triangle	2.72 ± 9.32	1.17 ± 7.58	1.89 ± 10.37	2.23 ± 7.86	0.6
Proximal radius and ulna	0.94 ± 10.88	−0.64 ± 5.60	−0.19 ± 2.51	0.25 ± 2.26	0.8
Proximal radius	−0.69 ± 3.60	−1.69 ± 2.53	−0.34 ± 2.20	0.01 ± 2.43	0.007

[1] $\bar{x} \pm$ SD. There were no significant interactions between the effects of time and treatment during 2 y.

[2] Comparison of year 1 with year 2 (ANOVA).

[3] Significantly different from Ca group, $P = 0.02$.

TABLE 6
Changes in the serum and urine variables relevant to calcium metabolism in the postmenopausal women supplemented with either calcium (Ca group) or calcium and vitamin D (Ca+D group) for 2 y[1]

	Ca group (n = 80)	Ca+D group (n = 73)	P Treatment	P Time × treatment
Serum				
Calcium (mmol/L)	−0.02 ± 0.10	−0.04 ± 0.10	0.6	0.7
25(OH)D (nmol/L)	−13.4 ± 23.70	−1.10 ± 21.30	0.02	<0.001
1,25(OH)$_2$D (pmol/L)	−1.10 ± 41.80	−4.50 ± 43.80	0.6	0.4
Phosphate (mmol/L)	−0.04 ± 0.12	−0.04 ± 0.14	0.5	0.2
PTH (ng/mL)	−0.004 ± 0.18	−0.02 ± 0.23	0.3	0.4
ALP (U/L)	−4.50 ± 13.20	−7.80 ± 13.10	0.4	0.2
Osteocalcin (ng/mL)	0.50 ± 2.10	0.02 ± 2.60	0.8	0.5
Urine				
DPYR (nmol/mmol creatinine)	−0.24 ± 3.50	0.10 ± 2.10	0.03	0.5
Calcium (mmol/d)	−0.60 ± 1.60	−0.50 ± 1.70	0.2	0.8

[1]$\bar{x}$ ± SD. 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; PTH, parathyroid hormone; ALP, alkaline phosphatase; DPYR, deoxypyridinoline.

concentration was low, presumably because a greater decrease in serum PTH occurred in this situation.

In a study by Lips et al (13) of elderly (aged 81–84 y), the initial serum 25(OH)D concentration was inversely correlated with the serum PTH concentration, and there was an inverse relation between the change in serum 1,25(OH)D and the pretreatment serum 25(OH)D after supplementation with vitamin D$_3$ (13). In the older women in that study, vitamin D$_3$ supplements decreased the serum PTH, whereas in our study, the serum PTH concentration actually increased during the first

FIGURE 1. Serum 25-hydroxyvitamin D [25(OH)D] concentrations in the postmenopausal women at baseline (year 0) and during years 1 and 2 of supplementation with either calcium (Ca group; ▣) or calcium and vitamin D (Ca+D group; ▣). In the Ca+D group, concentrations increased 5.3 ± 18.1 nmol/L ($\bar{x}$± SD; P < 0.05) in the first year and then decreased 6.4 ± 15.6 nmol/L (P < 0.05) in the second year. In contrast, concentrations in the Ca group decreased significantly (P < 0.05, Bonferroni's adjustment for multiple comparisons) during the 2-y study at an average annual rate of 6.7 ± 20.7 nmol/L. SEs for the Ca group were 3.039 for year 0 (n = 94), 2.858 for year 1 (n = 84), and 2.849 for year 2 (n = 79); those for the Ca+D group were 3.192 for year 0 (n = 93), 3.002 for year 1 (n = 75), and 2.164 for year 2 (n = 72). There was a significant (P < 0.001) interaction between the effects of time and treatment.

year of treatment with vitamin D$_2$, a change in our study that is not explained. Thomas et al (26) also found that, in hospitalized patients, the serum PTH concentration was significantly higher when the serum 25(OH)D concentration was < 37.5 nmol/L. Malabanan et al (27) found that, when the baseline serum 25(OH)D concentration was < 50 nmol/L, the serum PTH concentration increased and then fell significantly when 50 000 U vitamin D$_2$ was given weekly for 8 wk. It is interesting that the serum PTH did not decrease if the baseline serum 25(OH)D had been > 50 nmol/L.

In the design of the present study, it was thought that a weekly dose of 10 000 U vitamin D$_2$ given for 2 y would provide a reasonable supplement of vitamin D without causing adverse effects and would allow a conclusion as to whether supplementation of younger postmenopausal women with vitamin D could be associated with a preservation of or increase in BMD. An unexpected finding was the small increase in the serum 25(OH)D concentration at the end of the first year after weekly 10 000 U vitamin D$_2$ supplementation. At that time, the difference in serum 25(OH)D concentration between the Ca and the Ca+D groups was 12.0 nmol/L, and, at 2 y, it was 12.3 nmol/L. Lips et al (13), however, found that in older women (aged 81–84 y) supplemented with vitamin D$_3$, the increment in the serum 25(OH)D concentration was no greater with a daily supplement of 800 U vitamin D$_3$ than with that of 400 U vitamin D$_3$. In a subsequent study (14), the increase in serum 25(OH)D after 400–600 U vitamin D was given daily depended on the initial serum 25(OH)D concentration. When the baseline serum 25(OH)D concentration was < 25, 25–50, and > 50 nmol/L, the increments in serum 25(OH)D were 58.4, 39.4, and 12.5 nmol/L, respectively.

In the present study, the initial serum 25(OH)D concentration was 81 nmol/L and the increment with weekly supplementation with 10 000 U vitamin D$_2$ in the first year was 5.3 nmol/L, which implies that there are mechanisms that accelerate the metabolic clearance of vitamin D when concentrations of serum 25(OH)D in the blood begin to rise. This mechanism may accelerate with time because, during the second year of supplementation, the serum 25(OH)D concentration actually decreased toward baseline again. In the study by Hunter et al (22), the serum 25(OH)D concentration decreased in the supplemented patients between 6 mo and 24 mo of treatment.
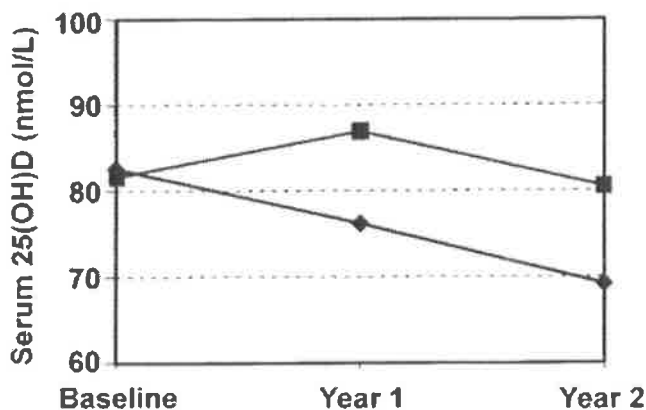
These observations led to further consideration of the controversial concept of what constitutes the vitamin D–replete state, which has been defined in terms of simultaneous serum PTH concentrations. For example, Thomas et al (26) found that, in an inpatient population study, the serum PTH began to rise when the serum 25(OH)D was < 37.5 nmol/L. Gloth et al (12) found that the serum PTH was below the upper limit of normal if the serum 25(OH)D was > 40 nmol/L. However, Dawson-Hughes (20) found that a nadir of suppression of serum PTH occurred when the serum 25(OH)D was 110 nmol/L, and the decrease in the serum PTH (remaining within the normal range) was achieved by giving small amounts of vitamin D to postmenopausal women in winter which reduced bone loss (16).

In any case, in our study, increments in the serum 25(OH)D concentration to > 80 nmol/L did not lead to better preservation of BMD than did calcium supplements alone. It is possible that greater effects on BMD would have been achieved if the serum 25(OH)D concentration had been sustained at a higher value, but this would seem unlikely because, in the study of Peacock et al (21), the achievement of serum concentrations of 25(OH)D of 118 nmol/L caused a smaller reduction in BMD loss than was seen when calcium alone was given.

In summary, the present study failed to show any additional benefit in preservation of BMD in postmenopausal women ($\bar{x}$ age: 56.1 y) when 10 000 U vitamin D$_3$/wk was added to daily calcium supplementation of 1000 mg. The use of calcium alone over a 2-y period was associated with no significant loss in BMD in the spine or femoral neck, a significant gain in BMD in the femoral trochanter and Ward's triangle, but a significant loss in BMD in the proximal radius and ulna.

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Paper 21. Fibroblast growth factor 23. A new clinical marker for oncogenic osteomalacia. AE Nelson, R Clifton-Bligh, M Mirams, A Gill, A Au, A Clarkson, H Juppner, R Ruff, P Stalley, R Scolyer, BG Robinson, RS Mason, P Clifton-Bligh. Journal of Clinical Endocrinology and Metabolism 2003; 88:4088-4094

P Clifton-Bligh made a significant contribution to this paper with respect to the assessment, investigation, treatment and follow up of the patient described. All investigations were initiated and supervised by P Clifton-Bligh. The patient presented with multiple rib fractures after exercise in the gym. A soft 2cm lump was noted in the lateral aspect of the thigh. Whole body scintigraphy confirmed multiple fractures as well as abnormal uptake in the right superior pubic ramus.Biochemistry showed a low serum phosphate and inappropriately low normal serum 1,25-OH vitamin D. Excision biopsy of the left thigh lump showed a benign angiolipoma. CT guided biopsy of the right superior pubic ramus was non-diagnostic. He was treated with calcitriol and the serum phosphate increased but tubular reabsorption of phosphate remained low. An MRI of the right superior pubic ramus 18 months after presentation showed a 2.7cm lesion. The tumour was removed *en bloc.* A trephine bone biopsy of the iliac crest after tetracycline labelling showed the features of osteomalacia. Calcitriol was discontinued before surgery. By 5 days after surgery the serum 1,25-OH vitamin D had risen to 408pmol/L and the serum phosphate had risen to normal, 1.71mol/L. He became free of pain and the serum phosphate remained normal 12 months after surgery. The tubular maximum for phosphate absorption became normal The histopathology of the removed tumour was described as a fibrohistiocytic giant cell tumour. The serum FGF-23 was elevated before surgery and was normal 5 days after surgery. Serum from the patient taken before surgery inhibited phosphate uptake by OK3B2 cells in culture. Positive staining for FGF-23 protein by immunohistochemistry was demonstrated in the giant cell tumour but not in the angiolipoma. Expression of FGF-23 mRNA and FGF-23 protein was demonstrated in the giant cell tumour. No mutations were found in the FGF-23 DNA amplified from tumour cells. Both the serum phosphate and the serum1,25-OH vitamin D rose rapidly after removal of the tumour suggesting that the humoral factor produced by the tumour independently regulated both phosphate transport and 1,25-OH vitamin D metabolism.

This paper is considered to be a significant contribution to the literature about oncogenic osteomalacia especially as measurements of FGF-23 have been made in the serum, and FGF-23 mRNA and protein have been detected in the tumour and that the patient's serum collected before surgical removal of the giant cell tumour inhibited phosphate uptake by OK3B2 cells.

CLINICAL CASE SEMINAR

Fibroblast Growth Factor 23: A New Clinical Marker for Oncogenic Osteomalacia

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The phosphate-wasting condition, oncogenic osteomalacia, is problematic to diagnose and manage clinically due to difficulty in locating the causative tumor. Fibroblast growth factor 23 (FGF23) has recently been implicated in the pathogenesis of oncogenic osteomalacia. In this case the patient presented with clinical features typical of oncogenic osteomalacia. Removal of an angiolipoma from the thigh did not correct the clinical or biochemical abnormalities. Subsequent identification and removal of a benign giant cell tumor in the pubic ramus, however, did result in normalization of his symptoms and signs. Positive staining for FGF23 protein by immunohistochemistry was demonstrated in the giant cell tumor, but not in the angiolipoma. The serum concentration of FGF23 was elevated in preoperative serum, then normalized after removal of the giant cell tumor. Expression of both FGF23 mRNA and protein was demonstrated in the giant cell tumor tissue, and FGF23 mRNA expression and renal phosphate uptake inhibitory activity were also detected in cultured giant cell tumor cells. This case provides further evidence for the involvement of FGF23 in the pathogenesis of oncogenic osteomalacia and for the utility of serum FGF23 measurement and immunohistochemical detection of FGF23 in the diagnosis and clinical management of this condition (*J Clin Endocrinol Metab* 88: 4088–4094, 2003)

ONCOGENIC OSTEOMALACIA IS a condition that commonly presents clinical difficulties in diagnosis and management. The condition is characterized by osteomalacia due to renal phosphate wasting and low serum concentration of 1,25-dihydroxyvitamin D occurring in the presence of a tumor that, if located and completely removed, results in rapid resolution of the symptoms and signs (reviewed in Ref. 1). The causative tumor may be small, slow growing, and clinically unapparent. Furthermore, these tumors have occurred in many different locations, often making their detection difficult. This clinical problem may now be assisted by investigations for the recently described fibroblast growth factor 23 (FGF23).

FGF23 was originally identified as the gene mutated in autosomal dominant hypophosphatemic rickets (ADHR) (2) and was also independently isolated by homology searching as a novel FGF expressed in the thalamus (3). ADHR is characterized by defective bone mineralization caused by renal phosphate wasting that results in hypophosphatemia and by inappropriately normal 1,25-dihydroxyvitamin D serum concentrations (4). The identification of mutations in

FGF23 that segregated with the disease in affected individuals with ADHR suggested a role for this gene in phosphate homeostasis (2). The mutations identified in ADHR occur in a consensus proteolytic cleavage site and result in a mutant FGF23 protein that is resistant to degradation (5).

The biochemical phenotype of ADHR is similar to that of the sporadic condition oncogenic osteomalacia (OOM). There is evidence that the tumors responsible for OOM secrete circulating factor(s) that results in renal phosphate wasting and abnormal vitamin D metabolism (reviewed in Ref. 1). Inhibition of renal phosphate reabsorption has been demonstrated *in vitro* by ourselves and other groups using conditioned medium from cultured OOM tumor cells (6–10). Several genes have recently been reported that are overexpressed by tumors responsible for OOM including FGF23 (11–13), thereby implicating FGF23 in the pathogenesis of this phosphate-wasting condition.

In this study FGF23 was examined in a patient who presented with symptoms and signs typical of OOM. The causative tumor was finally located and surgically removed, resulting in rapid resolution of both the patient's symptoms and biochemical abnormalities, which is characteristic of OOM. FGF23 was measured in pre- and postoperative serum, and expression of FGF23 mRNA and protein was examined in the tumor and in cultured tumor cells. The results

support the proposal that FGF23 is expressed by OOM tumors and that elevated circulating concentrations of FGF23 may be responsible for the renal phosphate wasting and abnormal vitamin D metabolism in OOM. Furthermore, the detection of elevated FGF23 in preoperative serum and of FGF23 expression in the causative tumor demonstrates the clinical utility of these tests in the diagnosis of patients with oncogenic osteomalacia.

Materials and Methods

Materials

The OK 3B2 cells were provided by Prof. Heini Murer (Zurich, Switzerland). Tissue culture media and additives were obtained from Life Technologies, Inc. (Gaithersburg, MD) and Trace Biosciences (Melbourne, Australia). The isotope [^{32}P]orthophosphoric acid was obtained from PerkinElmer (Rowville, Australia). Primers for PCR were synthesized by Sigma Genosys (Castle Hill, Australia). Antibodies used for immunohistochemistry and Western analysis were rabbit polyclonal antibodies raised against human [Tyr^{224}]FGF-23$_{225-244}$ amide (14). For immunohistochemistry, target retrieval solution S1699 for antigen retrieval and a biotin-free detection system (EnVision Plus and diaminobenzidene plus chromogen) were obtained from DAKO (Carpenteria, CA).

Cell culture

Cultures of cells from the giant cell tumor (designated FR cells) were established from minced tissue pieces immediately postoperatively and grown in low calcium DMEM as previously described (9). Serum-free conditioned medium was collected from the cells. Serum-free medium collected from cultured skin fibroblasts and unconditioned medium were used as controls.

Phosphate uptake bioassay

The measurement of phosphate uptake was carried out using OK 3B2 cells as previously described (10). The response to conditioned medium was measured after preincubation with confluent OK cells for 20 h and was determined as the percent inhibition of the uptake by cells incubated with control unconditioned medium. Serum was tested in the assay at concentrations from 5–20% (vol/vol) compared with serum from two age- and sex-matched controls at the same concentration, as previously described (15). Statistical analysis was performed using a two-sample *t* test. As there were four possible comparisons, and to avoid the possible false positive significant results, each *P* value from the *t* test was adjusted by dividing it by 4 according to the Bonferroni's procedure of multiple comparisons.

Amplification of FGF23 from genomic DNA

Genomic DNA was extracted from cultured tumor cells using the Puregene DNA Purification System (Gentra Systems, Minneapolis, MN). Primers that spanned the intron/exon boundaries were used to amplify the three exons of FGF23 with the 5'-3' sequences: FGF23 1F, aatctcagcaccagccactc; FGF23 1R, agatggacaacaagggtgct; FGF23 2F, ggaattggatggcaatgagt, FGF23 2R: cagggtacactgcaaatgga, FGF23 3.1F: ctcaacgccctaagaactgc; FGF23 3.1R, ggtatggggggtgttgaagtg; FGF23 3.2F, tcacttcctggtcagtctgg; FGF23 3.2R, tgctgagggatgggttaaag.

Expression of FGF23 mRNA by RT-PCR

Total RNA was extracted by Tri-Reagent (Sigma-Aldrich Corp., St. Louis, MO). The RNA was reverse transcribed to cDNA using Superscript II RNase H reverse transcriptase (Invitrogen, Groningen, The Netherlands) and oligo(deoxythymidine) primer following the manufacturer's protocol. FGF23 was amplified from cDNA using the forward primer 5'-TACCACCTGCAGATCCACAA-3' and the reverse primer 5'-GTTTGCTGAGGGATGGTTA-3'. Automated sequencing was carried out by the Australian Genome Research Facility (Brisbane, Australia).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections using the rabbit polyclonal anti-FGF-23 antibody. Formalin-fixed (and no decalcified) paraffin-embedded blocks were used. The tissues were sectioned onto positively charged slides (SuperFrost Plus, Menzel-Glaser Freiburg, Germany) and deparaffinized with xylene and alcohol. Nonpressurized water bath antigen retrieval at 97 C for 55 min was employed using target retrieval solution S1699, pH 6. Slides were incubated with the primary antibody for 60 min at a dilution of 1:200. The EnVision Plus, rabbit nonbiotin, detection system, and diaminobenzidene plus chromogen were used according to the manufacturer's protocol for immune complex detection. Strong granular cytoplasmic staining was interpreted as positive whereas a weak diffuse cytoplasmic blush or nuclear staining was interpreted as negative. The slides, including four negative tissue controls (two meningiomas and two schwannomas) and a negative control of the tumor without incubation with the primary antibody, were interpreted by a pathologist blinded to other data.

Western blotting

Tumor lysate was prepared by homogenization in lysis buffer containing Nonidet P-40 and protease inhibitor (Sigma-Aldrich Corp.). The protein concentration was determined using the Bradford protein assay (Bio-Rad, Inc., Hercules, CA); 25–50 µg protein were electrophoresed on 8–16% sodium dodecyl sulfate-polyacrylamide gel (Gradipore, Frenchs Forest, Australia) and electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were probed using the rabbit anti-FGF23 antibody, then incubated with antirabbit immunoglobulin horseradish peroxidase antibody (Amersham Pharmacia Biotech,) and visualized using ECL Plus (Amersham Pharmacia Biotech).

Results

Case reports

A 52-yr-old man was first evaluated when he developed fracture of the right third metatarsal after prolonged walking and multiple rib fractures after gym exercise. His past medical history was unremarkable. He was a nonsmoker, with no relevant family history of illness, and he worked as a physiotherapist. Clinical examination was consistent with the symptomatic fractures, and a 2-cm soft lump was noted in the lateral aspect of the left thigh. Whole body bone scintigraphy confirmed multiple rib fractures as well as increased tracer uptake in both sacroiliac joints and the right superior pubic ramus. Plain radiography of the pelvis demonstrated a small area of lucency in the right superior pubic ramus.

Initial biochemical evaluation revealed hypophosphatemia and inappropriately low normal 1,25-dihydroxyvitamin D concentrations (Table 1). Hyperphosphatasia hyperparathyroidism, hypocalcemia, and low serum 25-hydroxyvitamin D concentrations were also noted (Table 1). Osteodensitometry by DEXA (XR26, Norland, Fort Atkinson WI) showed reduced bone density in both lumbar spine (0.92 g/cm^2; T-score, -1.14) and left femoral neck (0.73 g/cm^2 T-score, -2.22). Whole body scintigraphy with octreotide was negative. Excision biopsy in November 2000 of the soft tissue lesion in the left thigh showed a benign angiolipoma Computed tomography-guided biopsy of the lucent area within the right superior pubic ramus was paucicellular and nondiagnostic. There were no subsequent changes in the patient's biochemistry after removal of the angiolipoma. The patient declined further surgical intervention at that time.

After 1-month treatment with ergocalciferol (vitamin D$_2$ 1000 U daily), he remained symptomatic with left chest wall

TABLE 1. Biochemical measurements in the patient after various treatments

Analyte	At presentation (05 Oct 2000)	After ergocalciferol (05 Jan 2001)	After calcitriol (09 Mar 2001)	After surgery (08 Aug 2002)	Normal range
Phosphate	0.53	0.53	0.75	1.34	0.78–1.43 mmol/liter
Calcium	2.19	2.23	2.25	2.35	2.20–2.55 mmol/liter
PTH	118	118	67	47.1	12–72 pg/ml
25(OH)D	39	100	85	54	42–169 nmol/liter
1,25(OH)$_2$D	38	16	28	116	38–162 pmol/liter
Alkaline phosphatase	156	139	56	70	30–115 U/liter
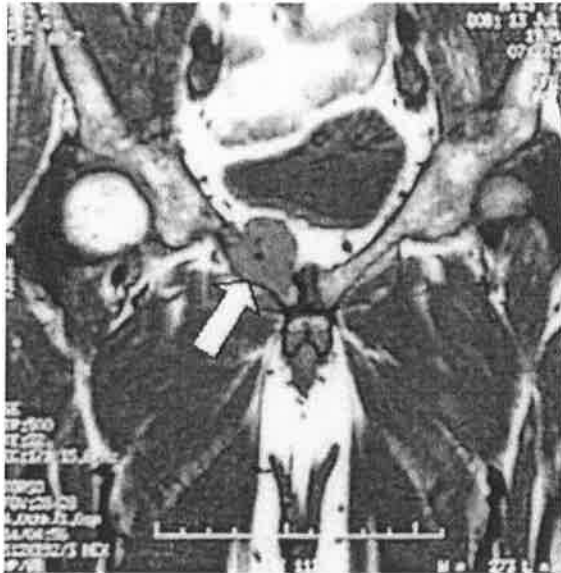
FIG. 1. Magnetic resonance image from patient FR, showing tumor in the right superior pubic ramus. T1-weighted magnetic resonance imaging scan of the pelvis, showing a tumor involving the right superior pubic ramus (*white arrow*) measuring 2.7 × 4 cm. The bulk of the tumor lies on the posterior aspect of the superior pubic ramus, and its medial portion bulged posteriorly and superiorly.

pain, and no significant change in his biochemical parameters occurred other than an increase in 25-hydroxyvitamin D to 100 nmol/liter (Table 1). However, 2 months after commencing calcitriol (1,25-dihydroxyvitamin D$_3$, 0.25 μg twice daily), his bone pain had improved, serum phosphate increased, and serum intact PTH had fallen to be within the normal range (Table 1). Eight months after commencement of calcitriol, the tubular transport maximum for phosphate/glomerular filtration rate was calculated as approximately 0.38 mmol/liter (normal range, 0.8–1.35 mmol/liter).

A magnetic resonance imaging scan of the pelvis performed 18 months after his initial clinical presentation showed growth of the lesion in the right superior pubic ramus, then measuring 2.7 × 2.7 × 4 cm and extending medially within the pelvic cavity to abut the bladder wall (Fig. 1). The tumor was surgically removed *en bloc* with the entire superior ramus. A trephine biopsy obtained from the left iliac crest at the time of surgery, doubly labeled with tetracycline, had features of osteomalacia, including increased osteoid area, surface, and seam width; mineralization lag time could not be calculated due to smearing of the tetracycline label. The patient made an uneventful recovery and was discharged on no medication. Calcitriol had been discontinued preoperatively, and within 5 d postoperatively

serum 1,25-dihydroxyvitamin D had risen to a peak of 408 pmol/liter (normal range, 38–162 pmol/liter), whereas 16 d postoperatively serum phosphate rose to a peak value of 1.71 mmol/liter (normal range, 0.78–1.43 mmol/liter; Fig. 2). Serum 1,25-dihydroxyvitamin D and phosphate concentrations declined into the normal range thereafter. The clinical outcome for the patient after removal of the giant cell tumor has been excellent. At follow-up 6 months after surgery, he had no fractures and no pain even with vigorous exercise, and his serum biochemistry was normal (Table 1). At 12 months follow-up, he remained well, with tubular transport maximum for phosphate/glomerular filtration rate calculated as approximately 1.17 mmol/liter (normal range, 0.8–1.35 mmol/liter).

Histology

The tumor was composed of closely packed oval to plump spindled cells with scattered osteoclast-like giant cells. In some areas an open staghorn (hemangiopericytomatous) vascular pattern was evident. Some osteoid was present and there was focal infiltration of extraskeletal adipose tissue. The mitotic rate was 2–3/10 high power fields. Areas of secondary aneurysmal bone cyst formation were noted. After review by multiple pathologists and radiological correlation, the preferred diagnosis was fibrohistiocytic variant of giant cell tumor of bone. Electron microscopy supported the impression of fibrohistiocytic differentiation.

Measurement of FGF23 and bioactivity in patient serum

FGF23 was measured by C-terminal ELISA (Immutopics Inc., San Clemente, CA). In preoperative serum, FGF23 was elevated at 477 reference units (RU)/ml (normal range, 21 ± 11SD RU/ml). The day following surgery to remove the giant cell tumor, the FGF23 serum concentration fell to 40 RU/ml and 5 d postoperatively it was 22 RU/ml. Serum from the patient was tested in the renal phosphate uptake assay and compared with age- and sex-matched controls. At a 20% concentration, significant inhibition of renal phosphate uptake by about 30% was detected compared with control serum in response to prelipoma removal serum (November 2000) and in response to pre-giant cell removal serum, and was not significantly detected in serum 5 d post-giant cell tumor removal. Inhibition of phosphate uptake by diluted preoperative serum is shown in Fig. 3, which is representative of four separate experiments.

Genomic sequence of FGF23 and cytogenetic analysis of cultured tumor cells

The three exons of FGF23 were amplified from genomic DNA extracted from cultured FR giant cell tumor cells, and

FIG. 2. Time course of perioperative biochemical changes. Perioperative serum concentrations of 1,25-dihydroxyvitamin D (£), phosphate (Œ), and PTH (▫). After surgical excision of the giant cell tumor on March 21, 2002 (*arrow*), serum 1,25-dihydroxyvitamin D and then phosphate concentrations increased within days. Normal ranges: 1,25-dihydroxyvitamin D, 38–162 pmol/liter; phosphate, 0.78–1.43 mmol/liter; PTH, 12–72 pg/ml.
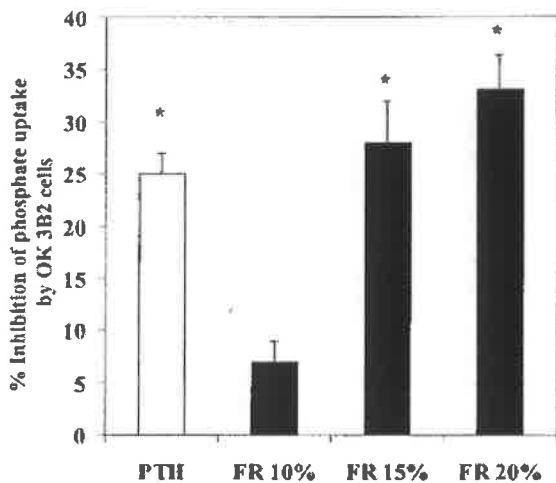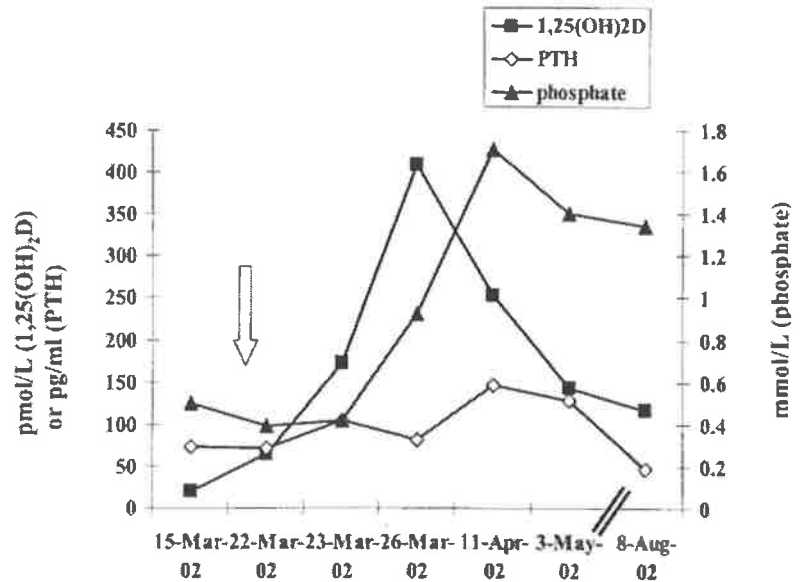

FIG. 3. Inhibition of phosphate uptake by renal OK 3B2 cells by preoperative serum from patient FR. Phosphate uptake was measured in triplicate in OK 3B2 cells after preincubation of confluent cells for 20 h with heat-inactivated prelipoma removal serum (November 2000) at a 5–20% (vol/vol) concentration and was corrected for total protein content per well. Inhibition in response to FR serum was calculated as the percent inhibition of uptake by cells incubated with the same concentration of control serum. The response to PTH (100 ng/ml) is shown as the control. *, Significantly different from vehicle control or control medium ($P < 0.05$).

the products were sequenced. No mutations were detected in FGF23 amplified from FR tumor cells; specifically, those mutations previously described in ADHR (involving nucleotides encoding arginines at codons 176 and 179) were absent. A polymorphism previously reported (2) was found in FGF23 exon 3 from both the tumor DNA and genomic DNA extracted from peripheral blood leukocytes. This C to T nucleotide variation is predicted to change threonine to methionine at codon 239 (T239M) in the coding sequence of FGF23.

The three exons of FGF23 were amplified from cultured OOM tumor cells from two other patients we have previously reported (9, 15), and the sequence of FGF23 was iden-

tical to the wild-type sequence (GenBank accession no AF262537). Specifically, no mutations were detected ir FGF23 amplified from OOM tumor cells in the [176]RHTR[17] sequence.

Cytogenetic analysis of metaphase spreads prepared from cultured FR cells detected no structural or numerical chromosome abnormalities.

Expression of FGF23 mRNA in FR tumor and detection of FGF23 protein by Western blotting and immunohistochemistry

RNA was extracted from fresh-frozen FR giant cell tumor FGF23 mRNA expression was demonstrated by RT-PCR (Fig 4A). A product of the predicted size of 649 bp was amplifiec by PCR from cDNA reverse transcribed from the RNA, anc the identity of the product was confirmed by sequencing.

Western blotting analysis of FR tumor extract using anti-FGF23 antibody detected an immunoreactive protein of approximately 32 kDa (Fig. 4B). The tumor showed widespreac positivity for FGF-23 by immunohistochemistry (Fig. 5A) Anti-FGF23 staining was cytoplasmic and distinctly granular, with some perinuclear accentuation. Numerous positive cells were found in every high powered field. In experiment: using an antibody raised in a different animal, positive staining was markedly reduced by preincubation of the antibody with peptide. Staining of the tumor with secondary antibody alone was negative. Anti-FGF23 staining was absent in four control tumor samples (two meningiomas and two schwannomas). The angiolipoma removed from the patient 1£ months earlier did not stain with the anti-FGF23 antibody although the high percentage of fat cells made interpretatior difficult.

Expression of FGF23 mRNA in cultured tumor cells

Expression of FGF23 mRNA was detected in RNA extracted from cultured FR tumor cells (Fig. 6) that had beer maintained for up to 6 wk and up to two passages in culture RNA from HEK293 cells was used as a positive control for
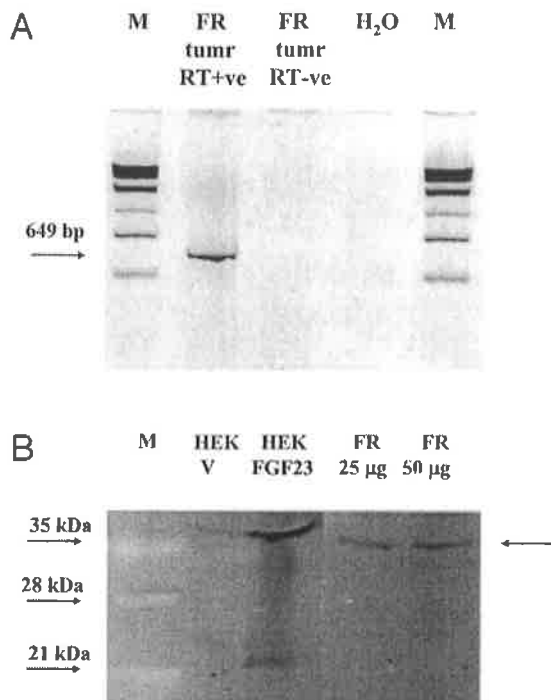
FIG. 4. Expression of FGF23 mRNA and protein in FR tumor. A, *FGF23* was amplified by RT-PCR from RNA extracted from FR tumor tissue using primers that spanned from exons 1–3 of FGF23. Controls without reverse transcriptase (RT−) and no cDNA (H_2O) were negative. B, Western analysis of FR tumor lysate using anti-FGF23 antibody. Analysis of 25 and 50 μg FR tumor lysate showed an immunoreactive band of approximately 32 kDa. Media from HEK 293 cells transiently transfected with FGF23 or vector were used as controls.

FGF23 mRNA expression (16). The identity of the 649-bp product was confirmed by sequencing.

Bioactivity of conditioned media from cultured tumor cells

Serum-free conditioned media from cultured FR cells significantly inhibited renal phosphate uptake by OK 3B2 cells compared with control unconditioned medium (Fig. 7), whereas there was no significant inhibition in response to serum-free fibroblast conditioned medium. Immunoassays for PTH and PTHrP detected no significant immunoreactivity in FR cell-conditioned medium that inhibited phosphate uptake by renal OK 3B2 cells.

Discussion

The clinical management and confirmation of the diagnosis of oncogenic osteomalacia in this case presented difficulties that commonly occur in patients with this phosphate-wasting disorder (15, 17, 18). Although the clinical presentation was suggestive of oncogenic osteomalacia, the removal of the initial tumor located, a benign angiolipoma in the left thigh, did not correct the clinical or biochemical abnormalities in this patient. A second tumor was identified within the right superior pubic ramus 18 months after initial presentation. Removal of this benign giant cell tumor resulted in rapid normalization of the patient's symptoms and signs. In this case, as reported previously (15, 19, 20), mag-
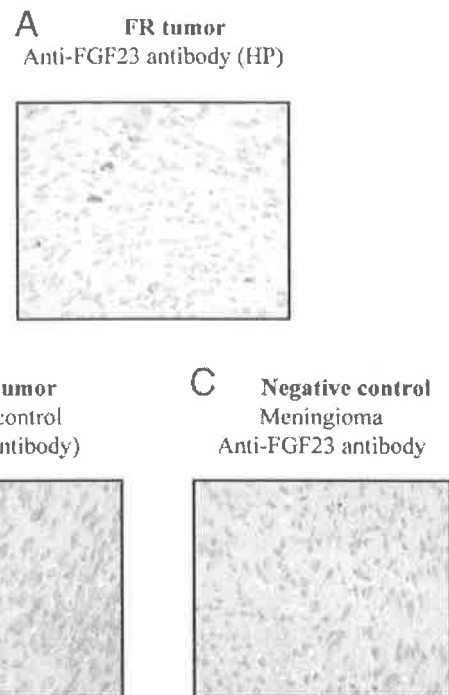

FIG. 5. Immunohistochemistry of FR tumor and negative control using anti-FGF23 antibody. Formalin-fixed paraffin-embedded sections were used, deparaffinized, then incubated with primary antibody EnVision plus and diaminobenzidene plus chromogen were used for immune complex detection. A, Positive staining of FR tumor with anti-FGF23 antibody under high power. Staining was cytoplasmic and granular, with some perinuclear accentuation. B, Negative staining of FR tumor incubated without primary anti-FGF23 antibody under high power. C, Negative staining of control meningioma tumor with anti-FGF23 antibody under high power.
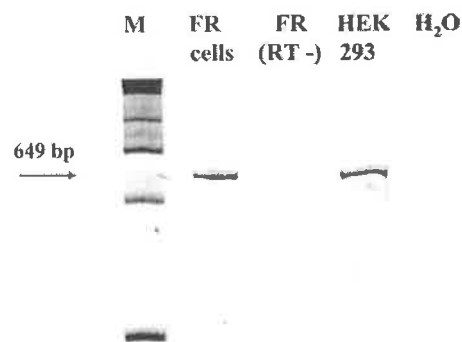

FIG. 6. Expression of FGF23 mRNA in cultured FR tumor cells. FGF23 was amplified by RT-PCR from RNA extracted from cultured FR tumor cells using primers that spanned from exons 1–3 of FGF23 cDNA from HEK 293 cells was used a positive control. Controls without cDNA (H_2O) and without reverse transcriptase (RT−) were negative.

netic resonance imaging assisted in localization of the giant cell tumor. Somatostatin receptor imaging, which has been reported to locate tumors causing oncogenic osteomalacia (21–23) and which identified the tumor in five of seven patients in a series recently reported (24), failed to locate the tumor in this case. The detection of FGF23 by immunohistochemistry in the causative giant cell tumor, but not in the angiolipoma first suspected, emphasizes that FGF23 expres-
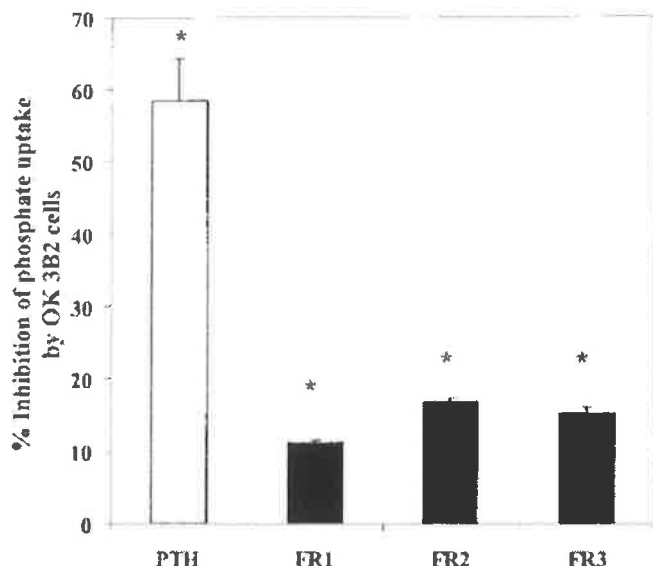
FIG. 7. Inhibition of phosphate uptake by renal OK 3B2 cells by conditioned media from FR tumor cultured cells. Phosphate uptake was measured in triplicate in OK 3B2 cells after preincubation of confluent cells for 20 h with serum-free conditioned medium collected from three different subcultures of FR tumor cells (FR1–FR3). Inhibition in response to FR-conditioned media was calculated as the percent inhibition of uptake by cells incubated with control unconditioned medium. The response to PTH (100 ng/ml) is shown as a positive control. *, Significantly different from control ($P < 0.05$).

sion may be a useful tumor marker in oncogenic osteomalacia.

The initial clinical presentation of our case was accompanied by biochemical abnormalities typical of oncogenic osteomalacia, namely hypophosphatemia and low normal serum 1,25-dihydroxyvitamin D concentrations. Consistent with previous reports (reviewed in Ref. 25), remission of clinical and biochemical abnormalities occurred after replacement with 1,25-dihydroxyvitamin D, but not with ergocalciferol alone. Notably, despite near-normal serum phosphate concentrations for 12 months during treatment with calcitriol, transiliac bone biopsy showed features consistent with osteomalacia.

Hyperparathyroidism was a feature of this case, which was apparent at presentation in the presence of subnormal serum 25-hydroxyvitamin D concentrations. There was no particular history of low sun exposure, but generalized bone pain and muscle weakness may have limited outdoor activities. In view of the hyperparathyroidism, treatment was commenced with calcitriol only, with no phosphate supplements. Hyperparathyroidism has been reported in other oncogenic osteomalacia cases commonly after treatment (17, 24) and in one case after removal of the oncogenic osteomalacia tumor (26). The mechanism of hyperparathyroidism, which is not always a feature of oncogenic osteomalacia, is not well understood.

The demonstration of FGF23 expression in the giant cell tumor of this patient provides further support for the involvement of FGF23 in the pathogenesis of oncogenic osteomalacia as suggested by previous reports of FGF23 expression in this condition (12, 27). Our results extend the findings of these previous studies by showing not only *FGF23* mRNA

expression, but also protein expression, by immunohistochemistry in the giant cell tumor. Western analysis of tumor tissue also demonstrated an immunoreactive protein of approximately 32 kDa, but not the smaller band detected in transfected cell conditioned medium, which was consistent with previous findings in an oncogenic osteomalacia tumor (12). Preoperative serum from our patient significantly inhibited phosphate uptake in a renal cell bioassay, consistent with the presence of a circulating phosphaturic factor. Moreover, the FGF23 concentration was elevated in preoperative serum and then returned to normal after tumor removal, in parallel with resolution of the clinical and biochemical abnormalities and consistent with a recent report (28). Finally both renal phosphate uptake inhibitory activity and FGF23 expression remained detectable in cultured tumor cells.

Of particular interest was the rapid increase in serum 1,25-dihydroxyvitamin D concentrations that preceded a parallel increase in serum phosphate levels after removal of the giant cell tumor. This phenomenon has been noted previously (17, 29, 30) and suggests that the humoral factor(s) produced by the tumor independently regulates both phosphate transport and 1,25-dihydroxyvitamin D metabolism. In a recent study in which mice were injected with purified FGF23, serum 1,25-dihydroxyvitamin D concentrations declined before a corresponding fall in serum phosphate (31). The clinical data from our case would be consistent with a role for FGF23 in suppressing 1,25-dihydroxyvitamin D levels in a manner independent of its phosphaturic effect.

It is not clear that FGF23 is the only (or even the major) mediator of reduced renal phosphate transport in oncogenic osteomalacia. Overexpression of other genes has been reported in oncogenic osteomalacia tumors (13). These include the genes encoding matrix extracellular phosphoglycoprotein and frizzled related protein 4, and some evidence has been reported that these proteins affect renal phosphate transport (32, 33). It is possible that the phenotype of oncogenic osteomalacia may be due to a multiplicity of factors that alone or together inhibit phosphate reabsorption and/or 1,25-dihydroxyvitamin D production. Nevertheless, a recent preliminary report that mice homozygous for targeted FGF23 gene disruption were hyperphosphatemic and had elevated serum 1,25-dihydroxyvitamin D concentrations provides compelling evidence that FGF23 is crucial for normal phosphate homeostasis and vitamin D metabolism (34).

This case report provides further evidence for the involvement of FGF23 in oncogenic osteomalacia. The elevated FGF23 concentration in the preoperative serum that normalized after resection of the causative tumor and specific FGF23 immunostaining of the causative tumor indicate that FGF23 will be a useful clinical marker for the diagnosis and management of oncogenic osteomalacia.

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Paper 22. Intravenous pamidronate in the treatment and prevention of osteoporosis. SS Chan, LM Nery, A McElduff, EG Wilmshurst, GR Fulcher, BG Robinson, JN Stiel, JE Gunton , PB Clifton-Bligh. Internal Medical Journal 2004; 34:162-166

P Clifton-Bligh made a significant contribution to this study in the formulation and conduct of the study, in the supervision of many patients and in the assessment of the data and in the writing of the paper.This stud y was carried out before intravenous zoledronate became generally available t to treat osteoporosis. The study was carried out in subjects who could not tolerate oral bisphosphonates or who failed to qualify for PBS subsidised oral bisphosphonate therapy because of lack of a documented minimal trauma fracture. The effect of 30mg of intravenous pamidronate given every three months, or 60 mg given every six months on bone mineral density was studied. The average duration of treatment wasm16.8 months. 84 patients were treated. The mean baseline t score at the lumbar spine was -1.54 and at the femoral neck -2.87. After pamidronate treatment there was a 3.5% mean increase in lumbar spine bone mineral density (P<0.001), in the femoral neck bone mineral density, 2.1% (P=0.001) and in the trochanteric bone mineral density, 3.1% (P<0.001). One third of the patients were on oral glucocorticoid therapy at the time of the study and responded in the same way as those not on glucocorticoids. No patient discontinued treatment because of side effects. There was no significant difference in the change in bone mineral density in those receiving 30mg of pamidronate every three months compared to those receiving 60mg every six months.

This paper is considered to be an important contribution describing the effect of intravenous pamidronate in a large number of patients at risk for minimal trauma fracture, one third of whom were receiving oral glucocorticoids. A significant increase in bone mineral density was seen with this regime.

Citations.

ORIGINAL ARTICLE

Intravenous pamidronate in the treatment and prevention of osteoporosis

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Abstract

Background: Potent oral bisphosphonates are the mainstay of therapy for osteoporosis. However, there are patients who cannot have oral bisphosphonates (e.g. because of gastrointestinal side-effects). Therefore, we wanted to examine the effects of intermittent i.v. pamidronate (APD) on bone mineral density (BMD) in patients who needed bisphosphonate therapy but could not have oral bisphosphonates.

Aim: To assess BMD before and after intermittent i.v. APD in patients requiring a bisphosphonate either for the prevention of osteoporosis on concurrent steroid therapy or for the treatment of osteoporosis.

Methods: This was a retrospective audit of 84 consecutive patients at risk of fractures commencing APD between October 1997 and May 2000. Patients were treated with intermittent i.v. APD. BMD as measured by dual-energy X-ray absorptiometry before and after APD was the main outcome.

Results: The mean length of treatment and mean total APD dose were 16.8 ± 7.0 months and 186.1 ± 79.5 mg respectively. The reasons for using APD were failure to qualify for oral bisphosphonates on the pharmaceutical benefits scheme due to lack of documented minimal trauma fractures (58%), symptomatic gastro-oesophageal disease (20%), intolerance of oral bisphosphonates (18%) and lack of efficacy of calcitriol (4%). Mean baseline *T*-score at lumbar (L) 2–4 spine and femoral neck were -1.54 ± 1.22 and -2.87 ± 1.19, respectively.

From baseline to after APD treatment, there was a significant increase in L2–4 BMD (0.883 ± 0.175 vs 0.912 ± 0.176 g/cm^2, $P < 0.001$, mean increase +3.5%), in femoral neck BMD (0.667 ± 0.137 vs 0.680 ± 0.134 g/cm^2, $P = 0.001$, mean increase +2.1%) and in trochanteric BMD (0.549 ± 0.129 vs 0.566 ± 0.132 g/cm^2, $P < 0.001$, mean increase +3.1%). One-third of the patients were on oral glucocorticoids at the time of the present study and they had a similar increase in BMD compared to patients not on glucocorticoids. Mild side-effects occurred in seven patients, none of whom discontinued treatment.

Conclusion: Intermittent APD increases BMD and may be a suitable alternative for patients who cannot have oral bisphosphonates. (Intern Med J 2004; 34: 162–166)

Key words: diphosphonates, bone density, osteoporosis.

INTRODUCTION

Bisphosphonates are stable analogues of pyrophosphate and are potent inhibitors of bone resorption. Potent oral bisphosphonates such as alendronate have proven efficacy in fracture prevention.[1,2] However, one of the side-effects of alendronate is that of oesophagitis, which limits its use in patients with symptomatic gastro-oesophageal disease.[3] In a short-term endoscopic study, 10 mg/day of alendronate was associated with a 13.2% incidence of gastric ulcers.[4]

Intravenous pamidronate (3-amino-1-hydroxypropylidene-1,1-biphosphonate (APD)) is of proven value in the management of multiple myeloma, metastatic breast cancer and symptomatic Paget's disease. There has not been a large multicentre clinical trial of i.v. APD with fracture reduction as an end-point. However, there have been some small studies demonstrating the efficacy of i.v. APD in increasing bone mineral density (BMD) in postmenopausal women with osteoporosis and also in glucocorticoid-induced osteoporosis.[5–10]

In this study, we report our experience using i.v. APD to improve BMD in patients who could not have oral bisphosphonates because of gastro-oesophageal disease or who failed to qualify for subsidy of oral bisphosphonates on the pharmaceutical benefits scheme because of lack of documented minimal trauma fractures. The aim was to compare BMD measurements before and after treatment with intermittent i.v. APD.

METHODS

Patient population

The present study was conducted at the Northern Metabolic Bone Centre of Royal North Shore Hospital, a tertiary referral centre in Northern Sydney. It was a retrospective study of 84 consecutive patients commencing i.v. APD therapy between October 1997 and May 2000. Patients with Paget's disease and primary hyperparathyroidism were excluded.

Pamidronate treatment

The decision to treat patients with APD and the regimen used were according to the treating specialist. Patients were mostly treated with 30 mg of APD every 3 months or 60 mg every 6 months. APD was administered as an i.v. infusion over 2–4 h on an outpatient basis.

Bone mineral density measurements

BMD at the lumbar spine (LS), femoral neck (FN) and trochanter was measured using dual-energy X-ray absorptiometry (DEXA) (Norland XR26, Norland Corporation, Fort Atkinson, WI, USA). The coefficient of variation (CV) of BMD measurements was obtained from a previous study using replicate analyses.[11] The CV of the measurements was 1.0% at the L2–4 spine, 1.2% at the hip and 1.8% at the trochanter. The Norland phantom (Norland Corporation, Fort Atkinson, WI, USA) was used as quality control over the study period. Twenty-six measurements of BMD using the phantom were performed over each 6-month period. For each 6-month period between October 1997 and May 2000, the mean BMD using the phantom was 0.920 ± 0.006, 0.915 ± 0.005, 0.915 ± 0.007, 0.910 ± 0.005 and 0.910 ± 0.007. The overall CV was 0.46%. Previous established age- and sex-adjusted BMD reference ranges were used to calculate T- and Z- scores, as provided by the manufacturer. BMD was expressed as the number of standard deviations from age- and sex-matched controls (Z-score). T-scores represent the difference in standard deviations from the mean BMD of a young normal population of the same gender. Osteopenia is defined as a T-score of –1 to –2.5 and osteoporosis as a T-score of $\le$– 2.5. BMD was measured prior to starting APD and repeated approximately 12–18 months later.

Three patients did not have repeat LS BMD measurements done because of significant artefacts (from osteoarthritis and aortic calcification), which made interpretation of DEXA unreliable. One patient did not have FN and trochanteric BMD measurements done because of the presence of bilateral hip replacements. These patients were excluded from the relevant analyses.

Biochemistry

Biochemical screening tests for secondary causes of low bone mass had been performed. As a minimum, all patients had blood collected for full blood count, electrolytes, liver function tests, calcium and 25-hydroxyvitamin D3, which was measured by ^{125}I radioimmunoassay (DiaSorin, Stillwater, MN, USA). Prior to commencing APD, 24 subjects were already taking 1000 units of ergocalciferol because of previously documented low 25-hydroxyvitamin D3 levels. If 25-hydroxyvitamin D3 levels were low at the baseline of the present study, ergocalciferol was given. Adequate calcium intake was encouraged and calcium supplementation was given if dietary calcium intake was insufficient.

Data collection and ethics approval

Data from the medical records were extracted by two of the authors of the present study using a standardised data collection form. Information was obtained on patients' demographics, medications, reason for APD treatment, regimen of APD treatment and adverse events. BMD at baseline and the most recent BMD were recorded. The number of APD doses between the baseline BMD and the most recent BMD was recorded. Biochemical investigations from baseline and from the end of the study period were documented.

The study was approved by the institutional ethics committee.

Statistical analyses

Statistical analysis was performed using SPSS for Windows version 10.1 (SPSS, Chicago, IL, USA). Data were expressed as mean $\pm$ standard deviation. BMD measurements before and after APD treatment were compared using the paired t-test. The mean percentage change in BMD from baseline to the end of the study was calculated. We also calculated the annual percentage increase in BMD when the follow-up BMD was not done at exactly 12 months. Student's two-sample t-test was used to compare the means of the absolute change in BMD between two groups. A P-value of <0.05 (two-tailed) was considered statistically significant.

RESULTS

The data obtained from 84 subjects (22 males and 62 females) whose mean age was 64.9 ± 15.1 years, form the basis of this report. The characteristics of the subjects at baseline are presented in Table 1. The majority of subjects ($n = 56$) were prescribed APD 30 mg every 3 months and the mean total APD dose was 186.9 ± 79.5 mg (Table 2). Reasons for the use of i.v. APD were as follows: failure to qualify for subsidy of oral bisphosphonates on the pharmaceutical benefits scheme because of a lack of documented minimal trauma fractures (58%), symptomatic gastro-oesophageal reflux disease/peptic ulcer disease (20%), intolerance of oral bisphosphonates previously (18%) and lack of efficacy of calcitriol (4%). Seventy per cent of patients were on intermittent pamidronate because of documented osteoporosis (L2–4 spine or FN T-score of $\le$ –2.5 or documented minimal trauma fracture). Thirty per cent of patients were on intermittent pamidronate for prevention of osteoporosis as they were on oral steroids and/or had osteopenia.

Following treatment with APD, BMD increased significantly at L2–4 spine, FN and trochanter (Table 3). The mean per cent increases at L2–4 spine, FN and trochanter were +3.5, +2.1 and +3.1%, respectively. The

annual mean per cent increase at L2–4 spine, FN and trochanter was +3.0, +1.9 and +2.7%, respectively.

Side-effects of APD treatment were seen in seven patients and these all occurred after the first dose. Four patients had mild flu-like symptoms, one had constipation, one had erythema at the injection site and one complained of a headache after the infusion. These side-effects did not recur with the second dose and no patient discontinued the treatment. There were no significant changes in full blood count, serum creatinine, alkaline phosphatase or calcium from baseline to the end of the study (data not shown).

Thirty milligrams of APD every 3 months ($n = 54$) compared to 60 mg every 6 months ($n = 21$) produced similar mean change in LS BMD (0.028 ± 0.047 vs 0.032 ± 0.039 g/cm^2, $P = 0.42$). Thirty milligrams of APD every 3 months ($n = 55$) compared to 60mg every 6 months ($n = 22$) also produced similar mean change in FN BMD (0.015 ± 0.032 vs 0.013 ± 0.037 g/cm^2, $P = 0.51$). Patients who were currently taking oral glucocorticoids ($n = 26$) compared to those who were not ($n = 55$) had similar mean change in LS BMD (0.030 ± 0.044 vs 0.026 ± 0.047 g/cm^2, $P = 0.72$). Patients who were currently taking oral glucocorticoids ($n = 26$) compared to those who were not ($n = 57$) had similar mean change in FN BMD (0.012 ± 0.033 vs 0.014 ± 0.033 g/cm^2, $P = 0.37$).

DISCUSSION

The present study shows that intermittent APD treatment over a mean period of 16.8 ± 7.0 months is effective in increasing BMD. APD treatment was generally well tolerated.

Peretz *et al.*[5] in 1996 reported that intermittent pamidronate increased LS BMD by 2.9% after 1 year of treatment in 36 women with postmenopausal osteoporosis. The optimal dose of APD in the treatment of osteoporosis is not clear. Sarli *et al.* studied 20 women with postmenopausal osteoporosis with active gastro-oesophageal disease.[6] Ten patients received 30 or 45 mg of APD weekly until they achieved an average dose of 157.5 ± 9.3 mg/year in 1 month (group A), or 30 or 45 mg every 3 months or 90 mg every 6 months until they achievied an average dose of 166.5 ± 6.9 mg/year (group B). Lumbar spine BMD increased by 4.4% and 8.5% after 1 year in groups A and B, respectively. Femoral neck BMD only increased significantly (4.8%) in group A. Parameters of bone remodelling such as osteocalcin, pyridinoline and deoxipyridinolin decreased progressively during APD treatment. Gerber *et al.*[7] studied 44 postmenopausal women and showed that treatment with 30 mg of APD given 3 monthly is effective in increasing BMD at the spine and hip, whereas 60 mg given every 3 months and 60 mg as a first infusion

Table 1 Baseline demographics of study population ($n = 84$)

Characteristics	Data
Age (years)	64.9 ± 15.1†
Body mass index (kg/m^2)	24.7 ± 4.6†
Male : female	22:62
Postmenopausal (% of female)	82.3
Documented minimal trauma fractures (%)	42
Past oral steroid use (%)	40.5
Current oral steroid use (%)	31.0
Current inhaled steroid use (%)	9.5
Hormone replacement therapy (% of female)	17.7
Smokers (%)	4.8
Calcium supplementation (%)	42.9
Low 25-hydroxyvitamin D (%)‡	8.3
Ergocalciferol use (%)	28.6
Osteopenia of lumbar 2–4 spine (%)§	45.2
Osteoporosis of lumbar 2–4 spine (%)¶	21.4
Osteopenia of femoral neck (%)§	23.8
Osteoporosis of femoral neck (%)¶	61.9
Lumbar 2–4 spine *T*-score	-1.54 ± 1.22†
Femoral neck *T*-score	-2.87 ± 1.19†

†Mean ± standard deviation. ‡Low 25-hydroxyvitamin D = <42 nmol/L. §Osteopenia = *T*-score of –1 to –2.5. ¶Osteoporosis = *T*-score of ≤ –2.5.

Table 2 Pamidronate (APD) treatment ($n = 84$)

Characteristics	Data
Length of treatment (months)	16.8 ± 7†
Total APD dose (mg)	186.1 ± 79.5†
30 mg every 3 months (%)	66.7
60 mg every 6 months (%)	26.2
60 mg every 3 months (%)	7.1

†Mean ± standard deviation.

Table 3 Bone mineral density (BMD) before and after pamidronate treatment (mean length of treatment is 16.8 ± 7.0 months)

Parameter	Before	After	P
Lumbar 2–4 BMD (g/cm^2)†	0.883 ± 0.175	0.912 ± 0.176	<0.001
Lumbar 2–4 *T*-score	-1.528 ± 1.225	-1.207 ± 1.271	<0.001
Lumbar 2–4 *Z*-score	-0.478 ± 1.148	-0.323 ± 1.098	0.005
Femoral neck BMD (g/cm^2)‡	0.667 ± 0.137	0.680 ± 0.134	0.001
Femoral neck *T*-score	-2.852 ± 1.194	-2.515 ± 1.439	0.003
Femoral neck *Z*-score	-1.197 ± 1.102	-1.011 ± 1.054	<0.001
Trochanteric BMD (g/cm^2)	0.549 ± 0.129	0.566 ± 0.132	<0.001

Data are presented as mean ± standard deviation and *P*-value is for paired *t*-test. †$n = 81$. ‡$n = 83$.

followed by 15 mg every 3 months increases spinal BMD only. We were unable to demonstrate that the regimen of APD had differential effects on BMD in the present study.

There is a number of small studies demonstrating the efficacy of i.v. APD in patients with glucocorticoid-induced osteoporosis.[8-10] In a controlled study, Boutsen *et al.* showed that intermittent APD (90 mg at baseline followed by 30 mg every 3 months for the duration of steroid use) was effective in increasing BMD at the spine and hip in 27 subjects starting at least 10 mg of pred-nisolone for the first time.[9] In a second primary prevention study of glucocorticoid-induced osteo-porosis, the same group found that a single 90-mg infusion of APD at the start of glucocorticoid therapy compared with intermittent APD therapy (90 mg followed by 30 mg every 3 months) produced similar BMD increases after 1 year.[10] Similarly, several small studies have demonstrated that i.v. APD is effective in preventing bone loss associated with glucocorticoids and other immunosuppressive agents after lung, heart and liver transplantation.[12-14]

In a recent large multicentre trial in postmenopausal women, 10 mg/day of alendronate increased LS, FN and trochanteric BMD by 5.0, 2.3 and 4.1%, respectively from baseline to 12 months.[15] In our study, the annual mean per cent increase at the L2–4 spine, FN and trochanter were +3.0, +2.2 and +2.7%, respectively. One-third of our patients were on oral steroids and they had similar mean increases in BMD compared to patients not on steroids. In patients receiving glucocorticoid therapy, Saag *et al.* showed 10 mg/day of alendronate increased BMD at the LS by 2.9%, FN by 1.0% and trochanter by 2.7% from baseline to 48 weeks.[16] Over this period, patients on placebo had –0.4, –1.2 and –0.7 change in LS, FN and trochanter BMD, respectively.

In contrast to the oral bisphosphonates,[1,2,17-19] there have been no large clinical trials of i.v. APD in fracture prevention. Therefore, evidence-based medicine would support the use of potent oral bisphosphonates such as alendronate as first-line therapy in patients for whom a bisphosphonate is indicated either for the treatment of osteoporosis or to prevent bone loss while receiving glucocorticoids.[16]

Of considerable interest is the efficacy of i.v. zoledronic acid in increasing bone mineral density in postmenopausal women.[20] Zoledronic acid is the most potent bisphosphonate studied in clinical trials. A single annual dose of 4 mg intravenously of zoledronic acid was as effective as other dosing schedules in increasing BMD.[20] There has been no direct comparative study of zoledronic acid and APD on BMD. If the efficacy of zoledronic acid is borne out in ongoing trials of fracture prevention, it would represent an effective and conven-ient way to treat and prevent osteoporosis.

Our study was a non-randomised, observational study of usual clinical practice. It was retrospective, lacked a control group and was not designed to examine the important end-point of fracture. Nevertheless, it is one of the largest series of patients treated with intermittent APD reported in the literature and it provides useful clinical information that APD is an important alternative to oral bisphosphonate therapy for patients for whom such treatment is contraindicated or not accessible. Our study demonstrates that i.v. APD increased BMD at the LS, FN and trochanter. Intravenous APD is particularly useful in patients on glucocorticoid treatment as many have not yet had a fracture. Further studies of i.v. APD and its more potent relation, zoledronic acid, in fracture prevention are needed.

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Paper 23. Prevention of osteoporosis as a consequence of aromatase inhibitor therapy in postmenopausal women with early breast cancer: rationale and design of a randomized controlled trial. S Kilbreath, KM Refshauge, J Beith, L Ward, K Sawkins, R Paterson, P Clifton-Bligh, PN Sambrook, JN Simpson, L Nery. Contemporary Clinical Trials 2011; 32:704-709

P Clifton-Bligh made a significant contribution to this work with respect to the design of the protocol, the formulation of methods of measuring bone density and serum bone cell markers. Aromatase inhibitors have improved the prognosis in breast cancer survivors whose tumours are estrogen receptor positive. However, the use of aromatase inhibitors is associated with a fall in bone mineral density and an increase in fracture risk. An option designed to prevent bone loss is to combine an exercise program with vitamin D and calcium supplementation. The plan is to randomize 30 of 60 patients with early breast cancer on aromatase inhibitor therapy to a structured exercise program three times weekly for 12 months. All participants will receive calcium and vitamin D supplements. In healthy postmenopausal women, previous work has shown that high impact exercise, together with vitamin D and calcium supplements retards the loss of bone mineral. This paper describes in detail the exercise protocol which will be used. Blood will be taken at times 0,6 and 12 months to measure serum 25-OH vitamin D, intact amino-terminal propeptide of type 1 collagen (P1NP), carboxyterminal telopeptide of type 1 collagen (CTX), and tartrate resistant acid phosphatase (TRACP). P1NP measures bone formation and CTX and TRACP measures bone resorption . It is expected that the outcome of the study will show that exercise at a certain level will reduce the rate of bone loss in potmenopausal women receiving aromatase inhibitors.

This paper describes an important randomized study of the impact of high intensity exercise on the rate of bone mineral density loss in postmenomausal women receiving aromatase therapy for breast carcinoma.

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Prevention of osteoporosis as a consequence of aromatase inhibitor therapy in postmenopausal women with early breast cancer: Rationale and design of a randomized controlled trial

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ARTICLE INFO

ABSTRACT

Background: Aromatase inhibitors (AIs) have improved the prognosis for breast cancer survivors and are now standard of care for postmenopausal women with hormone receptor positive early stage breast cancer. One side-effect, however, is a decrease in bone mineral density (BMD) and increased fracture risk. Since hormone replacement therapy (HRT) is contraindicated in these women, one prevention option is exercise combined with vitamin D and calcium. The effect of this intervention on drug-induced osteoporosis is unknown.
Methods: A single-blind randomized controlled trial will be undertaken to test the hypothesis that exercise combined with vitamin D and calcium can prevent the decrease in BMD associated with the use of AIs. Sixty postmenopausal women prescribed an AI for the treatment of breast cancer will be randomized into either an exercise or control group. Participants randomized to the exercise group will undertake a 12-month gym-based exercise program, 3 times per week involving resistance and impact training. Participants in the control group will be advised on the benefits of exercise for preventing osteoporosis, but not prescribed exercise. Both groups will receive vitamin D and calcium supplements. The primary outcome will be total hip bone mineral density measured via dual energy X-ray absorptiometry (DXA). Study outcomes will be compared between groups at baseline, 6 months and 12 months.
Summary: This study will investigate the effect of exercise in combination with vitamin D and calcium on prevention of drug-induced osteoporosis in postmenopausal women prescribed AIs for the treatment of breast cancer.

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1. Introduction

Breast cancer is the most frequently diagnosed cancer among women in the United States [1]. Approximately 75% of breast cancers are positive for the estrogen receptor, progesterone receptor or both [2]. These cancers depend on circulating estrogen for survival [2]. Estrogen deprivation, therefore, is the primary goal of hormone therapies used in the treatment of hormone receptor positive breast cancers.

Tamoxifen citrate (Tamoxifen) has been considered the standard adjuvant endocrine therapy for postmenopausal women with early stage hormone receptor positive breast cancer [3,4]. It limits the amount of estrogen available to the tumor through competitive antagonism of estrogen at its receptor [2]. The long-term use of tamoxifen, however, has been associated with side effects such as vaginal discharge and bleeding, cerebrovascular events, venous thromboembolic events and endometrial cancer [5,6].

The use of third generation aromatase inhibitors (AIs) has significantly improved the prognosis of women with breast cancer, with fewer serious adverse events than tamoxifen [6–11]. AIs minimize circulating estrogen by blocking the conversion of androgens to estrogen, substantially reducing estrogen synthesis, and thus the amount of estrogen available to the tumor [12]. The main side-effect of third generation AIs is an increased risk of osteoporosis and fracture [4,6,8,13–16]. Circulating estrogen also plays a crucial role in the maintenance of bone remodeling by stimulating bone growth and inhibiting bone resorption [17]. AIs minimize circulating estrogen, resulting in reduced bone remodeling and consequent bone loss. In postmenopausal women who are already at risk of osteoporosis, the reduction in BMD from AIs may result in a more rapid progression to osteoporosis.

In healthy postmenopausal women, high-impact exercise in combination with vitamin D and calcium supplements retards bone resorption, stemming the progression to osteoporosis [18–20]. In addition, in healthy women, the effect of exercise on BMD can be augmented by hormone replacement therapy [21]. For women with estrogen positive breast cancer in whom HRT is contraindicated, the combination of exercise with vitamin and mineral supplements has not been explored. To date, only one preliminary study of 16 participants has investigated the benefits of exercise in breast cancer survivors with accelerated bone loss precipitated by medication [22].

The overall aim of this study, therefore, is to determine the effect of exercise on BMD in postmenopausal women prescribed an AI for the treatment of breast cancer. We hypothesize that total hip and lumbar spine BMD will be higher in the exercise group than in the control group, markers of bone formation and bone resorption will more closely approximate normative values in the exercise group than in the control group, and measures of quality of life and general health will be higher in the exercise group than in the control group.

2. Research design and methods

A single-blind randomized controlled trial will be conducted with blinded outcome assessments at baseline, 6 months and 12 months. The trial will be performed according to the CONSORT guidelines [23].

2.1. Participants

Sixty postmenopausal women who have been treated for breast cancer and have recently been prescribed an aromatase inhibitor will be recruited for the study. Participants will be recruited via advertisements in the media and through referrals from medical oncologists and breast care nurses at hospitals in the Sydney metropolitan region.

Participants will be included in the study if they are postmenopausal, have early stage breast cancer, have recently commenced taking an aromatase inhibitor (no more than 12 weeks prior to baseline assessment), are sedentary (low-moderate on the International Physical Activity Questionnaire (IPAQ) [24]) and have an Eastern Collaborative Oncology Group Performance Status ≤ 2 [25]. Participants will be excluded from the study if they have a T score < -3 (i.e. three standard deviations below the average bone density for a healthy young adult of the same sex and ethnicity), have clinical or radiological evidence of distal spread of the disease, have taken HRT within the past 12 months, have taken bisphosphonates within the past 12 months, have been treated with intravenous bisphosphonates, have taken bisphosphonates for >2 years, have been treated with continuous systemic oral glucocorticoids within the past 6 months, are currently taking medications known to affect the skeleton, have a history of diseases that influence bone metabolism, have previous or concomitant malignancy within the past 5 years (excluding adequately treated basal or squamous cell carcinoma of the skin or in situ carcinoma of the cervix), or have had a recent fracture or trauma to the spine or a long bone in the past 6 months or to a short bone within the past 3 months. The study has been approved by the University of Sydney human research ethics committee and the relevant New South Wales Health human research ethics committees. Written informed consent will be obtained from all participants prior to data collection. The study will be conducted in accordance with the appropriate guidelines for the ethical conduct of research in humans. In addition, the protocol has been registered with the Australian Clinical Trials Registry (ACTRN12608000220369).

2.2. Randomization

Following the baseline assessment participants will be randomly assigned to either the exercise or control group. An individual not involved in the study will oversee the computer-generated randomization list, in permuted blocks of 6 or 8, and stratified by whether the participant has previously used tamoxifen. Sequentially numbered opaque randomization envelopes will be prepared by an individual not involved in the study.

2.3. Protocol

All participants will receive daily vitamin D (1000 IU) and calcium carbonate (1200 mg) supplements in accordance with current guidelines for women taking an aromatase inhibitor [2,26–28]. A diary will be provided for participants to record their use of vitamin D and calcium and all unused pills collected monthly. An information brochure developed by Osteoporosis Australia outlining the benefits of exercise in

the prevention of osteoporosis will be provided to all participants [29]. Participants will record all health care contacts and additional exercise undertaken during the course of the study in a treatment diary.

2.3.1. Control group

Participants in the control group will communicate with a research assistant monthly to monitor health status and to receive vitamin D and calcium supplements. No further advice will be given about exercise. Control participants will be instructed to continue their usual daily activities.

2.3.2. Exercise group

Participants in the exercise group will be prescribed a 12-month gym-based exercise program consisting of impact and resistance training. The exercise program was specifically developed for the study based on programs which have been most successful in increasing or maintaining bone mineral density in healthy postmenopausal women [30–38].

Participants will complete the program 3 times per week for approximately 1 h per session at a gym of their choice. A 12-month gym membership and personal trainer will be provided. The personal trainer will supervise between 4 and 12 training sessions in the first month depending on the support required by the participant. Participants will then continue the training independently with a review by the personal trainer monthly. The personal trainer will teach the participant the exercise program, evaluate the participant's technique and safety with the exercises, progress the participant's exercises as required, and maintain the participant's motivation.

Exercise compliance will be monitored with a training diary completed by the participant. The training diary will be collected by a research assistant monthly. Participants will receive weekly telephone calls from a research assistant to monitor compliance with the program and maintain motivation. Additional personal training sessions will be scheduled for non-compliant participants.

Training sessions will consist of four components: 1) warm-up (5 min), 2) impact training (25 min), 3) resistance training (25 min), and; 4) cool-down (5 min).

2.3.2.1. Warm-up.
The warm-up will consist of 5 min on the treadmill, stepper or exercise bike. Participants will be instructed to work at an intensity of 8–10 (very light) on the rating of perceived exertion (RPE) 6–20 scale [39].

2.3.2.2. Impact training.
Impact training will consist of a combination of aerobic weight-bearing exercises, and an impact exercise circuit. Participants will first complete 5 min of an aerobic weight-bearing exercise, alternating monthly between the treadmill and the stepper. Participants will be progressed either by increasing their speed on the treadmill or increasing the difficulty level on the stepper. The duration of this component of the program will not be increased. The impact exercise circuit will consist of 3 impact exercises. The exercises will be varied each month to increase difficulty of the exercise and to provide variety in the types of loading on the participant's bones. The impact circuit will include exercises such as skipping with a rope, hopping, jumping, bounding, step-

ups, side step-ups, jumping jacks/star jumps and high-knees. Participants will initially perform 1 repetition of each of the 3 impact exercises. One repetition is defined as performing the exercise for 1 min. As participants improve they will increase the number of repetitions of each exercise to 2 or 3 repetitions. Participants will rest for 1 min between each repetition of the exercise and between exercises. For both the aerobic weight-bearing exercise and the impact circuit, participants will be instructed to work at an intensity of 13–15 ("somewhat hard"–"hard") on the RPE scale [39]. Repetitions and RPE for each exercise will be recorded in a training diary.

2.3.2.3. Resistance training.
High-load resistance training will be carried out using a combination of free weights and machine weights. Participants will complete 5 resistance exercises each session. Exercises will include: squats (free weights or machine), leg press (machine), standing row (free weights), lat pull-down (machine) or seated cable row (machine) and back extensions. Prior to performing the upper body exercises, participants will complete a warm-up set of each exercise. The warm-up set will consist of 15–20 repetitions at 25–50% of one-repetition maximum (1-RM). The 1-RM was defined as the maximal weight a participant could lift once with proper body alignment and correct lifting technique [40]. Training sets will consist of 8–12 repetitions of the exercise. As participants will be unaccustomed to exercise training, sets will begin at 50–60% of 1-RM in the first week and progress to 70–80% of 1-RM by the end of the first month. The participant's 1-RM will be established for each exercise during the first training session. The 1-RM will be used by the trainer to calculate training weights for each exercise. Participants will complete 2 training sets of each exercise. Each warm-up and training set will be followed by a 1 min rest period. The program will utilize the principles of power training (i.e. fast concentric contraction followed by a slow eccentric contraction) as power training has been shown to be more effective than strength training in maintaining or increasing BMD in postmenopausal women [41,42]. Weights for each exercise will be progressed to maintain an intensity of 70–80% of 1-RM. The personal trainer will guide the participant with weight progression and will reassess strength at the monthly training sessions. Participants will be encouraged to progress their weight independently throughout the month.

2.3.2.4. Cool-down.
To cool-down participants will perform 1 min of a lumbar rotation exercise in the supine position. They will then perform stretches targeting the major muscle groups used during the training session. Stretches will be performed for 30 s each side for the quadriceps, hamstrings, gastrocnemius, gluteals, and posterior shoulder muscles.

During periods of absence from the gym (e.g. holidays), participants will be encouraged to continue the aerobic weight-bearing and impact exercises independently. They will also be provided with an alternative resistance training program using Theraband®, with the prescribed exercises designed to replicate those performed in their usual training sessions. Participants will continue to keep their training diary over such periods.

3. Outcome measures

All measurements except that related to assessment of lymphoedema will be undertaken by a blinded assessor. For each participant outcomes will be measured at baseline and at 6 and 12 months.

3.1. Baseline measures

Demographic information, details of breast cancer treatment regimen, pathology details, age, weight, height and menopausal history will be obtained from all participants at the baseline assessment. All primary and secondary outcome measures will also be obtained at the baseline assessment.

3.2. Primary outcome measure

Total hip bone mineral density (BMD) as measured using a dual energy X-ray absorptiometer (DXA), the gold standard for diagnosing osteoporosis [43,44], will be the primary outcome measure. All bone mineral density scans will be performed at the Northern Metabolic Bone Centre at Royal North Shore Hospital on the same DXA machine (Hologic Explorer QDR series JT1).

3.3. Secondary outcome measures

i) Trochanteric, femoral neck, lumbar spine and whole body BMD will also be measured using a dual energy X-ray absorptiometer (DXA) (Hologic Explorer QDR series JT1).
ii) Blood samples will be collected to assess biochemical markers of bone remodeling. Intact amino-terminal propeptide of type I procollagen (PINP) concentration will be assessed as a measure of bone formation. Carboxy-terminal telopeptide of type I collagen (CTX) and tartrate-resistant acid phosphatase (TRACP) will be used to quantify bone resorption. Serum calcium will be monitored at baseline, 6 months and 12 months, and 25(OH)-vitamin D will be monitored at baseline and 12 months for safety purposes.
iii) Self-reported quality of life will be assessed with the European Organization for Research and Treatment of Cancer Quality of Life Questionnaire Version 3 (QLQ-C30) and breast module (BR23) [45–47]. The Medical Outcomes Survey Short Form-36 will be used to assess self-reported general health status [48].
iv) Lymphoedema status will be monitored non-invasively using bioimpedance spectroscopy (BIS) (Imp SFB7. Impedimed Ltd.). BIS will be used to quantify the volume of extracellular fluid in the arms, from which a ratio can be derived comparing the 'at risk' arm to the non-'at risk' arm. Ratio means of 1.139 for at risk dominant arms and 1.066 for at risk non-dominant arms are indicative of lymphoedema [49]. Participants who develop lymphoedema during the study will be referred to an appropriate health practitioner for further assessment and treatment. BIS has been shown to be a sensitive and reliable method for the early detection of lymphoedema [49].

4. Data analysis

Sample size calculations were based on findings from the study by Kerr et al. (1987) who found a mean difference (±standard deviation) between the control and exercise group of 2.3 (±4.3)% in BMD at the greater trochanter in postmenopausal healthy women. In contrast to the women from Kerr et al.'s study, the women in the present study will be taking an aromatase inhibitor which accelerates bone loss [50]. The rate of bone loss in women taking an AI after one year was an additional 1.7% at the hip. Thus, the difference expected in the cohort will be $4.0 \pm 4.3\%$, requiring 30 participants per group [51,52]. This assumes a 5% 2-sided significance, 85% power 15% loss to follow-up and 5% lack of compliance with the exercise program.

Data will be scrutinized to ensure that there are no errors or omissions, and coded to enable blinded analysis. Intention-to-treat analysis will be used as the primary analysis; however, secondary analyses will include a per protocol analysis. Primary analysis will be a t-test comparing the change in each outcome variable between the exercise and control group. For the secondary analysis, the effect of exercise on change in each outcome variable will be analyzed using a linear regression model of final value on initial value and exercise treatment group, adjusted for confounders such as age and weight, as required.

5. Discussion

Breast cancer is the most common cancer affecting women in Australia, with 1 in 11 women being diagnosed with breast cancer by the age of 75 [53]. With the majority of women now being cured, the impetus is to eliminate the harmful effects of treatments without sacrificing the effectiveness of the treatment.

The introduction of aromatase inhibitors for the treatment of breast cancer has significantly improved prognosis [6–11], at the cost of diminishing skeletal health in postmenopausal women [4,6,8,13–16]. Without an effective intervention to prevent this, the incidence of osteoporosis, and therefore, fractures, long-term dependence and mortality will increase resulting in a significant financial burden on the health care system. It is imperative that we identify preventative strategies rather than deal with the consequences of reduced bone mineral density.

In healthy post-menopausal women, exercise in combination with vitamin D and calcium supplementation has been shown to prevent or minimize the loss of bone density associated with menopause. Since HRT is contraindicated in women treated for breast cancer, exercise in combination with vitamin D and calcium is an alternative primary prevention option. The effect of these interventions on drug-induced osteoporosis, however, remains unknown. This study will determine whether exercise in combination with vitamin D and calcium is effective in preventing a reduction in bone mineral density in postmenopausal women prescribed an aromatase inhibitor for the treatment of breast cancer.

The outcome of this study will enable best practice guidelines to be formulated for the prevention of osteoporosis in postmenopausal breast cancer survivors treated with

aromatase inhibitors. Furthermore, the findings will have direct relevance to clinicians prescribing aromatase inhibitors following breast cancer and will provide evidence of management.

Acknowledgments

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Paper 24. Red cover isoflavones enriched with formononetin lower serum LDL-cholesterol-a randomized, double blind, placebo-controlled study. PB Clifton-Bligh, M-L Nery, RJ Clifton-Bligh, S Visvalingam, GR Fulcher, K Byth, R Baber. European Journal of Clinical Nutrition 2015; 69:134-142.

This was a major study involving 147 post-menopausal women studied over 2 years. The recruitment phase lasted several years. P Clifton-Bligh formulated, initiated and progressed the study Almost all the patients were examined as per protocol by P Clifton-Bligh. The collection, analysis of the data and the writing of the manuscript was carried out by P Clifton-Bligh. The integrity of the protocol was maintained by P Clifton-Bligh and by M-L Nery. Isoflavones have a higher affinity for the estrogen receptor-beta, expressed predominantly in trabecular bone, than the estrogen receptor-alpha expressed in breast, endometrium and bone. The purpose of the study was to determine whether treatment with an isoflavone extracted from red clover, containing mostly formonometin (Rimostil) was associated with beneficial responses in lipid and bone metabolism. 50mg of Rimostil/day given in a randomized, double- blind, placebo controlled format for a 2 year period was associated with a 12% fall in the serum LDL-cholesterol which was highly significant when compared to the placebo response. There was no significant impact of Rimostil on the bone density of the spine,femoral neck, or forearm. The serum 25-OH vitamin D concentrations fell significantly over the two year period but there was no significant difference between the active treatment and the control group. The serum HDL-cholesterol showed a significant increase with time but there was no significant difference between the active treatment group and the control group. The fall in the apolipoprotein B with the treatment arm was of borderline signigicance compared to the placebo arm. There was no significant increase in endometrial thickness in either the placebo or the active treatment group over the two year period of observation. The study confirmed that a beneficial effect on serum LDL-cholesterol couold be achieved with Rimostil at this dose.

This double-blind placebo-controlled study of the effects of 50mg/day of formononetin given for two years showed a significant beneficial lowering of serum LDL-cholesterol but no significant preservation of bone mineral density.

ORIGINAL ARTICLE

Red clover isoflavones enriched with formononetin lower serum LDL cholesterol—a randomized, double-blind, placebo-controlled study

PB Clifton-Bligh[1,2], M-L Nery[1], RJ Clifton-Bligh[1,2], S Visvalingam[3], GR Fulcher[1,2], K Byth[4] and R Baber[2,3]

BACKGROUND: Although postmenopausal combined hormone replacement therapy reduces the risk of hip fracture, long-term use may be associated with an increased risk of breast cancer, and in women more than 10 years after menopause it is associated with an increased risk of cardiovascular disease. Isoflavones, because of preferential binding to estrogen receptor beta, may retain the beneficial effects on bone but lessen the adverse effects on the breast.
OBJECTIVE: The objective of this study was to study the effects of an isoflavone obtained from red clover (Rimostil) on bone mineral density, and on low-density lipoprotein (LDL) cholesterol.
DESIGN: In a double-blind, randomized, placebo-controlled trial, 50 mg of Rimostil was given to women who were menopausal for at least 1 year. Bone mineral density of the spine, femoral neck and forearm and serum LDL cholesterol were measured at baseline and at 6-month intervals. The duration of follow-up was 2 years.
RESULTS: There was no beneficial effect of Rimostil on bone density at any site. There was a 12% fall in serum LDL cholesterol in the Rimostil-treated arm, which was significantly greater than the 2% drop seen in the control arm ($P = 0.005$).

INTRODUCTION

After the onset of menopause, there is a rise in the serum cholesterol and an increased risk of cardiovascular disease.[1] Menopausal women also experience frequent troublesome episodic hot flushes. The most effective therapy to diminish both the frequency and severity of hot flushes is exogenous estrogen. Estrogen therapy, when given early in menopause, is also associated with a reduction in cardiovascular events,[2,3] but when given more than 10 years after the onset of menopause it is associated with an increased risk of cardiovascular events, including an increased risk of stroke.[4,5] In contrast, the risk of hip fracture is reduced significantly. In addition, the use of combined estrogen and progestin for more than 5 years is associated with an increased risk of breast cancer.[4] Estradiol interacts with both the estrogen receptor α (ERα), produced in the breast and the endometrium, and the estrogen receptor β (ERβ), predominantly in trabecular bone.[6] The above considerations have led to a search for substances that interact preferentially with the estrogen receptors in bone but to a lesser extent with the estrogen receptors in the breast and endometrium. In this respect, the isoflavones produced in soy and red clover have seemed promising despite the fact that they have much less affinity for the ERβ than estradiol itself,[6] although s-equol, a metabolite of daidzein produced in the intestine, has an affinity for ERβ as high as estradiol itself but a low affinity for ERα.[7] Soy protein extracts contain mostly genistein and daidzein, whereas red clover extracts contain more biochanin A and formononetin (the precursors for genistein and daidzein, respectively).

In a previous study using red clover extract enriched with formononetin, we found that the forearm bone mineral density (BMD) increased and the serum low-density lipoprotein (LDL) cholesterol was significantly decreased in a group of postmenopausal women.[8] This study had several flaws: the absence of an appropriate placebo group, the unexpected large increases in BMD in the forearm in a 6-month period and the absence of a dose–response relationship between the fall in the serum LDL cholesterol and increasing amounts of red clover extract.

With this in mind, we have undertaken a larger double-blind, placebo-controlled study over a 2-year period, in which BMD was measured in the lumbar spine, femoral neck and forearm together with measures of serum LDL cholesterol concentrations.

Thus, the primary aim of this study was to evaluate the efficacy of Isoflavones (Formononetin enriched, P-081, Rimostil, Sydney, NSW, Australia) on vertebral body, femoral neck and forearm BMD and on serum LDL cholesterol, as measured by the within-patient changes from baseline at 24 months. Secondary end points were changes in HDL cholesterol, ApoA, ApoB, Lp(a), triglycerides, serum 25OH-vitamin D, serum calcium and urinary deoxypyridinoline (DPYR). The hemoglobin and serum alanine aminotransferase (ALT) were measured to assess potential adverse effects of the active treatment.

PARTICIPANTS AND METHODS

Participants were recruited by newspaper advertisements between the years 2000 and 2006 in the local geographical community. To be included in the study, the women had to be at least 1 year postmenopausal, have a serum follicle stimulating hormone level of > 30 U/ml, have a serum

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25OH-vitamin D level > 40 nmol/l and a BMD T-score > − 2.0 in the spine and hip. Nine participants whose serum 25OH-vitamin D levels were subsequently found to be less than 40 nmol/l were inadvertently randomized and continued in the trial. The mean 25OH-vitamin D levels at baseline and at 6, 12, 18 and 24 months were not significantly different between active treatment and placebo groups. The mean age of participants was 54 ± 4 (s.d.) years.

Participants were excluded if hormone replacement therapy had been taken within 2 months of screening into the study, if there was a history of clinical vertebral fracture or other minimal trauma fracture or if they had any intercurrent medical condition, such as celiac disease, primary hyperparathyroidism, renal impairment or thyroid overactivity, that would be likely to confound the study outcome. Participants were also excluded if they had previously taken bisphosphonates, strontium, fluoride or raloxifene or if they were a vegetarian consuming more than 10 gm of legume daily. None of the participants took any of these bone-preserving medications during the study, and participants were instructed not to take soy or red clover products during the 2-year study period and to keep a food diary so that food intake could be assessed.

A total of 183 participants were initially screened for the study; 30 participants were excluded because of failure to meet the study's inclusion/exclusion criteria and 6 participants withdrew consent before randomization. In all, 147 participants entered into the study and were randomized: 75 participants in the active treatment group and 72 participants in the control group.

A full medical examination was performed on each participant at entry into the study and annually throughout the 2-year period. BMD measurements were performed every 6 months on the AP spine (L2–L4) and femoral neck using the Norland XR36 densitometer (Fort Atkinson, WI, USA), and the proximal and distal radius using the Norland pDEXA scanner (Fort Atkinson, WI, USA). Both scanners were calibrated and an external phantom was used to monitor quality control on a daily basis. From 2002, because of the involvement of the site in an international clinical trial, an additional external Hologic phantom (Bedford, MA, USA) was also incorporated into the Norland XR36 daily quality control regimen and monitored by Synarc (Portland, OR, USA) (coefficient of variation 0.26–0.55%). The monthly average measurements of the Hologic phantom between 2002 and 2007 varied between 99.6 and 100.4% of the designated value. A thoracolumbar spine X-ray was performed at entry into, and at the conclusion of, the study.

Fasting blood samples were collected every 6 months for the measurement of serum total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, ApoA, ApoB, triglycerides, Lp(a), serum calcium and serum 25OH-vitamin D. Biochemical safety measures to assess the safety of the active medication included serum ALT and hemoglobin. A fasting 2-hour second void morning urine sample was also collected every 6 months for the measurement of urine DPYR.

A trans-vaginal ultrasound to measure endometrial thickness was performed at entry into, and at the conclusion of, the study.

Study participants were also asked to monitor their menopausal symptoms throughout the study using a Greene Climacteric Scale[9] diary; each of the 21 items in the Greene Climacteric Scale were graded 0–3 in severity so that the maximum score possible was 63; and they were also encouraged to write down any adverse events or the use of concomitant medications in the diary.

Laboratory Assays: The serum cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, ApoA and ApoB were measured using the Roche modular assay system (Roche Diagnostics, Basel, Switzerland). The Lp(a) was measured initially with a Biopool ELISA assay (Umea, Sweden) and subsequently with a Mercodia (Upsala, Sweden) ELISA assay (CSLS). Serum 25OH-vitamin D was measured between 2000 and 2005 using the Nichols Advantage (San Clemente, CA, USA) assay (inter-assay variation 10–14%) and from the 22 July 2005 using the Diasorin Liaison (Saluggia, Italy) assay. The assays were cross-correlated (Diasorin Liaison = 1.07 Nichols+18, $r = 0.794$). The Nichols serum 25OD-vitamin D values were transformed into Diasorin Liaison equivalents using this equation. Urinary DPYR was measured using the Siemens (Erlangen, Germany) Immulite assay with an inter-assay variation of 9–15%.

Each study participant was asked to take two tablets of the study drug daily: this was either P-081 (marketed since as Rimostil), with each tablet containing 25 mg of isoflavone predominantly formononetin and biochanin, or placebo, which was identical in shape, size and color. Both groups of women were given supplemental calcium (CalSup) at a dose of 1000 mg per day. Compliance with the study medication was assessed by pill counts at each study visit.

Randomization was performed in blocks of 6 in a 1:1 design. The study participants and all of the study staff members were blinded to the treatment.

The study was approved by the Royal North Shore Hospital Human Research Ethics Committee and conducted according to ICH-GCP guidelines. Each study participant provided written informed consent.

Sample size calculation

The five primary outcome variables were the within-patient change from baseline at 24 months for BMD of the spine, femoral neck, proximal and distal radius and for serum LDL cholesterol. Differences between the active and control arms were tested at the 1.0% significance level for each of these outcomes in order to maintain an overall 5% significance level for the primary outcomes. Previous experience in our department suggested that the standard deviation of the within-patient percentage change from baseline BMD at any of the four sites would not exceed 3.4%.[10] A sample size of 65 per arm ensures 80% power to detect a difference of at least 2% between the mean within-patient percentage change from baseline observed in the active and control arms at 24 months. A sample size of 71 per arm was recommended to allow for up to 10% discontinuation.

Statistical analyses

Throughout the trial, the data were collected and collated by an independent manager. Novogen (Sydney, NSW, Australia), who supplied the study medication, had no role in the collection or analysis of the study data or in the submission of the manuscript for publication. The analysis was carried out by an independent professional statistician, and all authors vouch for the integrity of the data.

The statistical software packages S-PLUS Version 8 (TIBCO Software Inc., Palo Alto, CA, USA) and SPSS version 21 (IBM Corporation, Armonk, NY, USA) were used to analyze the data by the intention-to-treat principle. Two-tailed tests were used throughout. Each of the five primary outcome variables was tested at a significance level of 1% in order to maintain an overall 5% level. Exploratory analyses were used to test each of the secondary outcomes at the 5% significance level. Two-sample t tests (or Mann–Whitney tests for the highly skewed Lp(a) and ALT variables) were used to test for imbalances between the treatment arms at baseline (see Table 1).

Linear mixed-effects (LME) models[11] were fitted to the spine, femoral neck, proximal radius and distal radius BMD measurements, to the serum LDL, HDL and total cholesterol, to the triglycerides, ApoA, ApoB, serum calcium, serum 25OH-vitamin D, urine DPYR, hemoglobin and log-transformed Lp(a) and ALT measurements and to the Greene score, systolic and diastolic blood pressure measurements. Treatment (active or placebo), time and their two-way interaction were considered as fixed effects. The patient identifier and time were considered as random effects. Time was considered as a factor with five levels (baseline 0, 6, 12, 18 and 24 months). In the LME models used to assess linear trends within the patient over time, time was considered as a continuous covariate. Normal Q–Q plots were used to assess whether the residuals from the fitted models showed any obvious departure from the underlying assumption of normality.

The LME models included treatment and time as a five-level factor. The two-way interaction between these terms provided effect estimates which are summarized in Table 2. For all outcome measures, a significant interaction between the effects of treatment and time was interpreted as evidence of a difference between the within patient changes observed over time in both the control and active arms of the study.

Many exploratory analyses of secondary outcome measures were performed and false positive results may have occurred. Any such findings should be treated with caution and confirmed in further independent studies.

RESULTS

The patient characteristics at baseline by treatment arm are listed in Table 1. With the exception of triglycerides (control group lower than active), there were no significant differences between treatment arms at baseline.

A total of 20 participants in the active arm withdrew from the study before study completion. Of these, 15 dropped out in the first 6 months. In all, 30 participants in the control arm withdrew from the study, and of these 19 dropped out in the first 6 months. The most common reason for withdrawal from the study was

Table 1. Patient characteristics (mean and s.d.) by treatment group at baseline

Variable	Baseline values				
	Placebo		Active		P-value[a]
	Mean	s.d.	Mean	s.d.	
Age	54.1	3.7	54.6	4.0	0.433
BMI	25.3	4.5	26.3	4.1	0.161
Systolic BP	126.5	18.3	126.7	18.0	0.948
Diastolic BP	76.1	9.0	75.3	9.1	0.627
BMD					
Spine	1.04	0.14	1.02	0.12	0.357
Femoral neck	0.90	0.11	0.86	0.11	0.060
Proximal radius	0.76	0.07	0.77	0.08	0.944
Distal radius	0.33	0.05	0.32	0.05	0.188
Lipids					
Total cholesterol	5.80	0.88	5.91	1.05	0.505
Triglycerides	1.11	0.63	1.33	0.60	0.041
HDL cholesterol	1.82	0.49	1.67	0.35	0.052
LDL cholesterol	3.43	0.86	3.68	0.94	0.125
ApoA	1.75	0.25	1.69	0.21	0.155
ApoB	0.99	0.24	1.05	0.25	0.172
Lp (a)	207.00	252.00	271.00	294.00	0.139
Other					
Serum calcium	2.33	0.10	2.35	0.11	0.128
Serum ALT	23.4	10.9	22.8	13.1	0.544
Serum 25OH VitD	100.0	28.7	97.5	32.6	0.623
DPYR	8.9	3.4	8.4	2.7	0.406
Hemoglobin	134.5	8.0	133.9	7.6	0.643
Greene score	11.0	8.0	8.9	7.3	0.166

Abbreviations: BMI, body mass index; BMD, bone mineral density; BP, blood pressure; LDL, low-density lipoprotein; HDL, high-density lipoprotein; DPYR, deoxypyridinoline. Units used: Age, years; BMD, g/cms^2; total cholesterol, triglycerides, HDL and LDL, mmol/l; ApoA and ApoB, g/l; Lp (a), mg/l; serum calcium, mmol/l; serum ALP, U/l; serum 25OH-vitamin D, nmol/l; DPYR (urine deoxypyridinoline), nmol/mmol creatinine; Hemoglobin, g/l. [a]two-sample t test (or Mann–Whitney test for the skewed lipoprotein (a) and ALT).

worsening of menopausal symptoms. Study participants were not told to expect any change in the frequency of hot flushes during the study period. A total of 55 participants in the active arm and 42 in the control arm completed the 2-year study as per protocol. Figure 1.

If patients dropped out of the study in the first few weeks, they were excluded from the pill count analysis. Not all the patients were diligent in returning their study medications at the end of each 6-month period. Where data were available, compliance was almost identical in the active arm (88% ± 15%) and in the control arm (89 ± 13%). In all, 21 of those receiving active treatment and 29 of those receiving placebo did not record any pill count. Urinary isoflavones were not measured in this study.

In all, 110 participants had a pelvic ultrasound at the time of entry into the study. Endometrial thickness was normal (o 6 mm) in all except one participant, where it was noted to be 9 mm. A curette was performed by the participant's gynecologist; endometrial histology was found to be normal and the participant continued in the study. Fifty-eight participants had a further pelvic ultrasound at Month 24. One patient in the placebo arm had an endometrial thickness of 11 mm; a repeat ultrasound was performed a few months later and was normal.

A total of 142 participants had an X-ray of their thoracolumbar spine at the baseline visit. Vertebral wedging was reported in 14 participants: minor wedging in the thoracic spine was reported in

10 participants, significant wedging in the thoracic spine in 3 participants and significant wedging in the third lumbar vertebra in 1 participant. Ninety participants had further X-rays at Month 24. Thoracic vertebral compression not previously present at the baseline X-ray was reported in five participants: two in the active arm and three in the control arm. No incidence of a clinical vertebral fracture was reported in either arm.

There was no significant evidence of any difference between treatments in the within-patient changes observed over time for the primary outcomes of BMD of the spine, femoral neck, distal radius or proximal radius (all P-values for the time by treatment interactions > 0.25) (Table 2). Across both the active and control arms, there was a statistically significant decrease over time in the BMD of the spine, femoral neck, distal radius and proximal radius (test for linear within patient trend $P = 0.008$, Po 0.001, Po 0.001 and Po 0.001, respectively).

Five participants, four in the active arm and one in the control arm, commenced statin therapy during the study. The lipid parameters of these participants were excluded from all lipid analyses presented in Table 2 (the lipid results were virtually identical when the five participants were included). There was significant evidence of a difference between treatments in the within-patient changes observed over time for the primary outcome of serum LDL cholesterol ($P = 0.005$ for time by treatment interaction) and for serum total cholesterol ($P = 0.015$). In particular, a significant reduction was seen at each time point for the active arm, and this reduction was significantly greater than any change observed in the control arm at all time points except 12 months for both serum LDL cholesterol and for serum total cholesterol (Figure 2).

There was no significant evidence of any difference between treatments in the within-patient changes observed over time for urinary DPYR or serum 25OH-vitamin D (Table 2). Across both the active and control arms, there was a statistically significant reduction in serum 25OH-vitamin D over time (test for linear within-patient trend Po 0.001). There was no significant evidence of any difference between treatments in the within-patient changes observed over time for serum calcium, serum ALT (as a marker of liver function) and hemoglobin, nor was there any significant overall linear within-patient trend with time in any of these variables (data not shown).

There was no significant evidence of any difference between treatments in the within-patient changes observed over time for serum HDL cholesterol or for serum triglycerides (Table 2). Linear trend analyses showed a significant overall within-patient increase in HDL cholesterol and a significant overall within-patient decrease in serum triglycerides with time, neither of which differed significantly by treatment (Po 0.001 and $P = 0.012$, respectively, for linear within-patient trend with time across active and control arms). There was no significant evidence of any difference between treatments in the within-patient changes observed over time for serum ApoA, ApoB or serum Lp(a) (time by treatment interaction $P = 0.744$, 0.056 and 0.089, respectively).

Coagulation factors were measured in 15 participants in the active arm and 11 in the placebo arm. There was no significant difference between the two groups for the prothrombin time, APTT, serum fibrinogen and factor VIII (data not shown).

There was no significant evidence of a difference between treatments in the within-patient changes observed over time for the Greene climacteric scores (time by treatment interaction $P = 0.855$). The average body mass index of the control group at the completion of the study was 25.3 ± 4.1 (s.d.) compared with 26.3 ± 4.2 (s.d.) in the active group. ($P = 0.150$). Significant within-patient decreases from baseline to month 24 were observed for systolic and diastolic BP in both the active and control arms (Po 0.05 in all instances). However, the decreases were comparable in both arms ($P = 0.703$ for systolic and $P = 0.540$ for diastolic BP) (Table 2).

Table 2 Estimated mean within-patient change from baseline and associated 95% CI from the linear mixed-effects model involving time, treatment and their interaction

Variable	Treatment	Change from baseline at 6mths			Change from baseline at 12mths			Change from baseline at 18mths			Change from baseline at 24mths			Time by Treatment interaction P-value[a]
		Mean	(95%CI)	P-value[b]	Mean	(95%CI)	P-value[b]	Mean	(95%CI)	P-value[b]	Mean	(95%CI)	P-value[b]	
BMD														
Spine	Placebo	0.005	(−0.007 to 0.017)	0.353	−0.007	(−0.023 to 0.009)	0.236	−0.006	(−0.023 to 0.011)	0.791	−0.019	(−0.037 to −0.0002)	0.523	0.554
	Active	0.013	(0.001 to 0.024)		0.006	(−0.009 to 0.022)		−0.003	(−0.019 to 0.013)		−0.010	(−0.028 to 0.007)		
Femoral neck	Placebo	0.008	(−0.003 to 0.019)	0.321	−0.010	(−0.023 to 0.004)	0.288	−0.011	(−0.024 to 0.001)	0.602	−0.016	(−0.030 to −0.003)	0.754	0.265
	Active	0.0004	(−0.010 to 0.011)		0.000	(−0.012 to 0.013)		−0.007	(−0.018 to 0.004)		−0.013	(−0.025 to −0.001)		
Proximal radius	Placebo	−0.006	(−0.017 to 0.005)	0.404	−0.006	(−0.015 to 0.003)	0.782	−0.017	(−0.025 to −0.009)	0.960	−0.017	(−0.025 to −0.009)	0.983	0.942
	Active	−0.012	(−0.022 to −0.002)		−0.008	(−0.016 to 0.0003)		−0.017	(−0.024 to −0.010)		−0.017	(−0.024 to −0.009)		
Distal radius	Placebo	−0.003	(−0.008 to 0.002)	0.151	−0.005	(−0.010 to −0.001)	0.289	−0.010	(−0.016 to −0.004)	0.105	−0.010	(−0.016 to −0.004)	0.414	0.468
	Active	0.002	(−0.003 to 0.007)		−0.002	(−0.006 to 0.002)		−0.004	(−0.009 to 0.002)		−0.007	(−0.012 to −0.001)		
Lipids														
Total cholesterol	Placebo	−0.151	(−0.245 to 0.043)	0.018	−0.265	(−0.487 to −0.043)	0.741	−0.128	(−0.245 to 0.090)	0.033	−0.113	(−0.207 to 0.081)	0.07	0.015
	Active	−0.477	(−0.663 to −0.291)		−0.316	(−0.527 to −0.105)		−0.457	(−0.666 to −0.248)		−0.361	(−0.546 to −0.176)		
Triglycerides	Placebo	0.039	(−0.099 to 0.176)	0.905	−0.046	(−0.179 to 0.088)	0.375	−0.019	(−0.181 to 0.144)	0.957	−0.056	(−0.167 to 0.055)	0.137	0.560
	Active	0.027	(−0.103 to 0.157)		−0.130	(−0.258 to −0.001)		−0.025	(−0.182 to 0.132)		−0.173	(−0.279 to −0.066)		
HDL	Placebo	−0.061	(−0.137 to 0.016)	0.485	−0.042	(−0.113 to 0.030)	0.277	0.015	(−0.068 to 0.099)	0.592	0.049	(−0.033 to 0.132)	0.521	0.141
	Active	−0.098	(−0.172 to −0.025)		0.013	(−0.055 to 0.081)		−0.016	(−0.096 to 0.064)		0.087	(0.008 to 0.165)		
LDL	Placebo	−0.088	(−0.282 to 0.107)	0.008	−0.137	(−0.352 to 0.078)	0.386	−0.121	(−0.294 to 0.099)	0.031	−0.067	(−0.244 to 0.111)	0.004	0.005
	Active	−0.456	(−0.643 to −0.270)		−0.268	(−0.471 to −0.006)		−0.455	(−0.663 to −0.274)		−0.432	(−0.600 to −0.263)		
Apo A	Placebo	−0.074	(−0.132 to −0.016)	0.579	−0.084	(−0.142 to −0.026)	0.911	−0.049	(−0.108 to 0.010)	0.369	−0.018	(−0.069 to 0.033)	0.346	0.744
	Active	−0.097	(−0.153 to −0.040)		−0.079	(−0.134 to −0.024)		−0.086	(−0.143 to −0.029)		−0.052	(−0.101 to −0.003)		
Apo B	Placebo	0.018	(−0.017 to 0.062)	0.013	−0.059	(−0.111 to −0.006)	0.484	−0.016	(−0.064 to 0.032)	0.056	−0.032	(−0.080 to 0.016)	0.066	0.056
	Active	−0.061	(−0.104 to −0.018)		−0.085	(−0.134 to −0.035)		−0.081	(−0.127 to −0.035)		−0.095	(−0.141 to −0.049)		
log(Lipo (a))	Placebo	0.032	(−0.067 to 0.131)	0.091	0.079	(−0.028 to 0.186)	0.185	−0.009	(−0.103 to 0.116)	0.019	0.153	(0.009 to 0.298)	0.029	0.089
	Active	−0.086	(−0.182 to 0.009)		−0.022	(−0.126 to 0.082)		−0.214	(−0.333 to −0.105)		−0.074	(−0.218 to 0.007)		
Other														
Serum calcium	Placebo	0.005	(−0.024 to 0.034)	0.013	−0.002	(−0.035 to 0.030)	0.193	−0.005	(−0.034 to 0.023)	0.705	−0.006	(−0.037 to 0.026)	0.220	0.010
	Active	−0.046	(−0.073 to −0.020)		−0.032	(−0.063 to −0.001)		0.002	(−0.025 to 0.029)		−0.033	(−0.062 to −0.003)		
log(ALT)	Placebo	0.035	(−0.069 to 0.139)	0.188	−0.010	(−0.096 to 0.076)	0.753	0.048	(−0.072 to 0.168)	0.262	−0.041	(−0.130 to 0.048)	0.404	0.627
	Active	−0.061	(−0.159 to 0.037)		−0.029	(−0.110 to 0.052)		−0.047	(−0.160 to 0.066)		−0.093	(−0.177 to −0.009)		
Serum 25OH VitD	Placebo	−3.913	(−10.293 to 2.467)	0.461	−12.660	(−19.912 to −5.408)	0.131	−11.655	(−20.381 to −2.929)	0.051	−17.970	(−24.929 to −11.012)	0.452	0.338
	Active	−0.686	(−6.679 to 5.307)		−5.172	(−11.888 to 1.544)		0.059	(−8.096 to 8.214)		−14.383	(−20.886 to −7.879)		
DPYR	Placebo	−1.889	(−2.854 to −0.924)	0.252	−2.140	(−3.047 to −1.333)	0.021	−0.687	(−1.919 to 0.546)	0.802	−1.266	(−2.126 to −0.406)	0.87	0.051
	Active	−1.106	(−2.031 to −0.180)		−0.664	(−1.420 to 0.192)		−0.901	(−2.039 to 0.236)		−1.167	(−1.983 to −0.350)		
Hemoglobin	Placebo	−1.957	(−3.397 to −0.517)	0.103	−2.024	(−3.658 to −0.389)	0.188	−1.105	(−3.121 to 0.911)	0.689	−2.549	(−4.216 to −1.381)	0.021	0.061
	Active	−0.309	(−1.666 to 1.048)		−0.527	(−2.037 to 0.984)		−0.543	(−2.422 to 1.337)		0.153	(−1.406 to 1.711)		
Greene score	Placebo	NA	NA		0.099	(−1.945 to 2.142)	0.399	0.610	(−1.497 to 2.717)	0.567	0.782	(−1.548 to 3.112)	0.660	0.855
	Active	NA	NA		1.313	(−0.627 to 3.253)		1.459	(−0.534 to 3.453)		1.501	(−0.694 to 3.696)		
Systolic BP	Placebo	NA	NA		NA	NA		NA	NA		−6.1	(−10.7 to −1.6)	0.703	0.703
	Active	NA	NA		NA	NA		NA	NA		−5.0	(−8.7 to −1.4)		
Diastolic BP	Placebo	NA	NA		NA	NA		NA	NA		−4.1	(−6.5 to −1.7)	0.540	0.540
	Active	NA	NA		NA	NA		NA	NA		−3.0	(−5.6 to −0.4)		

Abbreviations: BMD, bone mineral density; BP, blood pressure; CI, confidence interval; DPYR, deoxypyridinoline; HDL, high-density lipoprotein; LDL, low-density lipoprotein. [a]test of difference between active and placebo within-patient response to treatment over time. [b]test of difference between active and placebo within-patient change from baseline at specified time.
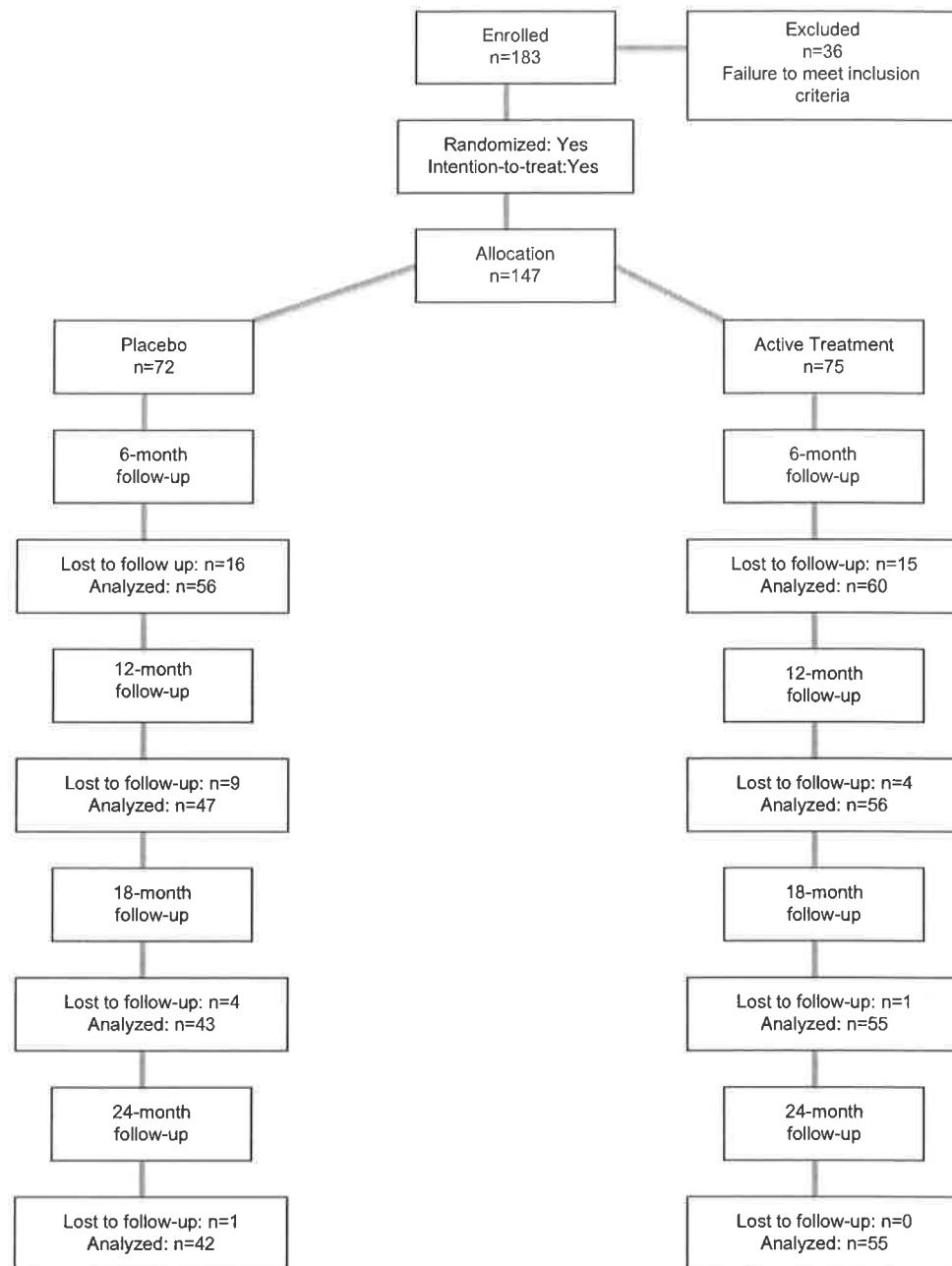
Figure 1. Flowchart of study participants and subject participation.

Adverse events

Several serious adverse events occurred during the study: in the active arm, one participant withdrew 3 weeks after randomization with a diagnosis of colon carcinoma, and another participant in this arm lost 22 kg in weight, and despite full investigation as to other probable causes, may have been due to voluntary excessive caloric restriction. Other reported adverse events in the active arm include biopsy-confirmed benign thyroid nodules ($n = 2$), bowel obstruction ($n = 2$, one small bowel and one large bowel) and type 2 diabetes, duodenal ulcer, gastric ulcer and celiac disease (all $n = 1$). In the control arm, one participant suffered a subarachnoid hemorrhage after a fall from which she recovered. Another patient died of a ruptured berry aneurysm. Other reported adverse events in the placebo arm include superficial thrombophlebitis ($n = 2$), biopsy-confirmed benign thyroid nodules ($n = 2$), deep-vein thrombosis, diverticulitis, gastric ulcer, biliary colic, cholecystitis,

abnormal LFTs of unknown cause, which recovered spontaneously, fractured L4 and fractured ribs (all $n = 1$).

Sinusitis, upper respiratory tract infection, urine tract infection, muscle and joint pain were common in both treatment arms. Several participants were troubled by constipation.

There was no instance of breast cancer in either treatment arm, and although participants were encouraged to maintain regular government-funded 2-yearly mammograms, systematic mammograms were not carried out during the study.

DISCUSSION

The findings of the studies showing an increased risk of breast cancer and cardiovascular disease in postmenopausal women receiving estrogen and progesterone therapy have led to the search for alternative agents, which might retain the benefits of
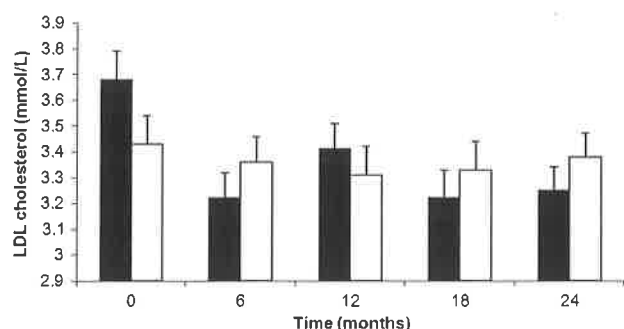
Figure 2 LDL cholesterol measurements by time in the active treatment group (closed bars) and the placebo group (open bars). Using a linear mixed-effects model, there was significant evidence of a difference between treatments in the within-patient change observed over time ($P = 0.005$).

estrogen therapy, with a concomitant reduction in the risk of adverse effects.

Estrogen exerts its effects by binding to ERα and ERβ[6,12] and also by influencing the function of cell kinases through interaction with the estrogen receptor.[13] ERβ is found predominantly in bone, whereas ERα is significantly expressed in the breast and endometrium but also significantly in bone. It is ERβ and not ERα that mediates the reduction in sclerostin with changes in estradiol and mechanical loading.[14]

Isoflavones have estrogen-like activity and bind preferentially to ERβ but with a much lower affinity than estradiol.[6] Soy and red clover extracts contain isoflavones; soy isoflavones consist predominantly of genistein and daidzein, whereas red clover isoflavones consist predominantly of formononetin and biochanin. Biochanin is converted into genistein and formononetin into daidzein, and it is not certain whether formononetin and biochanin have any intrinsic biologic activity before conversion. Genistein has 20–30 times the binding affinity for ERβ compared with ERα. The binding affinity of biochanin to estrogen receptors is 10 000 times less than estradiol.[6] Formononetin stimulated ERβ-dependent beta galactosidase production to a greater extent than via ERα.[15] However, formononetin stimulated osteoblast differentiation via the MAP kinase pathway and did not activate the osteoblast ER.[16] Genistein also enhanced osteoblast differentiation by the p38 MAP kinase pathway[17] and reduced the RANKL/OPG mRNA expression ratio in human osteoblasts.[18]

Early studies with soy isoflavones in ovariectomized rats showed the prevention of trabecular and cortical bone loss.[19,20] Biochanin maintained femoral BMD and formononetin restored femoral and tibial trabecular architecture[21,22] in ovariectomized rats. Red clover isoflavones, however, did not reduce bone resorption from bone prelabeled with [41]Ca.[23] Treatment of postmenopausal women with soy isoflavones and red clover isoflavones have produced variable results. A summary of the effect of isoflavones on BMD is shown in Table 3.[24–35] Some studies showed a preservation of particularly hip BMD,[29–33] especially with genistein.[29–31] Red clover isoflavones enriched with biochanin were associated with preservation of spinal BMD.[35] In our present study using red clover isoflavones enriched with formononetin, there was no impact on the rate of loss of BMD over a 24-month period at the spine, hip and forearm, which proceeded at a rate expected in the postmenopausal state. Zhang et al.[36] showed in a large Shanghai population study that high dietary soy protein intake was associated with a significant reduction in fracture rate. A typical soy diet consists of 15–50 mg/day of isoflavones.[37] Many of the studies using soy isoflavones involved much higher doses of isoflavones rich in genistein and daidzein. It is possible that other factors in soy extract apart from isoflavones may have a role in reducing incident fractures.

Another potential benefit of isoflavones might be to lower serum LDL cholesterol. The lipid-lowering effects of isoflavones have been variable in postmenopausal women, summarized in Table 4. In most studies using soy isoflavones, there was no change in serum LDL cholesterol,[25,31,38–41] whereas a significant fall occurred in two studies.[33,42] A meta-analysis of studies using soy isoflavones[43] showed a significant, albeit small, reduction in serum LDL of 0.13 mmol/l ($Po\ 0.0001$). With red clover isoflavones, there was no change in serum LDL cholesterol in five studies[44–48] and a significant fall of 11.6% in one study.[49] In our study using red clover isoflavones enriched with formononetin at 50 mg/day and conducted over a 24-month period, there was a significant fall in the serum LDL cholesterol, which was first observed at 6 months and maintained at 24 months. The serum LDL cholesterol in the treated arm fell by 12% from 3.68 mmol/l to 3.25 mmol/l, a drop of 0.43 (95% CI -0.26–0.60) versus a nonsignificant decline in the control arm of 0.07 ((95% CI -0.11-0.24).

Gould,[50] in a large population study, showed that for every 1 mmol/l reduction in serum LDL cholesterol there was a 15.6% reduction in all-cause mortality and a 28% reduction in coronary heart disease mortality. Bush et al.[51] followed up women aged 40-49 years for 8.5 years and there was a significant reduction in death from cardiovascular disease in estrogen users (RR = 0.34), and the reduction in mortality was related to a rise in the HDL cholesterol. When statins were used to reduce serum LDL cholesterol, for a 1 mmol/l reduction in serum LDL cholesterol, the risk ratio for mortality was 0.79 irrespective of the baseline serum cholesterol and the presence or absence of previous vascular disease.[52] In a Lipid Research Clinics' coronary primary prevention trial in men, a 12.6% reduction in serum LDL cholesterol was associated with a 19% reduction in myocardial infarction.[53] In a Shanghai cohort of middle- and older-aged women, the risk of coronary heart disease fell by 75% in those consuming a high-soy protein diet,[54] although this benefit cannot be attributed to the impact of isoflavones specifically. Isoflavones fed to cynomolgus monkeys was associated with a significant reduction in the extent of atherosclerosis.[55]

The exact mechanism whereby isoflavones may lower serum LDL cholesterol is unclear. In ovariectomized rats deprived of estradiol, hepatic expression of LDL receptor and PcsK9 is reduced and restored by estradiol supplementation.[56]

Soy isoflavones at 91 mg/day for 2.7 years reduced the rate of progression of atherosclerosis, as measured by a change in carotid artery intima-media thickness in postmenopausal women within 5 years of the onset of menopause.[57] Promensil and formononetin improved systemic arterial compliance.[45,58] Aortic stiffness measured as pulse-wave velocity is strongly associated with atheroclerosis[59] and with cardiovascular mortality,[59,60] and thus cardiovascular benefits of isoflavone therapy may occur independently of any change in serum LDL cholesterol.

We did find a significant lowering of blood pressure over the 24-month period of our study, but there was no significant difference between the active treatment and control groups. Red clover isoflavones enriched with formononetin[61] and a metabolite of formononetin[62] reduced systolic and diastolic blood pressure in postmenopausal women and formononetin had an antihypertensive effect in spontaneously hypertensive male rats.[63]

The isoflavone genistein, 54 mg/day, significantly reduced the frequency of hot flushes in postmenopausal women.[30,31] The red clover extract, Promensil, reduced the frequency of hot flushes.[64] Rimostil, 82 mg/day, given over a 12-week period had no impact on the frequency of hot flushes,[65] and in our study, Rimostil, 50 mg/day, also had no significant effect on menopausal symptoms evaluated by the Green Climacteric Scale.

There are several possible limitations to the interpretation of the results. The number of participants recruited may not have been large enough to allow the ascertainment of significant differences

Table 3. Effects of isoflavones on bone

Isoflavone type	Dose (mg/d)	Duration in weeks	Skeletal effects
Soy protein	40	24	BMD increased[24]
Soy isoflavones	110	52	BMD loss unchanged[25]
Genistein, Daidzein	300	104	BMD loss unchanged[26]
Soy isoflavones	200	104	BMD loss unchanged[27]
Soy isoflavones	105	52	BMD unchanged[28]
Genistein	54	104	BMD increased at femoral neck[29,30]
Genistein	93	104	BMD loss at femoral neck slightly reduced[31]
Soy isoflavones	120	156	BMD loss at femoral neck reduced[32]
Soy isoflavones	165	104	BMD loss of total hip reduced[33]
Soy isoflavones	120	104	Whole-body BMD loss slightly reduced[34]
Red clover isoflavones enriched with biochanin	43	52	BMD spine preserved[35]
Red clover isoflavones enriched with formononetin	50	104	BMD spine, hip and forearm not preserved (current study)

Abbreviation: BMD, bone mineral density.

Table 4. Effects of Isoflavones on LDL Cholesterol

Isoflavone type	Dose (mg/d)	Duration in weeks	LDL cholesterol effects
Soy isoflavones	110	52	No change[25]
Soy isoflavones (soy protein mostly genistein)	93	52	No change[31]
Soy isoflavones	165	104	6% reduction[33]
Soy isoflavones	80, 120	104	No change[38]
Soy isoflavones	75	52	No change[39]
Soy isoflavones (soy protein)	40	12	0.60 mm/l reductionl[42]
	69		
Genistein	54	52	No change[40]
Soy germ isoflavones (50–60% daidzein)	84, 126	12,24	No change[41]
Red clover isoflavones	43, 86	10	No change[44]
Red clover isoflavones biochanin enriched	40	10	No change[45]
	80		
Red clover isoflavones enriched with formononetin	40	6	No change[46]
Red clover isoflavones	80	13	11.6% reduction[49]
Red clover isoflavones enriched with formononetin	57.2	12	No change[47]
Red clover enriched with biochanin	43	52	No change[48]
Red clover isoflavones formononetin enriched	50	104	12% reduction (current study)

Abbreviation: LDL, low-density lipoprotein.

in BMD between the two treatment groups. However, the effect sizes measured for within-person changes would suggest that even if much larger numbers were studied a significant benefit of Rimostil on BMD would not have been seen. The larger number of dropouts from the inactive treatment group versus the active treatment group may have skewed results in an unpredictable way.

In summary, the present study has shown that, compared with placebo, 50 mg/day Rimostil significantly lowers the primary outcome variable of serum LDL cholesterol over 24 months. The improvement is evident by 6 months. Our previous study[8] also showed a significant reduction in serum LDL cholesterol with Rimostil. In contrast to our previous study, the use of Rimostil compared with placebo did not significantly alter the loss of BMD in the forearm, or in the lumbar spine and femoral neck. It is possible that the binding of the isoflavones in Rimostil to the ERβ was too weak to prevent loss of BMD. There was no adverse effect on endometrial thickness. The constellation of adverse effects in patients taking Rimostil was no different from those seen in the control group. It is uncertain whether the fall in the serum LDL cholesterol might lead to a change in the risk of cardiovascular disease, or whether the observed fall in the serum LDL cholesterol with Rimostil would augment any fall in the serum LDL cholesterol brought about by other agents such as statins.

DISCLAIMER

A partial professional salary was paid to MLN, the study coordinator who recruited and supervised all study participants. None of the other authors received any payment from Novogen Ltd. None of the authors or members of their families hold any stock in Novogen Ltd. This double-blind, placebo-controlled study was not registered with the clinical trials registry because the study commenced before the requirement was introduced to register all clinical trials.

AUTHOR CONTRIBUTIONS

PBC-B, GRF and RB developed the overall research plan and designed the protocol. MLN was responsible for participant recruitment and for the progress of each participant through the study protocol to the time of completion or withdrawal. PBC-B, RJC-B, SV, GRF and RB were responsible for ongoing clinical

assessment of each participant throughout the trial. PBC-B and KB made major contributions to the writing of the manuscript and take responsibility for the validity of the data. KB performed statistical analyses of the data. SV died during the course of the study. All other authors have read and approved the final manuscript.

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Paper 25. Mortality associated with primary hyperparathyroidism. PB Clifton-Bligh, ML Nery, R Supramaniam, TS Reeve, L Delbridge, JN Stiel, A McElduff, ES Wilmshurst, BG Robinson, GR Fulcher, D Learoyd, S Posen. Bone 2015; 74:121-124

P Clifton-Bligh made a major contribution to this paper with respect to the collection and analysis of the data, the writing of the manuscript and in the reading of the bibliography.This paper has identified all patients with primary hyperparathyroidism seen between 1961 and 1994 in the Department of Endocrinology at Sydney Hospital, and at the Royal North Shore Hospital. 561 patients were identified and a determination as to whether they were alive or dead at the end of 1994 was made. The ascertainment of mortality was further extended to the end of 2011. The interval from the time of diagnosis to the time of death was calculated.Each patient was compared with a control population matched for age, sex, and the year observation began and the duration of observation. Life tables were used for the Australian control population for the time interval 1961-1994. 113 patients with mild hyperparathyroidism were not subject to neck exploration. There was no significant difference in the age of those who had neck exploration and of those who did not. 124 patients died between 1961 and 1994. Relative survival over 10 years in those who had surgery compared to those who did not was not significantly different although the mean serum calcium in the non-operated patients was lower than in the operated patients. The average number of years lost by the hyperparathyroid population compared to the control population was 7.5 years. There was no significant difference in the death rate in those whose serum calcium was above 3 mmol/L compared to those whose serum calcium was below 3 mmol/L. A mulitivariate analysis in the surgically treated group showed that the serum calcium did not significantly influence survival. In the non-surgically treated group, the serum parathyroid hormone was significantly associated with mortality (HR 1.59, 95%CI 1.20-2.11, P=0.001). In the surgically treated group, risk factors associated with an increased risk of death were diabetes mellitus, coronary heart disease, congestive cardiac failure and hypertension. The study was continued until the end of 2011. Patients in the surgically treated group were compared to patients in the non-surgically treated group and matched for age, sex, serum calcium and for the presence or absence of hypertension. The mean serum calcium in this selected cohort was 2.88 mmol/L. There was no significant difference in the death rate between the two groups (P=0.732). At the end of 2011, 73 in the non-surgical group and 224 in the surgical group had died. The average age of death for the non-surgical group was 78.2 years and for the surgical group was 77.4 years, not significantly different.

This paper is considered to be a major contribution to the world literature in regard to the incidence of mortality in persons with hyperparathyroidism and involved an exhaustive study in a large number of patients over many years. The collection of the ongoing data was meticulous.

Original Full Length Article

Mortality associated with primary hyperparathyroidism ☆

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ABSTRACT

561 patients with primary hyperparathyroidism were followed between 1961 and 1994. Relative survival was compared to that of the Australian population studied during the same time interval. Mortality was significantly greater in the hyperparathyroid population (P < 0.001). Mortality was not greater in the patients with serum calcium levels >3.00 mmol/L compared to those with a serum calcium levels <3.00 mmol/L.

113 patients did not have parathyroid surgery. Their relative survival was not significantly different from those who had surgery but their mean serum calcium and parathyroid hormone (PTH) levels were significantly lower than those who had surgery.

A re-analysis of the 453 patients followed between 1972 and 2011 was carried out and a 20-year survival analysis made of those diagnosed between 1972 and 1981 and those diagnosed between 1982 and 1991. The latter group had significantly worse relative mortality than the former group (P < 0.001) but was significantly older at the time of diagnosis (56.94 ± 14.83 vs 52.01 ± 13.58, P < 0.001). The serum calcium and serum PTH levels were not significantly different between these two groups.

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Introduction

Hyperparathyroidism has been associated with an increased mortality both in surgically treated patients and in patients who have not undergone surgery [1–4]. In other studies, mortality in surgically treated hyperparathyroidism was not increased [3,5,6]. There is uncertainty as to whether surgery for hyperparathyroidism confers benefit in terms of survival. The present study was therefore undertaken to examine survival in a cohort of patients with hyperparathyroidism studied over a 50-year period between 1961 and 2011, and to determine whether hyperparathyroidism was associated with increased mortality and whether surgery made any impact on survival.

Patients and methods

The first patient diagnosed and treated for primary hyperparathyroidism in our institution was in 1961. Between this time and at the end of 1994, all patients diagnosed with primary hyperparathyroidism were identified, medical records were obtained and examined, and a determination was made as to whether or not they were alive at the end of 1994. In Australia, each state has access to Australia-wide date of death data although each state maintains its own death register. The patient cohort list was submitted to each local state death registry, dates of death recorded for any of those who had died, and relevant states were contacted in order to obtain a copy of each death certificate.

The interval from the time of diagnosis to the time of death was calculated. Each patient was compared with a control population matched for age, sex, the year observation began, and the duration of the observation. The control population was the Australian population at large for whom Life Tables from 1961–1994 existed at the time. Life Tables are published by the Australian Government Actuary. Taking into account age, sex, start year of observation and duration of observation, survival data of individuals with primary hyperparathyroidism compared with expected survival in the general Australian population was obtained from the Life Tables.

A second analysis of relative survival over a 20-year time interval was calculated for the patients studied between 1972 and 2011 using a new methodology. The group was divided into two cohorts, those

diagnosed between 1972 and 1981 and those diagnosed between 1982 and 1991, and a 20-year relative survival was calculated for each cohort.

Before 1972, the diagnosis of primary hyperparathyroidism was made if surgical removal of a parathyroid tumour restored eucalcaemia, or if a full investigation failed to find another cause for hypercalcaemia. After 1972, the diagnosis of primary hyperparathyroidism was made if the serum calcium and serum PTH was above the upper limit of the reference range. The PTH assay used was that of Kleerekoper et al. [7]. 561 patients were studied in the time interval 1961 to 1994 and 453 patients between 1972 and 2011. Because of the concept that persons with mild hypercalcaemia might not require surgery [3], 113 of our patients with mild hyperparathyroidism were not subjected to neck exploration. 448 patients had parathyroid surgery. The patients were predominantly of Anglo-Celtic ethnicity.

Statistics

In the first study of relative survival up to 1994, the statistical program developed by Guy Hedelin [8] was used. Cox's proportional hazard multivariate analysis was used to study the impact on survival of variables such as the presence or absence of known cardiovascular disease. In the later analysis up to 2011, the method outlined by Dickman et al. which uses a generalised linear model based on collapsed data using exact survival times and a Poisson assumption was employed [9].

Results

Table 1 shows the age, serum calcium and serum PTH in the surgically and non-surgically treated groups. There was no significant difference in age. As expected, the serum calcium and the serum PTH were significantly lower in the non-surgically treated group.

124 patients died between 1961 and 1994. The relative survival rate over a 10-year period for the total hyperparathyroid group was significantly lower than the control population, 86.8% (95% CI 84.9–86.2, P < 0.001). There was no significant difference in the relative survival between surgically treated and non-surgically treated patients over a 10-year period (Fig. 1). The average number of years of life lost by the hyperparathyroid patients compared to the control population was 7.5 years. There was no significant difference in the death rate between those with an initial serum calcium of >3.00 mmol/L compared with those with an initial serum calcium of <3.00 mmol/L. In a multivariate analysis in the surgically treated group, Table 2, the serum calcium did not significantly influence survival (HR 1.43, 95% CI 0.79–2.59, P = 0.236). In the non-surgically treated group, Table 3, the serum calcium did not significantly influence survival (HR 1.57, 95% CI 0.30–8.30, P = 0.593). In a multivariate analysis, risk factors associated with death in the surgically treated group were diabetes mellitus (HR 4.09, 95% CI 1.42–6.74, P = 0.001), congestive cardiac failure (HR 5.46, 95% CI 1.31–22.87, P = 0.002), coronary heart disease (HR 2.16, 95% CI 1.08–4.31, P = 0.03) and hypertension (HR 1.54, 95% CI 1.01–2.34, P = 0.044), Table 2. Paradoxically, the presence of kidney stones before surgery was associated with reduced mortality (HR 0.364, 95% CI 0.22–0.68, P = 0.001). The association of kidney stones with improved survival was also observed in another study [6]. In the non-surgically treated group, death was significantly associated with a high serum PTH (HR 1.59, 95% CI 1.20–2.11, P = 0.001), coronary heart disease
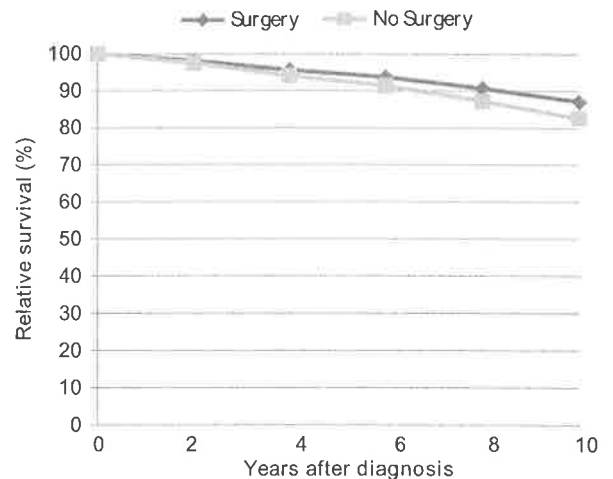

Fig. 1. Relative survival: Percent years after diagnosis, years 1961–1994. Relative survival for the surgically treated and non-surgically treated groups was not significantly different over 10 years of follow-up.

(HR 3.10, 95% CI 1.42–6.74, P = 0.004), and kidney stones (HR 2.48, 95% CI 1.07–5.76, P = 0.035), Table 3. This difference between the surgically treated and non-surgically treated group with respect to the impact of kidney stones is not clear. Compared with the non-surgically treated group, the hazard ratio of death for the surgically treated group adjusted for age, sex and time of diagnosis was 0.67 (95% CI 0.38–1.18, P = 0.167).

The study was continued until the end of 2011. Patients in the surgically treated group were compared with those in the non-surgically treated group matched with respect to age, sex, serum calcium and for the presence or absence of hypertension. This matching was performed before it was known whether any patient had died. 109 patients were matched; 44 in the non-surgically treated group and 65 in the surgically treated group. The serum calcium in this selected cohort was 2.88 mmol/L. In this cohort, 28 patients in the non-surgically treated group and 35 in the surgically treated group had died (HR 1.18, 95% CI 0.44–3.03, P = 0.732).

A reanalysis of the relative survival for the hyperparathyroid group from 1972 to 2011 showed that this group had a 20-year relative survival of 62.9% (95% CI 58.5–67.4, P < 0.001) compared to the general Australian population, Fig. 2. Relative survival appears to fall slowly

Table 1
Patient demographics: Baseline characteristics.

	Surgery n = 448	Non-surgery n = 113	p-value
Age	52.9 ± 14.7	55.5 ± 15.9	P = 0.117
Serum calcium (mmol/L)	3.04 ± 0.35	2.80 ± 0.18	P < 0.001
Serum PTH (ng/ml)	1.34 ± 1.12 (n = 344)	0.90 ± 0.74 (n = 98)	P < 0.001

Table 2
Overall relative survival for surgical group: Effect of possible risk factors for death.

Variable	P-value	Hazard Ratio	95% CI
Diabetes	0.001	4.085	1.836–9.089
Kidney stones	0.001	0.364	0.216–0.681
CCF	0.02	5.463	1.305–22.869
CAD	0.03	2.156	1.079–4.309
Hypertension	0.044	1.538	1.011–2.341
PTH	0.098	1.194	0.968–1.474
Paget's disease	0.137	1.653	0.853–3.204
CVA/PVD	0.141	1.974	0.797–4.889
Calcium	0.236	1.430	0.791–2.585
Fracture	0.283	1.585	0.684–3.673
Ulcer	0.431	1.306	0.672–2.536
Gender	0.493	1.185	0.729–1.927
Cigs/day	0.506	1.008	0.985–1.03
Smoking	0.511	1.203	0.694–2.086
Pancreatitis	0.600	1.365	0.427–4.367
Cholesterol	0.650	0.960	0.804–1.146
Cancer	0.896	1.048	0.520–2.112

Multivariate analysis of possible risk factors affecting relative survival in hyperparathyroid patients treated with surgery. Cox's proportional hazard analysis.
CCF: Congestive cardiac failure, CAD: Coronary artery disease, CVA: Cerebrovascular disease, PVD: Peripheral vascular disease.

Table 3
Overall relative survival for non-surgical group: Effect of possible risk factors for death.

Variable	P-value	Hazard ratio	95% CI
PTH	0.001	1.59	1.196–2.113
CAD	0.004	3.096	1.422–6.763
Kidney stones	0.035	2.481	1.068–5.763
CVA/PVD	0.061	2.752	0.953–7.947
Smoking	0.188	1.92	0.728–5.067
Cholesterol	0.190	1.255	0.893–1.764
Cancer	0.354	1.432	0.670–3.060
Paget's disease	0.397	0.592	0.176–1.994
Gender	0.399	0.730	0.351–1.516
CCF	0.439	1.468	0.556–3.877
Hypertension	0.444	1.328	0.643–2.740
Cigs/day	0.491	1.016	0.972–1.062
Calcium	0.593	1.574	0.298–8.306
Fracture	0.689	1.167	0.547–2.492
Ulcer	0.738	1.284	0.297–5.557
Pancreatitis	0.741	0.718	0.100–5.143
Diabetes	0.996	1.003	0.381–2.643

Multivariate analysis of possible risk factors affecting relative survival in hyperparathyroid patients not treated with surgery. Cox's proportional hazard analysis.
CCF: Congestive cardiac failure, CAD: Coronary artery disease, CVA: Cerebrovascular disease, PVD: Peripheral vascular disease.

between 0 and 12 years following diagnosis and then more rapidly between 12 and 20 years independently of the baseline serum calcium and serum PTH. Using a 20 year follow-up for the whole group, multivariate analysis did not show any survival difference between male and female, surgery versus non-surgery (P = 0.867), serum calcium >3 mmol/L versus <3 mmol/L (P = 0.794), or serum PTH analysed as quartiles (P = 0.317). The study group was separated into those diagnosed between 1972 and 1981 and those diagnosed between 1982 and 1991. Relative survival over a 20-year period was significantly better in the earlier group, 79.3% vs 45.9% in the later group (adjusted HR 0.20, 95% CI 0.06–0.62, P < 0.001). The later group contained significantly more females, was older at the time of diagnosis (56.94 ± 14.83 vs 52.01 ± 13.58 years, P < 0.001) but the serum calcium and serum PTH were not significantly different.
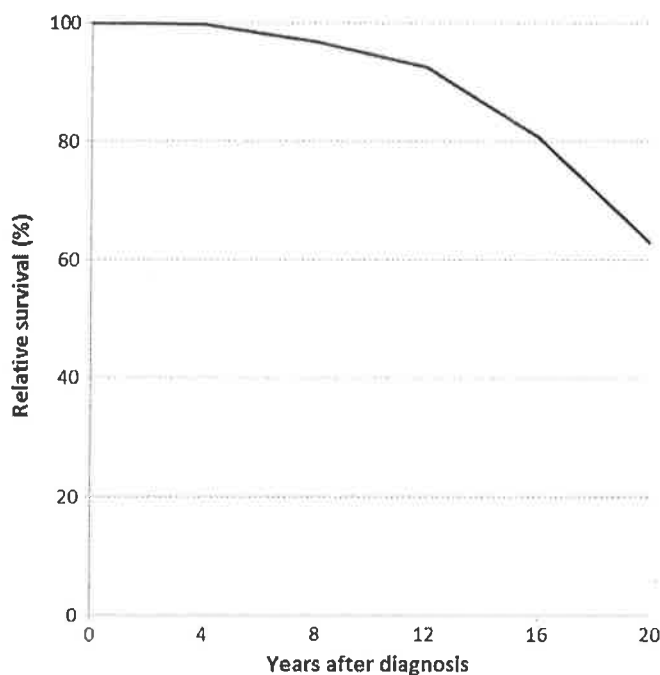

Fig. 2. Relative survival: Percent years after diagnosis, years 1972–2011. The 20 year relative survival of the hyperparathyroid patients studied between 1972 and 2011.

By the end of 2011, 73 in the non-surgical group and 224 in the surgical group had died. The average age of death in the non-surgical group was 78.2 ± 11.07 years and in the surgical group, 77.40 ± 10.82 years (P = 0.5523).

Discussion

In our cohort of 561 patients with primary hyperparathyroidism studied between 1961 and 1994 and then until 2011, there was a significantly increased risk of death compared to the general population both in those who had parathyroid surgery and in those who did not. The patients who did not undergo parathyroid surgery were the same age as those who underwent surgery but their serum calcium was significantly lower. There was no difference in mortality in those with serum calcium >3.00 mmol/L compared to those with a serum calcium of <3.00 mmol/L. In multivariate analysis, the baseline serum calcium did not influence the risk of death in the surgically or non-surgically treated group, and rendering the serum calcium normal by surgical removal of a parathyroid adenoma did not significantly influence relative survival. This is similar to the findings of Wermers et al. [3]. In the surgically treated group, the relative mortality was not significantly different from the non-surgically treated group. An attempt was made to match the surgically treated and the non-surgically treated group for age, sex, serum calcium and the presence of hypertension and again, there was no significant difference in mortality between the two groups. However, the numbers studied may not have been large enough to detect a significant difference even when deaths up until 2011 were included in the analysis.

In other studies, non-surgically treated mild primary hyperparathyroidism was associated with an increase in all cause mortality [4] and mortality was best correlated with the serum PTH and not with the serum calcium [10]. In other studies, serum calcium was an independent predictor of mortality [2,3], whereas the preoperative serum calcium did not predict survival in another study [11]. Hedback et al. [2] found that in all patients diagnosed with primary hyperparathyroidism in Sweden between 1985 and 1987, there was a significantly increased risk of death during follow-up compared to the normal Swedish population. Norenstedt et al. [12] found in another Swedish study of 14,635 patients subject to parathyroidectomy between 1961 and 2004 that the mortality between 31 and 365 days after surgery from 1987 onwards was not increased. Previously it had been found that if patients dying in the first year after parathyroidectomy and those having surgery after 1985 were excluded, there was an increased risk of dying for persons of all ages and that the increased risk persisted beyond 15 years [5]. The preoperative serum calcium was not known in this study. There was a significant increase in mortality in another study in patients followed up after parathyroid surgery compared to the general population (HR 2.3, P = 0.00024) [13]. However, in another study of 1052 patients who had parathyroid surgery between 1980 and 1984, there was no subsequent increase in mortality compared to the general population [6]. Mortality has been linked with serum calcium even in persons without documented hyperparathyroidism [14–18] and serum PTH has also been a strong predictor of death in eucalcaemic populations independent of serum 25OH-vitamin D levels [19–25]. In heart failure, serum PTH and 25OH-vitamin D were independently associated with increased mortality [26]. A large component of the mortality described is from cardiovascular disease and there are several possible mechanisms whereby circulating PTH may be linked to cardiovascular disease. Serum PTH was correlated with lower levels of serum HDL and higher levels of VLDL [27] known to be associated with an increased risk of coronary heart disease [28]. Insulin resistance was increased in hyperparathyroidism which improved after parathyroidectomy [29]. In a non-hyperparathyroid population, serum PTH was inversely correlated with insulin sensitivity [30]. Serum PTH corrected for age, sex and BMI was significantly correlated with mean arterial blood pressure [31] and also with the presence of hypertension, which is common in hyperparathyroidism [32]. In a long term follow-up study, a serum PTH of

>75 ng/L was highly correlated with the first appearance of hypertension [24]. Stefenelli et al. found that 81% of his hyperparathyroid cohort had left ventricular hypertrophy. In this cohort, only 55% had hypertension [33]. In another study, left ventricular hypertrophy in a non-hyperparathyroid group was a strong predictor of all cause mortality [34].

The shortened life expectancy of hyperparathyroid patients, which has been described by several investigators, has been attributed to the effects of hypercalcaemia or some as yet undiscovered effect of parathyroid hormone other than hypercalcaemia. In our study the serum calcium was not associated with increased mortality. We found that other factors had a significant impact such as the presence of diabetes mellitus, coronary heart disease and hypertension and the difference in mortality described in different studies examining the impact of hyperparathyroidism on morbidity may be due to a different incidence of these conditions in the populations studied.

In summary, our study confirmed the findings of other studies in that patients with primary hyperparathyroidism, both surgically treated and non-surgically treated, have an increased risk of mortality [1,2,4,5,11–13] and that the increased mortality is independent of serum calcium. This is in contrast to other studies in which surgery for hyperparathyroidism was not associated with a reduced risk of survival [5,6]. In a further study, the presence of hyperparathyroidism when the serum calcium was less than 2.80 mmol/L was not associated with a reduced survival and parathyroid surgery did not influence survival [3]. The question as to whether parathyroid surgery improves survival has not been resolved and would require a randomized trial in which additional risk factors such as the presence of coronary artery disease, diabetes mellitus and hypertension are matched between the surgical and non-surgical groups.

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Paper 26. Bone mineral density in postmenopausal women heterozygous for the C282Y HFE mutation. JE Gunton, F Gates, GR Fulcher, P Clifton-Bligh. Journal of Osteoporosis 2016; doi:10.1155/2016/5638273

P Clifton-Bligh made a significant contribution to this study with respect to the conception of the study, the collection and analysis of the data, the writing of the manuscript and the reading of the bibliography. Osteoporosis associated with haemochromatosis was first described in 1960. Haemochromatosis due to excess tissue iron storage is frequently due to a homozygous mutation C282Y in the HFE gene. In men with haemochromatosis and a serum ferritin between 35 and 8410 microgram/L the presence of osteoporosis was associated with a lower serum free testosterone. In a three year longtitudinal study in postmenopausal women, higher serum ferritin levels were correlated with increased bone loss at the femoral neck. In a study of postmenopausal women mean age 73 years, who had sustained a hip fracture those whose serum ferritin was above 150 microgram/L had a lower bone density than those in whom the serum ferritin was less than 150 microgram/L. The question arises as to whether a heterozygous mutation C282Y in the HFE gene in postmenopausal women is associated with a lower bone mineral density.In a previous study in postmenopausal women aged 54-64 there was a slight increase in the serum ferritin in those heterozygous for the mutation compared to those without the mutation. The present study examined bone mineral density in postmenopausal women heterozygous for the C282Y mutation whose average age was 62.3 years. The mean serum ferritin of the group was 220.1 microgram/L. The measures of bone mineral density were recorded as z scores, that is, the number of standard deviations the value was above or below age-matched controls. The average z score for the lumbar spine L2-L4 was -0.44 significantly different from 0 (P=0.016). The average z score for the femoral neck was -0.19, not significantly different from 0 (P=0.221). The correlation co-efficients relating the serum ferritin to the bone mineral density of the lumbar spine and the femoral neck were not significant. The correlation co-efficient relating the serum iron to the femoral neck bone mineral density was significant (r=-0.436, P=0.048).

This study is considered important because although the number studied is small, it is the first to examine bone mineral density in postmenopausal women heterozygous for the C282Y mutation in the HFE gene and has shown a significant reduction in the L2-L4 bone mineral density compared to age matched controls.

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Research Article

Bone Mineral Density in Postmenopausal Women Heterozygous for the C282Y HFE Mutation

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Mutations in the HFE gene may be associated with increased tissue iron stores reflected in an elevated serum ferritin. With homozygous mutation C282Y, the increase in serum ferritin may be associated with tissue damage in the liver, pancreas, and pituitary and with a reduced bone mineral density. With heterozygous mutation C282Y, the degree of iron retention is less but information relating to how a heterozygous C282Y mutation might impact bone mineral density is uncertain. The present study was undertaken to study the relationships between bone mineral density measured by dual energy X-ray absorptiometry and the serum ferritin and serum iron in postmenopausal women heterozygous for the C282Y mutation. The spinal bone mineral density, L2–4, was significantly less than age matched community controls ($P = 0.016$). There was no significant change in the femoral neck bone mineral density compared to age matched community controls. The correlation between the spinal bone mineral density, L2–4, the femoral neck bone mineral density, and the serum ferritin was not significant. The serum iron correlated significantly inversely with the femoral neck bone mineral density ($P = 0.048$). The heterozygous C282Y mutation may be associated with impairment of bone cell function in postmenopausal women when only small increases in the serum iron or serum ferritin have occurred.

1. Introduction

Osteoporosis associated with hemochromatosis in men was first described by Delbarre in 1960 [1] and confirmed by Pawlotsky et al. in 1979 [2]. Hemochromatosis due to excess tissue iron storage is frequently due to a homozygous mutation, cysteine to tyrosine, C282Y, in the HFE gene. The prevalence of osteoporosis in hemochromatosis and the pathogenic mechanisms involved are not completely understood, for example, whether there is a relationship between the serum ferritin and bone mineral density.

In men with hemochromatosis and a serum ferritin between 350 and 8410 μg/L, the presence of osteoporosis was associated with a lower serum free testosterone [3]. However, in men with the homozygous HFE mutation, the serum ferritin and the serum testosterone did not differ between those with and without osteoporosis. Most osteoporotic men

were not hypogonadal (76.9%) [4]. Interestingly, in men heterozygous for the HFE mutation, C282Y, but without iron overload, the serum SHBG was elevated but the serum testosterone was normal [5].

Significant iron overload may cause chronic liver disease. Liver disease was associated with a lower bone mineral density in men of all ages and in women older than 60 years [6]. In a three-year longitudinal study of postmenopausal women, higher serum ferritin levels were correlated with increased bone loss at the femoral neck [7]. The mean serum ferritin in these women was 76.9 ± 50.6 μg/L. However, in postmenopausal women a higher serum ferritin was not correlated with a lower bone mass [8, 9]. In postmenopausal women with and without osteoporosis the serum ferritin was not significantly different between the two groups [10], but in another study of postmenopausal women, mean age 73 years, who had sustained a hip fracture, those who had a serum

ferritin above 150 μg/L had a lower bone density than those in whom the serum ferritin was less than 150 μg/L [11].

In a study that looked at transiliac biopsy samples the concentration of iron in the cortical bone was greater in osteoporotic participants than in nonosteoporotic participants ($P \leq 0.01$) [12]. In a rat study, ovariectomy was associated with an increased concentration of iron in bone and the loss of bone mass and the deterioration of bone microarchitecture was ameliorated by oral treatment with an iron chelator [13]. In postmenopausal women, the serum ferritin was higher, 71 μg/L, than in premenopausal women, 37 μg/L. The postmenopausal estradiol levels were not correlated with the serum ferritin [14].

A question arises as to what happens to the serum ferritin in postmenopausal women with the heterozygous C282Y mutation in the HFE gene and whether the serum ferritin is related to bone mineral density in this group of women. The serum ferritin levels were not different between those heterozygous for the C282Y HFE mutation and those without the mutation between the ages of 20 and 70. In women aged 50–79 the geometric mean for the serum ferritin was 87 μg/L [15]. In postmenopausal women aged 54–64 there was a slight increase in the serum ferritin in women heterozygous for the HFE mutation, 113.5 μg/L (median), compared to 101.0 μg/L (median) in those without the mutation [16].

In another study in women aged 61–90 with the heterozygous C282Y HFE mutation the serum ferritin was significantly higher than in women without the mutation (geometric mean 79 μg/L compared to 48 μg/L). The 95% confidence interval for serum ferritin in women with the heterozygous C282Y HFE mutation was 61–98 μg/L [17]. Relatively small increases in the serum ferritin in postmenopausal women may be associated with an increased rate of loss of bone mineral density [7]. With this in mind we studied bone mineral density in postmenopausal women heterozygous for the C282Y HFE mutation.

2. Methods

All postmenopausal women in our institution who had had an HFE gene analysis between 1999 and 2012 were identified. Women homozygous for the C282Y mutation and women who were either homozygous or heterozygous for the H63D (His → Asp at residue 63) mutation were excluded. Women with celiac disease, hyperparathyroidism, or hyperthyroidism were also excluded. At the same time, the serum iron and serum ferritin were measured and liver function tests were carried out. Between 1999 and 23 March 2001, the serum ferritin was measured by the Roche immunoturbidimetric assay method (reference range 15–150 μg/L), and between 23 March 2001 and 26 October 2012, Roche E170 chemiluminescent assay was used (reference range 15–400 μg/L). Between 1999 and 23 March 2001 the serum iron was measured by Roche Hitachi 747 method (reference range 8–30 μm/L) and between 23 March 2001 and 26 October 2012 Roche Modular was employed (reference range 8–30 μm/L).

Of the 137 postmenopausal women who were heterozygous for C282Y, 26 also had bone density measurements of the lumbar spine (L2–L4) and femoral neck. The measurements were made on a Norland XR26 bone densitometer and converted to Hologic equivalents using the formula of Genant et al. [18]. Using the data from the National Health and Nutritional Examination Survey 2005–2008 for white non-Hispanic women [19], z-scores for the lumbar spine and femoral neck bone densities were calculated to determine if the age matched bone density measurements (z-scores) were significantly different on average from the general population. Bone mineral density measurements in Australian women were previously found to be very similar to those of women in the USA using NHANES data [20]. The relationship between the serum iron and serum ferritin and bone mineral density at the lumber spine and femoral neck was also studied. The pathology testing was supported by the hospital pathology laboratory (PaLMS).

3. Statistics and Ethics

Two-sided t-tests were used to compare z-scores with z-scores for the general population. The Pearson correlation test was used to study the relationship between the serum iron and serum ferritin and bone mineral density. A P value of <0.05 was considered statistically significant. Unless otherwise specified, the data are presented as mean ± standard deviation (SD). The study was approved by the Northern Sydney Local Health District Human Research Ethics Committee (NSLHD HREC). Because this was a retrospective study of data already accumulated by treating clinicians as part of general medical care, informed consent from individual participants was not required.

4. Results

The age of the study participants ranged between 48 and 81 years (average 62.3 ± 9.3 SD). Women over the age of 60 or in whom one year had elapsed since the last period were assumed to be menopausal. Those under the age of 60 not receiving oestrogen replacement had serum oestradiol levels of 38 pmol/L, 39 pmol/L, and 45 pmol/L, within the menopausal range. All women were white Caucasian. Of the 26 women, 6 were currently receiving estrogen therapy, 3 had previously received estrogen therapy, and 8 were currently on bisphosphonate therapy (alendronate, risedronate, or etidronate). One participant had received regular venesection because of raised serum iron and raised iron binding saturation. Three were receiving thyroxine therapy and had normal thyroid function tests. Three women with significant liver function abnormality and two who were currently receiving prednisone were excluded from the study. Finally 21 women were included in the analysis.

The mean serum ferritin for the group was 220.1 ± 129.8 μg/L. The mean serum iron was 17.3 ± 7.5 μm/L. The z-scores for bone density at the lumbar spine and femoral neck are shown in Table 1.

The average z-score for the lumbar spine, L2–L4, was −0.44 ± 0.77 and was significantly different from 0 ($P = 0.016$). The average z-score for the femoral neck was −0.19 ±

TABLE 1: Postmenopausal women heterozygous for the C282Y HFE mutation bone mineral density z scores and serum ferritin and serum iron.

	L2–4	FN	Ferritin	Iron
(1)	−0.95	−0.06	155	12
(2)	−0.27	+0.10	155	11
(3)	−0.68	−0.03	241	17
(4)	+0.56	+0.01	331	16
(5)	+0.93	−0.24	148	12
(6)	−0.91	−0.81	196	12
(7)	+0.16	−0.61	234	26
(8)	−0.94	−0.30	166	33
(9)	−0.86	+0.76	417	12
(10)	−0.12	−0.91	543	30
(11)	−1.02	−0.51	12	12
(12)	−0.93	−0.55	220	31
(13)	−1.70	+0.39	240	16
(14)	−0.41	−0.63	274	16
(15)	−0.30	+0.86	92	12
(16)	+0.10	+0.84	411	8
(17)	+0.28	+0.86	28	14
(18)	−1.50	−1.38	177	17
(19)	−1.06	−1.46	312	17
(20)	+1.23	+0.16	135	10
(21)	−0.89	−0.46	66	29

Serum Ferritin μg/L.
Serum Iron μmol/L.
L2–4: BMD of lumbar spine.
FN: BMD of femoral neck.

TABLE 2: Mean value for bone mineral density z-scores for lumbar spine and femoral neck.

	Mean	Standard deviation
L2–L4 z-score	−0.44	0.77
FN z-score	−0.19	0.69

0.69, not significantly different from 0 ($P = 0.221$) (Tables 2 and 3).

The correlation coefficients relating the serum ferritin to the bone mineral density of the lumbar spine, L2–L4, and femoral neck were not significant for the lumbar spine ($r = 0.019$, $P = 0.936$) or for the femoral neck ($r = -0.093$, $P = 0.688$) (Table 4).

The correlation coefficients relating the serum iron to the bone mineral density of the lumbar spine and femoral neck were not significant for the lumbar spine ($r = -0.236$, $P = 0.303$) but were significant for the femoral neck ($r = -0.436$, $P = 0.048$) (Table 4).

In this study, women receiving estrogen replacement or bisphosphonate therapy were included in the analysis. Therapy with either of these agents would have a tendency to increase bone mineral density so that any reduction in bone density is likely to have been greater before therapy.

5. Discussion

The link between excess tissue iron stores and osteoporosis was first noted by Delbarre in 1960. Excess tissue iron accumulation as reflected in an elevated serum ferritin is frequently seen in persons homozygous for the C282Y HFE mutation and many of these patients may develop osteoporosis [21]. Additional risk factors for the development of osteoporosis are hypogonadism in men and chronic liver disease in men and women [21].

The question arises as to whether milder degrees of tissue iron storage are associated with reduced bone density. In a group of postmenopausal women, the serum ferritin in those with osteoporosis was not different from those who were not osteoporotic [10]. Also in postmenopausal women a higher serum ferritin was not correlated with a lower bone mass [8, 9]. However, in a longitudinal study over 3 years in postmenopausal women, the serum ferritin was correlated with an increased loss of bone mineral density in the femoral neck with serum ferritin levels within the normal range [7], and in another study of postmenopausal women, mean age 73 years, who had sustained a hip fracture, those with a serum ferritin above 150 μg/L had a lower bone density than those in whom the serum ferritin was less than 150 μg/L [11].

In the present study of postmenopausal women heterozygous for the C282Y HFE mutation, the spinal bone mineral density of L2–L4 was significantly less than that of the average population mean corrected for age. The femoral neck z-scores for bone mineral density were not significantly lower than the average population mean. The serum ferritin was not correlated with the bone mineral density at the lumber spine or the femoral neck, a finding similar to that found in postmenopausal women studied by Lee et al. in whom the average serum ferritin was 77.1 μg/L [9]. The average serum ferritin in the present study was 220.1 μg/L, considerably higher than that found in the study of Kim et al. [7] and higher than average serum ferritin levels of 64 μg/L found in women with average age 52.8 years heterozygous for the C282Y HFE mutation in the study of Rossi et al. [15].

In another study of women aged 61–90 with the heterozygous C282Y HFE mutation the serum ferritin was 79 μg/L (geometric mean) [17]. In a longitudinal study of C282Y HFE simple heterozygotes followed over 12 years the serum ferritin levels of postmenopausal women did not change significantly and were not significantly different compared to the serum ferritin levels of postmenopausal women without the C282Y HFE mutation [22].

In a population study of Australian women greater than 50 years the median serum ferritin was 80.5 μg/L [23]. A further study of postmenopausal women with osteoporosis found no difference in the serum ferritin between those with and without osteoporosis but the serum iron was slightly lower in those with osteoporosis ($P = 0.047$). This was linked to a variation in the haptoglobin molecule which binds heme more avidly and reduces the amount of free iron available to tissues and which protected against fracture in postmenopausal women with osteoporosis [24].

Excess tissue iron has been implicated in impairment of bone metabolism. In the zebra fish exposure to ferric

TABLE 3: t-test of whether z-scores for bone mineral density significantly differ from 0.

| | t | df | P value (2-tailed) | Mean difference | 95% confidence interval of the difference | |
					Lower	Upper
L2–L4 z-score	−2.633	20	0.016	−0.44	−0.79	−0.09
FN z-score	−1.263	20	0.221	−0.19	−0.50	0.12

TABLE 4: Pearson correlations between serum ferritin, serum iron, and z-scores.

	L2–L4 z-score	FN z-score
Pearson correlation	$r = 0.019$	$r = -0.093$
Serum ferritin P value (2-tailed)	$P = 0.936$	$P = 0.688$
Pearson correlation	$r = -0.236$	$r = -0.436$
Serum iron P value (2-tailed)	$P = 0.303$	$P = 0.048$

iron increased the expression of TRACP-5b of osteoclast origin and treatment with deferoxamine increased bone mineralization [25]. In mice homozygous for the C282Y HFE mutation excess iron was seen on the surfaces of bone trabeculae and there was a decrease in the number and thickness of trabeculae. There was no change in osteoblast surface but osteoclast numbers were increased [26]. In a rat study, ovariectomy was associated with an increased concentration of iron in bone and the loss of bone mass and the deterioration of bone microarchitecture was ameliorated by oral treatment with an iron chelator. Bone resorption was reduced. The serum ferritin was not measured in this study [13].

In a study which looked at transiliac bone biopsy samples, the concentration of iron in cortical bone was greater in osteoporotic participants than in nonosteoporotic participants [12]. Hepcidin of hepatic origin influences gastrointestinal iron absorption. Increased levels of serum hepcidin decrease iron absorption and low levels increase iron absorption. BMP-2 increased hepcidin mRNA expression in liver cells and this was enhanced by hemojuvelin which acts as a coreceptor for BMP-2 [27] and which was independent of the effect of HFE [28]. Higher serum ferritin levels were associated with particular SNPs in the BMP-2 gene [29]. BMP-2 has a role in the differentiation and function of osteoblasts in bone so that diminished function may reduce bone mass and diminish hepatic hepcidin secretion. Iron absorption may increase as a result. Serum hepcidin was not measured in the present study.

In a mouse study using C2C12 preosteoblasts in culture, increased iron and ferritin in the cell culture media in combination with low estradiol levels inhibited the effect of BMP-2 on cell differentiation. Also mice heterozygous for the HFE mutation had lower femoral bone mineral density compared to wild type mice [30].

The present study is the first to examine bone mineral density in postmenopausal women heterozygous for the C282Y HFE mutation which showed a significant reduction in the lumbar spine bone mineral density when compared to age matched community controls. However, there are several important limitations in this study which must be regarded only as exploratory. Firstly, the C282Y HFE heterozygotes were identified from an already established data base and it is not clear why in individual cases the gene analysis was done in the first place. Because the mean serum ferritin was 220.1 μg/L, higher than the average values expected in postmenopausal women, it is likely that the HFE mutation was sought following the finding of a higher than average serum ferritin. Also it is likely that the HFE analysis was carried out in relatives of known C282Y HFE homozygotes. If the HFE mutation was sought specifically in postmenopausal women with known low bone mineral density then this would introduce substantial bias.

Women receiving estrogen or bisphosphonates were included in the analysis even though these medications were likely to have increased the bone mineral density and make it less likely to detect a significant reduction in age matched bone mineral density. Even so, despite the inclusion of these participants, in this small number of 21 postmenopausal women there was a highly significant ($P = 0.016$) reduction in the L2–L4 bone mineral density compared to age matched controls, strengthening the concept that C282Y HFE heterozygosity is associated with reduced bone mineral density in the lumbar spine.

Nevertheless, in order to confirm or refute the results of the present study, postmenopausal women selected randomly from the population at large should have measures of bone mineral density, serum iron, and serum ferritin and also studies for the heterozygous C282Y HFE mutation so that unbiased comparisons could be made between those with and those without the mutation.

It is not known if there is a threshold for serum ferritin above which there is an impairment of bone formation or an increase in bone resorption or if C282Y HFE heterozygotes who are osteoporotic respond to therapy designed to increase bone mineral density in the same way as persons without the mutation. Although venesection of one premenopausal woman with an initial serum ferritin of 513 μg/L in a separate study was associated with a significant increase in the bone mineral density of the lumbar spine and the femoral neck after one year [31], additional studies are required to determine at what levels of serum ferritin venesection may be helpful in increasing bone mineral density in postmenopausal women with osteoporosis and mild increases in the serum ferritin. In the present study, the serum ferritin in C282Y HFE heterozygotes ranged between 12 and 543 μg/L. Three patients

were excluded from analysis because abnormal liver function tests had serum ferritin levels of 540, 595, and 634 μg/L.

In conclusion, in the present study, C282Y HFE heterozygous postmenopausal women were shown to have significantly lower bone mineral density of the lumber spine, L2–L4, compared to age matched community controls. In this small study the serum ferritin was not correlated with measures of bone mineral density in the spine or femoral neck.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Paper 27. Clinical,cellular, microscopic and ultrastructural studies of a case of fibrogenesis imperfecta ossium. ML Barron, MS Rybchyn, S Ramesh, RS Mason, SF Bonar, P Stalley, S Khosla, B Hudson, C Arthur, E Kim,, RJ Clifton-Bligh, PB Clifton-Bligh. Bone Research 2017; Mar 14;5:16057.doi10.1038/boneres.2016.57

P Clifton-Bligh supervised the assessment diagnosis and clinical management of this patient and organised each investigation and special investigations. He collated the data, wrote the manuscript and researched and read the bibliography. This is a unique study. It describes the most extensive investigation yet described of a patient with fibrogenesis imperfecta ossium. New information is presented eg the finding of persistent reductions in serum C3 and C4 concentrations. Osteoblastic cell cultures were established from bone obtained by biopsy from affected bone and compared with osteoblasts in culture obtained from an appropriate normal control. Osteoblasts from the patient responded in an exaggerated way to 1,25-OH vitamin D, and produced excess amounts of osteoprotegerin. In a gene expression study, G-protein coupled receptor 128 of unknown function, was markedly upregulated. When normal osteoblasts were exposed to the patient's serum, aquaporin 1, was markedly upregulated. This information has provided new insights into the pathogenesis of the disease process.

This paper describes unique information from a patient with osteogenesis imperfecta ossium and is considered to make a major contribution to the understanding of the pathogenesis of this rare disorder.

Some of the experimental cell culture studies were carried out by ML Barron as part of her PhD project, University of Sydney.

ARTICLE

Clinical, cellular, microscopic, and ultrastructural studies of a case of fibrogenesis imperfecta ossium

Melissa L Barron[1], Mark S Rybchyn[1], Sutharshani Ramesh[1], Rebecca S Mason[1], S Fiona Bonar[2], Paul Stalley[3], Sundeep Khosla[4], Bernie Hudson[5], Christopher Arthur[6], Edward Kim[7], Roderick J Clifton-Bligh[7,8] and Phillip B Clifton-Bligh[7,8]

Fibrogenesis imperfecta ossium is a rare disorder of bone usually characterized by marked osteopenia and associated with variable osteoporosis and osteosclerosis, changing over time. Histological examination shows that newly formed collagen is abnormal, lacking birefringence when examined by polarized light. The case presented demonstrates these features and, in addition, a previously undocumented finding of a persistent marked reduction of the serum C3 and C4. Osteoblasts established in culture from a bone biopsy showed abnormal morphology on electron microscopy and increased proliferation when cultured with benzoylbenzoyl-ATP and 1,25-dihydroxyvitamin D, contrasting with findings in normal osteoblasts in culture. A gene microarray study showed marked upregulation of the messenger RNA (mRNA) for G-protein-coupled receptor 128 (GPR 128), an orphan receptor of unknown function and also of osteoprotegerin in the patient's osteoblasts in culture. When normal osteoblasts were cultured with the patient's serum, there was marked upregulation of the mRNA for aquaporin 1. A single pathogenetic factor to account for the features of this disorder has not been defined, but the unique findings described here may facilitate more definitive investigation of the abnormal bone cell function.

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INTRODUCTION

The case is presented as an example of a rare bone disorder, fibrogenesis ossium imperfecta. In addition to a description of the clinical features at presentation and subsequently, hitherto unreported blood test findings are described and an analysis of the function of osteoblasts cultured from affected bone is given. A gene microarray was also performed on the cultured osteoblasts looking at the expression of genes that might be involved in the bony abnormalities described.

The patient, a male student, aged 17 years, was first seen on 14 August 2002 with a painful right knee incurred while playing soccer. A magnetic resonance imaging of the right knee showed an anterior cruciate ligament tear and adjacent bone bruising but no other abnormality in the distal femur or proximal tibia. The ligament was surgically repaired and he recovered well from this injury. In June 2006, he had a febrile illness with a sore throat treated with amoxicillin. One month later, he developed polyarthralgia involving the knees, ankles, elbows, and wrists. There was no early morning stiffness or swelling of the joints. Over the next 2 years, he had several more episodes of sore throat treated with injections of procaine penicillin. During this time, the knee and ankle pain became more severe and constant made worse by drinking alcohol and was less after receiving penicillin. The last course of penicillin, given orally, was in May 2008. On the 19 July 2007, he was seen by a rheumatologist. An examination of his joints was

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Table 1. Investigations

Test	Results	NR
Hemoglobin	119 g·L^{-1}	130–160
White cell count	Normal	4–11
ESR	45 mm·h^{-1}	< 15
Hepatitis B surface antigen	Negative	—
Hepatitis C antibody	Negative	—
Serum C3	0.38 g·L^{-1}	0.83–1.46
Serum C4	0.04 g·L^{-1}	0.16–0.45
TreponemaAb	Negative	—
ASOT	345	< 300
ANA	Negative	—
HIV Ab	Negative	—
TSH	2.05 mU·L^{-1}	0.4–4.0
BorreliaAbIgG, IgM	Negative	—
Serum creatinine	70 μm·L^{-1}	60–100
Serum calcium	2.34 mmol·L^{-1}	2.20–2.55
Serum alkaline phosphatase	206 U·L^{-1}	41–119
Serum phosphate	2.14 mmol·L^{-1}	0.78–1.43
Serum 25OH Vitamin D	49.8 nmol·L^{-1}	33.1–129
Serum 1,25OH Vitamin D	109 pmol·L^{-1}	38–160
Serum PTH	29.8 ng·L^{-1}	< 50.0
24-hour urine calcium	7.29 mmol	1.25–7.50
Serum testosterone	15.5 nmol·L^{-1}	8.5–55.5
Urine deoxypyridinoline	11.4 nmol per mmol Cr	—
TRACP-5β	10.1 U·L^{-1}	< 4.8
Serum calcitonin	< 5 ng·L^{-1}	—
Serum ferritin	167 μg·L^{-1}	30–400
CRP	44 mg·L^{-1}	< 5
Rheumatoid factor	< 20 IU·mL^{-1}	—
Serum osteocalcin	4.9 μg·L^{-1}	3.7–10.0
Q fever and rickettsial antibodies	Negative	—
Leptospira IgM	Negative	—
IgG kappa monoclonal protein	3.9 g·L^{-1}	—
Serum-free kappa light chains	26.3 mg·L^{-1}	3.3–19.4
Anti-transglutaminaseAb	< 3 U·mL^{-1}	—
Serum acid phosphatase	10.1 U·L^{-1}	< 6.6
16srRNA	Not detected	—
Serum vitamin A	2.2 μmol·L^{-1}	0.7–3.0
Blood lead, cadmium, and mercury	Negative	—
Urine fluoride	4.60 mmol per mol Cr	Biological occupational exposure limit 42

normal. A serum C reactive protein (CRP) was 35 mg·L^{-1}, elevated. Tests for rheumatoid factor and for antinuclear antibody were negative. He was thought to have a post-streptococcal syndrome. He was seen by a microbiologist on 13 September 2007. At this time, the serum C3 and C4 were found to be markedly reduced. No evidence for a persisting infection was found.

On examination at presentation to us on 5 April 2008, his blood pressure was 100/70 mmHg. Examination of the heart, neck, lungs, and abdomen was unremarkable and the tendon reflexes were normal. There was no joint swelling, tenderness, or limitation of movement and muscle power was normal. There was loss of lordosis of the lumbar spine. There was no family history of bone or joint disorder. His father had been treated successfully for chronic myeloid leukemia. Investigations carried out in 2007 and 2008 are shown in Table 1. The tests focused on the possibility that an infection might be relevant to the joint disorder or that a generalized bone disorder might be present. The markedly reduced serum C3 and C4 was confirmed on repeated testing. There was a circulating IgG kappa monoclonal protein that has shown no tendency to rise progressively over the subsequent several years of testing.

Plain radiographs showed a marked increase in bone density in a bilateral and symmetrical manner affecting the metaphyseal regions of the long bones including the distal femora (Figure 1a), proximal and distal tibiae, greater trochanteric region of the proximal femora, distal humeri, proximal radius, and ulnae. In the spine, marked osteopenia was evident with central collapse of the endplates that had a concave appearance (Figure 1b and c). A nuclear bone scan showed increased uptake in the bones around the knees and ankle joints in a symmetrical pattern at the sites of increased bone density. The bone mineral density of the lumbar spine and femoral neck was measured by dual X-ray absorptiometry (DXA) (Hologic Explorer). The spinal bone mineral density (BMD L1–4) was 0.532 g·cm^{-2} (T score − 5.1) and the left femoral neck BMD was 0.631 g·cm^{-2} (T score − 2.3). He received alendronate 70 mg per week, for 3–4 weeks.

A bone marrow biopsy and aspirate from the right posterior superior iliac spine carried out on the 10 April 2008 to further evaluate the significance of the circulating IgG kappa monoclonal protein showed that the marrow was normocellular with all cell lines present to maturity. There was a slight increase in megakaryocyte numbers. Very mild plasmacytosis was seen (3%) and kappa IgG was predominant. Chromosomal analysis was normal. A diagnosis of monoclonal gammopathy of uncertain significance was made. The bone biopsy obtained by trephine comprised cancelous bone and hemopoietic marrow. In some regions, sparse very slender trabeculae were noted and elsewhere the trabeculae had a thicker appearance. In the latter, widened osteoid seams were prevalent in which irregular calcification was noted giving the impression of prominent and wide cement lines. These were bordered by plump osteoblasts and admixed osteoclasts were present. On polarization microscopy, there was a paucity of collagen fibers within the thickened osteoid seams. In some of the thickened seams, residual spicules of original normal lamellar bone were identifiable (Figure 1d and e). Features suggesting microtrabecular fracture were also identified.
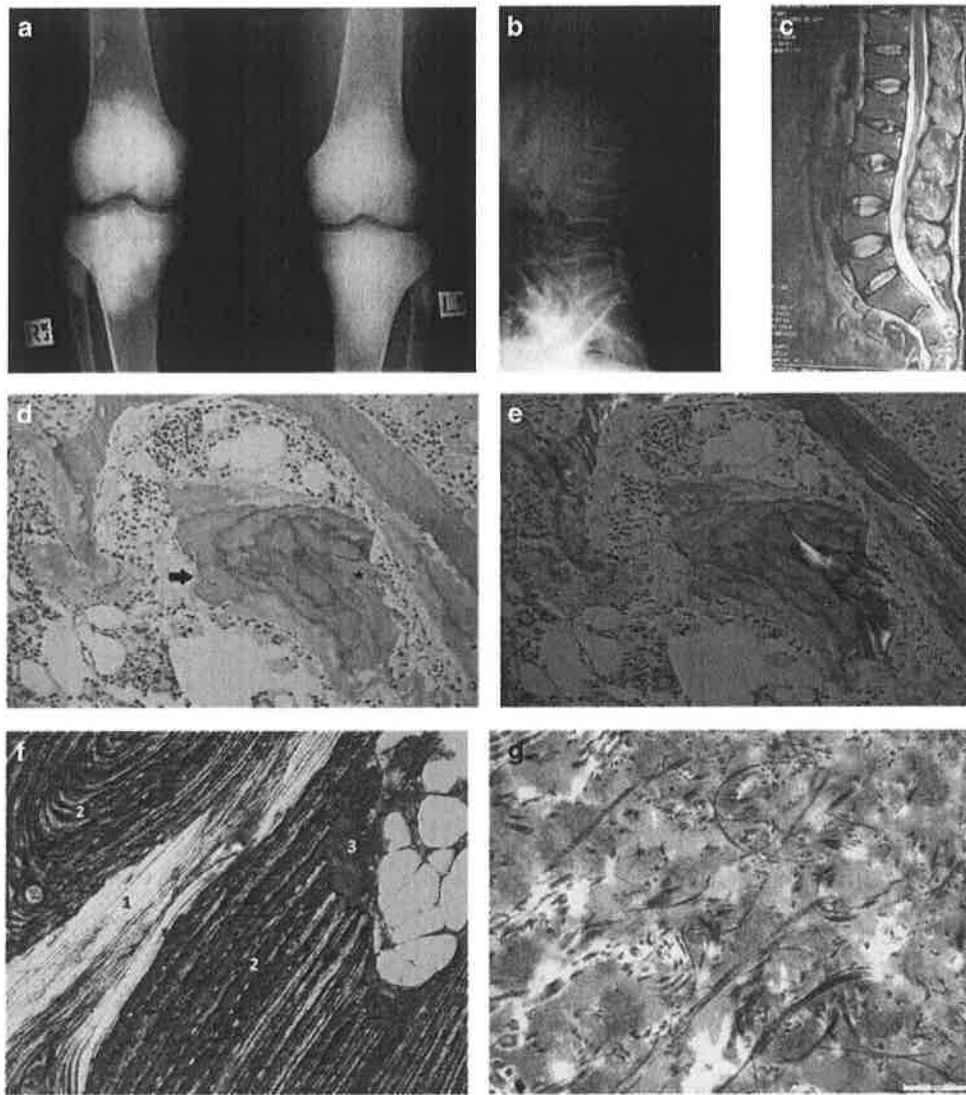
Figure 1. Radiograph of the knees (**a**) shows marked bilateral symmetrical homogeneous sclerosis involving the epiphyses and metaphyses, involving all bones related to the joint. Radiograph and T2-weighted MR image (**b** and **c**) shows central collapse of the vertebral end plates associated with osteoporosis. In the iliac crest bone marrow trephine, variably sized bone trabelculae are noted with intervening haemopoeitic marrow (**d**). In thicker trabeculae, wide irregular osteoid seams with calcification are noted (arrowed). Normal slender trabeculae of lamellar bone are presented focally. The intervening immature osteoid has irregular zones of calcification stained in blue. Centrally, a residual spicule of more dense pink normal lamellar bone (star) is bordered by zones of immature pink osteoid with irregular poorly defined zones of calcification, the calcification predominating where the osteoid abuts the bone trabeculum and diminished at the perimeter (arrowed). On polarization microscopy of the same area (**e**), the original host lamellar bone with generous quantities of collagen birefringence is minimal. In the femur biopsy, a Masson trichrome stain examined under polarized light (**f**) shows original host bone with cohesive and generous collagen content (zone 1). This is bordered by a thick area of coarse lamellar bone in which the blue areas represent the separation between the collagen bundles (zone 2). At the top right, a small area of immature osteoid (zone 3) is evident that is largely devoid of collagen. Electron microscopic findings in the femur biopsy (**g**) show abnormally curved and randomly oriented fibres of variable diameter that are easily identifiable within the matrix.

Congo red stain for amyloid was negative, granulomas were not noted, and mast cell numbers were unremarkable.

The radiological findings of axial osteopenia and appendicular sclerosis were considered highly unusual and the differential diagnoses considered included osteopetrosis, other sclerosing dysplasias, and osteogenesis imperfecta, all of which were considered unlikely given the presence of normal bone modeling. Sarcoidosis and mastocytosis (which can cause osteosclerosis) were considered unlikely given the bilateral symmetrical distribution of the abnormality and the bone marrow biopsy failed to support these. Fluorosis was considered unlikely as the abnormality was not confined to the axial skeleton and urinary fluoride was within normal limits. In juvenile Paget's disease and hyperostosis in

hyperphosphatemia, an earlier onset would be expected and involvement of the bone ends only would not be seen. Features of POEMS syndrome were excluded as there was no polyneuropathy, organomegaly, skin, or endocrine abnormalities, and myeloma, lymphoma, and myelofibrosis were excluded by bone marrow examination.

The main differential diagnoses ultimately included low-grade chronic osteomyelitis and a form of histiocytosis (Erdheim Chester disease specifically), although the symmetry of involvement made the former unlikely and the young age of the patient made the latter unlikely.

A subsequent open distal femoral biopsy to include cortex and medulla within the metaphyseal sclerotic area was performed on 14 June 2008 to further characterize the bony abnormality and to obtain bone for cell culture. At surgery the surgeon documented very hard bone. The biopsy comprised periosteum, cortex, and medulla measuring $30 \times 10 \times 4$ mm and was suitable for morphometric, microbiologic, and electron microscopic examination. Cultures for aerobic and anaerobic bacteria were negative. Fungal elements were not seen. A stain for spirochetes was negative. Several fragments were placed in transport medium for cell culture. Despite the hardness of the bone documented at surgery, decalcification of the specimen was brisk, occurring within 4 h, suggesting ease of release of the mineral component. The component bony trabeculae within the medullary cavity were thick with a varying lamellar architecture. They were comprised of central zones of mature lamellar bone in which densely packed collagen with a smooth homogeneous polarization pattern was identifiable. This was bordered by broad zones of thick coarse lamellar bone in which the collagen fibers, although arranged in a lamellar pattern were distinctly separate one from one another. In some regions, these were in turn bordered by a third wave of ossification in which even less collagen content was demonstrable on polarization microscopy. These findings were confirmed with Masson Trichrome (Figure 1f) and reticulin staining. Scattered foci of osteoclast resorption were noted and in some regions resorption pits were filled with immature osteoid with diminished collagen content. Osteoblasts were inconspicuous throughout in contrast to the plump osteoblasts seen in the bone marrow biopsy.

The accompanying marrow was hemopoietic and somewhat hypocellular in nature. However, all cell lines were present to maturity. Plasma cells were identifiable with scattered areas in which increased numbers of plasma cells could be seen. These were polyclonal.

Electron microscopic examination showed seams of osteoid at the surface of the lamellar bone in which very sparse collagen fibrils of varying diameters were present. The collagen fibers were randomly oriented and many were small, irregularly shaped, and curved. Scattered irregular mineralization foci were present and mineral lay within a pale amorphous matrix (Figure 1g).This contrasts with normal bone where there is an orderly parallel orientation of collagen which is tightly arranged. The constellation of changes reflect those of diminishing collagen content within the osteoid of each modeling cycle accompanied by abnormal collagen fibers on electron microscopy. These features are consistent with those documented in fibrogenesis imperfecta ossium.[1–4] Subsequently, from 2 months after the femoral bone biopsy, the patient received infusions of zolendronate, 4 mg, every 6 months. The spinal BMD showed a step-wise increase from 0.532 to 1.111 g·cm^{-2} (T score 0.2) over 7 years to 2015. In the same time interval, the left femoral neck BMD increased to 0.986 g·cm^{-2} (T score 0.4). In September 2009, the patient developed a fever and endocarditis due to salmonella. An aortic valve replacement was performed soon after.

In the interim, 10 000 units of vitamin D3 daily had been prescribed by an outside physician for a period of 2 years. Ultimately at 3 years after presentation to us, the serum 25OH vitamin D was 190 pmol·L^{-1} (NR 50–130) and the serum 1,25OH vitamin D was 370 pmol·L^{-1} (NR 38–160). At that time, the serum calcium was 2.32 mmol·L^{-1}, normal. Thereafter, the dose of vitamin D was reduced to 1 000 units daily. Since that time, he has been pain free and fully mobile.

MATERIALS AND METHODS
Cell culture conditions
Osteoblasts were generated from bone biopsies obtained from the distal femur of the patient and from the distal femur of a male age 19 years (control) at the time of elective repair of an anterior cruciate ligament. Written informed consent was obtained from the patient and the study was approved by the Northern Sydney Local Health District Human Research and Ethics Committee. Cells derived from bone fragments were collected using trypsin (0.25%) and cultured in a 25 cm^2 flask with high glucose Dulbecco's modified Eagle medium and 10% (v/v) heat-inactivated fetal bovine serum, supplemented with penicillin (0.03 g·L^{-1}) and streptomycin (0.04 g·L^{-1}), and maintained at a 37 °C environment containing 5% (v/v) CO$_2$.

In a separate experiment, the control osteoblasts were cultured with 10% normal serum or 10% patient's serum instead of fetal bovine serum. In this latter culture system, the serum was not heat-inactivated.

Biopsy cell morphology—scanning electron microscopy
Scanning electron microscopy was conducted to examine any morphological differences between the two biopsy-

derived cells following long-term culture, as previously described by Slater *et al.*[5] Cells were plated on Thermanox coverslips at a density of 1.25×10^5 cells per well in six-well plates with culture medium and grown to confluence, with media changes every 3 days. Once cells were 30 days post confluence and had formed multilayers, cultures were fixed in 2.5% (w/v) glutaraldehyde in $0.1 \, mol \cdot L^{-1}$ cacodylate buffer for 1 h at 4 °C, and washed three times with $0.1 \, mol \cdot L^{-1}$ cacodylate buffer. Each coverslip was then post-fixed in 1%(w/v) osmium tetroxide in $0.1 \, mol \cdot L^{-1}$ cacodylate buffer for 2 h at room temperature, washed three times with water, before dehydration with 30%, 50% and 70% (v/v) ethanol, three times each for 5 min. On the third wash, cells were left overnight in 70% (v/v) ethanol at 4 °C. Further dehydration with 90%, 95% and, finally, 100% (v/v) ethanol followed, each time for 10 min at room temperature. Cells underwent critical point drying, sputter-coated with 15 nm of gold palladium.

Biopsy cell ultrastructural analysis—transmission electron microscopy

Transmission electron microscopy was employed to analyse the ultrastructural differences between the two biopsy-derived cells. Methodology was similar to that described above for scanning electron microscopy, with the following differences: cells were dehydrated using a graded series of ethanols as above, then placed in 25% and 50% (v/v) Spurr's resin in ethanol for 1 h, followed by 2 h incubation in 75% (v/v) Spurr's resin mixture. The multilayers were then left in 100% Spurr's resin overnight at room temperature. At this stage, the coverslips were embedded in BEEM capsules and curing was carried out at 60 °C for 18 h.

Biopsy cell proliferation assay (thymidine incorporation)

Various bone active agents were tested on both biopsy-derived cells to determine differences in cell proliferation and treatment responses. Cells were plated into 96-well plates at 1×10^5 cells per well and incubated at 37 °C for 24 h. The medium was then replaced with serum-reduced OptiMEM media and further incubated for 24 h, before the addition of treatments (benzoylbenzoyl-ATP (BzATP) or 1,25-dihydroxyvitamin D in vehicle) for another 24 h. Tritiated thymidine (final concentration $20 \, nCi \cdot \mu L^{-1}$) was added to each well and incubated for 4 h. Cell proliferation was indirectly determined by the incorporation of tritiated (methyl,1'2-^{3}H) thymidine, as previously described.[6] Scintillation vials were read for 5 min in a liquid beta-scintillation counter (Packard Tri-Carb 1990CA, Packard Instrument Company, Downers Grove, IL, USA). A bicinchoninic acid (BCA) assay was also carried out for cell proliferation measuring the amount of protein per well, in accordance with the manufacturer's instructions.

Osteoblast differentiation (alkaline phosphatase activity)

Cells were plated in 24-well plates and grown for 7 days, without additional treatments, to compare cell differentiation between the two biopsy-derived osteoblasts. Alkaline phosphatase was determined as previously described,[6] using a modification of the method of Lowry[7] that uses *p*-nitrophenyl phosphate as the substrate of alkaline phosphatase. Plates were read at 405 nm to detect the alkaline phosphatase product (*p*-nitrophenolate). Alkaline phosphate measurements were corrected for total cell protein using the BCA assay.

RNA isolation and microarray analysis

DNA microarray technology was conducted to identify and classify a wide range of gene sequences expressed as messenger RNA (mRNA), to provide information on the differences in gene expression between the patient and control biopsy-derived cells. RNA was extracted from cells following 6 days in culture in six-well plates, using an RN easy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In a second experiment, RNA was extracted from the control osteoblasts after culture with normal serum or the patient's serum for 6 days. Quality control was performed to determine microarray suitability. RNA yield and integrity was evaluated by both NanoDrop spectrophotometry (Nanodrop Technologies, Wilmington, DE, USA) and 1%(w/v) agarose electrophoresis. Pure quality RNA samples were then dispatched to the Ramaciotti Centre at the University of New South Wales for a full service Affymetrix Gene Array analysis. Results were downloaded to the microarray program, GeneSpring GX. In the second experiment involving culture of control osteoblasts with either normal serum or the patient's serum, the Affymetrix gene microarray analysis was carried out by the Department of Bioinformatics, University of Queensland (QFAB).

Tumor necrosis factor receptor 11B (OPG)

Secreted osteoprotegerin (OPG) protein expression was examined for both the control and patient biopsy-derived cells. Cells were plated in 24-well plates in quadruplicates and grown for 7 days. Supernatant at both 4 and 7 days in culture were collected from each well and tested for OPG levels using an ELISA assay.[8] The Biomedica enzyme-linked immunosorbent assay (ELISA) test kits were enzyme immunoassays designed to determine OPG directly in cell culture supernates. Cell protein corrections were made using a BCA assay of cell lysates from the original plates.

Sclerostin and vitamin D receptor (western blot)

Cells were plated in 24-well plates and cultured for 20 days post confluence for sclerostin and for 7 days for the vitamin
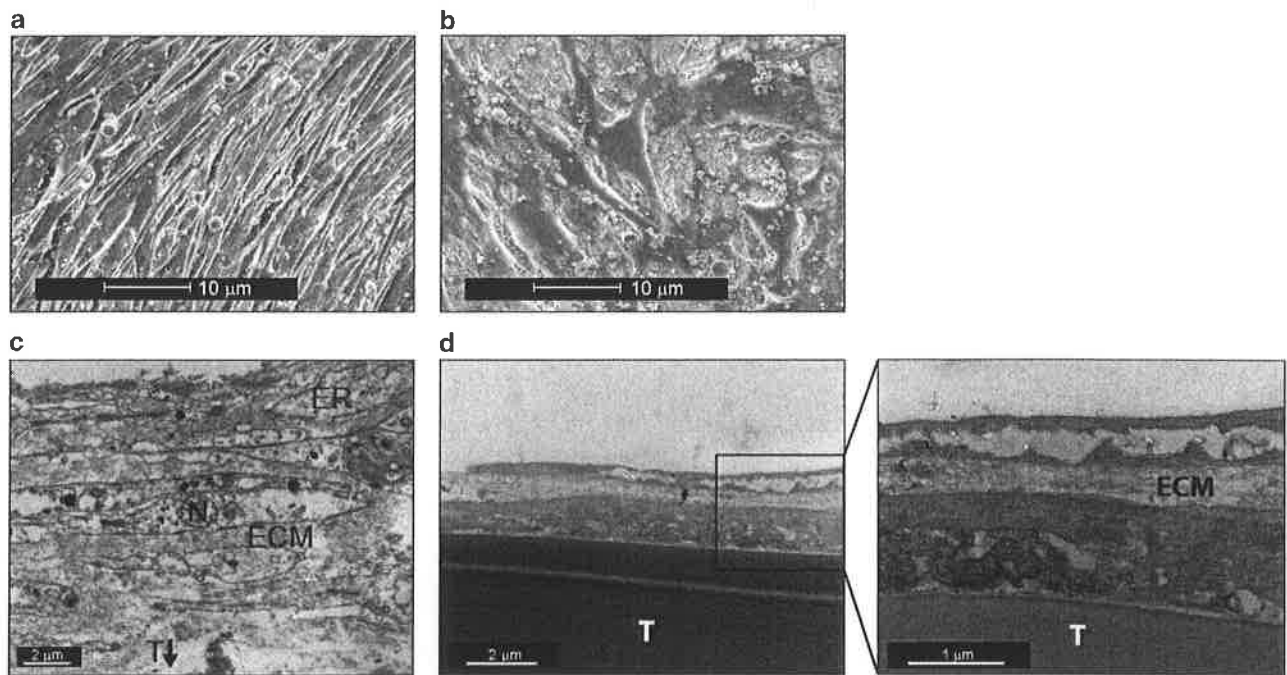
Figure 2. Scanning electron microscopy of control osteoblasts (**a**) cultured for 30 days post confluence. Scanning electron microscopy of the patient's osteoblasts (**b**) cultured for 30 days post confluence. Transmission electron microscopy of control osteoblasts (**c**) cultured for 30 days post confluence (ECM, extracellcular collagen matrix; ER, endoplasmic reticulum; N, osteoblast cell nucleus; T, thermanox coverslips). Transmission electronic microscopy of the patient's osteoblasts (**d**) cultured for 30 days post confluence.

D receptor (VDR). The attached cells were washed once with ice-cold phosphate-buffered saline (PBS) buffer before being lysed in ice-cold RIPA buffer (50 nmol·L^{-1} Tris, 150 mmol·L^{-1} NaCl, 1 mmol·L^{-1} EDTA, 1% NP40+ protease inhibitors (1 mmol·L^{-1} PMSF, 1 μmol·L^{-1} Becstatin, HCl, 1 μmol·L^{-1} Pepstatin A) for 5 min. The cell lysate was stored at −20 °C until needed for western blot analysis, as previously described.[8] The protein concentrations were determined by BCA assay and equal amounts of protein were subject to electrophoresis on 10% SDS-polyacrylamide gel electrophoresis for sclerostin and VDR and then transferred electrophoretically onto a nitrocellulose membrane and incubated in 1% (w/v) heat-denatured casein in PBS containing 0.04% (w/v) thymol (HDC) for 1 h at room temperature. Membranes were then exposed to primary antibody (1 μg·mL^{-1} anti-human sclerostin and 1 μg·mL^{-1} anti-human VDR) in HDC for 1 h at room temperature, washed several times with 0.05% (v/v) Tween-20 in PBS, and incubated with secondary anti-IgG horse radish peroxidase-conjugated antibody for 1 h at room temperature. Membranes were washed extensively and an enhanced chemiluminescence detection assay (Millipore, Millipore Corporation, Cork, Ireland) was performed according to the manufacturer's instructions. Band densities were measured using an Alpha Innotech Digital Imaging System (Genetic Technologies Inc, Miami, FL, USA).

RESULTS

Osteoblasts obtained from the patient's bone biopsy were established in culture. Osteoblasts obtained from a bone biopsy of the lower femur of a healthy 19-year-old male having elective knee surgery for a torn anterior cruciate ligament were used as a control.

Morphological and ultrastructural differences in biopsy cells following long-term culture
Long-term culture of osteoblasts results in the formation of multilayers on Thermanox consisting of a collagen-based extracellular matrix, presented in an organized manner. This phenomenon has been shown previously *in vitro* using fetal bone cells, with multilayers becoming macroscopic at ~20 days post confluence.[9] Scanning electron microscopy was conducted to give a more detailed view of the biopsy-derived osteoblasts (control and patient) and the morphologic differences of the surface layer after long-term culture. The control cells exhibited an elongated uniform structure very similar to fetal bone cells (Figure 2a). In contrast, the patient cells appeared very flat and disorganized, very different to any known or previously documented osteoblast (Figure 2b). These SEM images provide evidence of phenotypic differences between the two biopsy-derived cells in culture.

Transmission electron microscopy was employed to examine the multilayering of the biopsy-derived osteoblasts (control
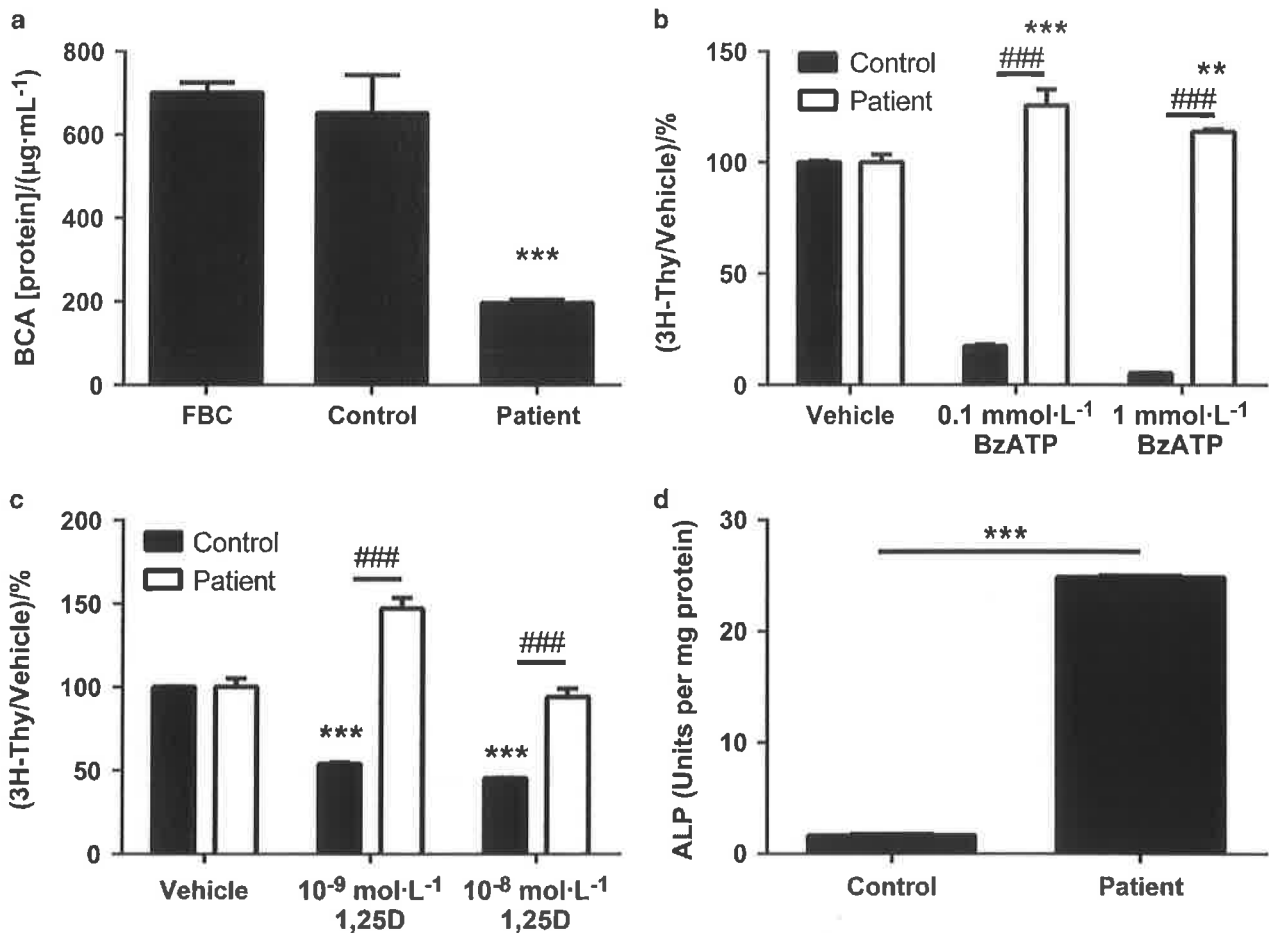
Figure 3. Protein production by cultured osteoblasts (**a**). FBC, fetal bone cells. ***$P < 0.001$ compared with control. 3H thymidine incorporation into osteoblasts in culture BzATP (**b**). ***$P < 0.001$ compared with vehicle; **$P < 0.01$ compared with vehicle; ###$P < 0.001$ compared with control. 3H thymidine incorporation into osteoblasts in culture (**c**). 1,25D, 1,25-dihydroxyvitamin D. ***$P < 0.001$ compared with vehicle; ###$P < 0.001$ compared with control. Alkaline phosphatase activity in cultured osteoblasts (**d**). ***$P < 0.001$ compared with control.

and patient) in finer detail. This was to investigate the ultrastructural organization of the collagen layering between these cells following long-term culture, using fetal bone cells as a positive control. The control biopsy-derived osteoblasts showed similar multilayers to those observed with fetal bone cells (Figure 2c). The patient-derived cells appeared quite disorganized and without any well-defined features, such as multiple cell layers and endoplasmic reticulum, both of which are seen in the other cell types (Figure 2d).

Cell proliferation is reduced in the patient biopsy-derived osteoblast cells
The growth of the patient-derived osteoblasts appeared much slower than the control cells under the specified culture conditions. Therefore, assays for protein (BCA) production by the cells and thymidine incorporation into the cells were performed to quantify these apparent cell replication differences. Cell proliferation between fetal bone cells, control, and patient cells were all compared by

protein measurement, following 5 days in culture without any treatment and also by the measurement of thymidine incorporation into the cells. Proliferation of patient cells was significantly lower in comparison with control biopsy cells and fetal bone cells. (Figure 3a).

Effects of bone active agents on the proliferation of biopsy-derived osteoblasts
Both the patient and control osteoblasts expressed the P2X7 receptor and the VDR.

The proliferation rate of the control biopsy-derived cells significantly decreased as BzATP concentrations increased ($P < 0.001$). In contrast, the patient biopsy-derived cells showed increased proliferation in response to BzATP, an agonist of the BzATP receptor, with significant increases at both the lower 0.1 mmol·L^{-1} BzATP dose ($P < 0.001$) and the higher 1 mmol·L^{-1} BzATP dose ($P < 0.01$; Figure 3b).

Similar to the effects seen with BzATP, the patient's cells responded in a paradoxical manner when treated with

Table 2. The fold change in gene expression

Gene status	Gene name	Gene symbol	Fold change
Genes upregulated	G-protein-coupled receptor 128	GPR 128	56.24
	Ankyrin repeat-domain-containing protein 1	ANKRD1	37.53
	EGF containing fibulin-like extracellular matrix 1	EFEMP1	36.28
	Secreted phosphoprotein 1 (osteopontin)	SPP1	32.06
	CD36 (collagen type 1 receptor)	CD36	30.59
	Vascular cell adhesion molecule 1	VCAM 1	21.68
	Cadherin 13	CDH 13	18.53
	Flavin containing mono-oxygenase 3	FMO3	18.51
	Collagen type IV alpha 1	COL4A1	17.39
	Tumour necrosis factor receptor 11B	TNRSF11B	17.26
	Lipid phosphate phosphatase related 4	LPPR4	14.18
	Secreted frizzled-related protein 2	SFRP2	13.22
	Collagen type IV alpha 2	COL4A2	9.25
	Cartilage oligomeric matrix protein	COMP	8.09
	Vitamin D receptor	VDR	2.58
Genes downregulated	Chemokine, cc motif, receptor-like protein	CCRL1	31.19
	Carboxypeptidase X, M_2	CPXM2	19.50
	Prostaglandin 12 synthase	PTG1S	14.48
	Zinc-finger protein FOG family member 2	ZFPM2	13.55
	T-Box5	TBX5	10.57
	Angiomotin	AMOT	9.72
	Calmegin	CLGN	9.41
	Scavenger receptor class A member 3	SCARA3	9.37
	Secreted frizzled-related protein 1	SFRP1	6.05
	T-Box3	TBX3	5.33

Patient's osteoblasts in culture compared with control osteoblasts after 6 days in culture.

1,25-dihydroxyvitamin D. Usually with increasing concentrations of 1,25-dihydroxyvitamin D, osteoblast cell proliferation decreases[6,10] and this was seen in the control biopsy cells. The patient cells, however, significantly increased proliferation in culture with a concentration of 10^{-9} mol·L^{-1} 1,25-dihydroxyvitamin D concentration($P < 0.001$; Figure 3c).

Alkaline phosphatase was used as a marker of osteoblastic differentiation for control and patient biopsy-derived bone cells, and was corrected for total cellular protein as measured by the BCA assay. These cells, similar to fetal bone cells, had a maximal alkaline phosphatase level after 7 days in culture. A comparison was made between the two biopsy osteoblasts, with no additional treatments. A significant increase in alkaline phosphatase activity was evident in the patient's osteoblasts ($P < 0.001$) in comparison with the control biopsy osteoblasts, a difference that reached 10-fold (Figure 3d).

Affymetrix gene array
Gene microarray analysis comparing the patient's osteoblasts with osteoblasts from a normal control in culture. A microarray was performed on mRNA extracted from the control and patient-derived osteoblasts after 6 days in culture to compare differences in gene expression levels between the two cell types. The mRNA underwent quality control before microarray analysis. The genes showing the most significant upregulation and downregulation are

shown in Table 2. The most upregulated gene in the patient's osteoblasts compared with the control osteoblasts was G-protein-coupled receptor 128, an orphan receptor of unknown function. The most downregulated gene was chemokine, cc motif, receptor-like protein 1 (CCRL1), of unknown relevance to bone cell function. RANK expression in osteoclasts is upregulated by chemokine receptor 2 (CCR2).[11] Tumor necrosis factor receptor 11b (OPG) gene expression was markedly upregulated. Secreted OPG protein was measured in both the control and patient biopsy-derived cells by ELISA. All OPG protein levels measured were corrected for total protein content, as assessed by BCA assay. OPG protein expression was increased significantly ($P < 0.001$) in the patient cells compared with control cells, confirming the microarray results for mRNA (Supplementary Figure 1). This increase resulted in a 10-fold difference. Because of the possibility that the diminished collagen observed in cell culture experiments might be due to reduced synthesis of collagen, the expression of collagen genes was examined.COL1A1, COL1A2, COL3A1, and COL3A2 were not significantly upregulated or downregulated. COL4A1, COL4A2, and COL11A1 were significantly upregulated, COL11A1 3.25-fold. Collagen protein synthesis was not measured. An immunostain of bone was negative for collagen 4.

The expression of the sclerostin (SOST) protein was examined in control and patient biopsy-derived cells

Table 3. The fold change in gene expression

Gene status	Gene name	Gene symbol	Fold change
Genes upregulated	Aquaporin 1	AQP1	8.28
	Cartilage intermediate layer protein	CILP	7.47
	Calsequestrin 2	CASQ2	7.21
	Retinol-binding protein 1	RBP1	7.11
	Nephronectin	NPNT	6.84
	Myosin light-chain alkali	MYL 1	6.22
	SRY-box 11	SOX 11	6.15
	Troponin C type 2	TNNC2	5.71
	Breast carcinoma amplifier sequence 1	BCAS1	5.32
	Heat shock 27 kDa protein 3	HSPB3	5.21
Genes downregulated	Metallothionein	MT1G	7.27
	Oligodendrocyte myelin glycoprotein	OMG	5.67
	Serpin peptidase inhibitor clade A	SERPIN A1	4.67
	Matrix metallopeptidase 3	MMP3	4.65
	Prostaglandin F receptor	PTGFR	4.38
	Fbj murine osteosarcoma viral oncogene	FOS	3.82
	R-spondin-3	RSPO3	3.71
	Aquaporin 9	AQP9	3.43
	Proteoglycan 4	PRG4	3.20
	Suprabasin	SBSN	3.05
	Secreted frizzled-related protein 4	SFRP4	2.68
	Bone morphogenetic protein 2	BMP2	2.15

Control osteoblasts cultured in patient's serum compared with control osteoblasts cultured in normal serum after 6 days in culture.

maintained in culture, by western blot analysis to test the microarray analysis which showed no significant fold change. The expression of sclerostin protein examined by this technique did not show any significant difference between the control and the patient biopsy-derived cells. (Supplementary Figure 2).

The expression of VDR gene was upregulated 2.58-fold. The VDR protein was also measured in control and patient biopsy-derived cells, maintained in culture, by western blot analysis of protein expression, and no significant difference between the cell types was seen (Supplementary Figure 3).

Gene microarray analysis comparing control osteoblasts cultured with the patient's serum vs the same normal osteoblasts cultured with control serum. The control serum was taken from a different healthy young male patient. The control serum and the patient's serum, after centrifugation to remove red cells and white cells, were added in equal amounts to separate incubation medium to comprise 10% of the incubation medium. The testosterone concentration was measured by Liquid Chromatography-Mass Spectrometry (LC-MS) of the incubation medium prepared with the patient's serum and with the control serum, and the values were 1.14 nmol·L^{-1} and 1.60 nmol·L^{-1}, respectively.

A microarray was performed on mRNA extracted from the control osteoblast after 6 days in culture where the culture had been performed with the patient's serum or control serum. The genes showing the most significant upregulation and downregulation are shown in Table 3. The gene expression seen in control osteoblasts cultured with the patient's serum was quite different from the gene expression seen when the control osteoblasts were cultured in normal media. The most upregulated gene was aquaporin 1 (AQP1) and the most downregulated gene was metallothionein (MT1G). The following genes which might be relevant to bone cell metabolism were neither significantly upregulated or downregulated: ectonucleotide pyrophosphatase 1 (ENPP1), osteopontin (SPP1), osteocalcin (GLAP), bone sialoprotein (IBSP), matrix extracellular matrix phosphoglyco-protein (MEPE), dentin matrix acidic phosphoprotein (DMP1), complement component 1, q1-subcompartment A and B chains (C1QA, C1QB). Selected genes were further studied by reverse transcription PCR. The data showed significant upregulation of AQP1 after 6 days of culture. A significant increase in cartilage intermediate layer protein (CILP) was confirmed. Significant downregulation of serpin peptidase inhibitor clade A (SERPINA1) and secreted frizzled-related protein 4 (SRP4) were seen. No significant changes in C1QA or C1QB were seen.

DISCUSSION
In 1950, Baker and Turnbull[12] described two patients in whom features resembling osteomalacia were present that pursued a relentlessly progressive course. The disorder,

initially referred to as "Baker's disease", was subsequently named fibrogenesis imperfecta ossium reflecting abnormal bone matrix lacking normal lamellar bifringence of collagen with polarized light and associated with a large amount of incompletely calcified osteoid. The histopathological findings in the disorder were initially described in 1956 by Baker[13] and subsequently expanded on by many authors.[1–4,14–15] All cases showed similar histological findings of varying degree of severity. Thickened trabeculae with pale wide osteoid seams were documented in which diminished or deficient collagen was associated with coarse granular calcification. Bone collagen only was affected and variable quantities of residual lamellar bone were identified depending on the stage of evolution. The calcified zones abut normal trabeculae with diminishing calcification in the outer zones and variable quantities of residual host lamellar bone. This entity differs from osteomalacia in that in osteomalacia the collagen content is normal. The abnormality spares periosteum, ligaments, tendons, and fracture callus. The changes in the cortex were less developed than in the medulla[15] and this was seen also in our case.

Electron microscopic findings showed that the numbers of collagen fibers are diminished, have a random orientation, and are commonly curved and irregular with a variable diameter of 22–136 nm. Commonly on electron microscopy random scattered calcified nodules were present. These were increased in the area near the calcified bone and decreased in the sparsely collagenized zones. Amorphous matrix predominated in the non-collagenous area. Osteoblasts, osteocytes, and osteoclasts have not previously been found to be abnormal.[1–4,14–15] The abnormal trabecular pattern of bone was noted on imaging by Golding in 1968.[16] Several authors have emphasized an altering appearance on imaging over time.[4,17] Collagen is required for mineralization. In the presence of residual collagen, the mineral content was found to be increased.[13,15] These authors suggested that the less collagen content initially allowed increased space for mineralization explaining the sclerosis seen on X-ray and the fact that it was extremely hard at biopsy. However, the mineral is poorly bound with ease of decalcification as exemplified in our case where there was easy rapid decalcification. The sclerosis reduces with diminution of collagen over time reflecting a lack of calcium binding.

Baker *et al* in 1966[18] described a third case emphasizing the abnormally coarse trabecular pattern of bone with increasing bone density particularly in the metaphyseal portion of long bones. In time, further cases were documented[19] and over the years it appears that of the order of 25–30 cases only have been described. An inhibitor of matrix calcification in the serum was not found[20] and the serum pyrophosphate was normal. Henneman *et al*[21] found that the soluble component of collagen, that is newly synthesized collagen, was increased but total mature collagen was reduced. It had been previously reported that cross-linking of collagen was associated with the mineralization process.[22] Stoddart *et al*[23] had documented a case with a circulating paraprotein, subsequently confirmed in several further cases in which a circulating paraprotein usually an IgG kappa or IgG lamda was found[3,17,24–26] Patients with the disorder have been treated with melphalan and prednisolone with histological improvement,[4,24] but others have not shown benefit with melphalan.[25] Bakos *et al*[26] described a patient whose severe diffuse skeletal pain was reduced by plasmapharesis.

In our case, the serum alkaline phosphatase was elevated, 206 U·L^{-1}, a consistent finding in other cases, with some values over 500 U·L^{-1}.[4,26] The serum levels of C3 and C4 were persistently diminished, a hitherto undocumented finding. There was no evidence of other disorders in which this may occur, including systemic lupus erythematosus and glomerulonephritis. The low level of C3 may have increased his susceptibility to the salmonella bacterial infection that damaged his aortic valve. Presumably the deficiency of C3 and C4 were acquired as there was no history of recurrent infections during childhood. Increased susceptibility to both Gram-positive and Gram-negative bacterial infection have been described with C3 deficiency.[27–28] Osteoblasts can be stimulated to produce C3 by 1,25-dihydroxyvitamin D.[29] In culture experiments when anti-C3 antibody was used, osteoclast formation was greatly inhibited.[30] There has been no evidence for cleavage of C3 in either osteoblasts or osteoclasts.[31] C1-s can cleave type I and type II collagen[32] but the possible role of any component of the complement system in the mineralization of collagen is unknown. In fibrogenesis imperfecta ossium, there appears to be a defect in the cross-linking of collagen and subsequent failure to appropriately calcify the collagen matrix. A somewhat similar histologic pattern has been observed in osteogenesis imperfecta type VI.[33] However, in that entity polarizable collagen is present, albeit in an abnormal distribution giving a so called "fish scale" appearance. A complete lack of collagen with diminution in quantity over time as seen in fibrogenesis imperfecta ossium (FIO) is not documented. The genetic mutation in osteogenesis imperfecta type VI has been identified as a homozygous mutation in SERPIN F1.

In our case, the combination of clinically diffuse bone pain, imaging findings of severe osteopenia in the axial skeleton, increased metaphyseal bone density in the long bones, and bone biopsy at both sites in which abnormal bone remodeling with increased osteoid seams comprising diminished collagen identifiable with polarized light, fits the

criteria for a diagnosis of fibrogenesis imperfecta ossium. A new finding, hitherto undocumented, of a low serum C3 and C4 in combination with a circulating IgG kappa paraprotein, a finding noted in prior cases, was identified, the significance of which to the pathogenesis of the disorder is unknown. Additional biochemical abnormalities were a significantly elevated serum TRACP-5B reflecting increased osteoclast activity, a feature not overtly identified in bone biopsy specimens, a persistently elevated serum inorganic phosphate, and an abnormal increase in 1,25-dihydroxyvitamin D after treatment with vitamin D. The serum calcium and serum phosphate have been consistently normal in other cases.

In an attempt to gain further insights into the pathogenesis of the bone disorder, osteoblasts were successfully derived and cultured from a biopsy obtained from the distal femur and compared with osteoblasts derived and cultured from the distal femur of a 19-year-old patient undergoing elective knee surgery deemed to be a normal control. The selected control was a healthy male close in age to our patient. Both had suffered a rupture of the anterior cruciate ligament. In both cases the biopsy was obtained from the lower femur. The osteoblasts obtained from the control biopsy behaved in culture in a remarkably similar manner to fetal bone cells (primary human osteoblasts),[9] and were markedly different in behavior to the osteoblasts obtained from the patient. This has led us to believe that the "control" osteoblasts in culture could be used as an appropriate control to compare with the patient's osteoblasts. In culture, the osteoblasts obtained from the patient showed marked morphological differences when compared with osteoblasts grown in culture from the control. The rate of proliferation of the patient's osteoblasts was much less than that of the control osteoblasts. Alkaline phosphatase activity was much higher in the patient's osteoblasts compared with controls. Paradoxically in contrast to the response in the control osteoblasts, 1,25-dihydroxyvitamin D markedly stimulated proliferation of the patient's osteoblasts.

In a gene microarray study carried out on mRNA extracted from the patient's osteoblasts and the control osteoblasts after 6 days in culture, OPG mRNA expression was markedly increased in the patient's osteoblasts when compared with the control osteoblasts and OPG protein measured by ELISA was also markedly increased in the patient's osteoblasts. This would be expected to diminish osteoclast activity but in fact the serum TRACP-5B was increased reflecting increased osteoclast activity. The expression of the VDR protein examined by western blot was not increased in the patient's osteoblasts, although the cells increased their rate of proliferation when cultured with 1,25-dihydroxyvitamin D.

Several other genes that might be involved in bone cell function were either markedly upregulated or downregulated in the gene microarray study. Osteopontin, upregulated, inhibits hydroxyapatite formation after phosphorylation. Alkaline phosphatase dephosphorylates osteopontin. Pyrophosphate upregulates osteopontin, and pyrophosphate and osteopontin together inhibit bone mineralization.[34] CD36 (collagen type 1 receptor), upregulated in the patient's osteoblasts, could be involved in the genesis of osteoporosis through stimulation of bone resorption.[35] The upregulation of collagen type IV alpha 1 and alpha 2 subunits has been linked to low bone mass.[36] Tumor necrosis factor receptor 11B (OPG) mRNA and protein expression were upregulated and by binding to RANKL could lead to unrestrained bone formation.[37] Secreted frizzled-related protein 2 (SFRP2) was upregulated. The expression of SFRP2 is strongly upregulated during osteoblastic differentiation[38] and inhibits Wnt3a/beta catenin signaling.[39] Cartilage oligomeric matrix protein (COMP), upregulated, has been implicated in the development of irregular ossification in epiphyses,[40] and in the development of abnormal collagen fibril morphology.[41] SFRP1 was downregulated. SFRP1$^{-/-}$ knockout was associated with increased osteoblastic proliferation and differentiation, and increased trabecular bone formation.[42] T-box 3 is a key promoter of osteoblast proliferation[43] and was downregulated in the present study. The bone biopsy was taken from an area in the femur of increased bone density and the increased expression of alkaline phosphatase and the paradoxical response to 1,25-didroxyvitamin D of the osteoblasts in culture suggest that factors facilitating bone formation were dominant.

In the study in which control osteoblastic cells in culture were exposed to the patient's serum AQP1 was the most upregulated mRNA. The function of this gene in osteoblasts is unknown but is highly expressed in osteosarcoma cells. Knockdown of AQP1 was associated with reduced proliferation of osteosarcoma cells.[44] R-spondin-3 was downregulated. R-spondins may enhance Wnt/beta catenin signaling.[45] SFRP4 was downregulated. SFRP4 has a phosphaturic action similar to that of FGF-23,[46] and its downregulation may be relevant to the persistent hyperphosphatemia seen in this patient. In the patient's poorly proliferating cells bone morphogenetic protein 2 (BMP2) was downregulated. BMP2 induces bone formation and osteoblastic differentiation in association with Runx2 and osterix expression.[47] BMP2 expression is activated by Wnt14 and Wnt14, and BMP2 synergize to promote osteoblastic differentiation.[48] Neither overexpression nor underexpression of any of the Wnt ligands was noted in the present study. Cross-linking of collagen is important for hydroxyapatite deposition on collagen fibrils. Lysyl hydroxylase is important in the cross-linking process.[49] Inhibition of lysyl hydroxylase gives irregularly shaped collagen fibrils. Lysyl oxidase is also involved in cross-linking of collagen and is

markedly upregulated in the differentiation of osteoblasts.[50] The genes coding these enzymes were neither upregulated nor downregulated in the present study. Collagen alone cannot initiate hydroxyapatite nucleation on collagen fibrils.[51] Bone sialoprotein (BSP) that is expressed in and secreted by osteoblasts is necessary to initiate hydroxyapatite deposition.[52] BSP binds to collagen1alpha 2 to stimulate matrix calcification.[53] BSP mRNA was neither upregulated nor downregulated in the present study. Another gene upregulated to a lesser extent was ROR2 (4.63-fold), which is a receptor for Wnt5a and activates the non-canonical pathway in osteoblasts. Overexpression of ROR2 increased osteoblastic differentiation[54] and this mechanism may be relevant in our patient with increased bone mass at the site of biopsy. Given that three to four tablets of alendronate were taken before the femoral bone biopsy, the possibility that these significantly influenced the gene expression in osteoblasts should be considered. Gene expression in osteoblasts has been studied in rhesus monkeys treated with alendronate.[55] The OPG gene was upregulated 16-fold compared with 17.26 upregulation in our study. ROR2 was downregulated in the rhesus monkey study but upregulated in our study in which the patient's osteoblasts were compared with control osteoblasts in culture.SPP1 (osteopontin) was downregulated 25-fold in the rhesus monkey osteoblasts compared with a 32.06 upregulation in our study. When control osteoblasts were cultured in the patient's serum SFRP4 was downregulated, a similar finding to that in rhesus monkey osteoblasts. None of the other genes listed in Tables 2 and 3 were significantly upregulated or downregulated in the rhesus monkey study.

In summary, this patient had wide spread bone pain, reduced vertebral bone density, marked sclerosis at the end of long bones, and a bone biopsy that showed diminished collagen content on light and electron microscopy, with reduced collagen birefringence on polarized light microscopy consistent with the diagnosis of fibrogenesis imperfecta ossium. A circulating IgG kappa paraprotein was identified and there was marked reduction in serum C3 and C4 a finding not previously reported in this disorder. More information about the possible role of C3 and C4 in the proliferation, differentiation and function of osteoblasts is required. Osteoblasts cultured from the femoral bone biopsy obtained from the patient showed abnormal morphology, reduced proliferation rates and responded abnormally to 1,25-dihydroxyvitamin D. A gene expression study performed on mRNA obtained from the osteoblasts showed marked upregulation of GPR 128 an orphan receptor of unknown function. Control osteoblasts in culture obtained from a normal male of similar age showed increased expression of AQP1 when cultured with serum from the patient. These are the first studies to examine proliferation, protein production and gene

expression in osteoblasts in this disorder. A single factor defining the pathogenesis of this disorder has not been discovered. A successful treatment for fibrogenesis imperfecta ossium has not been forthcoming. Treatment with melphalan has not been consistently effective. The use of bortezomib was considered but not pursued. The use of plasmapharesis has been described in one case. In the present case, the use of zolendronate infusions initially every 6 months has resulted in a marked increase in spinal BMD and a marked reduction in joint and muscle pain.

Acknowledgements

Author contributions
Melissa L Barron, Mark S Rybchyn and Sutharshani Ramesh carried out the cell cultures. Rebecca S Mason supervised and designed the cell culture experiments. S Fiona Bonar undertook the microscopic analysis. Paul Stalley performed the bone biopsy. Sundeep Khosla reviewed the case and further examined the patient. Bernie Hudson assessed the microbiological information. Christopher Arthur reviewed the significance of the circulating paraprotein. Edward Kim performed the quantitative PCR studies. Roderick J Clifton-Bligh had significant clinical and intellectual input. Phillip B Clifton-Bligh initiated and supervised all investigations and wrote the manuscript.

Competing interests
The authors declare no conflict of interest.

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Supplementary Information for this article can be found on the *Bone Research* website (http://www.nature.com/boneres)

Paper 28. Femoral neck x-ray absorptiometry parameters and peripheral quantitative computer tomography tibial cortical density predict survival in dialysis patients. N Yap, P Wong, S McGinn, ML Nery, J Doyle, L Wells, P Clifton-Bligh, RJ Clifton-Bligh. Nephron 2017;136:183-192

P Clifton-Bligh made a significant contribution to the conception and supervision of the study and in the collation of the data, review of the spread sheet, statistical revision, response to the reviewers comments, rewriting of the manuscript and research and revision of the bibliography.The study describes the longest follow up so far, 6 years, of mortality in dialysis patients in relation to their measured bone mineral density. Mortality over a six year period was highly correlated with femoral neck bone mineral density and remained significant when corrected for age, sex, transplant status, duration of dialysis, presence of diabetes, serum parathyroid hormone and smoking history. Tibial cortical density was also highly correlated with mortality but the relationship was less robust when corrected for the additional risk factors. The presence of cardiovascular disease was strongly correlated with mortality but the presence of cardiovascular disease was not associated with a lower bone mineral density compared to those without prevalent cardiovascular disease.The discussion is an important review of how bone mineral density may be a surrogate predictor of mortality and how other factors known to increase mortality in dialysis patients may impact on bone mineral density.

This paper is considered to be an important contribution to the understanding of factors which influence mortality in patients being dialysed for end stage renal disease.

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Femoral Neck X-Ray Absorptiometry Parameters and Peripheral Quantitative Computer Tomography Tibial Cortical Density Predict Survival in Dialysis Patients

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Abstract

Background: Low bone mineral density (BMD) is a known independent predictor of mortality in the general elderly population. However, studies in patients with end-stage renal disease (ESRD) are limited. The present study evaluated mortality during long-term follow-up in a population of patients having dialysis for ESRD, in whom BMD was also measured. ***Methods:*** Fifty-eight patients with ESRD were recruited consecutively from a dialysis clinic and followed prospectively for 6 years. Baseline BMD of the lumbar spine and femoral neck (FN) were measured by X-ray absorptiometry and by peripheral quantitative CT at the radius and tibia. Serum calcium, phosphate, parathyroid hormone (PTH), and albumin were measured at baseline. ***Results:*** During follow-up, 25 patients died. Univariate analysis showed that mortality was significantly associated with FN-BMD: hazards ratio (HR) per 0.1 g/cm^2 decrease 1.50 (95% CI 1.07–2.10), p = 0.019; FN-T score: HR per 1-SD decrease 1.84 (95% CI 1.16–2.92), p = 0.009; and tibial cortical density: HR per 10 mg/cm^3 decrease 1.08 (95% CI 1.02–1.14),

p = 0.010. In multivariate analysis with stepwise adjustment for age, sex, transplant status, albumin, PTH, phosphate, dialysis duration, diabetes, and smoking, FN-T score remained significantly associated with mortality: HR per 1-SD decrease 1.82 (95% CI 1.02–3.24), p = 0.044, whereas the HR for FN-BMD and tibial cortical density were no longer significant. When 4 patients who had peritoneal dialysis were excluded, the HR relating FN-BMD, FN-T score, and tibial cortical density to mortality remained significant but became insignificant when albumin was included in the multivariate analysis. ***Conclusion:*** Reduced FN-BMD, FN-T score, and tibial cortical density were significantly associated with an increased risk of death in patients with ESRD.   © 2017 S. Karger AG, Basel

Introduction

The complex mineral and bone disorders associated with chronic kidney disease (CKD-MBD) [1], previously known as renal osteodystrophy, comprise various combinations of high-turnover bone disease (hyperparathyroidism), adynamic bone disease, osteomalacia, and/or osteoporosis. In the general population, low bone mineral density (BMD) [2–4] and/or excess bone loss [4, 5] are

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known independent predictors of mortality. For patients on hemodialysis, total hip BMD [6], total body BMD [7, 8], and reduction in ultradistal radius BMD over 1 year [9] have each been shown to be predictors of all-cause mortality in studies of limited duration.

The use of X-ray absorptiometry (DXA) to assess bone density in CKD is limited by an inability to distinguish trabecular from cortical bone (the latter which is preferentially affected by hyperparathyroidism), and by the artefactual distortion of spine BMD by aortic calcification and/or CKD-MBD. Bone biopsy with tetracycline labeling remains the preferred standard for assessing CKD-MBD, but is invasive and impractical for repeated measurements, and widespread expertise in interpreting these specimens is lacking. Peripheral quantitative CT (pQCT) enables the cortical and trabecular components of bone to be studied non-invasively. Negri et al. [10] have previously demonstrated in a cohort of hemodialysis patients that distal radius cortical density and distal tibial cortical density measured by pQCT were significantly reduced compared with controls. No studies to date have looked at the relationship between pQCT parameters and mortality in this population to determine if the ability to differentiate cortical bone from trabecular bone offers greater sensitivity. We hypothesized that femoral neck (FN) BMD, as well as radius and tibial cortical density measured by pQCT might be predictive of mortality.

Materials and Methods

A total of 58 subjects with end-stage kidney disease were recruited consecutively through a single tertiary referral hospital in 2007 over 8 months and were then followed prospectively for 6 years to determine if BMD measured by DXA and pQCT was associated with all-cause mortality. At baseline, 54 patients were on in-center and satellite hemodialysis and 4 were on peritoneal dialysis. At the census date in 2013, 33 (57%) of the cohort had survived. The cause of death was ascertained through correspondence with treating physicians and general practitioners and/or review of medical records and death certificates, where available. The cause of death was sepsis in 10 (40%), cardiovascular disease in 6 (24%), withdrawal of dialysis for psychosocial reasons in 3 (12%), malignancy in 1 (4%), other (pulmonary embolism, hepatitis B, and sclerosing peritonitis) in 3 (12%), and unknown in 2 (8%).

Patients were examined and interviewed by a single investigator (P.W.) at baseline in 2007. Etiology of renal failure, duration and modality of dialysis, comorbidities, previous minimal trauma fractures, previous parathyroidectomy, smoking status, alcohol intake, and use of medications were ascertained. Documentation of renal transplant status included previous failed/non-functioning transplants and functioning transplants. For patients where dialysis modality changed or their transplant later failed, duration of dialysis was calculated from dialysis start date up to the census date

for those currently dialysis dependent or up to the transplant date for those with a currently functioning transplant.

Body mass index (BMI; kg/m^2) was calculated based on dry weight and blood samples were taken at the start of dialysis. Biochemistry included serum phosphate, calcium (corrected for albumin), albumin, alkaline phosphatase (ALP), and 25-OH vitamin D. Intact parathyroid hormone (iPTH) was measured using the IMMULITE 2000 solid-phase 2-site chemiluminescent enzyme-labeled immunometric assay (analytical sensitivity of 3.0 ng/L). Tartrate-resistant acid phosphatase 5b was measured using the BoneTRAP Assay (Immunodiagnostic Systems; CV 4.1%, limit of quantification <0.5 U/L). Calcium–phosphate product (Ca × Phos) was calculated.

AP and lateral thoracolumbar X-rays were performed on each patient to assess for prevalent vertebral fractures. Bone density was measured by DXA at FN, lumbar spine, 1/3 radius, and total body BMD, as well as body composition on Hologic Explorer ($n = 14$; CV 0.35%) or Norland ($n = 40$, CV 0.45%) densitometers. Four patients declined to participate in DXA scanning and one additional patient was unable to perform FN BMD DXA due to bilateral hip replacements. All 5 of these patients received hemodialysis. For purposes of comparison, Genant formulae were used to convert Norland measurements to Hologic equivalents [11]. FN-T scores were calculated using the NHANES III database [12]. Peripheral QCT (Stratec XCT 3000, Germany; CV ≤0.37%) was performed in the non-dialysis arm and contralateral tibia to measure trabecular area and density (measured at 4% proximal to the distal end) and cortical area and density (measured at 66% proximal to the distal end).

Patient baseline characteristics were compared using chi-square tests and independent samples t tests, with data presented as means with SDs. Mann–Whitney U tests were used to analyze non-parametric variables with data presented as medians with interquartile ranges. Univariate and multivariate Cox proportional hazards regression models were used to determine factors associated with survival. Cox regression was used to examine survival based on T score category, adjusted for age and sex. Statistical methods were performed using IBM SPSS (version 21, 2012).

Theory
Due to its ability to distinguish between cortical and trabecular components, we hypothesize that pQCT parameters, in addition to FN BMD, may demonstrate a relationship with mortality in our CKD-5D population.

Results

Baseline characteristics are shown in Tables 1 and 2 according to whether subjects were alive or deceased at follow-up. The causes of renal failure are included in Table 1. Significantly more patients in the deceased group had a history of cardiovascular disease (72%) compared with the survivor group (24%). Albumin, a known predictor of mortality in this population [13, 14], was lower in the deceased group. ALP was significantly higher in the deceased group and this has been shown to be a predictor of mortality in the hemodialysis population [15].

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Table 1. Baseline characteristics

	Alive ($n = 33$)	Deceased ($n = 25$)	p value
Demographic variables			
Age, years	58±15	65±16	0.075
Male	20 (61)	15 (60)	0.963
Caucasian	21 (64)	20 (80)	0.175
Previous transplant	11 (33)	3 (12)	0.060
Fracture	13 (39)	13 (52)	0.339
Vertebral fracture	6 (18)	3 (12)	0.718
Smoker	9 (27)	9 (36)	0.412
Diabetes mellitus	8 (24)	6 (24)	0.983
Previous history of CVD	8 (24)	18 (72)	<0.001
Parathyroidectomy	5 (15)	6 (24)	0.395
Duration of dialysis, months	122±70	114±82	0.59
BMI, kg/m^2	26±5	25±5	0.420
Weight, kg	73±15	68±13	0.206
Biochemical variables			
Phosphate, mmol/L	1.30 (1.07–1.72)	1.42 (1.07–1.87)	0.278
Ca × Phos, mmol^2/L^2	3.21±1.15	3.49±1.29	0.396
Albumin, g/L	41±5	36±7	<0.004
iPTH, ng/L	86.4 (13.3–157.0)	88.2 (31.2–329.0)	0.378
TRACP 5b, U/L	2.2 (1.9–4.1)	3.2 (2.2–4.7)	0.151
ALP, U/L	86 (73.5–94)	129 (84–164)	0.004
Causes of renal failure			
Hypertensive nephrosclerosis	2 (6.1)	1 (4)	
Diabetes mellitus	3 (9.1)	4 (16)	
Polycystic kidney disease	3 (9.1)	2 (8)	
Glomerular disease	14 (42.4)	8 (32)	
Tubulointerstitial disease	6 (18.2)	2 (8)	
Vasculitis	1 (3)	0 (0)	
Other/mixed	4 (12.1)	8 (32)	

Values are mean ± SD, median (range), or n (%).
Previous history of CVD, composite of cardiovascular disease, stroke and peripheral vascular disease; BMI, body mass index; Ca × Phos, calcium-phosphate product; iPTH, intact parathyroid hormone; TRACP 5b, tartrate-resistant acid phosphatase 5b; ALP, alkaline phosphatase.

Univariate analyses are shown in Table 3 (DXA and pQCT parameters) and Table 4 (demographic and biochemical variables). FN-BMD expressed either as a continuous variable, g/cm^2, or categorically by T score, was associated with significantly increased mortality (hazards ratio, HR 1.50 per 0.1 g/cm^2 decrease [95% CI 1.07–2.10], $p = 0.019$; and HR 1.84 per SD decrease [95% CI 1.16–2.92], $p = 0.009$, respectively). No association was seen between mortality and BMD measurements at the lumbar spine, forearm, or total body; and no association was seen with body composition. Mortality was also associated with tibial cortical density measured by pQCT (HR 1.08 per 10 mg/cm^3 decrease [95% CI 1.02–1.14], $p = 0.010$), although associations with tibial cortical area or radial cortical density did not reach significance (HR 1.05 per 10 mm^2 decrease [95% CI 1.00–1.10, $p = 0.06$] and HR 1.04 per 10 mg/cm^3 decrease [95% CI 1.00–1.09, $p = 0.07$], respectively). As expected, a previous history of cardiovascular disease and a low serum albumin were strongly predictive of mortality (HR 4.49 [95% CI 1.86–10.82] and HR 1.11 per g/L decrease [95% CI 1.04–1.18], respectively). Increased serum ALP was associated with a very small but significant increase in mortality (HR 1.002 [95% CI 1.001–1.004] per U/L increase).

The BMD of the FN and tibial cortex were measured separately in those with and without a previous history of CVD (Table 5). There was no significant difference for the FN-BMD, FN-T score, and the tibial cortical density

Table 2. Bone mineral density and body composition

	Alive (n = 33)	Deceased (n = 25)	p value
DXA parameters			
FN T-score	−1.22±0.91	−1.903±0.98	0.013
FN, gm/cm²	0.75±0.13	0.65±0.13	0.011
LS, g/cm²	1.12±0.19	1.04±0.19	0.148
Forearm, g/cm²	0.82±0.20	0.73±0.18	0.098
Total body, g/cm²	0.98±0.15	0.95±0.15	0.521
Total lean mass, g	43,665.00 (36,138.00; 51,466.00)	36,053.00 (30,605; 48,067.00)	0.157
Total fat mass, g	26,552.00 (19,072.00; 35,511.00)	32,590.50 (19,012.00; 36,158.00)	0.575
pQCT parameters			
Radius trabecular density, mg/cm³	177.12±51.76	169.60±53.86	0.595
Radius cortical density, mg/cm³	1,070.93±80.36	1,026.32±87.15	0.051
Radius cortical area, mm²	80.03±26.88	72.04±29.45	0.293
Tibia trabecular density, mg/cm³	207.45±61.08	201.83±47.90	0.701
Tibia cortical density, mg/cm³	1,060.04±63.97	1,013.5±66.36	0.011
Tibia cortical area, mm²	278.14±73.84	236.87±93.09	0.084

FN, femoral neck bone mineral density; LS, lumbar spine bone mineral density.

between those with and without a previous history of CVD, so that the increased hazard of death from pre-existing CVD does not seem to be reflected in a lower BMD. The relationship between BMD and death was adjusted for potential contributing factors using Cox proportional hazards multivariate analysis. When adjusted for age and sex, the relationship between FN-BMD, FN-T score, and tibial cortical density remained significant: HR for 0.1 gm/cm² decrease in FN-BMD 1.50 (95% CI 1.02–2.20), $p = 0.039$; HR for 1-SD decrease in FN-T score 1.86 (95% CI 1.13–3.05), $p = 0.015$; and HR for 10 mg/cm³ decrease in tibial cortical density 1.08 (95% CI 1.10–1.15), $p = 0.023$. When adjusted for a previous history of CVD, the significance of the relationship between BMD and death was reduced: FN-BMD HR 1.39 (95% CI 1.00–1.93), $p = 0.047$; FN-T score HR 1.62 (95% CI 1.04–2.54), $p = 0.033$; and tibial cortical density HR 1.06 (95% CI 1.00–1.13), $p = 0.054$, again suggesting that the impact of pre-existing CVD on mortality was at least partly independent of BMD. When adjusted for age, sex, transplant, albumin, PTH, phosphate, duration of dialysis, diabetes, and smoking; the HR for 1-SD decrease in the FN-T score remained significant 1.82 (95% CI 1.02–3.24), $p = 0.044$, but became insignificant for the FN-BMD 1.50 (95% CI 0.096–2.35), $p = 0.074$, and for the tibial cortical density HR for 10 mg/cm³ decrease in bone density 1.05 (95% CI 0.94–1.17), $p = $

0.414. However, the HR for a 1-SD decrease in the FN-BMD remained significant, 1.57 (95% CI 1.01–2.43), $p = 0.045$, until the last potentially confounding parameter, smoking, was added in the model. In the case of the tibial cortical density, significance was lost when albumin and PTH were added to the model adjusted for age and sex.

There were 4 patients who were treated with peritoneal dialysis and 54 patients with hemodialysis. Because of a possible difference in the effect of peritoneal dialysis on BMD compared to hemodialysis, the relationship between BMD and mortality was re-examined excluding the 4 patients on peritoneal dialysis. The HR for the FN-BMD, FN-T score, and tibial cortical density remained significant: HR for FN-BMD per 0.1 gm/cm² decrease 1.45 (95% CI 1.03–2.03), $p = 0.031$; HR for FN-T score per 1-SD decrease 1.76 (95% CI 1.11–2.81), $p = 0.017$; and HR for tibial cortical density per 10 mg/cm³ decrease 1.08 (95% CI 1.02–1.14), $p = 0.014$. The FN-BMD, FN-T score, and tibial cortical density HR became insignificant in the adjusted model when albumin was added as a covariate, thus confirming that a low serum albumin is a factor contributing to mortality independent of its effect on BMD. The significance of the HR was reduced by the addition of the presence of pre-existing CVD to the model: FN-BMD 1.34 (95% CI 0.97–1.86), $p = 0.079$; FN-T score 1.55 (95% CI 0.98–2.43), $p = 0.059$; and tibial cortical density 1.069 (95% CI 1.00–1.13), $p = 0.069$.

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Table 3. Univariate analyses – DXA and pQCT parameters and correlation with mortality

	HR (95% CI)	p value
DXA parameters		
FN BMD T-score	1.84 (1.16–2.92)*	0.009
FN BMD, g/cm^2	1.50 (1.07–2.10)[†]	0.019
LS BMD, g/cm^2	1.21 (0.96–1.54)[†]	0.111
Forearm BMD, g/cm^2	1.22 (0.95–1.57)[†]	0.116
Total body BMD, g/cm^2	1.10 (0.79–1.54)[†]	0.558
Total lean mass, g	1.00 (0.99–1.01)[‡]	0.168
Total fat mass, g	1.00 (1.00–1.00)[‡]	0.662
pQCT parameters		
pQCT radius trabecular density, mg/cm^3	1.02 (0.94–1.10)[⊦]	0.702
pQCT radius cortical density, mg/cm^3	1.04 (1.00–1.09)[⊦]	0.074
pQCT radius cortical area, mm^2	1.07 (0.92–1.25)[⊦]	0.360
pQCT tibia trabecular density, mg/cm^3	1.01 (0.94–1.09)[⊦]	0.728
pQCT tibia cortical density, mg/cm^3	1.08 (1.02–1.14)[⊦]	0.010
pQCT tibia cortical area, mm^2	1.05 (1.00–1.10)[⊦]	0.063

FN, femoral neck; LS, lumbar spine.
* Per 1 SD decrease; [†] per 0.1 g/cm^2 decrease; [‡] per 100 g decrease; [⊦] per 10 mg/cm^3 decrease; [⊦] per 10 mm^2 decrease.

Cox regression survival analysis for all-cause mortality by FN-T scores adjusted for age and sex showed significantly reduced survival for osteoporosis, HR 5.48 (95% CI 1.05–28.64), $p = 0.044$, compared to normal. At baseline, FN-BMD was normal (T score >–1 in 15 (28%), osteopenic (T score –1 to –2.5) in 30 (57%), and osteoporotic (T score ≤2.5) in 8 (15%). Thirty-three (57%) of the original cohort have survived after 6 years. Survival at 6 years for normal, osteopenic, and osteoporotic FN-BMD was 80, 60, and 25%, respectively.

Discussion

Our study has confirmed the association between BMD and mortality in the longest follow-up to date in a cohort of CKD-5D patients. We have shown that FN-BMD, FN-T score, and, for the first time, pQCT tibial cortical density are predictive of mortality. However, in the multivariate analysis, the HR for the FN-BMD became insignificant when the model was adjusted for age, sex, transplant status, albumin, PTH, PO4, duration of dialysis, diabetes, and smoking, but remained significant when smoking was excluded from the analysis, HR 1.57 (95% CI 1.01–2.43), $p = 0.045$. It is not clear why the HR for the FN-BMD and FN-T score should behave differently in the multivariate analysis. A negative T score is the

Table 4. Univariate analyses – demographic and biochemical variables and correlation with mortality

	HR (95% CI)	p value
Demographic variables		
Age, years	1.03 (1.00–1.06)	0.077
Previous transplant	0.33 (0.10–1.10)	0.072
Diabetes	1.01 (0.40–2.54)	0.979
Previous history of CVD	4.49 (1.86–10.82)	0.001
Duration of dialysis	1.00 (0.99–1.00)	0.426
Previous fracture	1.47 (0.67–3.22)	0.338
Previous vertebral fracture	0.65 (0.19–2.16)	0.479
Smoker	1.54 (0.67–3.53)	0.306
Previous parathyroidectomy	1.52 (0.61–3.81)	0.370
Biochemical variables		
iPTH, 10 ng/L	1.01 (1.00–1.02)	0.098
Phosphate, mmol/L	1.66 (0.74–3.70)	0.215
Ca × Phos, mmol^2/L^2	1.18 (0.85–1.65)	0.325
Albumin, g/L	1.11 (1.04–1.18)	0.001
25OH, nmol/L	1.01 (0.99–1.02)	0.278
TRACP 5b, U/L	1.14 (0.93–1.38)	0.209
ALP, U/L	1.002 (1.001–1.004)	0.004

Previous history of CVD, composite of cardiovascular disease, stroke and peripheral vascular disease; BMI, body mass index; Ca × Phos, calcium-phosphate product; iPTH, intact parathyroid hormone; TRACP 5b, tartrate-resistant acid phosphatase 5b; ALP, alkaline phosphatase.

Table 5. Correlation of DXA and pQCT parameters with known CVD

	No history of CVD	Previous history of CVD	p value
DXA FN BMD Hologic, g/cm²	0.73±0.14 $n = 25$	0.69±0.14 $n = 28$	0.310
DXA FN T score	−1.354±0.99 $n = 25$	−1.677±0.95 $n = 28$	0.231
pQCT tibia cortical density, mg/cm³	1,050.53±75.23 $n = 25$	1,028±58.17 $n = 31$	0.239

Previous history of CVD, composite of cardiovascular disease, stroke and peripheral vascular disease. FN, femoral neck. Values are mean ± SD.

number of SD below young normal means, and this categorical variable linked to average values for young normal may be more reliable in assessing the HR for mortality after correction for potential confounders than using measures of BMD. The HR for tibial cortical density became non-significant when albumin and PTH was added to the multivariate analysis after age and sex, suggesting that albumin and PTH were not exerting their effects on mortality through an impact on tibial cortical density. Also, the persistence of the significance for the HR for the FN-T score compared to the loss of significance for the HR for the tibial cortical density in the multivariate analysis suggests that the loss of the trabecular component in the FN bone architecture may be an important factor linking the FN-T score to mortality more powerfully than the loss of purely cortical bone in the tibial cortex.

The significance of the association between a reduced BMD and mortality was attenuated by the presence of pre-existing CVD indicating that pre-existing CVD influences mortality at least partly independently of its effect on BMD. There was no significant difference in the FN-BMD, FN-T score, and tibial cortical density in those with and without pre-existing CVD. When the relationship between BMD and mortality was re-examined excluding the 4 patients who received peritoneal dialysis, the HR for the FN-BMD, FN-T score, and the tibial cortical density remained significant but became insignificant when albumin was added to the multivariate model. We have insufficient numbers to compare the BMD in hemodialysis vs. peritoneal dialysis patients but in another study, there was no significant difference between LS-T scores, total hip-T scores, and tibial cortical volumetric BMD in hemodialysis vs. peritoneal dialysis patients [16]. A low serum albumin was a significant factor for death in peritoneal dialysis patients surviving longer than 24 months from the commencement of dialysis [17]. Oth-

er studies have shown that hemodialysis and peritoneal dialysis patients have the same long-term survival [18].

There are a number of possible mechanisms for the association between FN-BMD, FN-T score, and pQCT tibial cortical density and death. First, these could simply be surrogates for frailty and therefore increased mortality. We do not have baseline measurements of frailty such as muscle power to examine further the possible link between frailty and death, but there was no significant difference in lean mass between those who died and those who survived. Our study population was taken largely from an in-center dialysis unit and hence a more frail population when compared to those who dialyze by home-based therapies. However, studies in the postmenopausal and general elderly population have shown consistently that low BMD or excess rate of bone loss is predictive of mortality despite adjusting for markers of frailty such as age, BMI, and comorbidities. The increased mortality reported in these studies is predominantly due to vascular diseases [3, 5, 19, 20], including cardiovascular disease and stroke. Cardiovascular disease is the predominant cause of death in the end-stage renal disease (ESRD) population [21], and almost all patients with ESRD have some degree of CKD-MBD. Both low and high bone turnover are associated with reduced bone mass and increased vascular calcification [22]. BMD has been shown to be inversely associated with vascular calcification [23] and arterial stiffness as measured by pulse wave velocity [24]. In our study, however, BMD in those with and without CVD was not significantly different.

Disturbances of mineral metabolism in renal failure contribute to the excessive cardiovascular calcification seen in CKD patients. Elevations in serum calcium, phosphate, and Ca × Phos can increase extra-skeletal calcification and are associated with all-cause and cardiovascular mortality [22]. In vitro studies have shown that high levels of extracellular phosphate directly stimulate vascular smooth muscle

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cells to undergo phenotypic changes that promote mineralization of the vascular system by upregulating osteoblast differentiation core binding factor α-1 (Cbfα-1) [25]. Hemodialysis itself may exacerbate the situation as local inhibitors of calcification may be removed and calcium phosphate deposition may be exacerbated by the transient alkalemia, which often occurs after hemodialysis.

We did not find a significant association between serum PTH measurements and mortality in our cohort. In the general population, hyperparathyroidism is associated with increased cardiovascular mortality and morbidity [26] and has been linked with atherosclerosis and increased carotid intima-media thickness and stiffness [27].

There is a relationship between serum PTH and mortality in patients receiving hemodialysis but the relationship is U-shaped with increased mortality at high and low levels [28]. However, patients on peritoneal dialysis have higher levels of serum PTH than those on hemodialysis [29, 30], but do not have reduced survival compared to hemodialysis patients [18]. Although earlier studies showed that peritoneal dialysis was associated with adynamic bone disease and low serum PTH [31], the subsequent use of low-calcium peritoneal dialysate was associated with a rise in the serum PTH [32]. Interestingly, when hemodialysis patients were given phosphate binders to increase the serum PTH, cortical bone porosity as assessed by bone biopsy increased [33] but it is not yet known whether this induced increase in the serum PTH will affect mortality. There is also recent data suggesting the role of matrix protein dysregulation [34] in vascular calcification. Calcified atherosclerotic plaques contain hydroxyapatite and express several bone matrix proteins including matrix GLA protein (MGP), osteopontin, and bone morphogenetic protein type 2 (BMP-2). Animal models have demonstrated that dysregulation of these matrix proteins may lead to abnormal mineralization of bone vasculature. MGP is widely expressed in many tissues, including bone and the vascular wall, and is upregulated in atherosclerotic plaques, suggesting an anti-mineralization role in vascular osteogenesis. Mouse models deficient in extracellular MGP have inappropriate mineralization of cartilage, including the growth plate, leading to short stature, osteopenia, and fractures, as well as extensive medial vascular calcification, resulting in ruptured aneurysms and death [35]. Disruption of MGP's inhibitory effect on BMP-2 is thought to be important in the pathogenesis of vascular mineralization [34, 36]. Enhanced expression of BMP-2 and Cbfα-1 is seen in human atherosclerotic lesions but is absent in normal arteries [37]. Osteoprotegerin (OPG) is a potent inhibitor of osteoclast activation and is found lin-

ing calcific deposits in advanced atherosclerotic lesions [38]. OPG knock-out mice have decreased total bone density, high incidence of fractures, and medial calcification of the aorta and renal arteries [39]. The role of OPG as a risk factor for low BMD and CVD in patients with renal failure is not well defined. Uremia may also contribute to vascular calcification through aberrant expression of matrix proteins. Moe et al. [40] have demonstrated that uremic serum, compared with normal serum, induced the expression of Cbfα-1 in vascular smooth muscle cells in a non-phosphorus-mediated mechanism, highlighting the role of Cbfα-1 as a regulatory factor in the prominent vascular calcification seen in the ESRD population.

Traditional cardiovascular risk factors such as dyslipidemia, inflammation, hyperhomocysteinemia, estrogen deficiency, hypertension, and diabetes also regulate bone remodeling [34]. Both statins and bisphosphonates affect the mevalonate pathway and have potentially beneficial effects on both atherosclerosis and osteoporosis. Statins have been shown in vitro to stimulate bone formation [34]; however, results from clinical trials in relation to BMD and fracture risk have been discrepant [41] and there is currently insufficient evidence to support their use to prevent osteoporosis and fractures in the general population. Data in the hemodialysis population is even more limited and studies to date have failed to show any significant mortality benefit with statin therapy in the hemodialysis population [42, 43]. Statin therapy has potent effects on dyslipidemia, improves endothelial function by enhancing nitric oxide formation, and confers anti-inflammatory properties. Bisphosphonate therapy has been shown to inhibit atherosclerosis in human and animal studies [34] and thus represents a potential therapy to target both cardiovascular disease and osteoporosis. However, ESRD and concerns about exacerbating underlying adynamic bone disease limit its use in this population, and studies are limited as the presence of kidney disease has often been an exclusion criterion in studies regarding bisphosphonate efficacy. Several small Japanese studies have demonstrated the efficacy of etidronate in decreasing the progression of vascular calcification [44–47]; however, the prolonged use of high doses suggested in these studies are concerning due to the risk of osteomalacia. Inflammation identified by an elevated C-reactive protein (CRP) is common in patients on hemodialysis [48] and may contribute to increased cardiovascular risk. The serum CRP reflects the generation of pro-inflammatory cytokines, for example, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-alpha [49]. The serum CRP may be a marker for the severity and progression of the atherosclerotic process in blood

vessels [50]. Vascular access infections in hemodialysis patients may cause repetitive bursts of inflammatory activity [51]. Inflammation may be an important cause of hypoalbuminemia [52]. Increased circulating levels of IL-1, IL-6, and TNF-alpha as a consequence of chronic inflammation may stimulate osteoclast function and bone loss [53–55]; so there may be a link between inflammation, atherosclerosis, and low BMD in patients receiving hemodialysis. We do not have measures of serum CRP to examine the link between inflammation and BMD. Lipopolysaccharide, derived from the cell wall of gram negative bacteria, may induce increased secretion of IL-6 from osteoblasts [56] and also inhibit osteoblastic differentiation [57], a further mechanism whereby hemodialysis patients, who are prone to gram negative infections, may develop low BMD. In our study, the cause of death in 40% of those who died was sepsis and inflammation related to sepsis may have contributed to the low bone density.

The strengths of the present study include a well-characterized cohort of dialysis patients in a single tertiary center with long-term follow-up. This is the longest follow-up to date of studies looking at the relationship between mortality and BMD in dialysis patients, and is the first study to examine the relationship between mortality and pQCT in these patients. The cause and date of death were meticulously followed up through contact with treating physicians and review of medical records. The limitations of this study include small numbers compared to previous studies and a lack of data on other cardiovascular markers such as serum lipids, CRP, and vascular calcification.

Conclusions

FN-BMD, FN-T score, and pQCT tibial cortical density were predictive of mortality in this dialysis cohort. Ongoing exploration of underlying pathological mechanisms will be important to develop possible therapeutic approaches.

Statement of Ethics

The study was approved by the Northern Sydney Local Health District Human Research Ethics Committee and subjects provided written informed consent.

Disclosure Statement

The authors have no conflicts of interest to declare.

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