USE OF CURRENTLY AVAILABLE DIAGNOSTIC AND THERAPEUTIC TOOLS IN THE INVESTIGATION AND TREATMENT OF DISORDERS OF BONE AND CALCIUM METABOLISM

© STEPHEN J GALLACHER M.B. Ch.B. M.R.C.P.(UK)

UNIVERSITY DEPARTMENT OF MEDICINE
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Declaration

The work described in this thesis was performed in the University Department of Medicine, Glasgow Royal Infirmary between 1987 and 1992. All of the studies were conceived, designed and analysed by the author in person and the opinions expressed are therefore those of the author. Some of the studies described in this thesis have already been published (reprints situated inside back cover of this thesis), or are about to be published, in peer-review journals. They include:-

Gallacher SJ, Ralston SH, Dryburgh FJ, Allam BF, Boyle IT. Immobilization-related hypercalcaemia - a possible novel mechanism and response to pamidronate. Postgraduate Medical Journal 1990;66:918-922.

Gallacher SJ, Fraser WD, Patel U, Logue FC, Soukop M, Boyle IT, Ralston SH. Breast cancer-associated hypercalcaemia: a reassessment of renal calcium and phosphate handling. Annals of Clinical Biochemistry 1990;27:551-556

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Summary

In the past decade considerable advances have been made in both the investigation and treatment of various disorders of bone and calcium metabolism. The progress in investigative techniques has taken place in many different directions including biochemical evaluation, parathyroid imaging and bone densitometric measurement. Advances have also taken place in therapeutics with, in particular, the introduction into clinical practice of the bisphosphonates. These drugs are potent inhibitors of bone resorption and are therefore potentially useful in conditions where bone resorption is increased such as in most cases of hypercalcaemia, Paget's disease of bone and some cases of osteoporosis.

Accurate evaluation of serum parathyroid hormone (PTH) concentrations and parathyroid hormone-like activity by use of indirect biochemical indices such as nephrogenous cyclic adenosine monophosphate (NcAMP) and renal tubular threshold for phosphate reabsorption (TmPO4), led to the discovery that in many instances cancer-associated hypercalcaemia is due to the production by the tumour of a factor with PTH-like activity. This, in turn, led to the identification of this factor as PTH-related protein (PTHrP), a peptide with significant structural homology to PTH itself.

It has generally become apparent that PTHrP driven hypercalcaemia is more common in the presence of squamous cancer whereas hypercalcaemia due to direct invasion of bone by lytic metastases is more common in conditions such as myeloma. Traditionally breast cancer-associated hypercalcaemia has been considered to be due to direct metastatic invasion of bone, though more recent evidence suggests that humoral factors, such as PTHrP, may also play a significant role. A retrospective analysis of 20 patients with breast cancer-associated hypercalcaemia was carried out (chapter 3.1). This study indicated

that on the basis of elevated levels of urinary cAMP (UcAMP) excretion and/or depressed TmPO4 (in the presence of undetectable serum PTH), hypercalcaemia associated with the presence of a PTH-like factor was present in between 45-60% patients.

Recent evidence has suggested a number of potential physiological roles for PTHrP. One such example is in the active transfer of calcium into milk in the breast and the active transfer of calcium from mother to fetus in pregnancy by an action at the placenta. In the physiological situation, where PTHrP and PTH concentrations are likely to be within the normal reference range, it is unlikely that measurement of UcAMP, NcAMP or TmPO4 will give accurate indication of plasma PTHrP concentrations. In this context direct measurement of PTHrP is essential. A prospective study of 10 normal women through pregnancy (chapter 3.2) showed that plasma PTHrP concentrations rise throughout pregnancy reaching their highest concentration around term. Thereafter levels continue to rise into the puerperium. PTHrP levels during pregnancy correlated closely with serum alkaline phosphatase (ALP) concentrations suggesting a placental source to this PTHrP. Post-partum, however, it is likely that the continued rise in PTHrP was due to the onset of lactation and production of PTHrP by the breast. As expected, concentrations of NcAMP did not change significantly during the period of study.

The importance of the vitamin D/PTH axis has been known for a number of years. The availability of precise and accurate biochemical assays for 1,25-dihydroxyvitamin D3 (1,25(OH)$_2$D$_3$) (the principal active metabolite of vitamin D3 in man) and intact PTH(1-84) has allowed detailed investigation of their inter-relationship in vivo. 1,25(OH)$_2$D$_3$ and other active metabolites of vitamin D3 such as 1α-hydroxycholecalciferol (alfacalcidol) have recently been used therapeutically in the treatment of hypocalcaemia (including that associated with

hypoparathyroidism), osteoporosis and renal osteodystrophy. Recent evidence

has suggested an advantage in using these drugs by intravenous routes such that

significant PTH suppression may be achieved though not at the expense of the

development of hypercalcaemia. The acute (first 24hours) effect of intravenous

administration of alfacalcidol upon serum concentrations of $1,25(OH)_2D_3$, PTH

and osteocalcin was studied prospectively in 6 normal males, 6 patients with

primary hyperparathyroidism (PHPT) and 6 women with osteoporosis (chapter

3.3). Significant increases in serum concentrations of $1,25(OH)_2D_3$ were seen in

each group 2-3 hours after administration of alfacalcidol. The increase in

$1,25(OH)_2D_3$ observed was greatest in those patients with PHPT. In the first 24

hours after administration, suppression of PTH concentrations or elevations in

serum concentration of osteocalcin were not observed. This would suggest that

changes normally seen in the longer term with these parameters are effected via

changes in hormone synthesis rather than secretion.

The introduction of dual-energy X-ray absorptiometry (DXA) in the late 1980's

allowed more accurate and precise measurement of bone density at both the

lumbar spine (L2-4) and neck of femur - both sites where osteoporotic fractures

are relatively common and may be associated with significant morbidity or, in

the case of neck of femur, mortality. DXA has the advantages of faster scan

times than was possible with dual-photon densitometry, and lower exposure to

ionising radiation. This technique has a precision of generally <1% at L2-4 and

an accuracy of <2%. This, therefore, allows cross-sectional studies to evaluate

bone density in a number of situations. Examples of this are described in chapter

4.1 and 4.2.

The association of diabetes mellitus with osteoporosis has been suggested for

many years, however the evidence for this remains inconclusive. In chapter 4.1,

L2-4 and neck of femur bone densities were measured in 20 premenopausal

females with type I diabetes mellitus and 27 age-matched controls. Results

showed that L2-4 bone density was significantly higher in the diabetic patients,

though no change was noted in the neck of femur densities. Serum ALP and

urinary excretion of hydroxyproline were also measured in these subjects as

biochemical markers of bone formation and resorption respectively and were

both significantly elevated in the diabetic patients indicating the presence of a

high bone turnover status.

Following the unexpected presentation of two young men suffering from severe

haemophilia A with, in one case, vertebral compression fractures and, in the

other, a neck of femur fracture, a similar study was carried out in a group of 19

males with severe haemophilia (chapter 4.2). This showed that patients with

haemophilia had significantly lower bone densities than age/sex matched

controls. In addition though serum testosterone levels were significantly higher

in the haemophiliac patients, sex hormone binding globulin was also

significantly elevated meaning that these patients had a reduced free androgen

index and relative hypogonadism.

Although the value of routine pre-operative imaging remains controversial in

primary hyperparathyroidism (PHPT), considerable advances have been made in

the technologies available. In chapter 5.1, a comparison was made between the

two most widely used methods of parathyroid imaging in the UK - 10MHz

ultrasound (US) and 201-thallium/99m-technetium (Tl/Tc) subtraction scanning.

These two techniques were compared prospectively in 25 patients with PHPT.

Results showed poor sensitivities with both techniques (38-42%), though

specificities were significantly better (89-97%). The low sensitivities of these

techniques means that their routine preoperative use cannot be justified.

An attempt was made to improve the sensitivity of Tl/Tc scanning in chapter 5.2 by augmenting PTH secretion by infusing the calcium chelating agent, trisodium edetate. Six patients with PHPT and previous normal Tl/Tc scans were studied. In three instances the repeat Tl/Tc scan carried out after infusion of trisodium edetate was positive. Two of these patients went on to surgery and in each instance a parathyroid adenoma was found at the site corresponding to the scan abnormality.

With the introduction of the bisphosphonates into clinical practice considerable improvements have been seen in the management of many different conditions. The bisphosphonates are potent inhibitors of osteoclastic bone resorption and have been shown to be effective in the treatment of various types of hypercalcaemia - including that associated with cancer.

Pamidronate is the most potent of these drugs currently licensed for use in the UK. The optimum treatment schedule in cancer-associated hypercalcaemia is, however, not yet defined. Chapter 6.1 describes a comparison between a single intravenous infusion of high (90mg) and low (30mg) dose pamidronate. This study showed that, on average, there was no difference between these two regimens, though there was a suggestion that in those patients where there was evidence of tumour production of a PTH-like factor, the response to pamidronate was less good. This was explored further in chapter 6.2 where a retrospective analysis of results from 35 patients with cancer-associated hypercalcaemia treated with three different doses of pamidronate (30mg, 45mg and 90mg) were reviewed. Results showed that where NcAMP was elevated or TmPO4 reduced prior to pamidronate therapy, the fall in serum calcium was less pronounced, with normocalcaemia unlikely to be achieved.

Studies investigating therapies for cancer-associated hypercalcaemia usually use serum albumin adjusted calcium for monitoring purposes. Since other factors might potentially alter the usual close relationship between ionised and albumin adjusted calcium, both these parameters were measured prospectively in 8 patients with cancer-associated hypercalcaemia treated with pamidronate (chapter 6.3). While there was some variation in the relationship between these two parameters, this was slight and unlikely to be of major clinical significance.

On the basis of the evidence presented in chapters 6.1 and 6.2, pamidronate might be expected to be particularly effective where PTH-like factors are not involved in the pathogenesis of hypercalcaemia. Chapter 6.4 describes the effect of intravenous pamidronate on 5 patients with immobilisation-related hypercalcaemia. As expected, since PTH-like factors are not involved in the pathogenesis of this condition, these patients respond particularly well even to very low doses of pamidronate.

Pamidronate is also effective in other conditions where bone resorption is frequently increased such as Paget's disease of bone and corticosteroid-associated osteoporosis. Definitive dose regimens with pamidronate in either of these settings, however, have yet to be established. A prospective study of 45 patients with Paget's disease treated with either weekly or 3 monthly infusions of pamidronate is described in chapter 7.1. This showed that 3 monthly infusions, at equivalent total dose, are likely to be as effective as weekly treatment. Furthermore, it was apparent that patients with more severe disease required a higher total dose of pamidronate suggesting the existence of a dose/response effect.

Chapter 7.2 describes the effect of intermittent (3 monthly) infusions of pamidronate on corticosteroid-associated osteoporosis. This study showed

significant increases in spinal L2-4 density (measured by DXA) and significant reduction in bone turnover as noted by a fall in serum ALP and urinary HP/Cr.

Altogether these studies illustrate how advances in the investigation and management of metabolic bone disease and disturbances of mineral metabolism have been effected in recent years.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 **Disorders of Bone and Calcium Metabolism**

1.1.1 Introduction

Bone is a complex living tissue whose structural strengths rely upon the presence of a mineralised extracellular matrix. The presence of normal bone formation and resorption are crucial to normal bone physiology and disruption to these processes constitute the general descriptive heading of "metabolic bone disease".

Hypercalcaemia or hypocalcaemia may occur when alterations in calcium flux occur at the gastrointestinal tract, kidney or bone. Calcium continuously enters the extracellular fluid from gut and bone, however serum calcium concentrations are maintained within remarkably tight limits due largely to the effects of parathyroid hormone (PTH) and 1,25 dihydroxycholecalciferol $(1,25(OH)_2D_3)(1,2)$.

1.1.2 Calcium homoeostasis - role of GI tract

The net daily absorption of calcium from the gastrointestinal tract to the extracellular fluid is approximately 300mg (7.5mmoles). In order to be absorbable calcium must be both soluble and in ionised form (3). A variety of factors are known to affect the availability of calcium in the intestine. Phosphate, phytate and oxalate, for example will decrease calcium absorption (4) whereas carbohydrates such as lactose will lead to an increase (5). The availability of dietary calcium in itself is of importance as the efficiency of absorption is increased when dietary calcium is low (6). The main hormonal influence on dietary calcium absorption is the presence of vitamin D. The earliest evidence for this came from studies on rickets in both animals and man in the 1920s (7). In the 1950s it was demonstrated that calcium was absorbed by an active transport

mechanism across the gastrointestinal mucosal cells (8) and that this was under

the control of vitamin D (9). It has more recently become apparent that vitamin

D does not in itself have a direct effect on calcium absorption, but it must first be

metabolised to $1,25(OH)_2D_3$ (10) which is the metabolite with the greatest

efficacy in stimulating calcium absorption. In conditions where $1,25(OH)_2D_3$

levels are low, for example due to chronic renal failure (11) or dietary

deficiency, calcium malabsorption and hypocalcaemia may occur (12). Other

calciotrophic hormones such as PTH and calcitonin do not appear to have any

direct effects on the control of calcium absorption (13), though PTH may have an

indirect effect by increasing $1,25(OH)_2D_3$ concentrations by stimulating the 1α-

hydroxylase enzyme (14).

Drugs are also of considerable importance in calcium absorption. It has been

demonstrated that calcium absorption is directly inhibited by corticosteroids (in

spite of normal $1,25(OH)_2D_3$ levels) (15), neomycin (16), and bisphosphonates

(17).

Subject age is also important as in man there is a progressive decrease in calcium

absorption with advancing age as shown by balance studies and radiocalcium

absorption (18). Part of this malabsorption, however, is probably due to vitamin

D deficiency from poor dietary intake or reduced endogenous synthesis.

1.1.3 Calcium Homoeostasis - Role of the Kidney

Calcium excretion is regulated by a combination of glomerular filtration and

tubular reabsorption, the calcium in the urine being the fraction of the filtered

load which is not reabsorbed. Work by McLean and Hastings in the 1930s

(19,20) showed that approximately 50% of calcium in serum is in ionised form, a

further 10% is ultrafilterable but ligand bound with the remaining 40% being

protein bound - mainly to albumin. Only 60%, therefore, of total serum calcium can be filtered and excreted by the kidney. In an average adult 10000mg calcium cross the glomerulus each day and 98% of this is reabsorbed in the renal tubules (21).

Glomerular filtration would appear mainly to depend on renal blood flow. When this is reduced as happens, for example, in hypercalcaemic states secondary to dehydration (22), calcium clearance is reduced.

The majority of calcium filtered by the glomerulus is reabsorbed by the tubules. Approximately 65% is reabsorbed in the proximal tubules and is not under hormonal regulation, 20-25% is reabsorbed in the ascending limb of the loop of Henle and 10% in the distal tubules (23). Calcium is reabsorbed by an active transport mechanism in the proximal tubule closely linked with sodium (24). This association is of critical importance with respect to net serum calcium levels, as when dehydration occurs sodium is conserved by an increase in proximal tubular reabsorption. Since calcium reabsorption accompanies sodium reabsorption dehydration may potentiate hypercalcaemia. The mechanism of calcium reabsorption at the loop of Henle is unclear, though this is the site where thiazide diuretics act to promote calcium reabsorption (25). Calcium reabsorption is more closely controlled at the distal tubules where it is under the control of PTH (26) and possibly also vitamin D (27).

The kidney is also the principal site of synthesis of 1,25(OH)$_2$D$_3$ (10) - the major active metabolite of vitamin D. Abnormalities of 1,25(OH)$_2$D$_3$ metabolism are commonly found in association with chronic renal failure (28). In this situation circulating levels of 1,25(OH)$_2$D$_3$ are generally low and secondary hyperparathyroidism is common (29). Oral 1,25(OH)$_2$D$_3$ therapy has been successfully used to suppress this excess PTH secretion, but the doses required

are generally associated with the development of hypercalcaemia (30). It has been noted, however, that more adequate PTH suppression can be achieved without this concomitant rise in serum calcium when 1,25(OH)$_2$D$_3$ or 1α-hydroxycholecalciferol (alfacalcidol) is given intravenously rather than orally (31).

Calcium balance studies have shown that the kidney has considerable reserve in calcium excretion, as in order to increase serum calcium by 0.1mmol/l, 5mmol of calcium would have to be released from bone. Measurement of calcium balance in normal individuals has shown that 1-2mmol of calcium/day may be released from bone (32,33) which only rises to levels of 5-10mmol/day in conditions such as myeloma and metastatic breast cancer (34) - conditions commonly associated with hypercalcaemia. Thus, in the presence of normal renal function, increases in bone resorption of up to 10 fold would not be expected to cause hypercalcaemia. This is illustrated by the fact that hypercalcaemia is extremely rare in benign disorders associated with increased bone resorption (35).

Many different hormones are now known to influence the urinary excretion of calcium either directly or indirectly. Patients with primary hyperparathyroidism have been shown to have reduced clearance of calcium relative to that in normal subjects with similar serum calcium levels - due to increased tubular reabsorption of calcium (36). Corticosteroids are associated with increased urinary calcium losses as demonstrated both in Cushing's syndrome (37) and with administration of exogenous steroids (38) - probably due to inhibition of tubular calcium reabsorption (39). Oestrogen administration has also been shown to decrease urinary calcium losses though this is mainly due to inhibition of bone resorption leading to a subsequent decrease in the filtered load of calcium (40).

Peacock and Nordin (2,36) have suggested that in steady state there is a curvilinear relationship between urinary calcium excretion (CaE) and serum calcium (figure 1.1) such that as gut calcium absorption and bone resorption increase changes in renal calcium excretion will determine the eventual serum calcium level reached. For any given level of serum calcium, if CaE lies to the left of the line defining the normal range this implies a decrease in renal tubular calcium reabsorption. This is seen, for example, in hypoparathyroidism. Conversely if CaE is to the right of the line defining the normal range this implies is an increase in calcium reabsorption. This is seen, for example, in hyperparathyroidism. Calcium kinetic studies using radioisotope tracers have measured gut absorption and bone calcium resorption and compared these to urinary calcium excretion (41). Assuming net gut calcium absorption in the fasting state is negligible, then under these circumstances calcium excretion - expressed as a ratio of urinary calcium to urinary creatinine - probably equals the net skeletal loss of calcium i.e. bone resorption.

1.1.4 Calcium Homoeostasis - Role of Bone

The prime role of bone in calcium homoeostasis is to act as a reservoir of calcium which may be used when gut absorbed calcium is insufficient to maintain serum calcium.

Parathyroid hormone has an important role in mobilising calcium from bone by stimulating osteoclastic bone resorption. The main target cell for PTH in bone is the osteoblast and increased resorption probably occurs by local paracrine signalling between the osteoblast and osteoclast (42). The effects of PTH on bone resorption may also depend, at least in part, on the presence of 1,25(OH)$_2$D$_3$ (43). This bone remodelling system, though, is unlikely to be of major importance in calcium homoeostasis as long as bone formation and
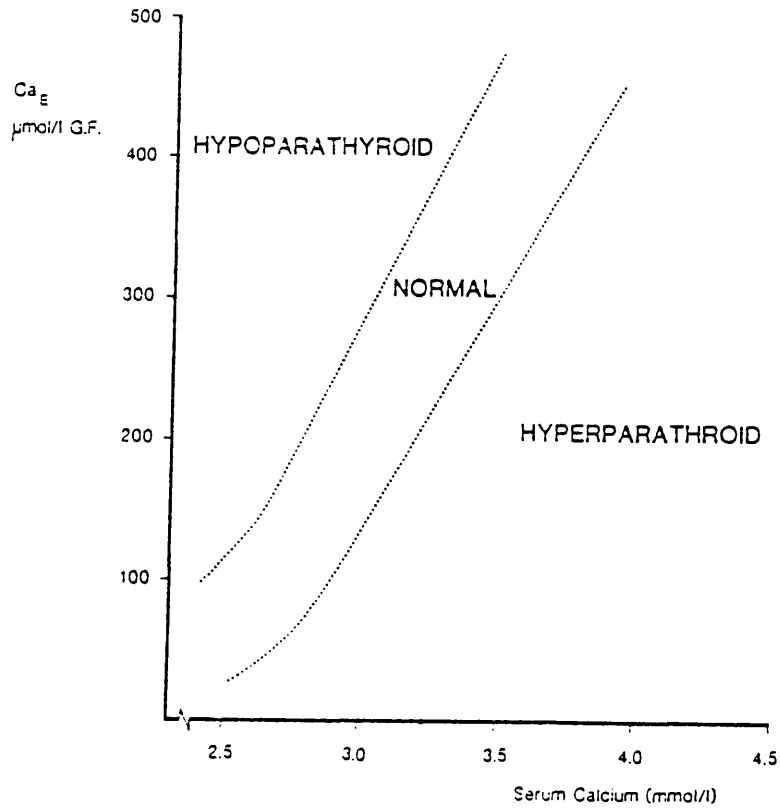
Figure 1.1

Relationship between urinary calcium excretion (μmol/litre glomerular filtrate) and serum calcium. Dotted lines indicated two standard deviations from the mean values obtained by Peacock et al.(36) in normal subjects during calcium infusions.

resorption remain coupled. When a pathological condition uncouples these processes, such as if bone resorption is increased in association with cancer (34) or immobilisation (44), hypercalcaemia may then ensue.

1.2 **Cancer-associated hypercalcaemia**

1.2.1 Differential Diagnosis

The most common cause of hypercalcaemia, certainly in hospital based studies, is that associated with the presence of cancer with an estimated incidence of approximately 150 new cases per million persons per year in the UK(45,46,47). The main differential diagnosis is from primary hyperparathyroidism with both this and cancer-associated hypercalcaemia making up approximately 90% of cases of hypercalcaemia (48). With the advent of highly sensitive and specific 2-site immunoradiometric assays for PTH, distinction between these two causes of hypercalcaemia has become easier with PTH low or undetectable in cancer-associated hypercalcaemia and elevated in primary hyperparathyroidism (49). Where PTH is low or undetectable other differential diagnoses to consider include: hyperthyroidism (50), immobilisation (51), sarcoidosis (52), vitamin D intoxication (53), milk-alkali syndrome (54), use of thiazide diuretics (55) and benign familial hypercalcaemia (56). Usually in these situations other clinical features alert one to the diagnosis with the possible exception of benign familial hypercalcaemia, where a high index of suspicion is required.

1.2.2 Pathogenetic Mechanisms

The mechanisms responsible for cancer-associated hypercalcaemia are heterogeneous and depend, to a significant extent, upon the type of malignancy present. In general terms two mechanisms are cited in the pathogenesis of this syndrome. Firstly, direct local invasion of bone by tumour cells leads to release of calcium into the blood at a rate exceeding the maximal rate of renal calcium excretion (35,57,58). Secondly, the tumour itself may produce a factor (or factors) which may act directly upon the bone to increase osteoclastic resorption

and/or at the kidney to increase renal tubular calcium reabsorption (59,60) - this mechanism has become known as humoral hypercalcaemia. It has become clear, however, that both mechanisms are of importance, though to a greater or lesser extent, depending both upon the tumour type (61) and the clinical progression of the hypercalcaemia (62). Humoral hypercalcaemia is more common with solid tumours such as bronchial carcinoma (58) and hypercalcaemia due to direct invasion of bone more likely with haematological malignancies such as myeloma (58) and carcinoma of breast (58,63).

As described in section 1.1.3 the kidney has considerable reserve to increase urinary calcium excretion until bone resorption is greatly elevated. Histological evidence has suggested that there is "uncoupling" of bone resorption and formation in cancer-associated hypercalcaemia (62) leading to a net loss of skeletal calcium into the extracellular fluid. Furthermore renal impairment is more likely, as these patients are more prone to vomiting and decreased fluid intake both associated with the tumour itself, or if treatment with chemotherapy and/or radiotherapy is used (58).

It appears that in some cases of cancer-associated hypercalcaemia, there is a combination of dehydration and increased bone resorption, such that the release of calcium from bone exceeds the renal capacity for calcium excretion and serum calcium rises. PTH secretion is therefore suppressed, renal tubular calcium reabsorption falls (36) and release of skeletal calcium from bone surfaces is reduced (64). In addition $1,25(OH)_2D_3$ synthesis is reduced and intestinal calcium absorption is reduced (65). These homoeostatic mechanisms, however, may only partially compensate and hypercalcaemia ensues. Severe hypercalcaemia in itself is associated with a reduction in renal blood flow and GFR (22). Furthermore, tubular abnormalities occur leading to further loss of sodium and water (66) exacerbating the dehydration and a metabolic alkalosis

may occur (67). A vicious circle then exists with worsening hypercalcaemia due to sodium linked calcium reabsorption at the proximal tubule (24) and further impairment in GFR.

1.2.3 Potential Humoral Mediators

In 1941 Albright suggested the possibility that cancer-associated hypercalcaemia may be attributed to ectopic PTH production when describing a patient with renal cancer, a raised serum calcium and low serum phosphate (68). Further evidence for this appeared in the 1960's when the first radioimmunoassays for PTH became available. Studies showed elevated levels of PTH in a group of patients with bronchial cancer (69) and in other non-parathyroid tumours (70). The presence of PTH was also demonstrated in tumour cells by immunohistochemical techniques (71). Lafferty, in 1966, (72) used the term pseudohyperparathyroidism to describe a series of 50 patients with these biochemical features. In this work he commented on the frequency of squamous and genitourinary cancers and the relative paucity of breast and haematological cancers in these patients. However, as the radioimmunoassays for PTH began to improve some doubt began to emerge about the involvement of PTH in cancer-associated hypercalcaemia. Powell et al.(73), in 1973, demonstrated that tumour extracts from several patients with humoral hypercalcaemia were capable of resorbing bone in vitro although serum PTH from these patients was consistently undetectable. It is now generally accepted that hypercalcaemia caused by ectopic PTH secretion by a tumour is exceedingly rare, and has only been convincingly described on two occasions (74,75).

The biochemical similarity of this syndrome to primary hyperparathyroidism, however, extended beyond an elevated plasma calcium and lowered plasma phosphate. Nephrogenous cyclic adenosine monophosphate (NcAMP) is that

fraction of cyclic adenosine monophosphate generated and excreted by renal tubular cells and is produced almost exclusively in response to circulating PTH in normal man (76). NcAMP, therefore, provides an index of biologically active PTH or PTH-like peptides. Since cancer-associated hypercalcaemia is generally associated with low or undetectable PTH and NcAMP is often elevated in this syndrome, (77,78,79) this was further evidence for the existence of a factor which was not PTH but which had PTH-like activity. Other features normally found in association with excess PTH secretion were also described in association with hypercalcaemia of malignancy such as decreased renal tubular threshold for phosphate reabsorption (TmPO4) and elevated threshold for renal tubular calcium reabsorption (TmCa) (80,81), though this latter index was shown to be highly dependent on renal sodium handling (82).

The presence of these features of PTH excess in the absence of detectable serum PTH led to the search for another factor which was not PTH but had PTH-like properties, was produced by a number of malignant tumours, would resorb bone and would cause hypercalcaemia. This search ended in 1987 with discovery of PTH-related protein (PTHrP) in a lung cancer (BEN) cell line. This protein has been isolated and its complementary DNA (cDNA) cloned and its gene mapped and isolated (83,84). The PTHrP gene is a complex transcriptional unit that by alternative splicing gives rise to messenger RNAs (mRNAs) encoding three related PTHrPs which differ only in their C-terminal regions. The initial peptide to be cloned was a 141 amino acid peptide in which 8 of the first 13 (N-terminal) amino acids were identical to PTH - this conserved sequence being common to all forms of PTHrP. After this N-terminus sequence, the homology with PTH is less striking with only 5 of the following 50 amino acids identical. Since this peptide was cloned, others which have been identified include 1-139 and 1-173 amino acid peptides (85,86).

PTHrP has been demonstrated to be of both similar (87) and increased potency

(88) in terms of cAMP production compared to PTH in various in vitro systems.

It is likely that differences in potency reflect both species differences and

differences in the lengths of the peptides tested (89). PTHrP has now been found

to be present both within the tumour cells (90) by immunocytochemistry and in

the serum of patients with humoral hypercalcaemia (91,92). In addition to being

produced by malignant tumours PTHrP has been found in normal keratinocytes

(93), lactating mammary tissue (94), placenta (95), parathyroid glands (96) and

central nervous system (97) - all of which would suggest that this protein has a

physiological role.

Whether or not the presence of PTHrP by itself explains all the manifestations of

humoral hypercalcaemia has been questioned. Certainly, compared with PTH,

osteoclastic bone resorption is more marked, bone formation rates are lower,

$1,25(OH)_2D_3$ levels are lower and the calcium lowering effect of

bisphosphonates in such patients greater and more sustained than in primary

hyperparathyroidism. Furthermore patients with humoral hypercalcaemia tend to

be alkalotic rather than acidotic as is seen in primary hyperparathyroidism (67).

It is possible, therefore, that in malignancy the effects of PTHrP are modified by

the presence of other factors such as tumour necrosis factor (TNF), interleukin-1

(IL-1) or transforming growth factor (TGF) - all of which are more potent bone

resorbing factors than PTH and are often produced by tumours (98,99,100).

There is also some evidence that tumours may produce another factor which

interferes with renal 1-alpha hydroxylase activity and explains the low

$1,25(OH)_2D_3$ levels often seen in patients with cancer-associated hypercalcaemia

(101). The differential effect on renal bicarbonate handling appears to be effected

via an independent action of a component of PTHrP beyond amino acids 1-34

(102).

Prostaglandins were first suggested as playing a role in the aetiology of this syndrome after the demonstration that they possessed potent bone resorbing activity in vitro (103). Raised levels of prostaglandin E2 and prostaglandin F were later found in tumour tissue from a patient with hypercalcaemia and renal adenocarcinoma (104) which seemed to respond to indomethacin therapy. Other workers, however have failed to confirm that prostaglandin synthetase inhibitors such as indomethacin have a significant calcium lowering effect in cancer-associated hypercalcaemia (105).

Cytokines such as interleukin-1 (IL-1) and tumour necrosis factor (TNF) have both been shown to increase bone resorption in vitro and may cause hypercalcaemia in animal models, however their role in human cancer-associated hypercalcaemia remains uncertain (106,107).

Abnormalities of 1,25(OH)$_2$D$_3$ production have also been described in association with hypercalcaemia of malignancy. Generally serum levels of 1,25(OH)$_2$D$_3$ are suppressed (65) with the exception of hypercalcaemia associated with lymphoma where levels may be elevated (108).

1.2.4 Management

Moderate hypercalcaemia (adjusted calcium 3.00-3.50mmol/l) is associated with a number of symptoms including polyuria, polydipsia, nausea, vomiting, lethargy and fatigue. CNS symptoms in the form of drowsiness, confusion and coma predominate in severe hypercalcaemia (calcium >3.50mmol/l), and may lead ultimately to death. In a recent review Ralston et al.(109) showed that efficient treatment of cancer-associated hypercalcaemia, with a reduction in serum calcium to below 2.80mmol/l, was associated with a marked improvement in symptoms and, in addition, more of these patients were able to be discharged

from hospital. The ideal treatment is that targeted specifically against the primary tumour i.e surgical resection or chemotherapy. Since this is not always feasible palliative, symptomatic treatment is often necessary.

As discussed in 1.1.3, patients with hypercalcaemia are usually dehydrated (22,66) and therefore rehydration is the first line in therapy. In most circumstances isotonic (0.9%) sodium chloride should be used since any measure that induces natriuresis will also induce calciuresis since tubular sodium and calcium reabsorption are linked (24). Detailed studies on the effects of rehydration have been carried out by Hosking et al.(110) who estimated an average sodium deficit of 500mmol in patients with cancer-associated hypercalcaemia. When this deficit was replaced with 4-8 litres of saline over 48 hours a mean decrement in serum calcium of 0.4mmol/l was seen.

The first effective pharmacological treatment for hypercalcaemia was inorganic phosphate which was first described in 1930 (111), and results of the effects of phosphate in a series of patients with cancer-associated hypercalcaemia was published in 1966 (112). Although this treatment was reasonably effective side-effects such as acute renal failure and extraskeletal calcification proved unacceptable (113,114), and phosphate, therefore, should be reserved solely for life-threatening episodes where standard therapies have failed.

Corticosteroids have been used for many years with variable success (115,116), however they cannot be recommended for routine use (117). It does appear, though, that some tumours are especially sensitive to corticosteroids such as haematological malignancies and tumours of the lymphoreticular system.

Mithramycin is a cytotoxic agent which has been shown to have a significant anti-hypercalcaemic effect (118). The main limitation in the use of mithramycin

in this setting is the high incidence of serious side effects which include a coagulopathy, liver function upset - occasionally leading to massive hepatic necrosis and upset in renal function (119,120).

Calcitonin has been shown to be an effective agent in the treatment of cancer-associated hypercalcaemia (121,122). The mechanism of action of calcitonin is thought to be a combination of inhibition of osteoclastic bone resorption and the presence of a powerful natriuretic and calciuretic action (121,123). Although calcitonin may be an effective agent, a significant number of patients are either resistant or gain only transient benefit (121). The short duration of action of calcitonin has been ascribed to "down regulation" of the calcitonin receptors (124). By combining calcitonin with corticosteroids this "escape" phenomenon appears to be, at least partially, prevented (124,125).

A more recently employed agent in the management of cancer-associated hypercalcaemia is gallium nitrate. Like calcitonin, it acts primarily by inhibiting osteoclastic bone resorption. In a direct comparison, Warrell et al (126) have shown gallium to be more effective than calcitonin in the treatment of this condition. Problems have been described, however, with nephrotoxicity, the development of anaemia and, rarely, optic neuritis. Its present role in the management of cancer-associated hypercalcaemia remains uncertain.

The bisphosphonates are a family of compounds that were introduced into the treatment of metabolic bone disease in the 1970s. These drugs are synthetic analogues of pyrophosphate which are resistant to pyrophosphatases (127). They inhibit growth and dissolution of hydroxyapatite crystals in vitro and inhibit osteoclastic bone resorption in vivo (128,129). These properties led to this group of drugs being used in conditions where bone resorption is elevated such as in hypercalcaemia associated with malignancy (130), immobilisation (131) and

hyperthyroidism (132) and in Paget's disease of bone (133). In view of their efficacy and safety profile, these drugs have now probably become treatment of choice in the management of cancer-associated hypercalcaemia (134). In a recent study comparing the three intravenous bisphosphonates currently licensed for this indication in the United Kingdom, pamidronate (aminohydroxypropylidene bisphosphonate, APD) at a dose of 30mg was superior in terms of calcium lowering effect to 600mg clodronate (dichloromethylene bisphosphonate, Cl2MDP) and standard doses of etidronate (ethanehydroxy bisphosphonate, EHDP) (135). It is generally accepted, however, that clodronate can also be very effective though multiple infusions (over several days up to a total of 3000mg) are often required (136). As pamidronate is considerably more potent in terms of osteoclast inhibition than these other two drugs (see 1.5.5), lower doses (30-90mg) can be used effectively. This confers the added advantage of a single intravenous infusion generally being effective (137). Clodronate and etidronate are both also available as oral preparations which are often used in an attempt to maintain normocalcaemia. Results with these are variable and the benefit of prolonged oral treatment, often necessitating the ingestion of a large number of tablets per day, remains in doubt (138,139) - particularly when if relapse of hypercalcaemia does occur a further single infusion of pamidronate will usually have a calcium lowering effect similar to the initial treatment (140).

The optimum dose regimen for pamidronate remains uncertain. It is currently recommended that the dose of pamidronate be titrated against the prevailing serum calcium level, with larger doses of pamidronate given where the pre-treatment serum calcium is highest. This is based on a single study by Thiebaud et al (141). Further evidence exists, however, that when doses of 15mg to 60mg pamidronate are used there is little difference in the calcium lowering effect, irrespective of the pre-treatment calcium level (140,142). A dose-response effect does appear to exist with pamidronate (like the other bisphosphonates), but only

at doses below 0.05mg/kg (143). It is certain, however, that a number of patients fail to respond or only respond incompletely to pamidronate (144,145). The reasons for this are not clear, however it has been suggested that where there is evidence of tumour production of PTHrP, the calcium lowering effect is less good. This was suggested by Gurney et al.(146) who showed, in a small series, that TmPO4 (a marker of PTH or PTH-like activity) was lower in patients with cancer-associated hypercalcaemia who responded less well to pamidronate.

Serum albumin is generally low in patients with cancer and it is important, therefore, that the total serum calcium be corrected for this low albumin. Failure to do so will mean that the incidence of hypercalcaemia will be significantly underestimated (147). It has been suggested, however, that the usual correction algorithms may not hold in critically ill patients (148) or in elderly hospital in-patients (149). Furthermore the binding affinity of calcium for albumin is not constant at albumin concentrations below 30g/l (150). In addition to the above, measurement of ionised calcium depends to an extent on the prevailing serum albumin concentration with measured ionised calcium levels rising where serum albumin is higher (151). The experience at Glasgow Royal Infirmary in patients with cancer, is that a close correlation is present between ionised calcium and albumin corrected calcium (152) (see 2.1.1). However, it is not yet clear whether this close correlation is maintained when calcium is lowered with a bisphosphonate or whether the degree of metabolic upset present alters the usual close relationship between ionised and albumin adjusted calcium.

Although side-effects are thought to be uncommon with bisphosphonates, these drugs are all associated with impairment of bone mineralisation to a greater or lesser extent. This is seen especially with etidronate where even low dose oral therapy for Paget's disease may be associated with osteomalacia (153). Other

side effects include gastrointestinal intolerance with oral therapy, fever
(following intravenous therapy) and rarely lymphopenia (154).

1.2a **Vitamin D Metabolism and Hypercalcaemia**

1.2a.1 Introduction

The synthesis of vitamin D and, in particular, of its active metabolite, 1,25-dihydroxyvitmin D3 (1,25(OH)$_2$D$_3$) is of importance in both normal calcium homoeostasis and in the pathogenesis of cancer-associated hypercalcaemia (11,43). The principal site of 1,25(OH)$_2$D$_3$ synthesis is the kidney (10,11). However, there is evidence that in certain situations extra-renal 1,25(OH)$_2$D$_3$ production is also of considerable importance (154a).

1.2a.2 Regulation of 1,25-dihydroxyvitamin D3

The conventional view is that 1,25(OH)$_2$D$_3$ is synthesised in accordance with the physiological demand of the body for calcium and phosphate. The main site of this regulation is at the level of the 1α-hydroxylase enzyme which controls the production of 1,25(OH)$_2$D$_3$ from 25-hydroxy vitamin D3 (25(OH)D$_3$). Activity of this enzyme is controlled by changes in serum calcium, phosphate, parathyroid hormone (PTH), calcitonin and other vitamin D metabolites (154b). The primary stimuli of the renal 1α-hydroxylase are PTH, low serum calcium and low serum phosphate. The resultant increase in serum 1,25(OH)$_2$D$_3$ concentrations observed following stimulation of 1α-hydroxylase then act to mobilise calcium from bone (by stimulation of osteoclastic resorption) and to increase intestinal calcium absorption. It also appears that 1,25(OH)$_2$D$_3$ can regulate its own biosynthesis by acting as a feedback regulator on either the parathyroid gland or the kidney or both. Furthermore, 1,25(OH)$_2$D$_3$ may stimulate the formation of renal 24-hydroxylase which metabolises excess 25(OH)D$_3$ to the relatively inactive metabolite, 24,25-dihydroxyvitamin D$_3$ (154c).

1.2a.3 Extra-renal Synthesis of 1,25-dihydroxyvitamin D3

Although the principal site of 1,25(OH)$_2$D$_3$ production is the kidney (10,11), it is now recognised that extra-renal 1,25(OH)$_2$D$_3$ production can be of importance in some situations. This is seen, for example, in pregnancy where 1,25(OH)$_2$D$_3$ is produced by the placenta (408).

Barbour et al.(154a) reported the finding of a high serum 1,25(OH)$_2$D$_3$ concentration in an anephric patient with sarcoidosis. Since then it has been shown that the elevated serum concentrations of 1,25(OH)$_2$D$_3$ seen in patients with sarcoidosis result from increased production of 1,25(OH)$_2$D$_3$ by the macrophage (a prominent constituent of the sarcoid granuloma) (154d). Although similar to renal 1α-hydroxylase, macrophage 1α-hydroxylase is immune to the stimulatory effects of PTH, is very sensitive to stimulation by interferon-gamma - a lymphokine produced by activated lymphocytes, is very sensitive to inhibition by glucocorticoid but is refractory to inhibition by 1,25(OH)$_2$D$_3$ (154e). The renal enzyme, on the contrary, is relatively insensitive to inhibition by glucocorticoid but is down-regulated by 1,25(OH)$_2$D$_3$.

From the above it might be expected that in patients with cancer-associated hypercalcaemia, serum concentrations of 1,25(OH)$_2$D$_3$ would be low since serum calcium is elevated and PTH suppressed. While this may generally be the case, a significant proportion of patients with cancer-associated hypercalcaemia have either normal or elevated serum concentrations of 1,25(OH)$_2$D$_3$. This appears to be the case both for lymphoreticular malignancies, (where 1,25(OH)$_2$D$_3$ appears to be synthesised within the tumour) (108) and for squamous cancer (65). The significance of this latter finding remains unclear as it would appear that those patients who have cancer-associated hypercalcaemia and normal or elevated serum concentrations of 1,25(OH)$_2$D$_3$, do not exhibit the expected increase in

intestinal calcium absorption (154f). In this situation (where PTH is suppressed) it has been suggested that the increased serum concentrations of $1,25(OH)_2D_3$ observed may be due to the stimulation of 1α-hydroxylase by a parathyroid hormone-like peptide - presumably parathyroid hormone-related protein (PTHrP) (85). However, it remains a possibility that the elevated concentrations of $1,25(OH)_2D_3$ found in these patients may derive from tissues other than kidney (65).

1.3 **Primary Hyperparathyroidism**

1.3.1 Introduction

Primary hyperparathyroidism (PHPT) is a common condition associated with excess and inappropriate production of parathyroid hormone (PTH) and characterised by the presence of hypercalcaemia. One of the earliest and most famous descriptions of PHPT was that of Captain Charles Martell who developed progressive crippling disease (osteitis fibrosa cystica) associated with recurrent renal stones (155). In modern clinical practice approximately 250 new cases of primary hyperparathyroidism per million population are seen per year (46) and the prevalence of this condition, based on a study measuring serum calcium in 26,000 consecutive samples, is about 1 in 1000 (156). As stated in 1.2.1 the main differential diagnosis is from cancer-associated hypercalcaemia where, certainly with the new assays for measuring the intact peptide, PTH is generally, though not always, undetectable (49).

PHPT may be due to the presence of a solitary parathyroid adenoma, hyperplasia of the parathyroid glands or, very rarely, a parathyroid carcinoma. In 85-90% of cases a parathyroid adenoma is the cause of this condition.

1.3.2 Clinical Features

Most clinical features such as thirst, polyuria, dyspepsia, lethargy and nausea are likely to be directly due to the effect of hypercalcaemia, as are complications such as renal stones and pancreatitis (157). The bone changes such as, in the most extreme cases, osteitis fibrosa cystica and increased bone resorption and osteoporosis (158) are more likely to be due to the direct action of PTH on the osteoblast (42). The hypercalcaemia of PHPT is remarkably constant with only

very small changes taking place with time. This is in contrast with cancer-associated hypercalcaemia where serum calcium rises progressively (35).

There has been considerable interest in recent years in the control of PTH secretion. A considerable weight of evidence now exists to show that the abnormal (adenomatous or hyperplastic) parathyroid gland(s) is not completely autonomous. Gardin et al (159) showed by using infusions of ethylenediamine tetra-acetate (EDTA) that the set point of parathyroid cells for calcium is elevated in PHPT - which explains why the elevation of serum calcium which occurs in this condition does not lead to PTH suppression as would be expected. It is possible, therefore, to augment PTH secretion still further in PHPT by lowering the serum calcium, even though absolute hypercalcaemia still persists (159,160). Grant et al.(161), in a study using intravenous citrate to create relative hypocalcaemia, however showed that the rise in PTH is dependent not only on the absolute fall in serum calcium but also upon the rate of change of calcium. Similarly, causing a further elevation in serum calcium by using an oral calcium load test may lead to at least partial suppression of PTH secretion (162,163).

1.3.3 Management

Parathyroidectomy is generally highly successful, usually being curative, especially when carried out by an experienced surgeon (164). The indications for surgery, particularly in asymptomatic subjects, remain controversial. This area has been reviewed recently by the National Institutes of Health in the United States who have issued a "Consensus Statement" listing suggested indications for undertaking parathyroid surgery (165) which include:

1.Markedly elevated serum calcium or previous life-threatening hypercalcaemia.
2.Reduced creatinine clearance.

3.The presence of a kidney stone or stones.

4.Markedly elevated 24 hour urinary calcium excretion.

5.Substantially reduced bone mass.

The risks of surgery in some instances, therefore, may outweigh the potential benefits. Furthermore the role of parathyroid surgery in the tertiary hyperparathyroidism which may follow successful renal transplantation is also controversial (166). For these reasons there has been continued interest in the non-surgical management of hyperparathyroidism.

Choice for medical therapy for primary hyperparathyroidism is limited (167). Treatments which have been used include oral phosphates (112), though some patients respond poorly to this regime and unacceptable diarrhoea is often a problem. Bisphosphonates have been used in the treatment of PHPT. Oral etidronate is of no real benefit, however intravenous clodronate has been used with some success, though the calcium lowering effect is often incomplete and short lived (168,169). Oestrogens or progestagens are useful in postmenopausal women (170,171) and may maintain serum calcium within the normal range for a number of years. Their mechanism of action is unknown, however they appear to act independently of changes in PTH secretion. Other agents which have been used include H2 blockers (172), the calcium channel blocker, diltiazem (173) and the betablocker, propranolol (174) - none of these agents, however, appear to be of any major, long-term, clinical value.

From the above it can be seen that at the present time, where active intervention is indicated, the treatment of choice is surgery. In an attempt to lessen operation time and improve the chances of finding an adenoma some form of parathyroid imaging is often carried out preoperatively (175) and evidence exists that the use

of pre-operative imaging may both reduce operating time and result in an increased number of successful operations (176).

Many different methods have been used to this end with variable degrees of success. Clinical examination is unsatisfactory as the abnormal gland is usually small and soft in consistency. Overall, less than 1% of parathyroid adenomas are palpable (177). However, several different parathyroid imaging techniques have been developed - these include 201-thallium/99m-technetium (Tl/Tc) subtraction scanning (178,179), high resolution (10MHz) ultrasound (US) (180), computed tomography (CT) (181) and magnetic resonance imaging (MRI) (182). The most widely available of these techniques in the UK are ultrasound (US) and Tl/Tc scanning. Tl/Tc scanning was first described by two groups in 1983 (178,179). This technique involves the use of thallium-201 (201Tl), a potassium analogue which is taken up by metabolically active tissue throughout the body. Thallium-201 appears to substitute for potassium in the ATP-ase dependent sodium/potassium pump (175). In a normal individual neck images obtained after 201Tl show thyroid activity but separate parathyroid activity is not discernible. Even when a large parathyroid adenoma is present it is often difficult to determine whether any extra-thyroidal activity is present. As Technetium-99m is taken up by the thyroid via the iodide trap but does not accumulate in the parathyroids, subtraction of a 99mTc image from a 201Tl image should remove thyroidal activity rendering residual activity in the parathyroid visible. Reported accuracy of localisation of these techniques varies from 65% to over 90% (178,179,183).

Krubsack et al.(184) prospectively compared US, Tl/Tc, CT and MRI scanning. Specificities of all four of these techniques were generally high (87-95%), however sensitivities were lower, with Tl/Tc scanning best at 73% and US poorest at 55%. Direct comparison between US and Tl/Tc scanning shows both

techniques are comparable with specificities very high and sensitivities in the range 74-80% (185,186).

Selective venous catheterisation with measurement of PTH levels throughout the neck has been shown to be useful in localising parathyroid adenomas (187,188), with an accuracy of around 80% (188). This technique, however, requires considerable skill, is time consuming and costly and is associated with a small, but definite, morbidity and therefore should probably be reserved for instances where there has been a previous unsuccessful neck exploration (188).

1.4 **Immobilisation-Related Hypercalcaemia**

1.4.1 Diagnosis and clinical features

Immobilisation-related hypercalcaemia was first described by Albright in 1941 (189). Although it is not often appreciated as a clinical problem, hypercalciuria is commonly associated with immobilisation - leading to a negative calcium balance and osteoporosis (190). Generally immobilisation-related hypercalcaemia is associated with conditions where bone turnover is already high such as in children, adolescents (191) and in patients with Paget's disease of bone (192). This syndrome was initially thought to be exceedingly rare. More recently this view has been challenged as work by Maynard et al.(193) showed hypercalcaemia to affect 11% of tetraplegic patients under the age of 21. In addition, measurement of ionised calcium may also identify milder examples of this condition (194). Immobilisation-related hypercalcaemia is characterised by increased bone resorption, with radiological evidence of osteopenia (195), increased osteoclastic activity on bone biopsy (196) and elevated urinary hydroxyproline excretion (197). In addition when tetracycline uptake into mineralising bone is studied it is apparent that bone formation rates may also be reduced (44). Parathyroid hormone levels have been reported as high, normal or low in this condition (195) and parathyroidectomy is ineffective. Serum levels of $1,25(OH)_2D_3$ are generally low (195).

1.4.2 Management

Optimal therapy for immobilisation-related hypercalcaemia is not well defined, though physiotherapy and re-mobilisation are important components. Dietary calcium restriction is irrational where intestinal calcium absorption is low (due to low $1,25(OH)_2D_3$ levels) and a negative calcium balance exists (198). Calcitonin

therapy may be effective but as with cancer-associated hypercalcaemia problems of "escape" may occur (124,197). Corticosteroids have also been shown to be of some value. However, their concomitant reduction of intestinal calcium absorption (199) and reduction in bone formation (200) make them far from ideal agents. Given that increased bone resorption is the principal abnormality that predisposes these patients to hypercalcaemia, the bisphosphonates are likely to be of value in the management of this condition. Preliminary evidence suggests this indeed is the case. A report by Haag et al (131) has demonstrated successful treatment with etidronate and Yates et al.(201) have similarly shown 2 patients responding successfully to clodronate. More recently a report by McIntyre et al.(202) has described a 16 year old male successfully rendered normocalcaemic with 30mg pamidronate. As is the case with cancer-associated hypercalcaemia it is likely these drugs will become the agents of choice in the treatment of this condition.

1.5 **Paget's Disease of Bone**

1.5.1 Introduction

Paget's disease is a focal disorder characterised by increased and disorganised formation and resorption of bone. This results in a dramatic increase in bone turnover (203). As a result of this increased metabolic activity, changes in bone shape may occur, which in turn has implications for the quality and competence of bone formed.

1.5.2 Epidemiology

The prevalence of Paget's disease is approximately 3-4% around the age of 40 and rises exponentially with age to around 10% or higher after age 90 years (204). There have been no studies reported examining the incidence of the disorder, but its prevalence might suggest a rising incidence with age, with an average of 0.3% per annum in the population over 55 (205). Paget's disease appears to be more prevalent in men with a male to female sex ratio of 1.4 to 1.9:1 (204,206).

Paget's disease would appear to be more common in Europe, North America and Australasia (207). A survey of 31 British towns showed the highest prevalence to be in Lancashire, with prevalences elsewhere in the UK being significantly lower (206). These features have lead to the search for a possible environmental "trigger".

1.5.3 <u>Aetiology</u>

The aetiology of Paget's disease is unknown however a "slow virus" infection has been considered likely. This is on the basis that characteristic inclusion bodies resembling paramyxovirus nucleocapsids are almost invariably present in pagetic osteoclasts (208). The identity of this proposed virus remains uncertain, though positive immunohistochemical staining has been described for both measles virus (209) and respiratory syncytial virus (210) antigens in pagetic osteoclasts. Measles virus RNA sequences have also been reported to be present in these cells by in situ hybridisation (211). More recently canine distemper virus (CDV) has been suggested as the putative agent on the basis of the apparent association of Paget's disease with dog ownership (212) and positive in situ hybridisation for CDV in pagetic bone (213). More recently some doubt has been cast on this hypothesis as, Ralston et al.(214) using the highly sensitive technique of the polymerase chain reaction (PCR), failed to detect viral sequences for a number of paramyxoviruses including measles, canine distemper virus, parainfluenza virus 3 and respiratory syncytial virus in pagetic bone.

1.5.5 <u>Clinical Features</u>

The proportion of the pagetic population with symptoms is difficult to establish. A widely quoted figure is 5%, though figures of up to 30% are described (215,216). Pain is the most common presenting feature accounting for around 50% of hospital referrals (217). The mechanism of the pagetic pain is not clear, however it has been suggested that it is due to increased vascularity or periosteal stretching. In addition, microfractures - which are a not uncommon accompaniment - are often painful and there is a high incidence of joint disease associated with Paget's (217).

Fractures, generally of long bones, are not uncommon. In up to 90% of cases these occur in the femur, tibia or forearm (218). They generally follow trivial injury, though in around half the patients with fracture no history of trauma is obtained. Non-union is a significant problem in the management of these fractures with Dove (219) describing an incidence of 40% in patients with femoral fractures.

Neurological complications are common. They generally arise from Paget's disease affecting the skull or spinal column, though bone enlargement may cause compression of peripheral nerves (220).

Rarely cardiac output may be increased in patients with Paget's disease associated with an increase in the capillary bed (221). This may in turn give rise to high output cardiac failure especially in those patients with more extensive disease (222).

Even more rarely, sarcomatous change may complicate Paget's disease. The true incidence of this is unknown, though likely to be less than 0.15% (223).

1.5.6 Management

A wide variety of agents have been used in the treatment of Paget's disease. Major interest has focussed on the use of calcitonin and the bisphosphonates which are now generally regarded as the agents of choice. Many other drugs have been tried including fluoride, mithramycin, actinomycin D, glucocorticoids, colchicine and glucagon and generally these agents are either ineffective, toxic or both (224).

Calcitonin is a 32 amino acid peptide secreted by the C cells of the thyroid (225).

The major stimulus to its endogenous secretion is an increase in serum calcium

(226), however it is also affected by gastrin, ß-adrenergic stimulation and alcohol

(227). The main physiological action of calcitonin is thought to be lowering of

serum calcium (228) which is effected via inhibition of skeletal mobilisation of

calcium by suppression of bone resorption (229). In addition to these features,

calcitonin has been claimed to possess potent analgesic properties (230), which

may have some relevance to its use in Paget's disease. The mechanism of this

action is not clear, though it may be via an increase in endogenous opiate

secretion (230).

Administration of a single dose of calcitonin to patients with Paget's disease

causes a decline in urinary hydroxyproline excretion within 2 hours (231)

followed by a later decline in serum alkaline phosphatase (232). With long-term

calcitonin therapy (up to 27 months), these biochemical parameters may fall by

up to 65% of their pre-treatment levels (233) though rarely return to normal

(234). Rebound in these biochemical parameters towards their pre-treatment

values is commonly seen on cessation of therapy (235). Often serum alkaline

phosphatase levels cannot be lowered below a certain level in spite of increasing

the dose of administered calcitonin (233) - a feature known as the "plateau

phenomenon".

Side effects are common soon after administration of calcitonin. Most common

are nausea, flushing, sweating and diarrhoea - these features appear to be dose

related (236). Furthermore calcitonin has to be given by subcutaneous or

intramuscular injection (though nasal preparations are currently being

developed). These features are all major drawbacks in the use of calcitonin in

treating Paget's disease.

As discussed in 1.2.4 the bisphosphonates are potent inhibitors of osteoclastic bone resorption and are, therefore likely to be of value in the treatment of Paget's disease. As in cancer-associated hypercalcaemia, the bisphosphonates most commonly used in the treatment of Paget's disease are etidronate, clodronate and pamidronate - although third generation derivatives such as alendronate, tiludronate and risedronate are now on trial.

Etidronate was the drug first studied in the management of Paget's disease (133). There are several disadvantages in using etidronate in this setting. Firstly it has generally only been used in oral form, and as these drugs are highly water soluble their bioavailability is very low. Recker and Saville (237) have studied the bioavailability of 14C-labelled etidronate and found bioavailability ranged between 1% and 10% - even on an empty stomach. Secondly, of all the bisphosphonates, etidronate has been shown to have the greatest inhibitory effect on bone mineralisation (129) and even at recommended therapeutic doses has been associated with significant osteomalacic change (154). Furthermore, with respect to inhibition of bone resorption, etidronate is significantly less potent than the other bisphosphonates and therefore higher doses need to be used in an attempt to obtain an adequate therapeutic response (238,239).

In spite of these apparent disadvantages, etidronate has been used with some success in the treatment of Paget's disease (240,241,242,243). Etidronate is generally given at a dose of 5mg/kg/day for 6 months or 20mg/kg/day for 1 month (244), with the latter dose being said to cause more effective suppression of biochemical markers of disease (245). While inhibition of bone resorption, as measured by suppression of urinary hydroxyproline excretion, occurs early after the administration of oral etidronate, suppression of urinary calcium excretion does not occur (246). This probably represents the mineralisation impairment associated with this therapy. Interestingly, this is not seen with intravenous

etidronate where urinary calcium excretion rises in spite of apparent inhibition of bone resorption (247).

Clodronate, which is a "second generation" bisphosphonate, is a potent inhibitor of bone resorption which has a less marked effect on inhibition of mineralisation than etidronate (128). Clodronate is highly effective as both oral (248) and intravenous (249) preparations and as with all bisphosphonates the treatment effect lasts for several months or years after cessation of therapy (250,251). Clodronate is significantly more effective than calcitonin and if clodronate is added to the therapeutic calcitonin regime when the "plateau" phase is reached further disease suppression can be achieved (250).

Pamidronate is also a potent inhibitor of bone resorption which like clodronate is said not to inhibit mineralisation. Pamidronate is 100 times more potent than etidronate (239) and 10 times more potent than clodronate (252). Like clodronate, pamidronate has been shown to be effective in both oral (251,253) and intravenous forms (254,255). There is, as yet, no consensus regarding the treatment regime to be recommended: most workers have used short courses of daily or weekly treatment (251,256) with good effect. Thiebaud et al.(257) suggested that a single intravenous infusion of 60mg is sufficient to achieve disease suppression lasting for 1 year. This study however, generally followed patients with mild Paget's disease and the one patient who had extensive disease did not respond to this regime. Similarly Vega et al.(255) found a regime of 25mg pamidronate per day for 7 days produced only a very transient response, with relapse occurring in 2-3 months. Most patients in this study, though, had very severe Paget's disease with pre-treatment alkaline phosphatase levels of up to 6-8 times the upper limit of normal. Harinck et al.(253) compared oral pamidronate, 600mg given daily until urinary hydroxyproline excretion returned to normal, with a 10 day course of intravenous pamidronate (20mg daily).

Studies on duration of remission in these patients showed this to be around 2 years in 50% and 4.5 years in 25%. In a further study (251) comparing two different groups, 1 treated solely with oral pamidronate and 1 with a combination of oral and intravenous pamidronate, the same authors concluded that intravenous therapy was more effective but that a 10 day course of 20mg per day was not enough to achieve remission in all patients.

Disease suppression after intravenous pamidronate is generally associated with decreased radioisotope uptake on bone scanning (258). Vallenga et al (258) reported that radionuclide uptake on bone scans drops by approximately 85% of pre-treatment value after 1 year with pamidronate therapy. Patel et al.(259) found that some lesions may show increased uptake and others decreased uptake in the same patient, even when biochemical evidence of disease suppression exists. Along with disease suppression, bone remodelling occurs which may be associated with dramatic radiographic improvement (260).

1.6 **Primary Osteoporosis**

1.6.1 Introduction

Osteoporosis is characterised by low bone mass, leading to an increased risk of fragility fractures (261). It can be thought of in terms of a disorder of remodelling (262) which involves not only thinning of trabecular and cortical bone but also, in many cases, destruction of the bone trabeculae themselves (262,263). Osteoporosis has been recognised to have become one of the major public health problems of the latter part of the twentieth century. It is thought to be responsible for at least 1.2 million fractures each year in the United States (264) where the direct and indirect costs were estimated at $6.1 billion annually in 1984 (265). In the United Kingdom in 1987/8 it was estimated that the direct costs of hip fracture alone was £160 million per year (266).

Involutional (or primary) osteoporosis generally begins in middle life and becomes progressively more frequent with advancing age. Riggs and Melton have suggested that this condition can be divided into two distinct syndromes, type I (postmenopausal) osteoporosis and type II (age-related/senile) osteoporosis (267,268).

Type I osteoporosis typically affects women within 15-20 years after the menopause. It is characterised by fractures at sites rich in trabecular bone such as vertebrae and Colles' fracture of the distal forearm. Type II osteoporosis affects both men and women usually over the age of 70 years, though is twice as common in women. It is characterised by fractures at sites which, in the young adult, contain substantial amounts of both cortical and trabecular bone. This is usually manifested by fractures of the hip and vertebrae.

These fractures, however, are not specific to osteoporosis, and to make this diagnosis with certainty, therefore, requires demonstration of decreased bone density (269). Many different techniques are now available to do this (270), some of which are discussed in more detail below.

1.6.2 Diagnosis

If measurements of bone mass are to be clinically useful, they must be safe, precise, accurate and of reasonable cost. Generally four methods are in common usage:

1.Single-photon absorptiometry (SPA).

2.Dual-energy photon absorptiometry (DPA).

3.Dual-energy X-ray absorptiometry (DXA).

4.Quantitative computed tomography (QCT).

Single-energy absorptiometry employing radionuclides has been available for nearly 30 years and is commonly used to measure appendicular bone mass (usually radius and ulna) (271). Dual-energy methods were developed later to permit correction for soft tissue and are used in order to measure bone density at axial sites such as the spine and hip. Until recently radionuclides, usually 153-Gadolinium or 125-Iodine, were the source of photons, however low energy X-rays are now being used because the higher photon flux permits greater precision and shorter scan speeds despite lower exposure to radioactivity (272,273). By replacing the 153-Gadolinium source with a dual-energy X-ray source, the precision error of measurements in vivo can be halved, while at the same time scans are rapid (5 versus 20 minutes for regional areas) and have better spatial resolution (2mm versus 4mm) (274). The results of these various techniques are expressed in units of mass (gm mineral) or units of mass corrected for area

(g/cm2). This is usually what is referred to as bone density however, as QCT

allows measurement of volume of bone, results with this technique can be

expressed as a true density (ie g/cm3) (275).

In order to be clinically useful these measurements must be able to predict the

likelihood of fracture. While this is still debated a number of prospective studies

have shown this to be true. Measurement of radial bone mass predicted the risk

of fracture in 366 Swedish women followed for 15 years (276) and measurement

of density at radius and spine predicted fractures at all sites in 1237 Japanese

American women followed for more than 4 years (277). In general terms a

decrease of 1 standard deviation in bone mass is associated with an increased risk

of fracture of the order of 50-100% (278).

1.6.3 Postmenopausal osteoporosis - pathogenesis

Postmenopausal osteoporosis (type I) is often associated with Colles' fracture of

the forearm and/or vertebral compression fractures. Women with this type of

osteoporosis have rates of trabecular bone loss of up to three times normal,

though cortical bone loss is only slightly increased (279,280). Riggs et al.(264)

have suggested that this is due to the following sequence of events: oestrogen

withdrawal at the menopause leads to increased bone loss with increased bone

resorption. This is associated with a slight but significant rise in serum calcium

which in turn leads to decreased secretion of PTH (281) and increased secretion

of calcitonin (282). Renal 1-alpha hydroxylase is suppressed, which results in

decreased $1,25(OH)_2D_3$ levels which in turn is associated with decreased calcium

absorption (283). This leads to a negative calcium balance which further

exacerbates bone loss (284). While this theory is attractive, serum levels of sex

steroids are similar in postmenopausal women with and without type I

osteoporosis (285). Other factors - perhaps peak bone mass in the individual -

must be involved, therefore, to determine individual susceptibility to this condition.

1.6.4 Postmenopausal Osteoporosis - Management

Management of postmenopausal osteoporosis can be considered both in terms of prevention and treatment of established disease. Dietary calcium, if low, should be increased at least to the recommended minimum daily allowance of 800mg per day (264). The National Institutes of Health Consensus Conference on Osteoporosis in the United states recommended a calcium intake of 1000mg to 1500mg per day for all postmenopausal women (286), although the evidence to support these figures is uncertain. As it is logistically impossible to investigate and treat the entire population at risk of osteoporosis, it will be necessary to develop criteria to identify those at risk. At the present time it is not clear how this should be done, although it seems likely that an assessment of osteoporosis risk factors, such as early menopause, small body build, family history excess alcohol etc, should be made and on the basis of this densitometry carried out on those apparently at greatest risk (264).

Treatment of postmenopausal osteoporosis can be divided into treatment with antiresorptive agents such as calcium, oestrogen, calcitonin and bisphosphonates and agents which stimulate bone formation such as fluoride and parathyroid hormone.

The role of calcium in the treatment of osteoporosis remains controversial. Several studies have shown that calcium supplementation will reduce bone loss from both cortical and trabecular bone (287,288) and suppress indices of increased bone turnover (289). On the basis of this, therefore, it would seem reasonable to use calcium, at least as an adjunct to other therapies.

Oestrogen therapy in postmenopausal osteoporosis is associated with a dose-dependent reduction in both bone loss (290) and fracture risk (291). To be effective in preventing fractures it probably has to be taken for at least 10 years after the menopause (292), which may cause problems with compliance which may be as low as 30% (293). Oestrogen therapy is usually combined with a progestogen to decrease the risk of endometrial carcinoma (294), though this is not necessary in those women who have had a hysterectomy. Regular menstrual bleeding is a major disincentive to compliance with hormone replacement therapy (295), though this may be improved by the use of continuous oestrogens and progestogens which generally are not associated with a withdrawal bleed (296).

As described previously calcitonin is a potent inhibitor of bone resorption (see 1.2.4 and 1.5.5) (229) and has been used with variable success in the management of cancer-associated hypercalcaemia and Paget's disease of bone (229,232,233). Parenteral calcitonin has been shown in a number of studies to increase bone mass (297,298), though data on long-term fracture rates is lacking. With the development of intranasal calcitonin long term therapy has become more convenient with recent studies suggesting salmon calcitonin may have a role in both prevention (299) and treatment of osteoporosis (300).

In recent years there has been considerable interest in the use of bisphosphonates in the prevention and treatment of postmenopausal osteoporosis. Initial work in this area was carried out in 1976 when Heaney and Saville (301) demonstrated that continuous treatment of osteoporosis with etidronate at a dose of 20mg/kg body weight resulted in a 50% reduction in bone resorption and a slight but significant increase in total body calcium. In 1984 Anderson et al.(302) reported results on a form of "coherence therapy" using etidronate and phosphate. The theory underlying "coherence therapy" is that sequential stimulation and

suppression of osteoclastic activity will induce synchronisation of bone

formation and resorption throughout the skeleton. In Anderson's study phosphate

was used to activate osteoclastic bone resorption then etidronate was given

depress osteoclastic activity. Treatment was then stopped for a period to allow

unopposed osteoblastic (bone formative) activity. In this study a marked

improvement in bone mass was noted on bone histomorphometry. In 1990 Storm

et al.(303) published results of a three year placebo controlled study on the use of

oral etidronate given at a dose of 400mg per day for 2 weeks followed by

calcium supplementation for 11 weeks to 66 women. Bone density at the spine

was measured by dual-photon absorptiometry and showed a mean increase of

5.3% at three years. Importantly a significant reduction in fresh, radiologically

apparent, vertebral fractures was noted from weeks 60 to 150 in this study. Later

the same year Watts et al.(304) reported results of a larger 2 year double-blind

placebo controlled study looking at a total of 429 women. This study included a

group given phosphate in an attempt to activate osteoclastic activity prior to

etidronate administration as described by Anderson et al.(302). This study used

the more precise technique of dual-energy X-ray absorptiometry to measure bone

density. After 2 years the etidronate treated patients showed an average increase

in their spinal bone density of the order of 5%. Furthermore, this was not

achieved at the expense of losing bone from the hip as neck of femur density was

unchanged. As in the study of Storm et al.(303) the incidence of new vertebral

fractures was reduced by half. Cyclical etidronate is therefore a highly promising

treatment however many questions remain unanswered. Firstly the long term

effect is not known, although early results suggest a plateau in bone density gain

of around 7% is reached (305). Secondly it appears that those patients with the

lowest bone densities respond best which raises some doubt about the

preventative value of this treatment. Thirdly, and perhaps most importantly,

questions have been raised about the quality of new bone formed. As bone

turnover is reduced a progressively greater proportion of the skeleton will be comprised of older bone which may be more liable to fracture (306).

Most studies to date with bisphosphonates have concentrated on oral therapy. In view of the very low oral bioavailability of these drugs, intravenous administration is likely to be of some advantage. There is little information available on the use of intravenous bisphosphonates in the treatment of postmenopausal osteoporosis. A recent study from Italy (307) using aminohydroxybutylidene bisphosphonate (AHBuBP) by intermittent intravenous infusions showed an average increase in spinal density of 9% in 1 year. The authors suggested that this result was better than with oral etidronate at least in part because of the greater bioavailability achieved by using this route of administration.

The alternative to using inhibitors of bone resorption to treat osteoporosis is to stimulate bone formation. Perhaps the best known agent in this class of drugs is sodium fluoride.

Fluoride is thought to act by direct osteoblastic stimulation leading to increased bone formation rates (308). Results of the long-term effects of fluoride therapy are conflicting. Results from France (309) using 50mg per day sodium fluoride in enteric coated capsules has shown that the probability of sustaining a new fracture was significantly lower compared to a group on placebo. More worrying are results from Riggs et al.(310) where women were treated with an average of 75mg per day of sodium fluoride for 4 years. In this study in spite of a 35% increase in bone density at the lumbar spine, the overall rate of vertebral fractures did not decrease suggesting that the new bone formed may be structurally unsound (311). Furthermore an increase (not statistically significant) in hip fractures was seen. This increased frequency of peripheral fractures has

also been described by another group (312) and until this controversy is sorted out the clinical role of sodium fluoride will remain in doubt.

If the role of sodium fluoride is in some doubt, the value of parathyroid hormone as a treatment for osteoporosis is even less clear. In a multicentre study Reeve et al.(313) using a 1-34 amino acid fragment of PTH, treated 16 patients with daily subcutaneous injection for 6 months. There was an increase in new bone formation histologically, but there was no improvement in either calcium balance or cortical bone density. This might suggest that any increase in trabecular bone is at the expense of cortical bone.

1.6.5 Senile osteoporosis - pathogenesis and management

As described above senile osteoporosis (or type II osteoporosis) is more common in men and women over the age of 70 years. This condition is manifest not only by vertebral and Colles' fractures, but also by a marked increase in femoral neck fractures - whose incidence increases exponentially with increasing age.

In type II osteoporosis bone density values for the femoral neck and vertebrae are generally in the lower part of the normal range, adjusted for age and sex (314). This implies a proportionate loss of trabecular and cortical bone has occurred. The two most important aetiological features in the pathogenesis of type II osteoporosis are decreased osteoblast function and impaired production of $1,25(OH)_2D_3$ (264,315). Impaired production of $1,25(OH)_2D_3$ is, in turn, associated with decreased calcium absorption and secondary hyperparathyroidism.

Generally the pharmacological treatment of type II osteoporosis is similar to type I, though most clinical studies have failed to distinguish between these two types of osteoporosis to any significant degree.

Some differences do, however, exist with respect to managing the patient with type II osteoporosis. Oestrogen replacement therapy acts mainly by suppressing the normal postmenopausal increase in bone turnover. For maximum effect, therefore, it should be commenced as soon as possible after the menopause as it has been disputed whether oestrogens have any effect in elderly postmenopausal women (292). Clear evidence does exist, though, that oestrogens prevent bone loss in all stages of postmenopausal life, at least up to the age of 70 years (316,317).

Given that decreased $1,25(OH)_2D_3$ production is associated with type II osteoporosis (315) and this may result in intestinal calcium malabsorption (199), vitamin D supplementation, with active metabolites of vitamin D, may be indicated. Orimo et al.(318) found that administration of 1ug 1-alpha vitamin D_3 to elderly patients with senile osteoporosis resulted in a significant reduction in vertebral crush fractures over a 2 year period. This study was in a relatively small number of patients and further studies in this area are awaited with interest.

1.7 **Secondary osteoporosis**

Secondary osteoporosis is usually considered to include endocrine causes of generalised osteoporosis such as hyperthyroidism (319), hypogonadism (320) and Cushing's syndrome (321). There has also been a suggested association with diabetes mellitus (322), and osteoporosis has rarely been described during or just after pregnancy (323). The most common cause of secondary osteoporosis is probably that associated with use of therapeutic doses of corticosteroids.

1.7.1 Corticosteroid-associated osteoporosis - pathogenesis

Soon after the introduction of corticosteroid drugs, it became apparent that patients on prolonged therapy developed vertebral fractures (324). The true incidence of osteoporosis in patients receiving corticosteroids is unknown. Available data suggests a 30-50% incidence of atraumatic fractures (325,326).

Corticosteroid-associated osteoporosis is a result of a number of factors that adversely affect calcium homoeostasis. Gonadal dysfunction is a common long term consequence of corticosteroid therapy. This is due to a combination of inhibition of pituitary gonadotrophin secretion (327) and a direct suppressive effect on the ovary or testis (328).

It is generally accepted that corticosteroids decrease intestinal calcium absorption in man (199). This appears to be due to a combination of a direct action on the intestinal mucosa decreasing active calcium transport (329) and decreased vitamin D activity (330).

Hypercalciuria with a resultant negative calcium balance is a common association with prolonged corticosteroid usage (331) and this probably plays an

important role in the development of secondary hyperparathyroidism which is also commonly present (332).

The most important actions of corticosteroids, however, in the pathogenesis of osteoporosis is their direct effect on bone. Histomorphometric studies have suggested that bone resorption is increased (332). This increased bone resorption may be, at least in part, due to the presence of secondary hyperparathyroidism and also to a direct effect in stimulating osteoclastic bone resorption (333). As well as increasing bone resorption corticosteroids also decrease bone formation. Histomorphometric studies show a marked decrease in the number of osteoid seams present (334) and, furthermore, the total amount of bone replaced each remodelling cycle has been shown to be reduced (335). Osteoblast-like cells have corticosteroid receptors and corticosteroids appear to have a direct inhibitory effect on osteoblast replication and differentiation (336).

1.7.2 Corticosteroid-associated osteoporosis - diagnosis

Not all patients on long term corticosteroid therapy develop osteoporosis, with premenopausal women in particular, apparently relatively protected (337). For this reason, it is important to identify persons at risk for closer follow up or treatment. Exactly how this should be done is not clear. Densitometry is likely to be helpful, though a recent study using dual-photon densitometry suggested that this technique was not sufficient to identify at risk patients as fractures appeared to occur at higher bone densities than is generally the case in involutional osteoporosis (338). This study is yet to be confirmed with dual-energy X-ray densitometry (DXA).

1.7.3 Corticosteroid-associated osteoporosis - management

In contrast to postmenopausal osteoporosis there is dearth of information of the prevention and treatment of corticosteroid-associated osteoporosis (339).

The most obvious way of attempting to lessen the effects of corticosteroids on bone is to try and reduce the dose of corticosteroid used. One way of doing this appears to be by using an alternate day dosing regime. This has been shown to result in less marked inhibition of intestinal calcium absorption and, at least in animals, reduces the incidence of osteoporosis (340). However, Gluck et al.(341) measured forearm bone density in a cohort of patients receiving either alternate day or daily corticosteroids and found no difference in the rate of bone loss. There is, therefore, little support for using alternate day dosing regimes to limit osteoporosis.

Since intestinal calcium malabsorption is thought to be one of the major factors involved in the pathogenesis of this condition (199), vitamin D and its active metabolites may be expected to be of some benefit. Hahn et al.(342) in 1979 showed that administration of vitamin D was associated with an increase of 46% in intestinal radiocalcium absorption and a decrease of 54% in PTH. This was associated with a reduction in the number of osteoclasts seen on bone histology. Two other studies have reported benefit in using 1-alpha hydroxyvitamin D on the bone mass of steroid-dependent patients (343,344), however these were both short term studies and the long term use of vitamin D and its metabolites in corticosteroid-associated osteoporosis remains uncertain.

Fluoride is a potent stimulator of bone formation (see 1.6.4). Since bone formation rates are low in corticosteroid-associated osteoporosis, fluoride might be expected to be effective. Little data on this is available. Rickers et al.(345)

randomly allocated patients commencing steroid therapy to fluoride and calcium, vitamin D, or placebo. Bone mineral content at the forearm declined 2.5% in all groups in the first 6 months and the authors concluded that fluoride was without benefit at least in the prevention of corticosteroid-associated osteoporosis.

Similarly, despite considerable interest in the use of calcitonin for the treatment of postmenopausal osteoporosis, little attention has been paid to its role in corticosteroid-associated osteoporosis. The only major study published to date is that of Ringe et al.(346) who demonstrated a beneficial effect with calcitonin in a six month prospective study.

The increased bone resorption normally seen with this condition implies that the bisphosphonates may also be effective. As is the case with the other agents described above, little information is available here. Reid at al.(347) in 1988, treated 16 patients on oral corticosteroids with oral pamidronate for one year. These 16 patients had a mean increase in spinal bone density of 19% (as measured by quantitative computed tomography) whereas bone density in controls was unchanged. Five of these pamidronate treated patients were followed for a further year and the initial gain in bone mass appeared to have been maintained (348). While this agent would appear to be promising further studies are awaited.

1.7.4 Diabetes mellitus associated osteoporosis

An association between diabetes mellitus and osteoporosis was first described by Albright and Reifenstein in 1948 (349). In the description of diabetic bone disease, a distinction must be drawn between insulin dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM).

In IDDM, a decreased bone mass has been frequently observed when measured by X-ray (350), single photon absorptiometry (351,352) and dual photon absorptiometry (353). The situation in NIDDM is less clear with both decreased (350) and increased bone mass (354) being described. As NIDDM is often associated with obesity, and obesity is associated with a reduced incidence of osteoporosis (355), an appropriate control group is necessary. Whether or not osteopenia is correlated with diabetic control is not clear as this information is lacking from many studies. In IDDMs, where osteopenia is present, diabetic control has generally been poor with glycated haemoglobin concentrations of greater than 10% (356,357). Similarly the extent of bone loss has been shown to be correlated with both the extent of glycosuria and fasting blood glucose concentration (321,358).

In addition to a suggested low bone mass, diabetic patients appear to have an increased risk of sustaining a fracture (359,360). When the various studies which have investigated this are pooled, the estimated increased risk of fracture in diabetes is approximately twofold (361). Against this a case-controlled study from the Mayo clinic failed to show any excess of fracture in diabetic patients (362). This study, though, has been criticised as, being retrospective some fractures may have been missed. Furthermore, it is likely that a number of undiagnosed diabetics were present in the control group; if this is the case the validity of the results from this study are open to question. It is possible, however, that fracture rates are increased in diabetics, not because of alterations to bone mass, but because of an increased incidence of falls, for example secondary to the presence of neuropathy. Indeed, Nabarro attributed the incidence of vertebral fractures in diabetics to hypoglycaemic seizures (363).

The mechanism of bone loss associated with diabetes has been investigated by histological and biochemical studies. It would appear that diabetes is associated

with a low bone turnover state with decreased bone formation and resorption (361). This would be compatible with the view that insulin is required for normal bone formation (364). One histomorphometric study has suggested that the initial problem in diabetic bone disease is increased bone resorption with the low turnover state a late finding (365). Further biochemical evidence has suggested this to be true with hypercalciuria frequently found (321,366), implying increased bone resorption (see 1.1.3). Hypercalciuria, however, in the setting of diabetes mellitus, may not be simply due to increased bone resorption. The osmotic diuresis associated with glycosuria may be associated with calciuresis (366) and changes in plasma glucose and insulin have been shown to alter renal tubular calcium handling (367,368). Studies are, therefore required looking at more specific markers of bone resorption such as hydroxyproline or collagen cross-link excretion.

The presence of secondary hyperparathyroidism has been suggested as a cause of the hypercalciuria of diabetes (366) and this certainly could contribute to increased bone resorption. The literature is once again conflicting on this issue with there also being studies suggesting the presence of relative hypoparathyroidism (369) and normal parathyroid function (370).

As can be seen from the above considerable uncertainty exists over the extent and pathogenesis of osteopenia in diabetic patients with further studies using today's more sensitive technologies required.

CHAPTER 2

TECHNICAL METHODS

2.1 **Biochemical Technical Methods**

2.1.1 Routine analyses

Serum calcium, phosphate, creatinine, electrolytes, gamma glutamyl transferase

(γGT) and alkaline phosphatase and urinary calcium, phosphate, sodium and

creatinine were measured by standard autoanalyser techniques (Technicon,

Tarrytown, USA). Serum total calcium was adjusted for albumin using the

algorithm: calcium (adjusted) = total measured calcium ± [47 - measured

albumin (g/l) x 0.019]. This method has been previously shown to correlate

closely with ionised calcium (152).

Reference ranges for our laboratory were:-

> serum adjusted calcium: 2.20-2.60mmol/l

> serum phosphate: 0.70-1.40mmol/l

> serum creatinine: 40-130umol/l

> gamma glutamyl transferase: <36U/l

> alkaline phosphatase: 80-280U/l

> urine calcium/creatinine: <0.50mmol/mmol

2.1.2 Parathyroid hormone (PTH)

Serum intact PTH(1-84) was measured in a two-site immunoradiometric assay,

using two monoclonal antibodies. PTH(1-34) is labelled with the rat/mouse

monoclonal antibody 3B3 which is radiolabelled. The C-terminus 74-84 amino

acids are labelled with the mouse monoclonal ESQ-1 (on solid phase). The assay

has a minimum detection limit of 0.5pmol/l and a range of 1.5-250pmol/l with an

intra-assay co-efficient of variation (cv) of less than 10%. Studies on clinical

samples indicate excellent discrimination with this assay between normal

subjects (mean 2.21; range 1.0-5.0pmol/l) and patients with primary

hyperparathyroidism (mean 21.0; range 5.8-100pmol/l) who in turn are well

separated from patients with cancer-associated hypercalcaemia (14/18

<0.5pmol/l) (371). In one study (3.1) PTH in the first seven hypercalcaemic

patients was measured in a N-terminal specific radioimmunoassay (372). This

assay used highly purified bovine PTH as standard and for radioiodination and a

guinea pig anti-bovine PTH serum which would recognise both ends of the intact

PTH molecule. The mean intra-assay cv for this assay was 7% and the normal

range was from the limit of detection (150ng/l) to 600ng/l.

2.1.3 Parathyroid hormone-related protein (PTHrP)

Serum PTHrP concentration was measured in a two-site immunoradiometric

assay (Nichols Institute, California, USA). In this assay affinity-purified anti-

PTHrP(37-74) bound to a solid phase is used as the capture antibody and

radiolabelled anti PTHrP(1-36) used as the signal antibody. The sensitivity of

this assay is 0.7 pmol/l. Human PTH(1-34), PTH(1-84), PTHrP(1-34) and

PTHrP(36-76) have less than 0.1% cross-reactivity. All samples were collected

into specific collection tubes, containing proteinase inhibitors, on ice and were

centrifuged immediately. This assay has a cv <10% in the PTHrP concentration

range 2-50pmol/l. A normal reference range has been established of <0.7-

2.6pmol/l (373)

2.1.4 Vitamin D metabolites

25-hydroxycholecalciferol (25(OH)D_3) and 1,25 dihydroxycholecalciferol

(1,25(OH)$_2D_3$) were measured using a modification of the method used by

Reinhardt et al.(374). The vitamin D metabolites were extracted from serum and

separated from other vitamin D metabolites by reverse phase high performance

liquid chromatography (HPLC). The purified 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ were then quantitated using competitive protein binding assays with charcoal separation. The mean between assay cv for 25(OH)D$_3$ was 10% and normal range 15-100nmol/l. The sensitivity of the assay was typically 5nmol/l. For 1,25(OH)$_2$D$_3$, between assay cv was 20% and sensitivity typically 15pmol/l. The normal range for our laboratory was 20-120pmol/l.

2.1.5 Osteocalcin

Serum osteocalcin was measured in a commercially available radioimmunoassay (Incstar, Minnesota, USA). This utilises anti-bovine osteocalcin antibody and 125I bovine osteocalcin. Within and between assay cv's were 10% and 15% respectively. The normal range was 1.8-6.6ng/ml. Typical sensitivity of this assay was 0.6ng/ml.

2.1.6 Ionised calcium

Serum ionised calcium was measured in a Radiometer ICA 1 and results were adjusted to pH 7.40 in order to minimise the effect of carbon dioxide loss from the sample (375). Samples were collected anaerobically into vacutainers without preservative and analysed within 1 hour. Normal range for our laboratory is 1.10-1.28mmol/l with a between assay cv of <2% and within assay cv of <1%.

2.1.7 Testosterone, sex hormone binding globulin and gonadotrophins

Testosterone was measured by radioimmunoassay using double antibody separation. This assay has an interassay cv of <10% and intra-assay cv of <8%. SHBG was measured using a "Delfia SHBG" kit (Pharmacia Wallac, Milton Keynes, UK). This assay is a solid phase two-site fluroimmunometric assay in

which polyclonal anti-SHBG antibodies (derived from rabbit) and monoclonal

antibodies (derived from mice) directed against the SHBG molecule are used.

Typical intra-assay cv is 8% and typical interassay cv is 6%. A measure of free

androgen index (FAI) was derived using the formula - testosterone/SHBG x100.

Leutinising hormone (LH) was measured using a commercially available

microparticle enzyme immunoassay (Abbot Laboratories, Illinois, USA). This

assay has a typical sensitivity of 0.5mU/l, interassay cv of 5.5% and intra-assay

cv of 6.7%. Follicle stimulating hormone (FSH) was also measured using a

microparticle enzyme immunoassay (Abbot Laboratories, Illinois, USA). This

assay has a typical sensitivity of 0.2mU/l, interassay cv of 7.0% and intra-assay

cv of 3.8%.

2.1.8 Cyclic adenosine 3' 5' monophosphate (cAMP)

Plasma cyclic adenosine monophosphate (PcAMP) was measured by a

commercially available radioimmunoassay (Amersham, Aylesbury, UK). Urine

cyclic adenosine monophosphate was measured, after appropriate dilution, by an

"in-house" radioimmunoassay as described by O'Reilly et al.(376). The between

assay cv was less than 10% and the limit of detection 2.6nmol/l. Urinary cyclic

AMP excretion (UcAMP) was expressed as a function of glomerular filtrate

(UcAMP = urine cAMP divided by urine creatinine multiplied by serum

creatinine) and the normal range was 22-66nmol/l GF.

The nephrogenous component (NcAMP) of UcAMP was calculated as UcAMP

minus PcAMP (normal range 5-27nmol/l GF) (76).

2.1.9 Hydroxyproline

Hydroxyproline (HP), an index of total bone resorptive activity (377), was measured in true fasting (second voided) morning urine specimens using a colourimetric technique similar to that described by Goverde et al.(378), followed by autoanalyser quantitation. Urinary HP was expressed as a molar ratio relative to urinary creatinine (Cr) excretion (mmol/mmol). The mean between assay cv was 8% and typical sensitivity 0.005mmol/l. The reference range for HP/Cr in our laboratory was 0.005-0.033 mmol/mmol.

2.1.10 Renal tubular reabsorption of phosphate

The notional threshold for renal tubular phosphate reabsorption (TmPO4) was derived from a nomogram (379) after measurement of urinary phosphate and creatinine and serum phosphate and creatinine. The normal range lay between 0.70-1.35 mmol/l GF.

2.1.11 Urinary calcium excretion

Urinary excretion of calcium was expressed as a molar ratio relative to urinary creatinine (Ca/Cr; mmol/mmol), with the normal range being up to 0.50. A value for calcium excretion (CaE) was also obtained by the equation (urine calcium/urine creatinine) x serum creatinine. Results were expressed as umol/l GF and the reference range for normocalcaemic subjects 7.5-37.5umol/l GF.

Renal tubular reabsorption of calcium was assessed by comparing CaE and the simultaneous serum calcium measurement with a normal range obtained by calcium infusion studies in healthy subjects (36) (figure 1.1). A value for the renal tubular threshold for calcium absorption (TmCa) was obtained from a

computer program produced by Rorer Pharmaceuticals Ltd and based on a nomogram (380) (normal range 1.98-2.61mmol/l GF).

2.1.12 Urinary sodium excretion

Urinary sodium excretion (NaE) was derived by the following equation: (urinary sodium/urinary creatinine) x serum creatinine. Results were expressed in mmol/l GF and the reference range in healthy normocalcaemic subjects 0.10-1.30mmol/l GF.

2.2 Radionuclide Bone Scans

Radionuclide bone scans were performed using standard techniques, three hours after the intravenous injection of 600 Megabequerels (MBq) of 99m-labelled methylene bisphosphonate.

2.3 Whole Body Retention of Radiolabelled Bisphosphonate

Whole body retention of radiolabelled bisphosphonate (WBR) was carried out as a further measure of bone turnover in patients with Paget's disease. A single intravenous injection of 2 MBq 99m-methylene bisphosphonate was given then 24 hours later the patient passed through a whole body counter. The normal range was 19-37%. Typical precision of this technique was <1%.

2.4 Dual-Energy X-Ray Absorptiometry (DXA)

Measurement of bone density was carried out at the lumbar spine (L2-4) and the neck of femur by the technique of dual-energy X-ray absorptiometry (DXA) on a Lunar DPX. All scans were carried out on the same machine by the same operator, using the same analysis software and results expressed as areal densities (g/cm2). Precision at L2-4 for 17 normal subjects scanned 6 times was 0.5% and at neck of femur was 1.05%.

2.5 Parathyroid Thallium/Technetium Subtraction Scans

A standard 201-Thallium(Tl)/99m-Technetium(Tc) parathyroid subtraction scan was carried out as described by Fogelman et al.(381). Images were acquired in 1 minute segments for 20 minutes after the intravenous administration of 80 MBq 201Tl then for 10 minutes after the administration of 180MBq 99mTc. Images

were stored in a computer interfaced to the gamma camera and a "subtraction"

scan then obtained.

EXPERIMENTAL WORK

CHAPTER 3

CLINICAL APPLICATION OF CURRENTLY AVAILABLE

BIOCHEMICAL TECHNIQUES

3.1 **An assessment of the pathogenesis of breast cancer-associated hypercalcaemia**

3.1.1 Introduction

Biochemical measurements can provide significant insight into the pathogenesis of many disorders of bone and calcium metabolism. This is particularly well illustrated by cancer-associated hypercalcaemia where, as a result of careful biochemical evaluation, it was well recognised that a substance with PTH-like activity was present in the circulation (68-73,77-81). This fact was appreciated for many years before the discovery of parathyroid hormone-related protein (PTHrP). It is now accepted that such humoral hypercalcaemia is more common with solid tumours (91,92) whereas hypercalcaemia due to local osteolysis by bone metastases is more common in haematological malignancy such as myeloma (91,92). The position with breast cancer-associated hypercalcaemia is somewhat less clear. Traditionally, it has been thought that breast cancer-associated hypercalcaemia occurred predominantly as the result of increased osteoclastic bone resorption due to extensive bone metastases (58,63). Recent evidence, however, has suggested that humoral factors may also play a significant role in the pathogenesis of this condition (382,383).

In this study, by using a combination of biochemical markers and radionuclide bone scans, an assessment was made of the relative contribution of humoral and local osteolytic mechanisms to the pathogenesis of hypercalcaemia in 20 patients with breast cancer.

3.1.2 Patients and methods

The study group consisted of 20 consecutive patients who presented with hypercalcaemia associated with breast cancer. Nine normocalcaemic patients with breast cancer and bone metastases, who also presented consecutively, served as disease controls. Hypercalcaemia, in this study, was defined as a serum total calcium adjusted for albumin of greater than 2.80mmol/l. All hypercalcaemic patients had been sodium repleted for 48 hours prior to study with a minimum of 6 litres 0.9% sodium chloride solution. At the time of study two hypercalcaemic patients were receiving tamoxifen and two megestrol, though both these treatments had been commenced at least 6 months previously. None of the patients included in this study received corticosteroids or any other cytotoxic chemotherapy around the time of these investigations.

Bone metastases were assessed by review of standard radionuclide bone scan films. Patients with less than six "hot spots" were considered as having a light tumour load and those with six or more "hot spots", a heavy tumour load - as previously described (384).

Tissue diagnoses were taken from histopathological reports of breast biopsies.

Statistical methods used were the Mann-Whitney U-test and Spearman rank correlations where appropriate.

3.1.3 Results

Hypercalcaemic patients were divided into two groups by the levels of urinary cyclic adenosine monophosphate (UcAMP); those where values fell within the reference range of 22-66nmol/l GF and those where UcAMP was greater than

66nmol/l GF (figure 3.1). UcAMP levels were within the reference range in all 9

normocalcaemic patients. While results of nephrogenous cAMP (NcAMP) were

available in only 9 hypercalcaemic patients, there was a close correlation

between NcAMP and UcAMP (r=0.90, p=0.01) and, for the purposes of this

study UcAMP was considered to be a reasonably accurate reflection of NcAMP.

Calcium excretion (CaE) when expressed against the prevailing serum calcium

(figure 3.2), was deviated to the right of the line defining their normal

relationship in 9 cases in the hypercalcaemic group - indicating that renal tubular

reabsorption of calcium (TmCa) was raised. Interestingly, these patients were not

necessarily the same as those who exhibited elevated UcAMP levels. This

relationship between CaE and serum calcium was generally normal in the

normocalcaemic group, though in one instance TmCa may have been marginally

increased.

In the hypercalcaemic group, UcAMP excretion tended to be higher in patients

with a light tumour load, though some patients with a heavy tumour load also

exhibited raised UcAMP levels. UcAMP values were (median, range) 70nmol/l

GF (33-131) in patients with a light tumour load and 38nmol/l GF (22-112) in

those with a heavy tumour load (p=0.07) (figure 3.3). Tumour load was

considered to be light in 7/9 patients in the normocalcaemic group; however,

UcAMP excretion was within the normal range in all patients in this group.

Other results are shown in table 3.1. Serum calcium, creatinine and urinary

calcium/creatinine ratio were similar in both the "normal" UcAMP and "high"

UcAMP hypercalcaemic groups. Urinary calcium/creatinine ratio (Ca/Cr) was

significantly lower in the normocalcaemic group, though it was still above the

upper limit of the reference range of 0.50 implying increased bone resorption in

these patients. Similarly, CaE was significantly lower in the normocalcaemic
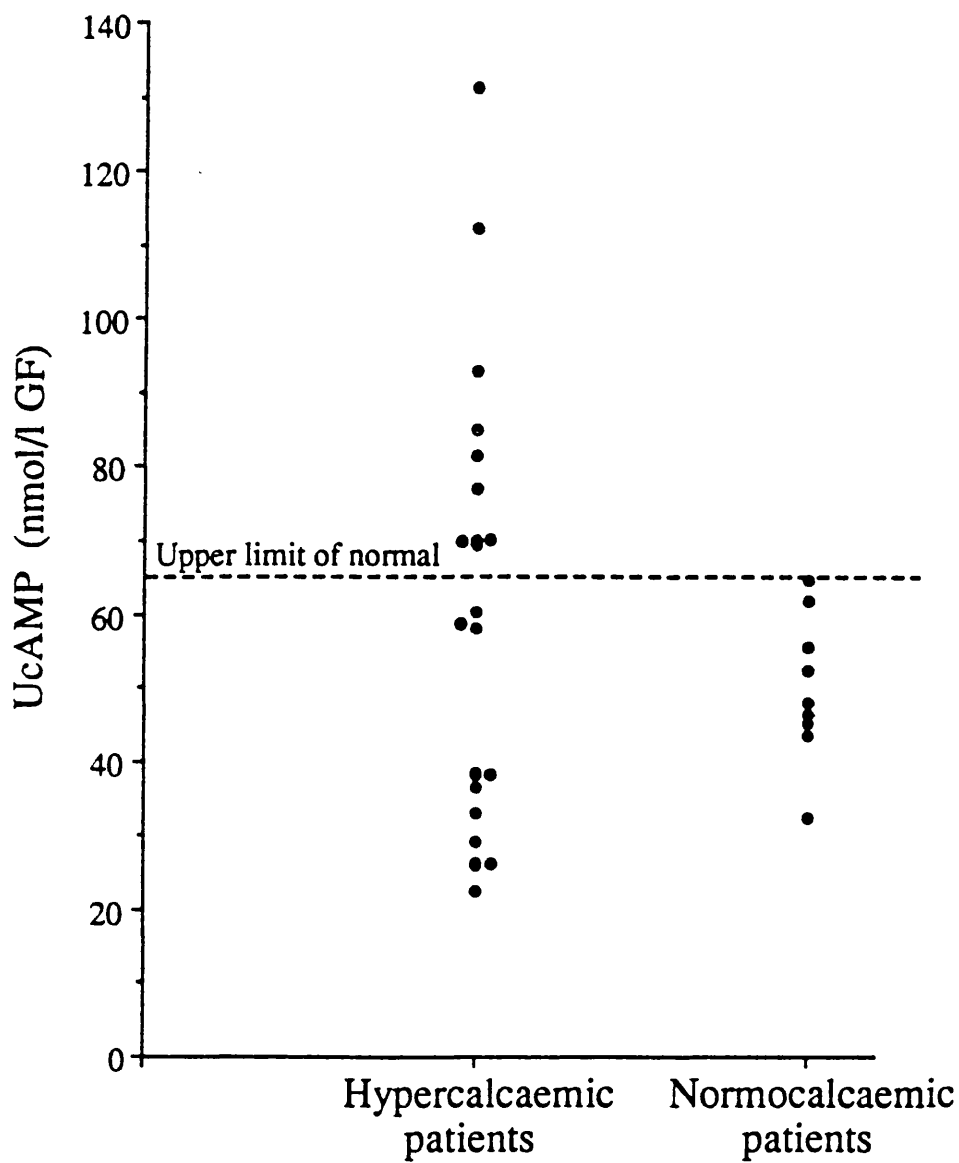
Figure 3.1

Distribution of urinary cyclic adenosine monophosphate (UcAMP) excretion in normocalcaemic and hypercalcaemic breast cancer patients.
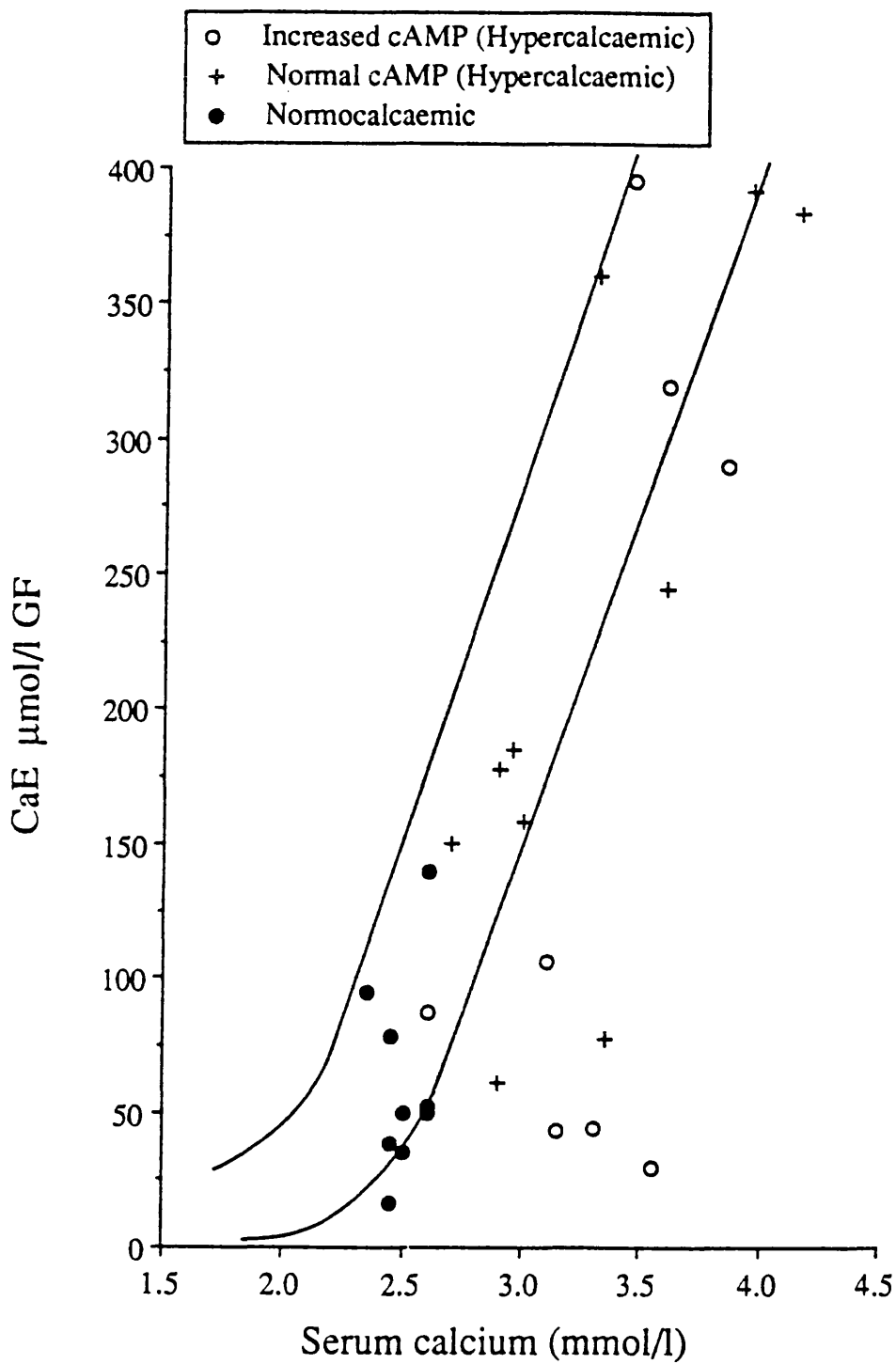
Figure 3.2

Relationship between serum calcium and calcium excretion (CaE). Lines indicate normal range. Values lying to the right of the normal range imply increased renal tubular reabsorption of calcium.
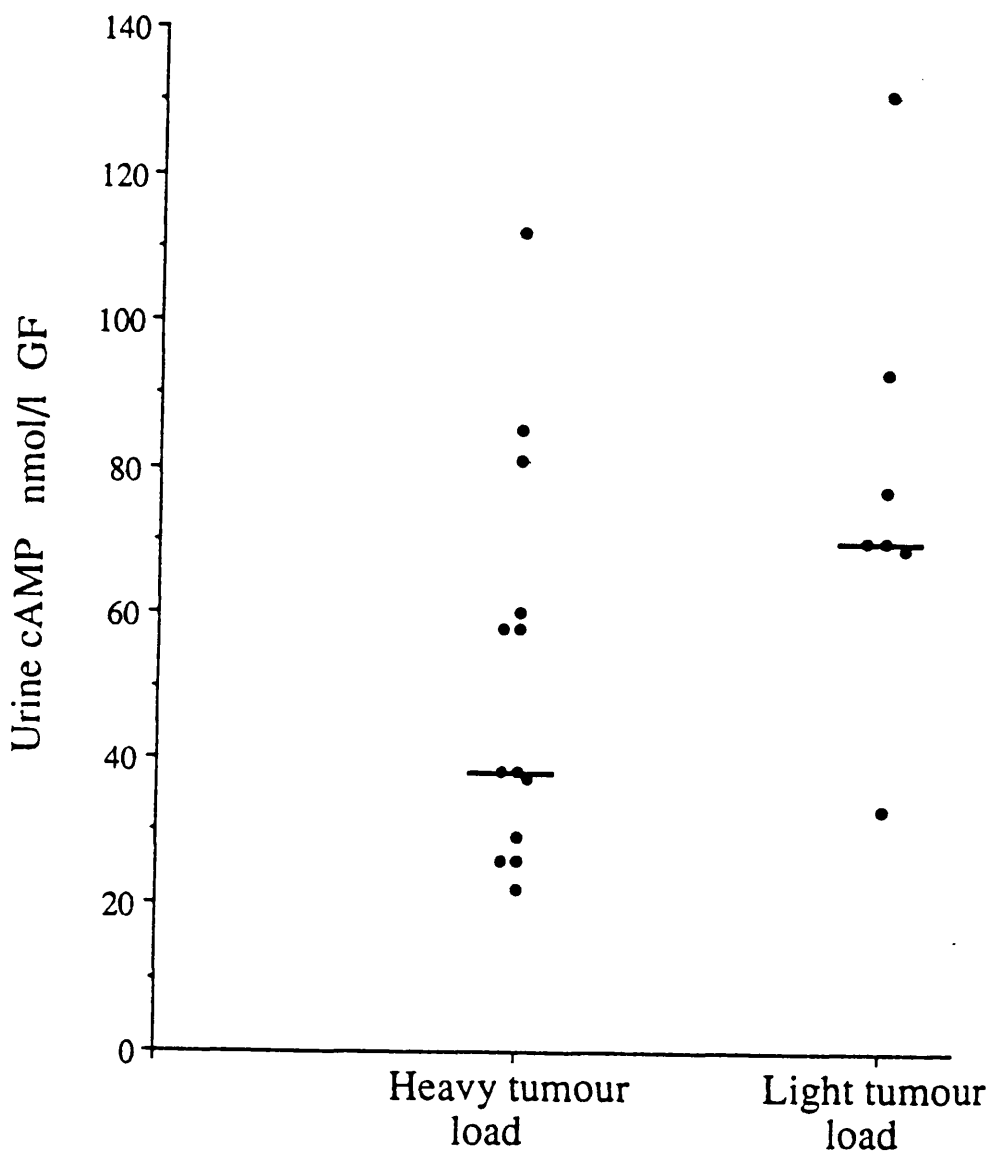
Figure 3.3

Relationship between urinary cyclic adenosine monophosphate (UcAMP) excretion and tumour load as assessed by radionuclide bone scans in the hypercalcaemic breast cancer patients. Bars represent means.

Table 3.1

Biochemical details of patients with breast cancer-associated hypercalcaemia

	Serum calcium mmol/l	Serum phosphate mmol/l	Serum creatinine umol/l	Urine calcium/creatinine mmol/mmol	$TmPO_4$ mmol/lGF	CaE umol/lGF	NaE mol/l GF
Normal UcAMP (n=11) Median	3.30	1.25	95	2.10	0.92	185	2.80
Range	2.80	1.0	55	0.87	0.39	61	0.2
	-4.15	-1.6	-260	-3.92	-1.76	-855	-10.4
Elevated UcAMP (n=9) Median	3.45	0.75*	75	2.85	0.39*	106	1.10
Range	2.80	0.4	70	0.56	0.14	29	0.001
	-3.85	-1.2	-120	-4.78	-0.84	-574	-5.9
Reference Values	2.20	0.7	40	<0.05	0.70	7.5	0.1
	-2.60	-1.4	-120		-1.35	-37.5	-1.3

* $p < 0.005$ versus normal UcAMP group

group. Sodium excretion (NaE) was significantly higher in the hypercalcaemic "normal" UcAMP group. The reason for this is not clear, though the data is skewed by one patient where NaE was very high (10.4mmol/l GF). Values for the renal tubular threshold for phosphate reabsorption (TmPO4) were decreased in 12 of the hypercalcaemic patients and both TmPO4 and serum phosphate were significantly lower in patients with raised UcAMP. There was a significant negative correlation between individual TmPO4 values and UcAMP levels (r= -0.54, p=0.02), but no significant correlation was found between UcAMP and serum calcium, serum creatinine, urinary Ca/Cr, CaE or NaE. In the normocalcaemic group 8/9 TmPO4 values were normal. In one patient it was low at 0.58mmol/l GF, though UcAMP excretion in this patient was normal at 55.5nmol/l GF.

Parathyroid hormone (PTH) levels in the hypercalcaemic patients were undetectable in 15 cases and just detectable at the lower limit of the reference range in 3. PTH was not measured in the remaining 2 cases in this group. In the normocalcaemic patients, PTH was within the reference range in 8/9 instances. In the abnormal case it was just above the upper limit of the reference range. PTH was undetectable in 1 patient in this group whose serum calcium was 2.60mmol/l.

The majority of tumours were invasive ductal carcinomas. There was no obvious association between the tissue type and level of UcAMP.

3.1.4 Discussion

In clinical practice, PTHrP mediated hypercalcaemia may be suspected by the finding of biochemical abnormalities suggestive of primary hyperparathyroidism in the absence of detectable PTH levels (58,59,60,73,77,78). While breast cancer

has traditionally been considered to be due to local osteolysis as the result of increased bone resorption from skeletal metastases (63), PTH-like biochemical abnormalities have also been observed in this situation and, furthermore, PTHrP has been extracted from the tumour of a patient with humoral hypercalcaemia associated with breast cancer (385). Hypophosphataemia and depressed renal tubular phosphate reabsorption have long been recognised to occur in hypercalcaemic breast cancer patients (386,387), but previous workers have attributed these findings to the effect of hypercalcaemia on the renal tubule per se (386). More recently, elevated levels of NcAMP have been found in between 35%-39% of patients with breast cancer and hypercalcaemia (79,382), as have increased levels of renal tubular reabsorption of calcium (383) - all features which would be consistent with the renal tubular effects of PTHrP (84,98).

NcAMP data was not available in all of the hypercalcaemic patients in this study, but in the presence of normal renal function UcAMP is thought to be a reliable indicator of NcAMP (388). This is borne out in this work by the close correlation found between UcAMP and NcAMP in those patients where full data were available.

In this study elevated UcAMP values were found in 45% of cases where hypercalcaemia was present, a slightly higher proportion than that which was noted by Isales et al.(382) and Rude et al.(79). It was not possible in this present study to define "high cAMP" and "low cAMP" groups of hypercalcaemic patients as originally described by Stewart et al.(77) because the UcAMP values followed a continuous distribution, with even the lowest values being clearly detectable within the normal range. Since the majority of hypercalcaemic patients had undetectable PTH levels, the abnormalities of renal tubular calcium and phosphate handling and UcAMP excretion may be explained by the action of

PTHrP. If this is the case, a higher percentage of patients with breast cancers may be expressing PTHrP than has generally been accepted.

The interpretation of the "normal" UcAMP values in the hypercalcaemic cancer patients is more difficult. However, continued UcAMP excretion in the presence of low PTH values may also have been due to the action of PTHrP. While levels of UcAMP excretion were also within the normal range in the normocalcaemic patients, this could have been explained by the effects of endogenous PTH. However, in one normocalcaemic patient PTH was undetectable and UcAMP at the upper limit of the reference range at 64.6nmol/l GF. It is possible, therefore, that this patient may also be expressing PTHrP and, one may speculate, might become hypercalcaemic in the future.

While TmPO4 and serum phosphate values were significantly lower in the hypercalcaemic patients with high UcAMP excretion, elevations in renal tubular calcium reabsorption were not confined to patients with elevated UcAMP levels. Thus, while 5/9 (56%) patients with high UcAMP had elevated renal tubular calcium absorption, 4/11 (36%) with normal UcAMP exhibited similar increases in renal tubular reabsorption of calcium. Furthermore, although most hypercalcaemic patients with a light tumour load had elevated UcAMP levels, raised UcAMP was also seen in patients with a heavy tumour load.

These data, in keeping with previous observations (389), indicate that some manifestations of PTH-like activity may be seen in the absence of others in patients with cancer-associated hypercalcaemia. Although the reasons for this are unclear, it has been suggested that some tumours associated with humoral hypercalcaemia release other factors which may interfere with PTHrP binding to the PTH receptor (101). An alternative possibility, and one which may be relevant to the renal calcium handling data, would be the presence of variations

in sodium excretion, which have been shown to interfere with the clinical

assessment of renal tubular calcium reabsorption (24,82).

Acute extracellular fluid expansion with 0.9% saline at a rate of 1litre/hour can

increase UcAMP excretion in normal subjects by causing a dilutional fall in

serum calcium and subsequent rise in PTH. However, when hypoparathyroid or

hypercalcaemic subjects are studied no rise in UcAMP is observed (390)

suggesting that natriuresis does not control UcAMP production. Since a rise in

serum PTH at the time these patients were studied was not observed, it is

unlikely that the fluid replacement regime used is the cause of the increased

UcAMP seen in these hypercalcaemic patients.

These results indicate that biochemical markers of a PTHrP-mediated

mechanism of hypercalcaemia may be observed in 45%-60% of patients with

breast cancer depending upon whether TmPO4, UcAMP or renal tubular calcium

reabsorption is assessed. Whether such a mechanism of hypercalcaemia is also

operative in patients with normal but "inappropriately detectable" UcAMP levels

will probably be defined in the future by the availability of immunoassays for the

direct measurement of PTHrP.

3.2 Changes in serum parathyroid hormone-related protein (PTHrP) concentrations in normal pregnancy

3.2.1 Introduction

The previous study illustrated the value of measuring indirect biochemical markers for parathyroid hormone-related protein (PTHrP). However, in the setting of humoral hypercalcaemia of malignancy, PTHrP plasma concentrations are likely to be significantly elevated (91,92) which in turn leads to elevation of nephrogenous cyclic adenosine monophosphate (NcAMP) (77,78,79) and depression of the renal tubular threshold for phosphate reabsorption (TmPO4) (80,81). In physiological situations (where PTHrP concentrations are not elevated) it is likely that direct measurement of PTHrP is required rather than the use of these indirect markers of PTH-like activity.

Following the discovery of PTHrP as the likely principal mediator of humoral hypercalcaemia of malignancy (83,84,85), subsequent studies suggested the possibility of a physiological endocrine or paracrine role for this peptide. PTHrP itself and its messenger RNA (mRNA) have been found in a number of normal tissues including skin, thyroid, parathyroid, pituitary, adrenal and stomach (96,391). In addition, it is likely that PTHrP plays a role in the maintenance of normal pregnancy and lactation. Very high levels of PTHrP have been found in human and other mammalian milk (94,392) and evidence now exists to suggest that PTHrP is the principal factor involved in the transfer of calcium from the breast into milk (393). PTHrP has also been shown to have a physiological role in controlling maternal/fetal placental calcium transport in sheep (95,394).

In this study a new two-site immunoradiometric assay was used to measure changes in plasma concentrations of PTHrP during normal pregnancy.

Concentrations of PTHrP found were compared with other indices of calcium homoeostasis.

3.2.2 Patients and Methods

10 women (mean age 26.9 years, range 17-32) were studied at presentation to the antenatal clinic (range 9-11weeks gestation, mean 9.9 weeks), at 2nd trimester (20-24 weeks, mean 22.2 weeks), 3rd trimester (33-34 weeks, mean 33.4 weeks), term (36-40 weeks, mean 38.1 weeks) and 6 weeks post-delivery. Gestational dates were confirmed by ultrasound. At each visit measurement was made of plasma PTHrP, PTH, cyclic adenosine monophosphate (PcAMP), osteocalcin , calcium, albumin and total alkaline phosphatase (ALP). Calcium was adjusted for the prevailing serum albumin as described in section 2.1.1. In addition, at each visit a true fasting (second voided) urine sample was obtained for measurement of cyclic adenosine monophosphate and urinary calcium and creatinine. Urinary calcium excretion was expressed as a ratio of urinary creatinine (mmol/mmol) (Ca/Cr) and a figure for nephrogenous cAMP (NcAMP) was derived as described in section 2.1.8.

No patient suffered from any other condition thought likely to interfere with their calcium homoeostasis and no patients were on any medication other than supplemental iron and folic acid. None of the patients studied developed pre-eclampsia or pregnancy-associated hypertension and each had an uneventful pregnancy. Onset of labour was spontaneous in 9 instances and in the remaining case an elective Caesarian section was performed on account of cephalo-pelvic disproportion. In each case a normal live child was delivered. After delivery only 1 mother elected to breast feed her child.

Statistical analyses of the above parameters was carried out using Scheffe's test

for multiple simultaneous comparisons. Results are presented as means ± SEM.

3.2.3 Results

Changes in plasma PTHrP concentrations are shown in figures 3.4 and 3.5.

PTHrP was undetectable (<0.7pmol/l) in 2 instances in 1st trimester, 1 instance

in 2nd trimester and 1 instance at term. PTHrP rose gradually from 0.8pmol/l ±

0.2 (1st trimester) to 2.7pmol/l ± 0.2 (post natal) (p<0.00001). Serum alkaline

phosphatase (figure 3.5) rose from nadir at 1st trimester of 94U/l ± 8 to a peak of

347 ± 25U/l at term (p<0.00001), thereafter levels fell to 184 ± 19U/l (p=0.04 vs

term) at post natal assessment. ALP and PTHrP were significantly correlated up

to term (r=0.44, p=0.005). Plasma PTH (figure 3.6) tended to fall slightly

through pregnancy to a nadir of 1.5 ± 0.1pmol/l in the 3rd trimester (p=NS),

though rose significantly at the post natal assessment from 1.8pmol/l ± 0.2 (1st

trimester) to 3.1pmol/l ± 0.5 (p<0.01). NcAMP (figure 3.4) was unchanged

during pregnancy, however showed a tendency to rise in parallel with PTH and

PTHrP post natally. 1st trimester NcAMP was 15.8nmol/l GF ± 2.3 and was

21.3nmol/l GF ± 3.4 at the post natal visit (p=NS). Neither PTH nor PTHrP alone

was significantly correlated with NcAMP. Osteocalcin (figure 3.6), though

initially lower than normal pre-pregnancy levels, rose through pregnancy from a

nadir of 1.2ng/ml ± 0.2 to a peak of 2.4ng/ml ± 0.3 (post natal) (p<0.05). While

the pattern of change of PTH and osteocalcin through pregnancy was similar, no

significant correlation was evident between these two parameters. Adjusted

serum calcium showed no significant change through pregnancy or the

puerperium (figure 3.7). Mean concentration at 1st trimester was 2.36 ±

0.02mmol/l, at term was 2.42 ± 0.02mmol/l and post partum was 2.35 ±

0.03mmol/l (p=NS). Changes in urinary Ca/Cr are shown in figure 3.8. Values
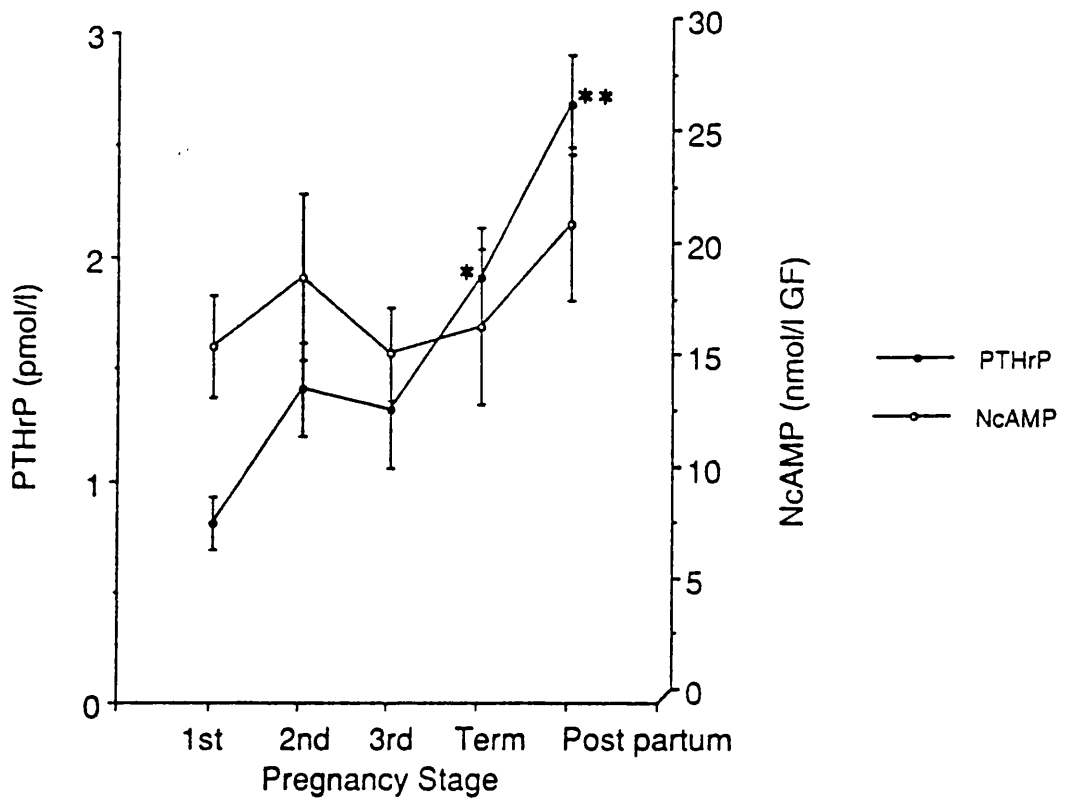
Figure 3.4

Changes in concentrations of nephrogenous cyclic adenosine monophosphate

(NcAMP) and plasma parathyroid hormone-related protein through normal

pregnancy. Points shown represent mean ± SEM.
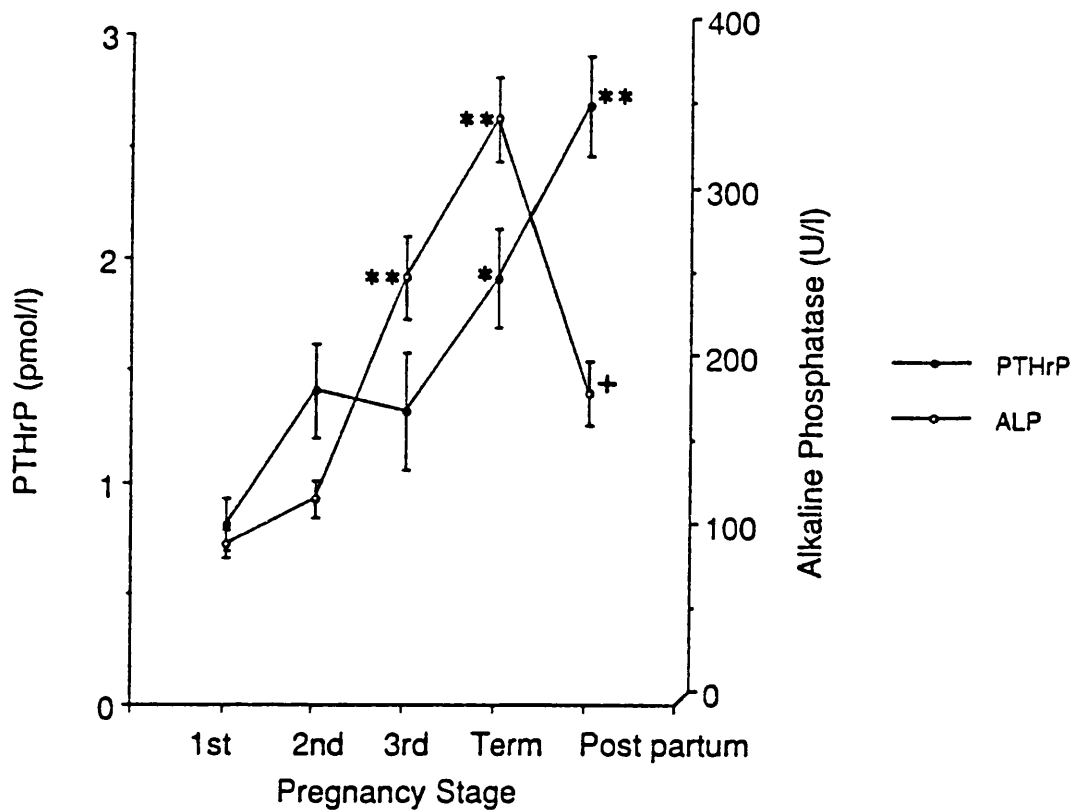
*p<0.02 **p<0.00001

Figure 3.5

Changes in serum concentration of alkaline phosphatase (ALP) and plasma parathyroid hormone-related protein (PTHrP) through normal pregnancy. Points shown represent mean ± SEM.

*$p < 0.02$ **$p < 0.00001$ +$p = 0.04$ vs term
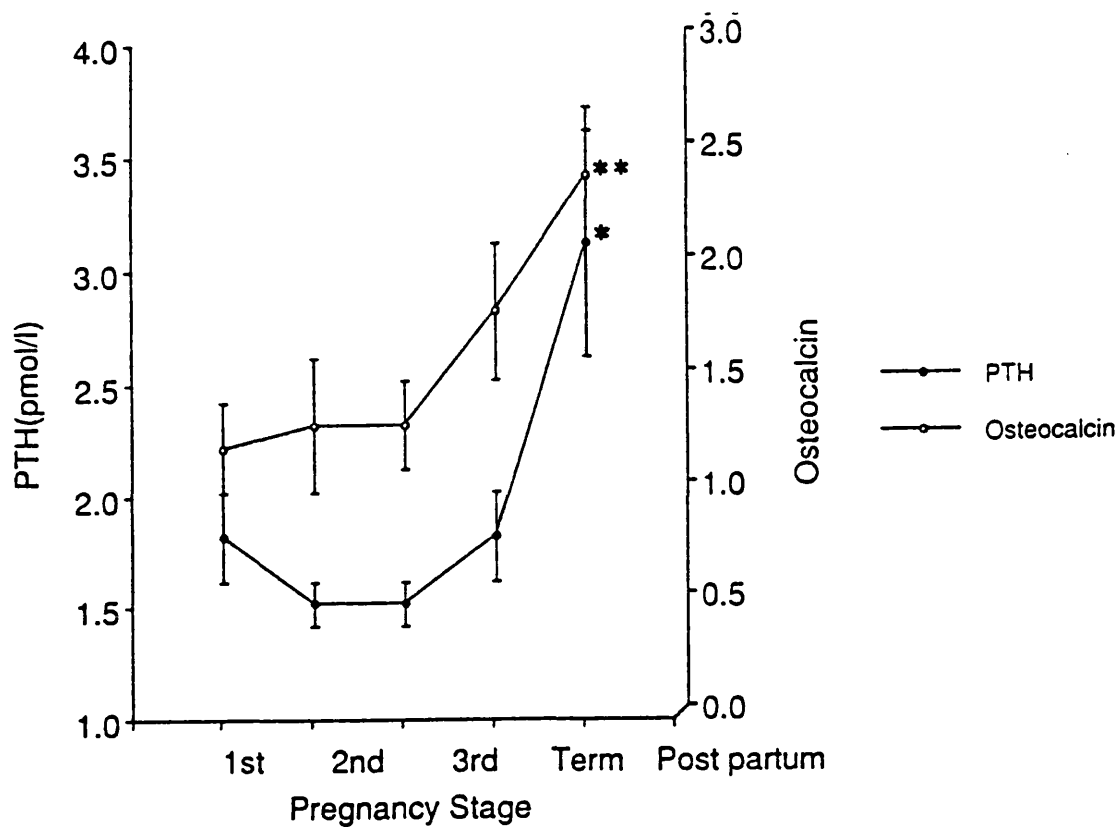
Figure 3.6

Changes in serum concentrations of osteocalcin and parathyroid hormone (PTH) through normal pregnancy. Points shown represent mean ± SEM.

*p<0.01 **p<0.05
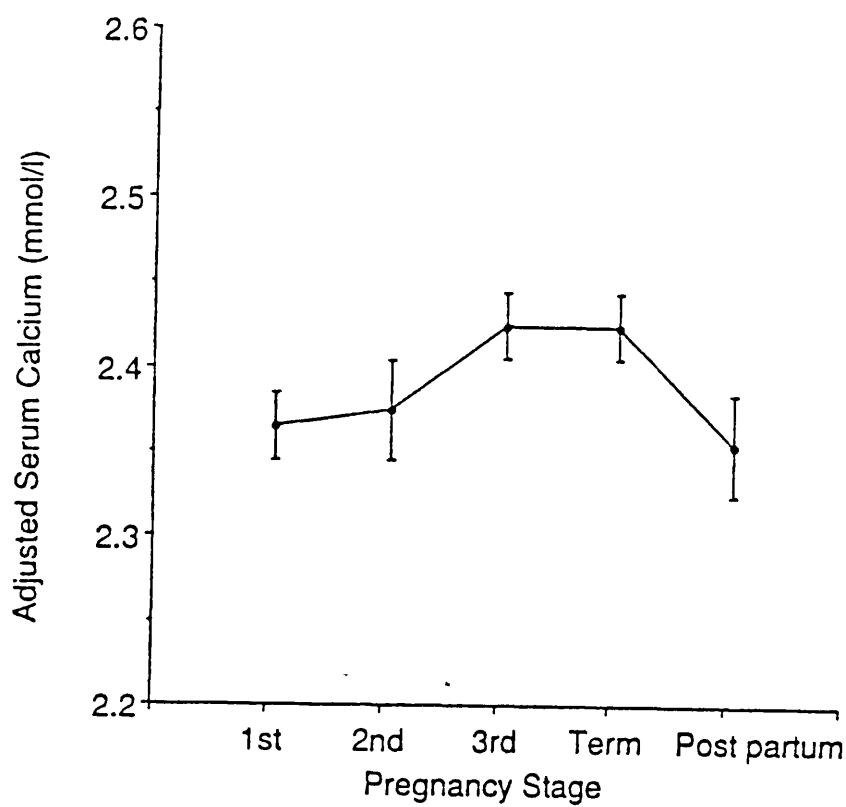
Figure 3.7

Changes in serum albumin adjusted calcium (mmol/l) through normal pregnancy. Points shown are means ± SEM
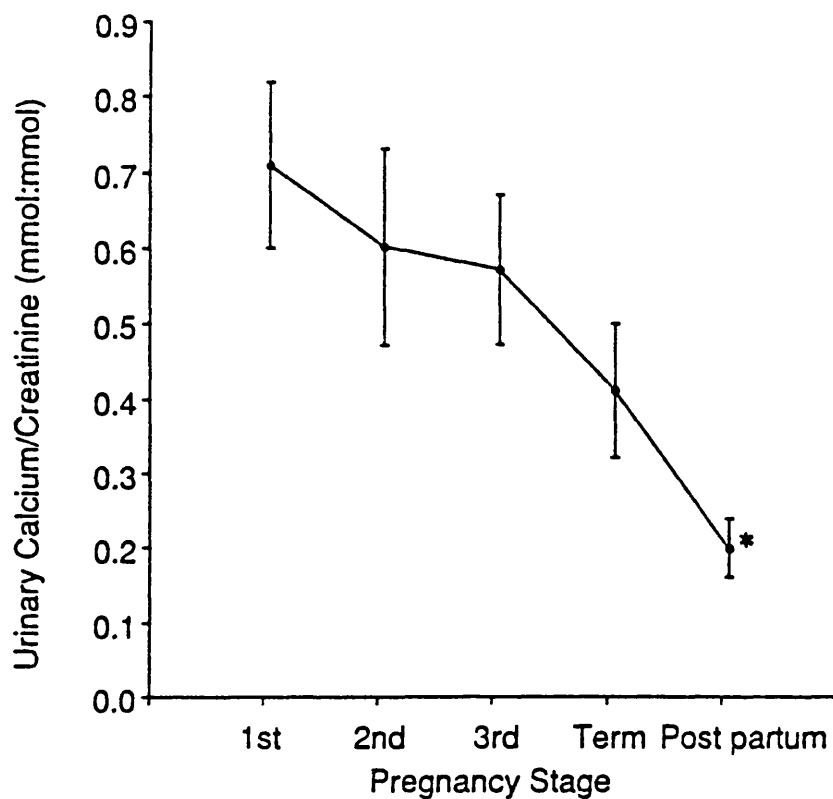
Figure 3.8

Changes in fasting urinary calcium/creatinine ratio through normal pregnancy.

Points shown are mean ± SEM.

*p<0.02

fell through pregnancy from a peak of 0.70 ± 0.11 at 1st trimester to a nadir of 0.19 ± 0.04 at post natal assessment (p<0.02).

3.2.4 Discussion

Results from this study show that plasma concentrations of PTHrP rise through pregnancy (albeit within the normal reference range) and continue to rise after birth at least as far as 6 weeks into the post natal period. It would appear likely that this post-natal rise in plasma PTHrP is secondary to the very high levels of PTHrP usually found in breast milk. Khosla et al.(395) reported that, though breast milk PTHrP levels may rise greatly, maternal plasma PTHrP concentrations usually remain within the reference range as was the case in this study. It has been suggested that suckling is the major stimulus to PTHrP secretion (396), however since only 1 of the 10 patients in this study was breast feeding, and this patient did not have the highest plasma PTHrP concentration, this would seem unlikely. More likely is the suggestion that PTHrP secretion may be associated with increases in circulating serum prolactin (397) independent of suckling.

Though the breast is the probable major source of plasma PTHrP in the puerperium, the placenta is the likely major source during pregnancy. This is suggested in this study by the close relationship up to the time of delivery between PTHrP and alkaline phosphatase which is known to be produced by the placenta (398). This would be consistent with the work of Rodda et al.(95) who showed PTHrP to be present in sheep placenta, with in that case highest levels found in mid gestation. After delivery, with placental separation ALP levels start to fall, however PTHrP levels continue to rise presumably due to breast production as suggested above.

NcAMP is generally considered to be a reliable indicator of PTH and/or PTHrP bioactivity (76,84). No significant changes were seen in NcAMP during pregnancy though levels did tend to rise in the post partum period at which point PTH and PTHrP levels were at their highest. In normal circumstances bioactive PTH is responsible for the production of over 90% of NcAMP (76). PTHrP, however, is also a potent stimulator of NcAMP production (84). When both PTH and PTHrP are present in the circulation together, such as is the case in this study, the usual close association between these parameters would appear to be lost - presumably as both are simultaneously exerting a potent stimulative effect on NcAMP production. Furthermore, concentrations of both PTH and PTHrP generally remain within their respective normal reference ranges and any stimulus to NcAMP production is likely to be weak.

Previous studies have suggested that while total serum calcium may fall slightly during pregnancy due to haemodilution, no significant changes are noted in ionised calcium (399,400). In this study albumin adjusted calcium was measured, which has been previously shown to correlate closely with ionised calcium in the non-pregnant state (152). As other workers have described with ionised calcium (399,400), no significant change in albumin adjusted calcium was noted in this study in pregnancy or the puerperium.

In studies where radioimmunoassays for PTH have been used, it has been suggested that PTH levels increased during pregnancy with a state of secondary hyperparathyroidism being present (401,402). In this study, however, and other more recent studies where immunoradiometric assays have been used (399,400), PTH levels tend to fall and rise again after delivery. This rise in PTH post delivery is likely to indicate a state of secondary hyperparathyroidism which may exist to compensate for the transfer of calcium into breast milk. This may also explain the low fasting urinary calcium/creatinine ratio seen at this time (403). It

is likely that bone resorption is increased after delivery in order to mobilise calcium. As bone resorption and formation are usually coupled, it is likely that bone formation rates are also increased. The finding of increased levels of osteocalcin in the puerperium in this study would be in keeping with this hypothesis (404). Similar increases in serum osteocalcin concentrations have been noted by other workers (405,406). Seki et al.(400) noted a significant correlation between PTH and osteocalcin; this was not a feature of this present study; though the pattern of change of PTH and osteocalcin through pregnancy and the puerperium was similar. 1,25-dihydroxyvitmin D_3 has also been shown to rise through pregnancy (400). This is likely to be due to both an increase in vitamin D binding protein (407) and an increase in the rate of conversion of vitamin D_3 to 1,25(OH)2D3 due to the action of placental 1-alpha hydroxylase (408). Furthermore, since PTH has a potent stimulative effect on 1,25(OH)2D3 production (14), 1,25(OH)2D3 levels are generally high in the puerperium (400,401). Osteocalcin production is in turn stimulated by 1,25(OH)2D3 (409) and this relationship may also contribute to the increases noted in serum osteocalcin concentrations.

Fasting urinary calcium/creatinine fell significantly during pregnancy and fell further in the puerperium. The reasons for this are not clear; hypocalciuria has previously been shown to be associated with pre-eclampsia (410,411,412), however none of the patients studied developed pre-eclampsia or hypertension. A more likely explanation is that the hypocalciuria present reflects the presence of a maternal negative calcium balance due to the transfer of calcium from mother to fetus in pregnancy and, as suggested above, mother to breast milk in the puerperium.

In summary it would appear that the changes in plasma concentrations of PTHrP may be observed in pregnancy and the puerperium. It is likely that the placenta

and breast are the main sources of this increased production of PTHrP. It is not clear, however, whether these changes in plasma PTHrP concentrations have a significant effect on maternal calcium homoeostasis independent of PTH and $1,25(OH)_2D_3$.

3.3 Acute effects of intravenous 1α-hydroxycholecalciferol (alfacalcidol) on parathyroid hormone, osteocalcin and 1,25-dihydroxyvitamin D₃

3.3.1 Introduction

The previous two studies were concerned principally with the direct and indirect assessment of parathyroid hormone-related protein (PTHrP). Currently available biochemical assays can also give useful information concerning the physiological and pathophysiological interactions between the other major hormones and proteins involved in the parathyroid hormone (PTH)/vitamin D axis both in the normal state and in patients with various disorders of bone and calcium metabolism.

In recent years 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) has been increasingly recognised as a key hormone in the control of PTH secretion (413). Conversely PTH is also of major importance in the metabolism of 1,25(OH)₂D₃, with one of its principal actions being stimulation of the 1α-hydroxylase enzyme which leads to increased conversion of 25-hydroxyvitamin D₃ (25(OH)D₃) to the active metabolite of vitamin D, 1,25(OH)₂D₃ (14).

Further evidence of the interactions between PTH and 1,25(OH)₂D₃ comes from the fact that chronic oral administration of 1,25(OH)₂D₃ has been successfully used to suppress excess parathyroid hormone (PTH) secretion in conditions such as chronic renal failure. However, the doses required are usually associated with the development of hypercalcaemia (30). It has been noted, however, that adequate PTH suppression can be achieved without this concomitant rise in serum calcium when either 1,25(OH)₂D₃ or 1α-hydroxycholecalciferol

(alfacalcidol) (which leads to an acute rise in 1,25(OH)$_2$D$_3$) is given intravenously rather than orally (31).

Administration of intravenous alfacalcidol is, therefore, a useful way of stimulating an acute rise in 1,25(OH)$_2$D$_3$ which would allow the acute effects of 1,25(OH)$_2$D$_3$ upon other peptides to be studied.

In this study the acute effects of a single intravenous bolus of alfacalcidol upon the PTH/vitamin D axis is described.

3.3.2 Patients and Methods

A single intravenous bolus of 2ug alfacalcidol was given to 6 normal males (mean age 33 years, range 30-36), 6 women with primary hyperparathyroidism (PHPT) (mean age 72 years, range 65-78) and 6 women with established osteoporosis (mean age 63 years, range 52-70). The diagnosis of primary hyperparathyroidism was made on the basis of the presence of hypercalcaemia associated with either elevated or inappropriately detectable PTH. The diagnosis of osteoporosis was made on the basis of the presence of a least one vertebral compression fracture on X-ray associated with a bone density, measured by DXA, of at least one standard deviation below that of age-matched controls.

All studies commenced at 9am with the subject fasting, blood was drawn through an indwelling peripheral intravenous cannula at time zero, just before administration of alfacalcidol, and at 1 hour, 2 hours, 3 hours, 5 hours, 8 hours, 12 hours and 24 hours afterwards. All subjects were ambulant during the day, took usual meals at 0900, 1200, and 1700 and went to sleep at midnight. Measurement was made at each time-point of serum calcium, albumin and alkaline phosphatase, intact PTH(1-84), 1,25(OH)$_2$D$_3$, and osteocalcin.

Statistical comparison within each given treatment group was carried out using Sheffe's test for multiple simultaneous comparisons, while one way analysis of variance was used to assess differences between the groups. Comparison between the baseline parameters in all three groups was carried out using a Mann-Whitney U-test.

3.3.3 Results

In each group $1,25(OH)_2D_3$ levels rose to a peak 2-3 hours after administration of alfacalcidol (figure 3.9). Thereafter levels fell slowly though mean values tended to remain above baseline even at 24 hours. As might be expected basal $1,25(OH)_2D_3$ concentrations were highest in the PHPT group (table 3.2). Highest peak levels were also found in PHPT group at (mean ± SEM) $150±15$pmol/l versus $114±15$ (controls) ($p<0.01$) and $127±15$ (osteoporosis) ($p<0.05$). One way analysis of variance showed that the rise in $1,25(OH)_2D_3$ was greater in the PHPT patients than in the normal subjects and osteoporotic patients ($F=4.1$, $df=1/64$, $p<0.05$). No statistically significant difference in the $1,25(OH)_2D_3$ response was evident between the normal control subjects and the osteoporotic patients.

Changes in PTH observed are shown in figure 3.10. Again as expected highest basal values are seen in the PHPT group (table 3.2). While there was an initial trend downwards in PTH concentrations in both the PHPT patients and controls, this was not statistically significant.

Changes in adjusted serum calcium are shown in figure 3.11. As with PTH and $1,25(OH)_2D_3$, basal serum calcium was highest in PHPT group (table 3.2). After administration of alfacalcidol no significant changes in serum calcium concentrations were noted during the period of monitoring in any of the three study groups.
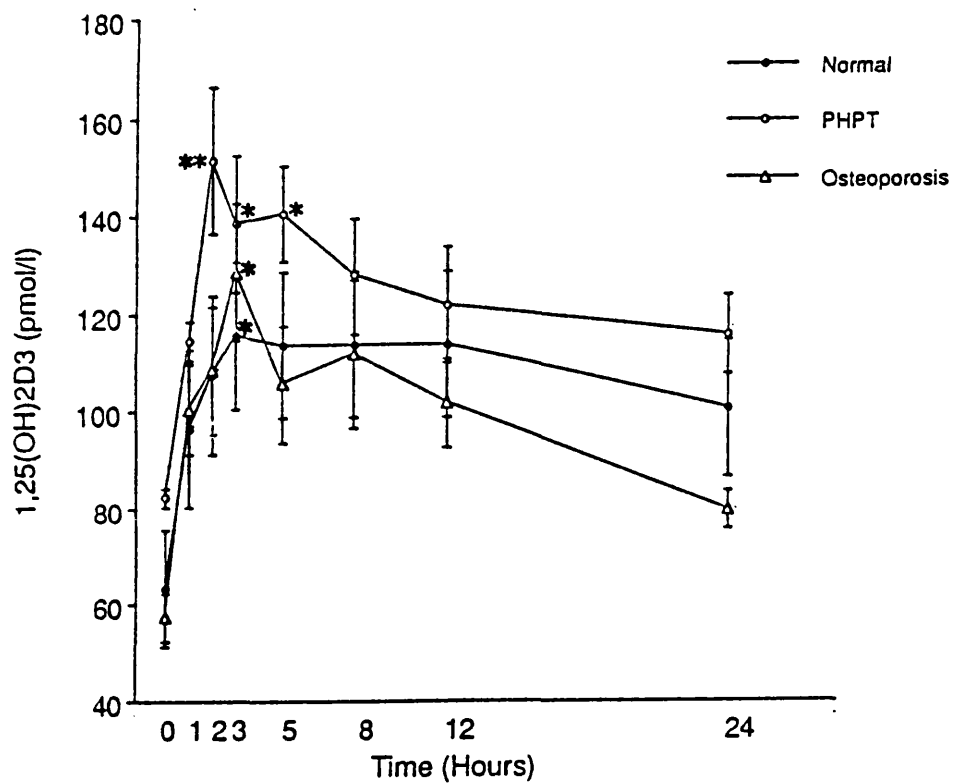
Figure 3.9

Changes in serum 1,25-dihydroxyvitamin D3 (1,25(OH)$_2$D$_3$) concentrations in the three treatment groups. Alfacalcidol was administered after time 0. Points shown are means ± SEM.

*p<0.01 **p<0.05 (versus baseline)
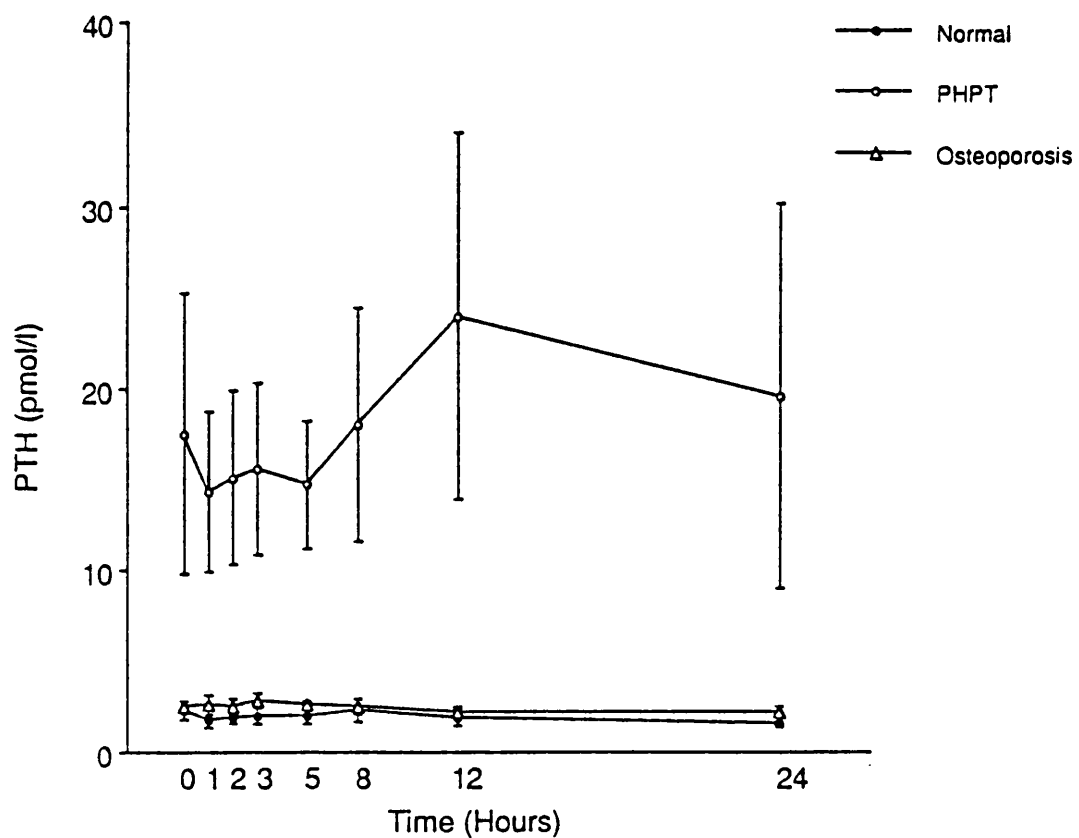
Figure 3.10

Changes in serum parathyroid hormone (PTH) concentrations in the three

treatment groups. Alfacalcidol was administered after time 0. Points shown are
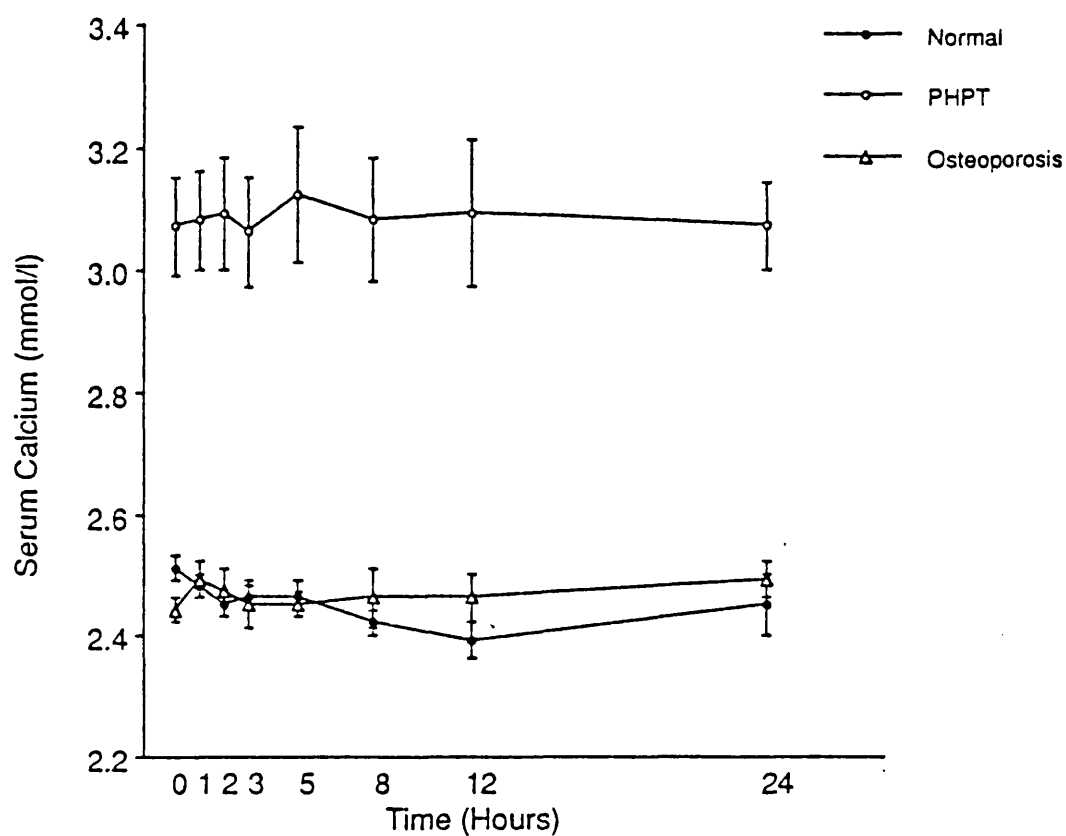
means ± SEM.

Figure 3.11

Changes in adjusted serum calcium in the three treatment groups. Alfacalcidol was administered after time 0. Points shown are means ± SEM.

Table 3.2

Baseline biochemical parameters prior to alfacalcidol admiistration in the three treatment groups studied

	Normal	Osteoporosis	PHPT
1,25(OH)2D3(pmol/l)	62	56	81*
SEM,range	12,27-88	5,42-74	2,74-88
PTH (pmol/l)	1.9	2.1	17.1**
SEM, range	0.5,0.8-4.2	0.3,1.2-3.4	7.7,8.5-48.0
Calcium (mmol/l)	2.50	2.43	3.06**
SEM, range	0.02,2.45-2.55	0.02,2.35-2.50	0.08,2.85-3.35
ALP (U/l)	170	182	259
SEM, range	11,130-210	16,135-245	72,100-520
Osteocalcin (ng/ml)	3.5	3.3	6.9+
SEM, range	1.0,0.7-6.9	1.3,0.8-6.7	0.5,5.8-8.1

*p<0.01 vs osteoporosis, p<0.05 vs normal   **p<0.01 vs normal and osteoporosis   +p<0.05 vs normal and osteoporosis

Changes in serum osteocalcin and alkaline phosphatase are shown in figures 3.12 and 3.13. While basal levels of ALP tended to be highest in the PHPT group this was not statistically significant (table 3.2). Basal levels of osteocalcin, however, were significantly higher in the PHPT group (table 3.2). Following administration of alfacalcidol no significant changes in either ALP or osteocalcin levels were seen and there were no differences in the response to alfacalcidol between the three groups.

3.3.4 Discussion

This study illustrates the changes which occur in $1,25(OH)_2D_3$ after a single intravenous injection of alfacalcidol. In all treatment groups peak concentrations of $1,25(OH)_2D_3$ were found between 2 and 3 hours after intravenous alfacalcidol with levels still tending to be elevated at 24 hours.

Prolonged courses of intravenous $1,25(OH)_2D_3$ or 1α-hydroxycholecalciferol (alfacalcidol) therapy have been shown to successfully suppress the excess PTH secretion associated with chronic renal failure without the development of concomitant hypercalcaemia (31). Where alfacalcidol is used 25-hydroxylation must first take place for the formation of $1,25(OH)_2D_3$ (414). It is generally believed that this metabolic step is not stringently regulated in human liver cells (415). Although, in contrast, activity of this enzyme may be more tightly regulated in some other species (416).

The results in this present study are similar to those described by Papapoulos et al.(417) in a group of patients with chronic renal failure on regular haemodialysis. In that study the response of $1,25(OH)_2D_3$ to intravenous alfacalcidol appeared to be linear in the dose range 1-4ug. Papapoulos et al.(417) also found that $1,25(OH)_2D_3$ remained significantly elevated for a prolonged
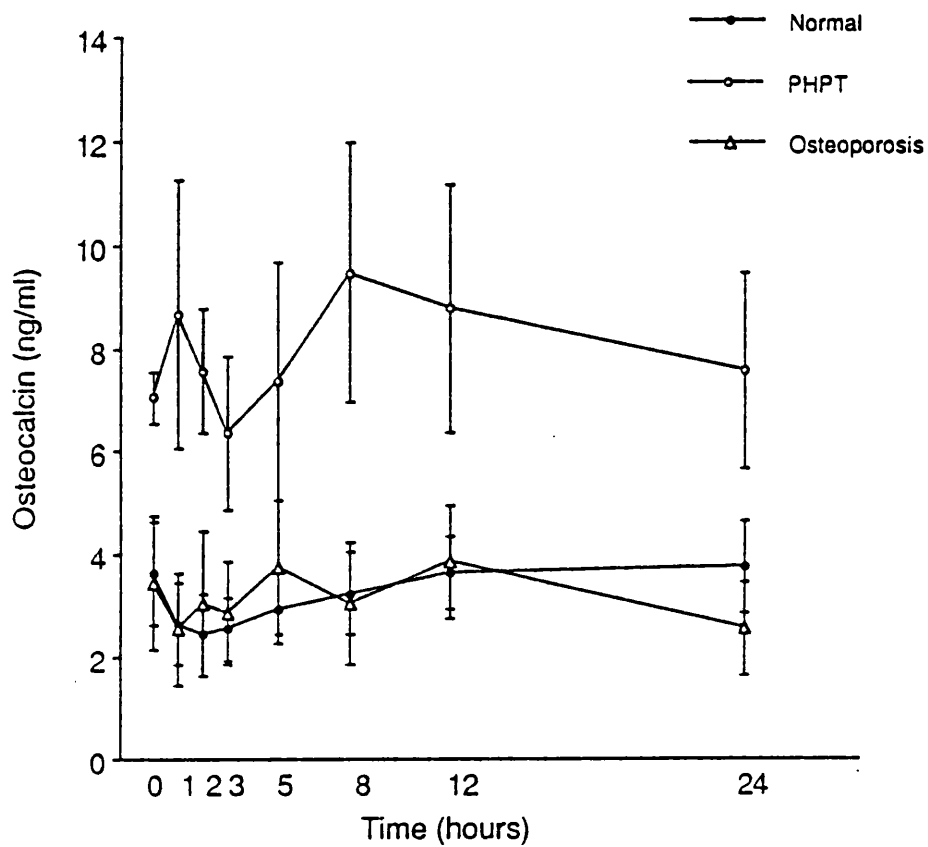
Figure 3.12

Changes in serum osteocalcin concentrations in the three treatment groups.

Alfacalcidol was administered after time 0. Points shown are means ± SEM.

period - returning to normal only after 1 week. Mawer et al.(418) described similar changes in serum $1,25(OH)_2D_3$ after the oral administration of $1,25(OH)_2D_3$. While peak changes in $1,25(OH)_2D_3$ have been shown to occur within 15 minutes of the intravenous administration of $1,25(OH)_2D_3$ (419), the slower time to peak $1,25(OH)_2D_3$ concentrations obtained in this study presumably reflects the time required for hepatic 25-hydroxylation to take place.

Both the basal concentration and the absolute increase in $1,25(OH)_2D_3$ was significantly higher in the PHPT group. Since all three groups received the same dose of alfacalcidol this would suggest that the activity of hepatic 25-hydroxylase is increased in patients with primary hyperparathyroidism. However, contrary to the experience of Silverberg et al.(420) there was no evidence of impairment of $1,25(OH)_2D_3$ formation in those patients with osteoporosis. This study, however, cannot exclude other explanations for the changes in $1,25(OH)_2D_3$ observed in the PHPT patients such as impaired $1,25(OH)_2D_3$ metabolism or clearance.

The activity of the renal 1α-hydroxylase enzyme has been shown to decline with increasing age (421), however similar age effects on the 25-hydroxylase enzyme would not explain the changes seen in this study. The greatest rise in $1,25(OH)_2D_3$ (and therefore the greatest 25-hydroxylase activity) was seen in the PHPT patients who were also the oldest.

No significant changes were noted in intact PTH concentrations in this present study. Llach et al.(422) similarly failed to demonstrate any changes in immunoreactive PTH concentrations after the acute administration of 2.7ug oral $1,25(OH)_2D_3$. The reasons for this apparent lack of effect on PTH secretion remain unclear particularly since Chertow et al.(423) showed that, in vitro, $1,25(OH)_2D_3$ inhibits the release of PTH from parathyroid slices within 2 hours.

This work was carried out in the rat and certainly species differences may, at least in part, explain this discrepancy.

Intravenous alfacalcidol and $1,25(OH)_2D_3$ are being increasingly used to treat the secondary hyperparathyroidism associated with chronic renal failure (31,417,424,425). These studies have generally been carried out over weeks or months and are associated with significant PTH suppression, usually without concomitant hypercalcaemia. This may be due either to a direct effect of $1,25(OH)_2D_3$ in suppressing PTH synthesis (426) or an alteration to the set point for serum calcium such that PTH is suppressed at a lower serum calcium concentration than pretreatment (31).

No significant rise in serum calcium was noted in any of the 3 groups studied. In fact, the mean serum calcium concentration tended to fall in the normal subjects in the initial 12 hours after administration of alfacalcidol. This was also noted by Llach et al.(422), where again this change was transient and not statistically significant.

In addition to suppressing PTH synthesis, an increase in the concentration of $1,25(OH)_2D_3$ is known to stimulate osteoblastic production of osteocalcin (409). In this study basal levels of osteocalcin were higher in the patients with PHPT, in keeping with their increased bone turnover. By contrast, as has been described previously (404), osteocalcin levels in the osteoporotic patients were no different from normal. The acute administration of intravenous alfacalcidol, however, was not associated with any increase in osteocalcin concentrations in any of the three groups studied. As is the case with PTH this may be because $1,25(OH)_2D_3$ is likely to exert its effect on osteocalcin by stimulating synthesis rather than secretion. Duda et al.(427) demonstrated significant increases in osteocalcin in both normal and osteoporotic postmenopausal women after oral administration of

2ug 1,25(OH)2D3 daily. In that study, however, increases were first described between days 1 and 3. No comment was made on changes within 24 hours. Similar results were noted by Gram et al.(428) where again 2ug 1,25(OH)2D3 was given orally, in that case to normal males. This led to significant increases in osteocalcin after 1 week, however again no measurements were made within this period. Duda et al.(427) also noted that while osteocalcin levels increased, serum alkaline phosphatase concentrations did not change. The reason for this discrepancy between alkaline phosphatase and osteocalcin was not apparent.

In conclusion this study describes for the first time the changes in 1,25(OH)2D3 which occur in normal subjects and patients with primary hyperparathyroidism and osteoporosis after a single intravenous injection of alfacalcidol. These results would suggest that liver 25-hydroxylase activity is increased in primary hyperparathyroidism. Furthermore 1,25(OH)2D3 does not appear to alter serum PTH concentrations in the acute situation. Similarly 1,25(OH)2D3 does not appear to have an acute effect on stimulating serum osteocalcin concentrations. These results would suggest that the changes of PTH suppression and osteocalcin stimulation seen in longer-term studies occur via changes in hormone synthesis rather than secretion.

CHAPTER 4

DUAL-ENERGY X-RAY ABSORPTIOMETRY (DXA) IN THE EVALUATION OF OSTEOPOROSIS

4.1 **Bone turnover and densitometry in type I diabetes mellitus**

4.1.1 **Introduction**

The development of dual-energy X-ray absorptiometry (DXA) has represented a significant technological breakthrough in the assessment of osteoporosis. This technique allows both accurate and precise measurement of bone density at the lumbar spine (L2-4) and neck of femur with negligible patient radiation exposure (274). Furthermore this technique allows significantly faster scan times than was the case with dual-photon absorptiometry which therefore permits access to this facility to greater number of patients (272,273).

The improved precision and accuracy of this technique over previously available methods means that reliable bone density data can be obtained in cross-sectional and longitudinal studies investigating both the effect of anti-osteoporotic therapy and the prevalence of osteoporosis secondary to other medical conditions.

One such example is in the assessment of patients with diabetes mellitus where a possible association with osteoporosis has been recognised for many years (349). However, densitometric studies carried out in patients with type I diabetes mellitus using either single photon absorptiometry (351,352) or dual photon absorptiometry (353) have, on the whole, been inconclusive with some confirming and others refuting this association. Some evidence also exists that diabetic patients have an increased incidence of bone fracture (359,360) but again this evidence is conflicting. In this study dual-energy X-ray absorptiometry (DXA) was used to assess lumbar and femoral neck bone densities in a cohort of 20 premenopausal women with type I diabetes mellitus. In addition, measurement was made of a number of biochemical indices of bone turnover.

4.1.2 <u>Patients and methods</u>

Twenty females with type I diabetes mellitus, mean age 32 years (range 23-42) and mean diabetes duration 8.8 years (range 2-22), presenting consecutively for review were recruited and compared with a group of 27 age matched female controls, mean age 34 years (range 24-42). These control females had been recruited by a local advertising campaign to take part in a study investigating normal bone density in the West of Scotland. Each diabetic patient was paired by age with at least one non-diabetic control. Where more than one age matched control was available, the mean of their bone densities was calculated and used to compare with the appropriate diabetic patient. None of the diabetic patients or control subjects suffered from any other medical condition or were taking any medication thought likely to interfere with their bone metabolism. All subjects were premenopausal at the time of study. Furthermore none of the diabetic patients had biochemical evidence of renal impairment and the mean daily dose of insulin in the diabetic patients was 44 units per day (range 18-86).

A single measurement was obtained of L2-4 spinal density and neck of femur density and a fasting blood and urine sample obtained. All bone density measurements obtained were adjusted for body weight using standard software. Assessment of medium-term diabetic control was made by measurement of glycated haemoglobin (HbA1). Body mass index (BMI) was calculated using the formula - weight (kg)/height(m)^2.

Statistical analyses between groups was carried out using a paired Student's t-test. 95% confidence intervals (95% CI) for differences between means are also shown. The results obtained for each group tended to be skewed and the data was, therefore, logarithmically transformed prior to a t-test being carried out. Correlations were performed using Spearman Rank Correlations.

4.1.3 Results

These patients were well matched in terms of size as the mean BMI of the diabetic patients was 26.5 ± 1.3 kg/m^2 versus 24.2 ± 1.2kg/m^2 for the controls (p=NS, 95% CI; -2.7 to 5.6). Mean HbA1 in the diabetic patients was 10.2% (range 6.7-19.5) (normal 4.9-8.3%) compared with 6.1% (range 4.4-7.4) in the controls (p<0.0001).

Results for L2-4 and neck of femur bone densities in the diabetic patients and their controls are shown in figures 4.1 and 4.2. Mean ($\pm$SEM) L2-4 density was significantly higher in the diabetic patients at 1.224 ± 0.021g/cm^2 versus 1.142 ± 0.015g/cm^2 (controls), p=0.016 (95% CI for difference between means: 0.011 to 0.110g/cm2). Mean ($\pm$SEM) neck of femur density, however, did not differ significantly between the two groups (0.963 ± 0.026g/cm^2 [diabetic] versus 0.960 ± 0.028g/cm^2 [controls]; p=NS)(95% CI for difference between means: -0.089 to 0.067g/cm^2).

Biochemical results for the diabetic patients and control subjects are shown in table 4.1. Mean ($\pm$SEM) serum alkaline phosphatase (figure 4.3) was significantly higher in the diabetic patients at 185 ± 16U/l versus 135 ± 10U/l (controls), p<0.01 (95% CI: 12 to 70U/l). Mean fasting HP/Cr ratio was also elevated in the diabetic patients at 0.028 ± 0.003 versus 0.017 ± 0.002 (controls): p=0.002 (95% CI: 0.004 to 0.015) (figure 4.4). Mean fasting urinary calcium/creatinine ratio was elevated in the diabetic patients at 0.40 ± 0.06 versus 0.21 ± 0.03: p=0.013 (95% CI: 0.05 to 0.33). No difference was evident, however, in serum calcium, PTH or 1,25(OH)$_2$D$_3$. In addition NcAMP was within the normal reference range in the diabetic patients indicative of normal PTH activity. Mean γGT was normal in both groups. No significant correlation was evident
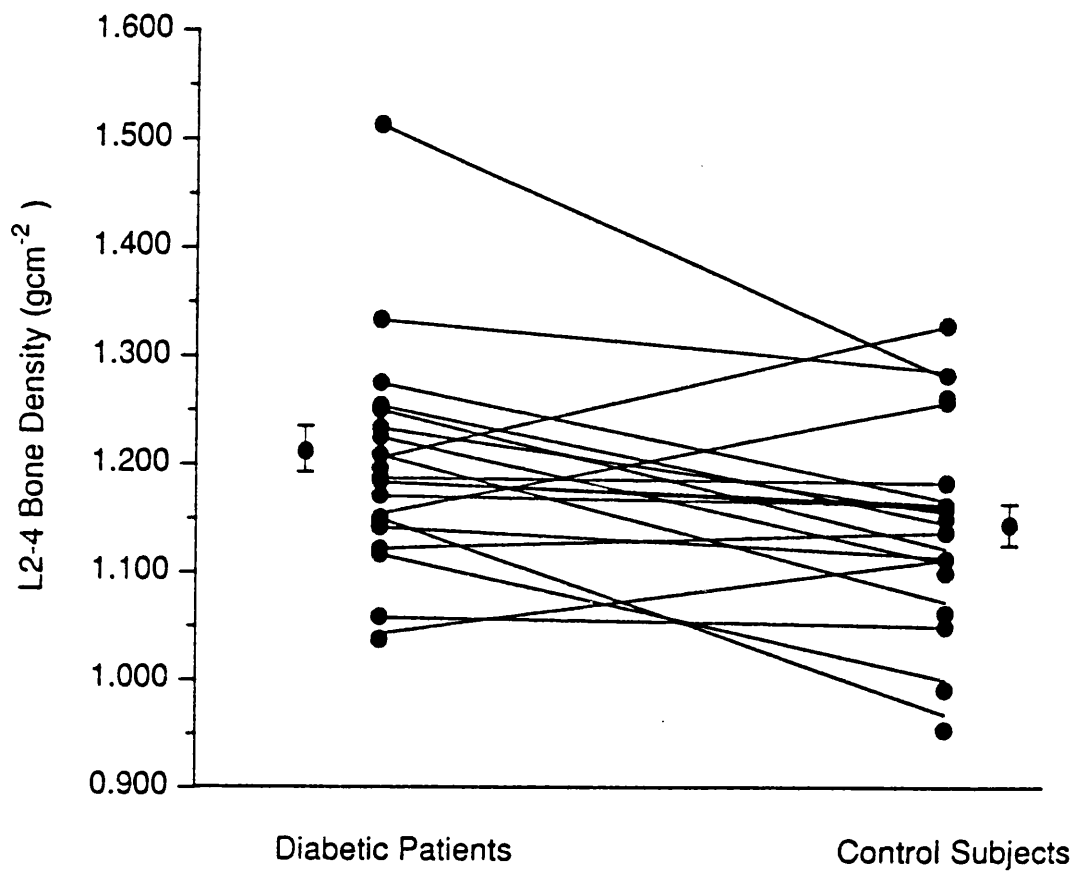
Figure 4.1

Spinal (L2-4) bone density in the diabetic patients and age-matched control subjects. Horizontal bars indicate means with standard errors (SEM) also shown. Difference between means: p=0.016.
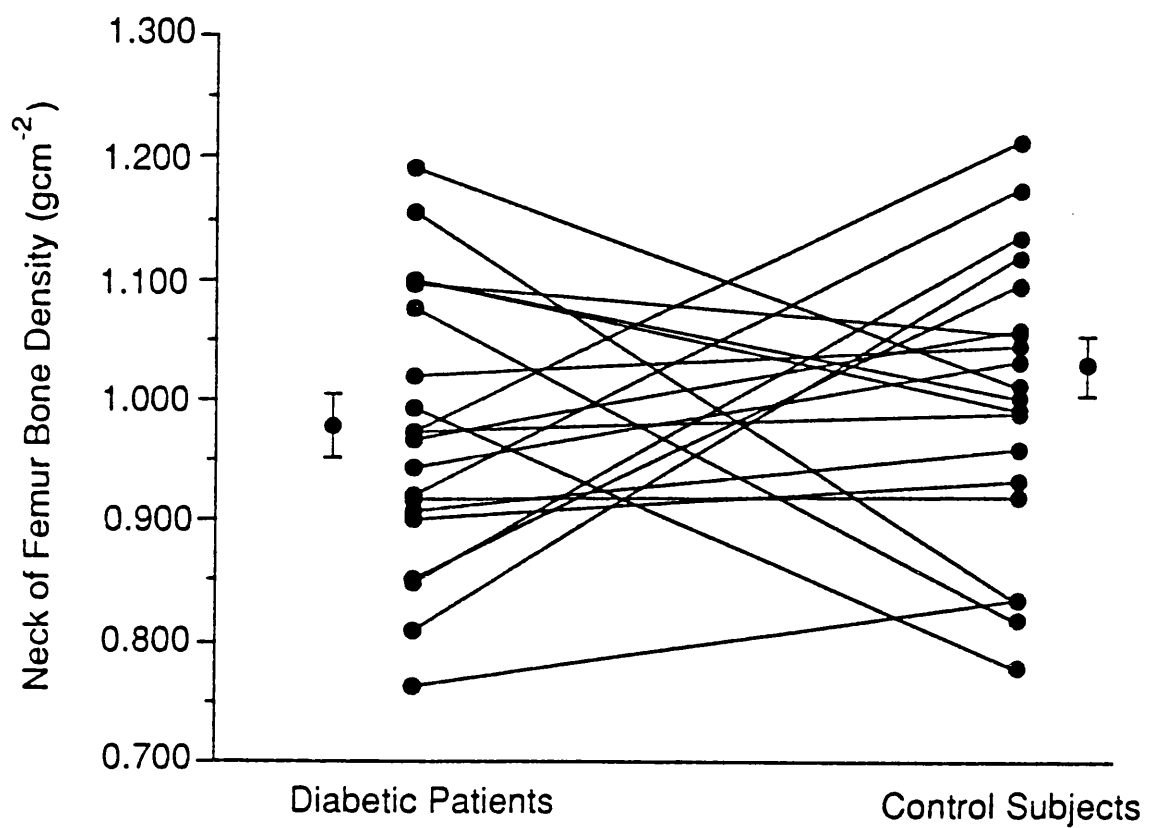
Figure 4.2

Neck of femur bone density in the diabetic patients and age-matched control subjects. Horizontal bars indicate means with standard errors (SEM) also shown. Difference between means: p=NS.
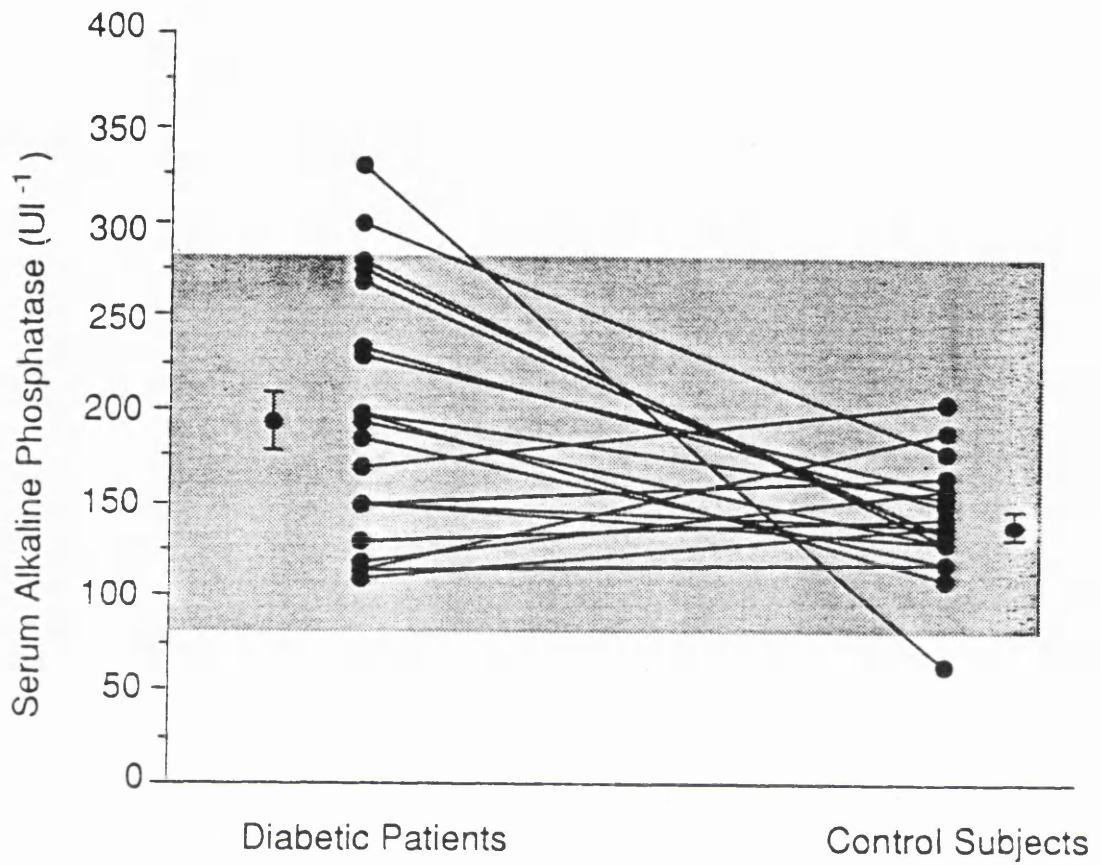
Figure 4.3

Serum alkaline phosphatase in the diabetic patients and age-matched control
subjects. Horizontal bars indicate means with standard errors (SEM) also shown.
Shaded area indicates normal reference range for alkaline phosphatase.
Difference between means: p<0.01.
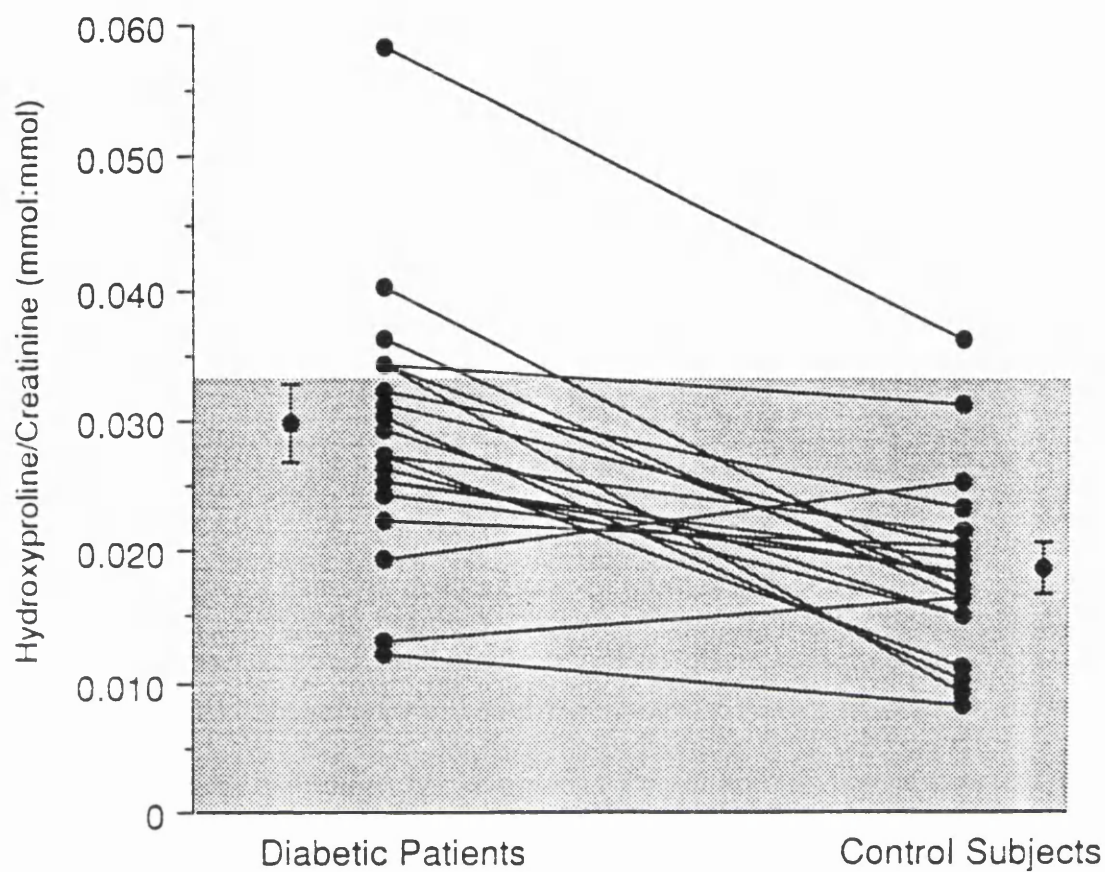
Figure 4.4

Urinary hydroxyproline/creatinine ratio (HP/Cr) in the diabetic patients and age-matched control subjects. Horizontal bars indicate means with standard errors (SEM) also shown. Shaded area indicates normal reference range for HP/Cr.

Difference between means: p=0.002.

Table 4.1

Biochemical results of the diabetic patients and non-diabetic control subjects (Values shown are means)

	Diabetic	Non-Diabetic	p
ALP (U/l)	181	135	<0.01
SEM, range	16,100-320	10,55-195	
γGT (U/l)	18	20	NS
SEM, range	2,10-33	1,11-30	
HP/Cr (mmol:mmol)	0.028	0.017	0.002
SEM, range	0.002,0.011-0.057	0.002,0.007-0.035	
Ca/Cr (mmol:mmol)	0.40	0.21	0.013
SEM, range	0.06,0.15-0.83	0.03,0.07-0.53	
Calcium (mmol/l)	2.41	2.39	NS
SEM, range	0.01,2.30-2.50	0.02,2.30-2.50	
Mean PTH (pmol/l)	2.4	2.7	NS
SEM, range	0.4,<0.8-9.3	0.2,1.5-4.3	
Mean NcAMP(nmol/l GF)	15.7	-	-
SEM, range	1.0,7.7-25.9	-	
Mean 1,25(OH)$_2$D$_3$ (pmol/l)	82	73	NS
SEM,range	11,31-158	12,43-115	

ALP - alkaline phosphatase   γGT - gamma glutamyl transferase
HP/Cr - urinary hydroxyproline/creatinine ratio
Ca/Cr - urinary calcium/creatinine ratio
PTH - parathyroid hormone   NcAMP - nephrogenous cyclic adenosine monophosphate
1,25(OH)$_2$D$_3$ - 1,25-dihydroxyvitamin D3

between the diabetic patients L2-4 density and HbA1 (r=0.41, p=NS), duration of diabetes (r=0.22, p=NS) or daily dose of insulin taken (r=0.13, p=NS).

4.1.4 Discussion

The results from this study suggest that while, on the basis of biochemical criteria, bone turnover is apparently increased in premenopausal female diabetics, these patients show no densitometric evidence of osteopenia and may even have higher than normal bone density at the lumbar spine.

In this study bone densities in the diabetic patients were paired with at least 1 non-diabetic control subject. While the mean bone density for the control subjects in this study was apparently a little lower than the UK reference range quoted by Lunar Corporation, this difference is small with overall the control subjects having a bone density of 97.2% of the Lunar reference population. It is possible this difference simply reflects that the norm for the Glasgow population is a little lower than the UK Lunar reference data.

Several studies in the past have suggested that type I diabetes mellitus is associated with an increased incidence of osteoporosis. These studies have generally been carried out in children and adolescents (350,351), and usually show only small reductions in bone density. These findings are not universal, however, as at least one recent study has failed to show any evidence of osteoporosis in young males with type I diabetes and bone densities below normal in this study were only noted in 6.6% of females (429). Furthermore, studies published to date have generally used single photon absorptiometry (SPA) which can only be applied to peripheral sites (most commonly forearm) and measures mainly cortical bone. Relatively little data exists on spine and hip density in patients with diabetes: in one study Auwerx et al.(353), using dual

photon absorptiometry (DPA), demonstrated mean L2-4 density to be below the age adjusted reference range in 14 diabetic women. However, this study did not include a non-diabetic control group. It is possible in this present study that other features such as the presence of large osteophytes or calcification in the aorta could give falsely elevated L2-4 density results. Though it is not possible to exclude these problems as spinal X-rays were not carried out, it would seem an unlikely explanation given the relative youth of the subjects studied.

Results from this present study indicate that bone turnover may be increased in the diabetic patients as illustrated by the elevated levels of serum alkaline phosphatase and urinary hydroxyproline excretion. However increased bone turnover need not necessarily always be associated with the development of osteopenia if bone formation and resorption rates are similar (430). Although further increases in bone resorption such as may be seen after the menopause might, in this situation, be expected to result in a period of rapid bone loss and osteoporosis.

Previously, bone resorption has been shown to be elevated in patients with diabetes, as assessed by urinary hydroxyproline excretion (431), urinary calcium excretion (357,369) and bone histomorphometry (365). Histomorphometric studies have also suggested the presence of a high turnover state (365) akin to primary hyperparathyroidism, though this has generally not been confirmed in biochemical studies where bone formation rates, as measured by osteocalcin, are low (432). However, serum concentrations of ALP in this present study were significantly higher in the diabetic patients and in some instances above the reference range. Since γGT was within the reference range in the diabetic patients and similar to that seen in the non-diabetic controls and since γGT rises in parallel with ALP due to their similar pathway of secretion, it is likely that this

elevation in serum ALP is of bony origin and indicative of increased osteoblastic activity.

Studies in animal models of diabetes have found low serum concentrations of $1,25(OH)_2D_3$ (433). However this observation has only been made in human diabetes in children or adolescents with poorly controlled diabetes or ketoacidosis (434). In contrast, levels of $1,25(OH)_2D_3$ in this study were not low and if anything tended to be a little higher in the diabetic patients. It is possible, though, that small differences in $1,25(OH)_2D_3$ concentrations may exist which have not been observed due to the relatively small numbers of patients in this study.

Early studies in diabetic rats suggested that PTH concentrations were elevated (435). This finding has not been confirmed in more recent studies (436) and was not observed in this study, nor would PTH activity appear to be increased as NcAMP levels were normal.

The reason why the diabetic patients in this study have increased bone density is unclear. Certainly insulin is known to increase serum levels of insulin-like growth factor-1 (IGF-1) (437) and it is known that both insulin and IGF-1 promote osteoblast replication and function (438). It is possible, therefore, that this in turn leads to an increased rate of bone formation.

Another feature of note is that in many previous studies diabetes related osteopenia has occurred soon after the onset of the disease (439) and bone loss did not increase further with increasing duration of diabetes (357). This may be due to the absolute or near absolute deficiency of insulin which accompanies the onset of diabetes mellitus, which is then corrected with the introduction of insulin therapy.

A number of epidemiological studies have suggested that diabetes mellitus is associated with an increased risk of fracture (359,360,440) and a pooled estimate of the published data suggests an increased risk of fracture of approximately twofold in patients with diabetes (361). Against this, however, a case control study from the Mayo clinic failed to show any excess fracture incidence in diabetic patients (362). It is possible that diabetes has an adverse effect on fracture incidence by mechanisms independent of bone mass such as the occurrence of an increased incidence of falls due to the presence of neuropathy or hypoglycaemia (361), indeed in cases described by Nabarro (363), he attributed the presence of vertebral compression fractures to hypoglycaemic seizures.

In conclusion this study shows that when a highly accurate and precise measure of bone density is used, there is no evidence of spinal or femoral neck osteopenia in premenopausal women with type I diabetes mellitus. These patients, however, do have biochemical evidence of increased bone turnover. Should this increased bone turnover persist in the very long term an increased rate of bone loss might be expected after the menopause. Further studies are required to address this question.

4.2 <u>Severe haemophilia A and osteopenia: a densitometric and biochemical assessment</u>

4.2.1 <u>Introduction</u>

The previous study illustrated the value of DXA in being able to confirm or refute the presence of osteopenia. In this next study DXA was used to assess bone density in a group of patients with severe haemophilia A - a condition which hitherto has not been known to be associated with osteopenia.

In 1989 a 31 year old man with severe haemophilia A presented to the regional adult haemophilia centre at Glasgow Royal Infirmary with severe back pain. X-rays of spine confirmed the presence of compression fractures of DV9, DV10 and LV2. There was no history of significant antecedent trauma nor did this patient have any apparent risk factors for osteoporosis. Following this, a 20 year old male with severe haemophilia A presented with a fractured neck of femur after an epileptic seizure. At this point, the question was therefore raised as to whether osteopenia may be associated with haemophilia A.

In this study bone density was measured at the lumbar spine (L2-4) and the neck of femur in 19 men with severe (factor VIIIc level <1iu/dl) haemophilia A.

4.2.2 <u>Patients and methods</u>

A total of 26 patients were identified as suffering from severe haemophilia A from the records of the regional adult haemophilia centre at Glasgow Royal Infirmary and these patients were invited to attend for measurement of bone densitometry and to have a blood sample taken. 19 patients (mean age 41 years, range 18-69) accepted this invitation and each of these brought a true fasting

(second voided) urine sample at time of attendance. Only those patients who did not suffer from human immunodeficiency virus (HIV) infection were considered for recruitment into this study, though 18/19 of these patients were hepatitis C antibody positive.

Bone density at the lumbar spine (L2-4) and neck of femur was measured, as was measurement of serum alkaline phosphatase (ALP), gamma glutamyl transferase (γGT) parathyroid hormone (PTH), 1,25-dihydroxyvitamin D3 (1,25(OH)$_2$D$_3$), testosterone, sex hormone binding globulin (SHBG), leutinising hormone (LH), follicle stimulating hormone (FSH), nephrogenous cyclic adenosine monophosphate (NcAMP) and urinary calcium/creatinine (Ca/Cr) and hydroxyproline/creatinine (HP/Cr) ratios. Using the values obtained for testosterone and SHBG a figure was obtained for the free androgen index (FAI) as described in section 2.1.7.

At the time of study a group of 19 age matched male control subjects were recruited from members of hospital staff. These subjects underwent similar investigation to the haemophiliac patients. For statistical analysis each subject was paired by age with one of the haemophiliac patients.

Other than severe haemophilia A, all of the patients (and control subjects) studied were otherwise well and suffered from no other condition (other than hepatitis - see above) nor were they taking any medication thought likely to interfere with their bone and calcium homoeostasis. No haemophiliac patient was a carrier of hepetitis B antigen and none had clinical evidence of liver disease.

Comparisons between the two study groups was carried out using a two-tailed paired Student's t-test. Results of SHBG and FAI were only available for 17 haemophiliac patients and 12 control subjects, results of γGT were only available

in 17 haemophiliacs and 17 controls and results for urinary HP/Cr were only available for 15 haemophiliacs and 15 controls. Statistical comparison between the two groups for these parameters was carried out using a Mann-Whitney U-test. Results described are means ± SEM.

4.2.3 Results

Bone density at both L2-4 and neck of femur was significantly lower in the haemophiliac patients compared with the control subjects. Density at L2-4 (figure 4.5) was 1.109g/cm2 ± 0.042 in the haemophiliac patients versus 1.234g/cm2 ± 0.027 (controls); p=0.018 and at the neck of femur (figure 4.6) was 0.877g/cm2 ± 0.034 (haemophiliacs) versus 1.067g/cm2 ± 0.032 (controls); p=0.0003. Serum alkaline phosphatase (ALP) (figure 4.7) was significantly higher in the haemophiliac patients at 200U/l ± 10 versus 158U/l ± 8 (controls); p=0.004. Gamma glutamyl transferase (figure 4.8) was significantly higher in the haemophilia patients at 42U/l ± 7 compared to the controls (20U/l ± 2); p=0.007. Serum testosterone (figure 4.9) was also elevated in the haemophilia patients at 26.0nmol/l ± 2.5 versus 17.4 ± 1.6 (controls); p=0.009. However along with this elevated testosterone, SHBG (figure 4.9) was also significantly higher at 56nmol/l ± 6 (haemophilia patients) versus 27nmol/l ± 3 (controls); p=0.0005 and FAI (figure 4.10) was significantly lower at 44 ± 5 (haemophiliac patients) versus 69 ± 7 (controls); p=0.008. No significant difference was evident between either group for LH, FSH, PTH, albumin adjusted calcium, 1,25(OH)$_2$D$_3$, NcAMP, urinary Ca/Cr or urinary HP/Cr (table 4.2).

Seven of the haemophiliac patients gave a history of having sustained a peripheral fracture requiring medical attention since the age of 16. These were fractures of femur, ankle (x2), wrist, clavicle (x2) and metacarpals. None of the control subjects gave a history of fracture since aged 16.
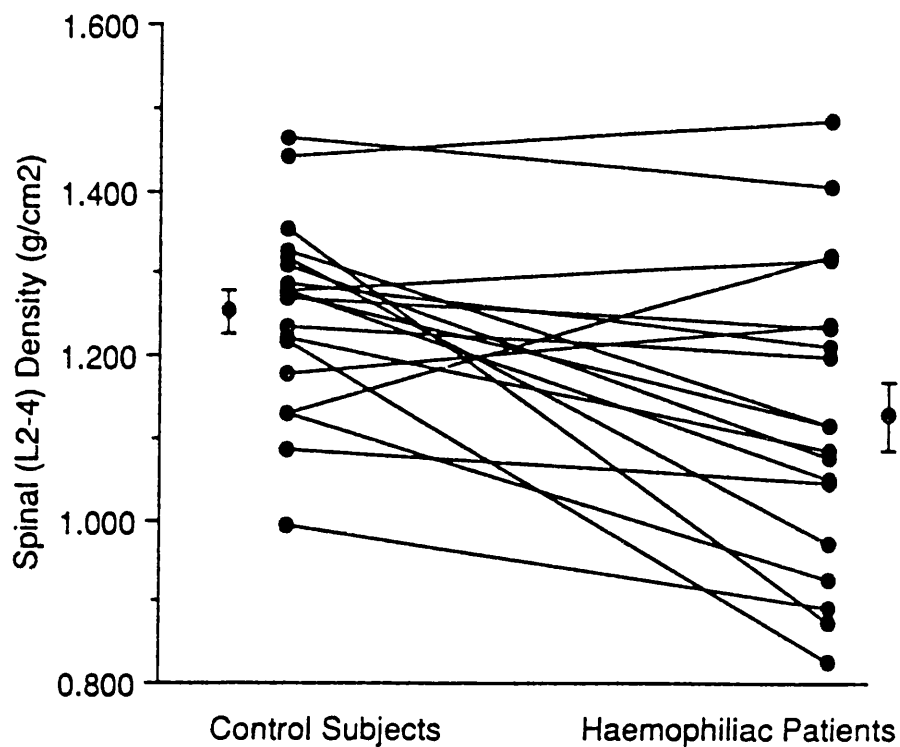
Figure 4.5

Spinal (L2-4) bone density in the haemophiliac patients and age-matched control subjects. Horizontal bars indicate means with standard errors (SEM) also shown. Difference between means: p=0.018.
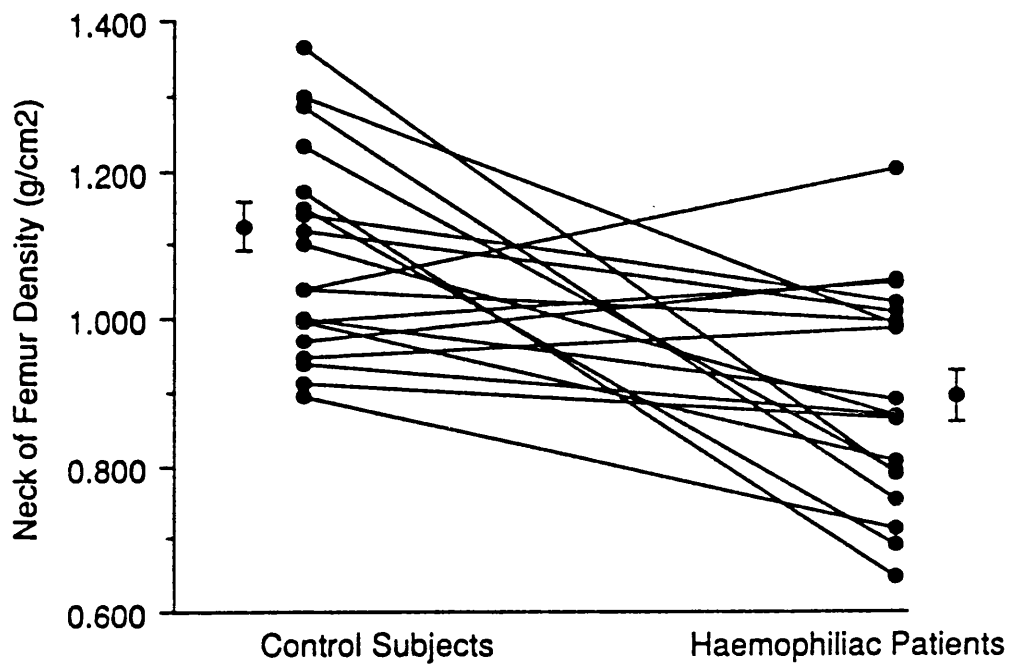
Figure 4.6

Neck of femur bone density in the haemophiliac patients and age-matched control subjects. Horizontal bars indicate means with standard errors (SEM) also shown. Difference between means: p=0.0003.
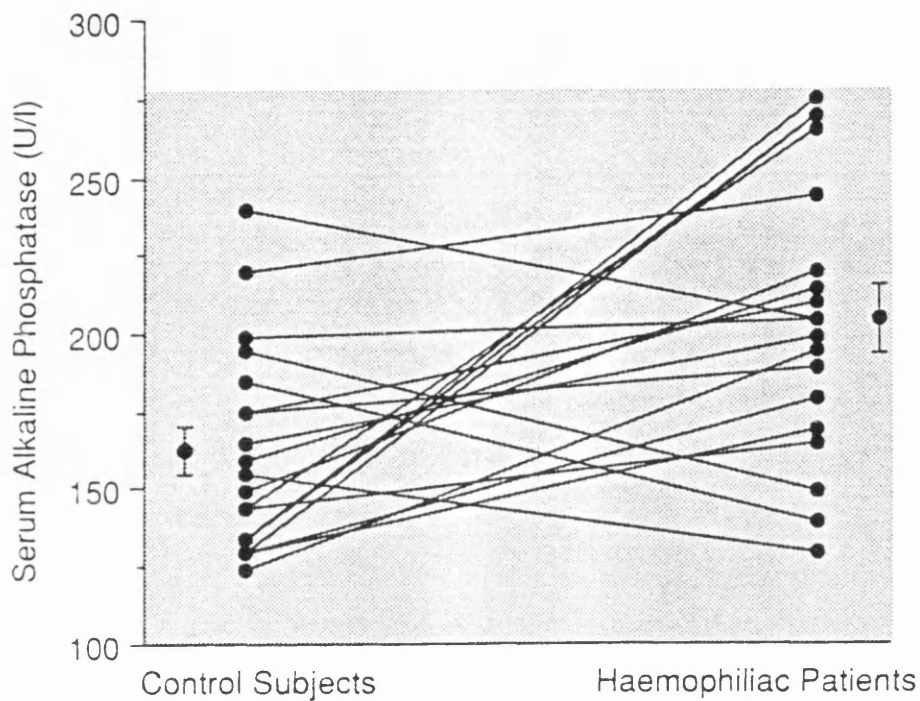
Figure 4.7

Serum alkaline phosphatase in the haemophiliac patients and age-matched control subjects. Horizontal bars indicate means with standard errors (SEM) also shown. Shaded area indicates normal reference range for alkaline phosphatase. Difference between means: p=0.004.
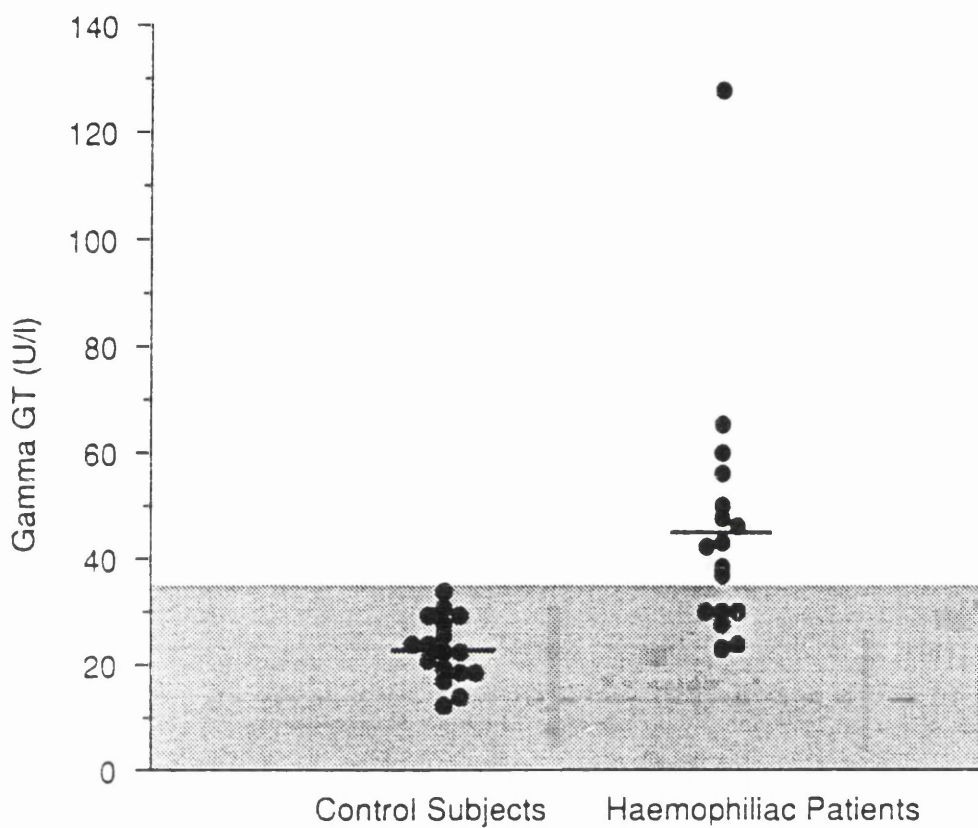
Figure 4.8

Serum gamma glutamyl transferase (gamma GT) in the haemophiliac patients and control subjects. Horizontal bars indicate means. Shaded area indicates normal reference range for gamma GT. Difference between means: p=0.009.
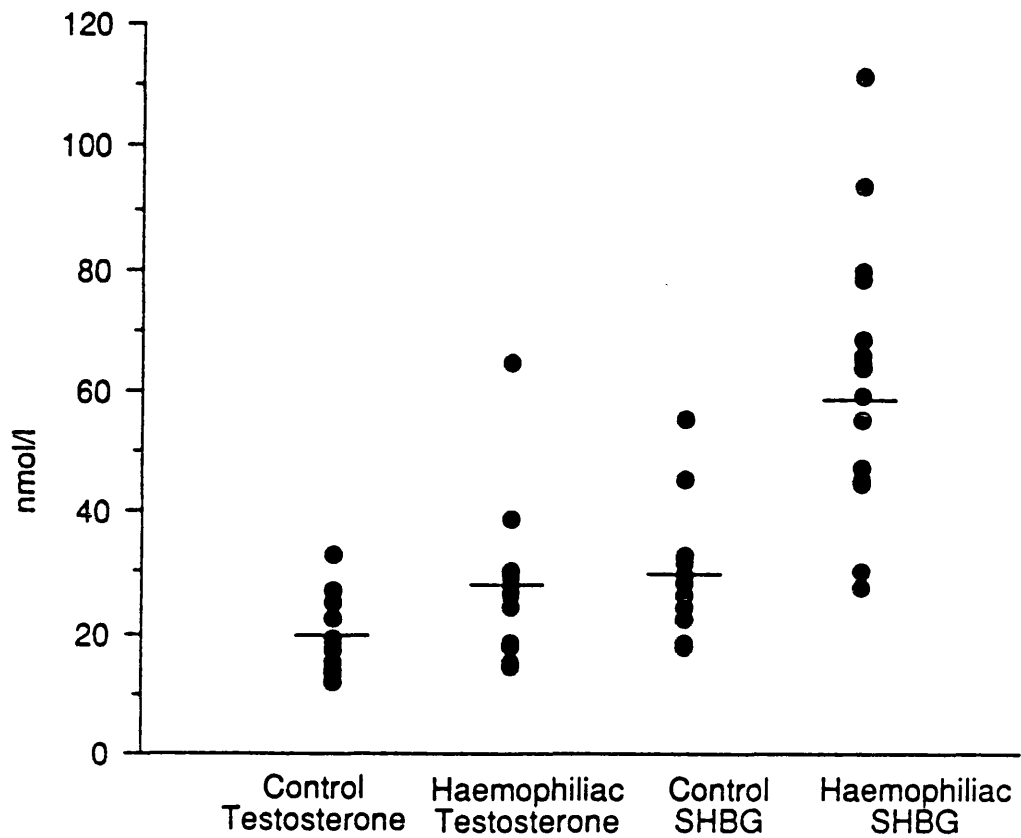
Figure 4.9

Serum testosterone and sex hormone binding globulin (SHBG) concentrations in the haemophiliac patients and control subjects. Horizontal bars indicate means.
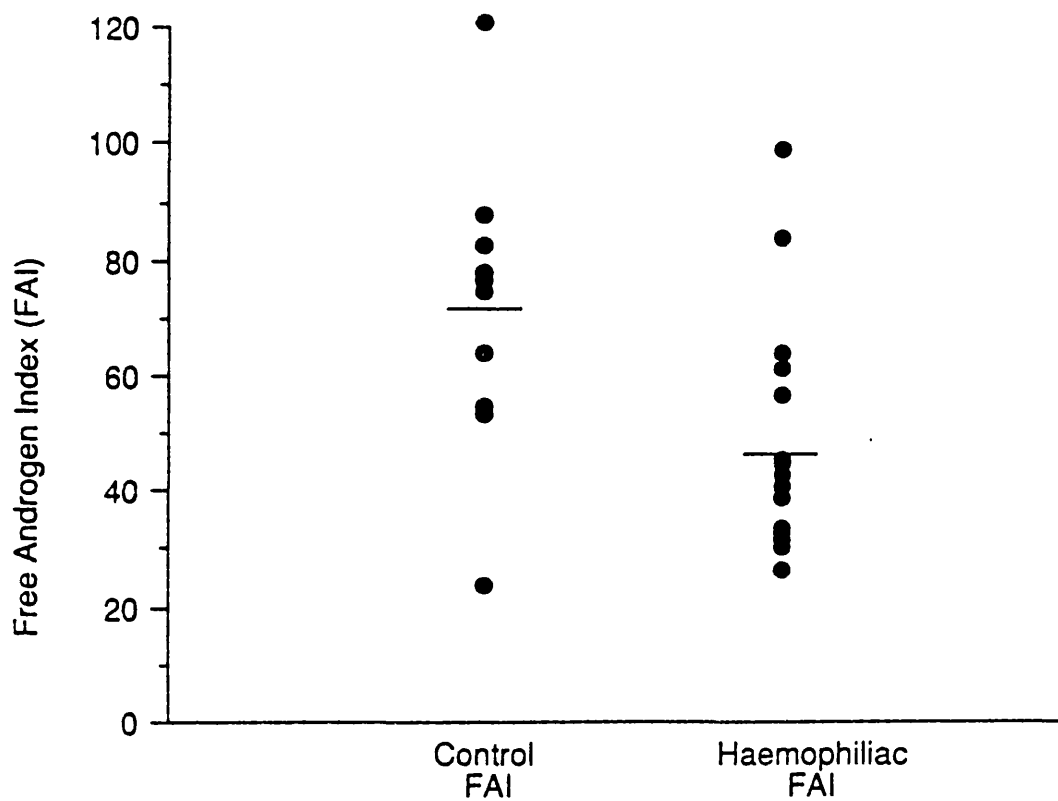
Figure 4.10

Free androgen index (FAI) in the haemophiliac patients and control subjects.

Horizontal bars indicate means. (FAI = serum testosterone/sex hormone binding

globulin x100).

Table 4.2

Biochemical parameters in the haemophiliac patients and control subjects. Points shown are means (SEM).

	Haemophiliac patients	Control subjects
LH(U/l) range	6.9(0.8) 2.3-11.0	5.6(0.9) 1.9-12.0
FSH(U/l) range	3.0(0.4) 1.3-7.6	2.2(0.2) 1.1-4.0
PTH(pmol/l) range (normal 1-5)	2.5(0.2) 1.5-3.9	2.5(0.2) 1.1-4.3
calcium(mmol/l) range (normal 2.20-2.60)	2.37(0.03) 2.31-2.41	2.36(0.04) 2.30-2.45
1,25(OH)$_2$D$_3$(pmol/l) range (normal 20-120)	65(7) 31-146	62(5) 34-113
NcAMP(nmol/l GF) range (normal 5-27)	14.4(2.5) 4.7-26.0	16.1(3.4) 7.7-23.9
Ca/Cr range (normal <0.5)	0.39(0.04) 0.18-0.46	0.38(0.05) 0.19-0.40
HP/Cr range (normal <0.033)	0.029(0.006) 0.010-0.059	0.021(0.005) 0.009-0.028

LH - leutinising hormone   FSH - follicle stimulating hormone
PTH - parathyroid hormone   1,25(OH)$_2$D$_3$ - 1,25-dihydroxyvitamin D3
NcAMP - nephrogenous cyclic adenosine monophosphate
Ca/Cr - urinary calcium/creatinine ratio
HP/Cr - urinary hydroxyproline/creatinie ratio

4.2.4 <u>Discussion</u>

The major complications of severe haemophilia are recurrent haemarthroses resulting in chronic arthritis and bleeding into muscles which may cause nerve compression (441). While radiological surveys of haemophiliacs have shown local osteopenia secondary to chronic arthritis (442), this study describes for the first time an association between haemophilia A and generalised osteopenia. This osteopenia being noted specifically at both the lumbar spine and neck of femur.

This study also suggests that the degree of osteopenia found in the haemophiliac patients will be associated with a significantly increased risk of fracture. At least 7 of the haemophiliac patients admitted to having sustained a significant bony fracture in their adult life (as opposed to none of the control subjects). However, it was not possible from the history to ascertain accurately if these were relatively "atraumatic" fractures; and further long-term prospective studies are required to address this issue.

Osteoporosis in males is uncommon (443) and the cause of the association between severe haemophilia A and osteopenia noted in this study is not immediately apparent. Hypogonadism is an important cause of male osteoporosis (444). While it would appear that the patients with haemophilia in this study had elevated levels of serum testosterone, the fact that SHBG was also significantly elevated means that the free androgen index in these patients was significantly below that in the control subjects. This increase in SHBG noted in the haemophiliac patients may be due to the presence of underlying hepatic disease which is a common feature in patients with haemophilia, with, in this study, there being evidence of previous hepatitis C infection in 18/19 of the haemophiliac patients studied. Hepatic dysfunction in non-haemophiliacs is known to stimulate increased production of SHBG (445), though the exact mechanism of this

remains unclear. The fact that γGT was significantly elevated in the haemophiliac patients would be consistent with this hypothesis of hepatic dysfunction having a causal role in increasing SHBG. Mean gonadotrophin levels were not significantly different between the haemophiliac patients and controls, however, the fact that the free androgen index was significantly lower in the haemophiliacs implies the presence of, at least, relative hypogonadism.

Associated with the presence of hypogonadism, it is possible that the haemophiliac patients fail to achieve their anticipated peak bone mass and, as is the case in post-menopausal osteoporosis, bone resorption is likely to be increased out of proportion to bone formation (444,446) - the consequence of both these potential problems being osteoporosis. Identification of those patients with significant osteopenia by measurement of bone density and FAI is potentially of some importance as preventive therapy with androgen replacement may reduce subsequent fracture risk (450).

Fasting urinary calcium/creatinine ratio is a relatively crude marker of bone resorption (41) and this was not elevated in the haemophiliac patients. Similarly urinary hydroxyproline excretion was not significantly elevated in the haemophiliacs. However since HP/Cr results were not available for all the subjects studied, it remains possible that the lack of a significant elevation in hydroxyproline excretion is due to a type 2 statistical error. In the previous study serum ALP was taken as a marker of bone formation, however in that study γGT was normal and the increase in ALP seen in the diabetic patients was therefore likely to be of bone origin. Alkaline phosphatase was also significantly elevated in the haemophiliac patients but since these patients also had elevated levels of γGT this increase in ALP is more likely to be of hepatic origin. Further studies using more specific markers of bone formation such as osteocalcin or ALP isoenzymes are required.

The major clinical feature of severe haemophilia is excessive bleeding due to impaired coagulation from deficiency of factor VIIIc. Long term treatment with heparin, a potent natural anticoagulant, is also associated with osteopenia (447). The pathogenesis of heparin-induced osteopenia is not clear, though it may be due to a direct effect of heparin on osteoclast activation (448). In addition heparin-induced osteopenia is also associated with low levels of serum $1,25(OH)_2D_3$ (449). It is possible, therefore, that severe chronic coagulation deficiencies (due to factors as yet unrecognised) may in themselves be associated with osteopenia. However, it should be noted that in the present study levels of $1,25(OH)_2D_3$ were normal, unlike the situation described with heparin (449).

Since patients with severe haemophilia are prone, from birth, to recurrent episodes of bleeding especially into joints and muscles, they may be less mobile than control subjects and this relative immobilisation may then lead to osteopenia (190). The two index cases described were certainly both confined to bed for prolonged periods in the past, however this was not a notable feature with the remaining patients. A more likely scenario is that periods of immobilisation (and therefore excessive increased bone resorption) have a detrimental effect on a skeleton already "at risk" from generalised osteopenia.

In conclusion, this study describes for the first time a possible association between haemophilia, premature fractures and osteoporosis possibly associated with chronic hepatitis. Further, larger, studies are required to confirm this association and to investigate the degree of fracture risk. Since bone biopsy for histomorphological analysis carries a risk of bleeding in such patients, further studies of biochemical indices of bone turnover are required. If increased fracture risk is substantiated then severe haemophiliacs should be considered for routine bone densitometric assessment and if necessary anti-osteoporotic therapy.

CHAPTER 5

IMAGING IN PRIMARY HYPERPARATHYROIDISM

5.1 **A comparison between 10MHz ultrasound and 201-thallium/99m-technetium subtraction scanning in primary hyperparathyroidism.**

5.1.1 Introduction

The value of routine pre-operative localisation of parathyroid adenomas and/or hyperplasia continues to be the subject of some controversy (165). High success rates of surgery with an experienced surgeon have cast doubt on whether any form of imaging technique is necessary (451). These techniques have, however, over the past decade improved in sensitivity (176,451,452) and there is some evidence now that pre-operative localisation may both shorten operation (and anaesthetic) time and reduce the number of unsuccessful operations (176).

Many different imaging modalities are now available and include thallium/technetium subtraction scanning (179), high resolution (10MHz) ultrasound (180), computed tomography (CT) (181) and magnetic resonance imaging (MRI) (182). In this study the efficacy of the two most widely available techniques - 10MHz ultrasound and thallium/technetium subtraction scanning - were compared prospectively.

5.1.2 Patients and methods

10MHz ultrasound (US) and 201-thallium/99m-technetium (Tl/Tc) subtraction scanning was carried out in 30 consecutive patients presenting with primary hyperparathyroidism (PHPT). The diagnosis of PHPT was made on the basis of the presence of hypercalcaemia in association with either elevated or unsuppressed parathyroid hormone (PTH) concentrations. All ultrasound scans were carried out using a Diasonic DRF 400 ultrasound machine and a 10MHz stand-off probe by one operator with the patient in a supine position with his

neck hyperextended. This operator was "blinded" to the results of the thallium/technetium scan which was generally carried out first. 25 of these initial 30 patients went forward to neck exploration. All surgery was carried out by the same experienced surgeon who had full access to both imaging results at the time of operation. All tissue removed at surgery was examined by a pathologist and any parathyroid tissue found was weighed in order to give an assessment of the gland size. Surgery was not carried out in 5 of the patients initially studied - in each case this was because conservative management was felt to be more appropriate than surgery.

Details of patients studied and their pre-operative biochemical parameters are shown in table 5.1. Size of the abnormal parathyroid gland removed is also shown.

Since the data on the size of the abnormal parathyroid glands is skewed, the results are shown as medians and their associated range. For statistical comparison these data were logarithmically transformed and a Student's t-test carried out. Correlations where shown were made by regression analysis. Sensitivities and specificities for each technique were calculated assuming that in each patient there were four possible sites for a parathyroid adenoma.

5.1.3 Results

The operative findings were the standard against which these two imaging methodologies were compared. Since only 25 of the original 30 patients went on to neck exploration, the data analysis is confined to these 25 patients.

A single parathyroid adenoma was identified at surgery in 23/25 patients. In one instance 2 hyperplastic parathyroids were removed (with subsequent restoration

Table 5.1

Clinical and biochemical parameters of patients studied (values shown are medians).

Number of patients	30 (23 female, 7 male)
Age (years)	62.5
SEM, range	2.3,30-83
Serum calcium (mmol/l)	2.93
SEM, range	0.05, 2.70-4.00
PTH (pmol/l)	10.5
SEM, range	1.8, 2.7-56.0
Median gland size (mg)	540
range	50-6820

PTH - parathyroid hormone

of normocalcaemia) and in one no abnormal parathyroid tissue was found (and the patient remained hypercalcaemic). Post-operatively normocalcaemia supervened in each of the 23 patients where a parathyroid adenoma was removed.

Thallium/technetium subtraction scanning correctly localised the abnormal parathyroid tissue in 10/24 instances. The sensitivity of this technique was 42% and specificity 97%. 10MHz ultrasound correctly localised the abnormal parathyroid tissue in 9/24 instances (in one patient no abnormal parathyroid tissue was found at surgery). The sensitivity of this technique was 38%, with a specificity of 89%. In 9 instances both these imaging modalities were positive together and were in agreement, and in 8 of these instances correctly predicted the localisation of the parathyroid adenoma. However, in 11 patients (44%) both modalities were negative - though in 2 of these patients the thallium/technetium scan was difficult to interpret due to the presence of a multinodular thyroid. These two patients had large parathyroid adenomas at surgery (2500mg, 600mg).

The ability of these modalities to correctly identify abnormal parathyroid tissue tended to vary with the size of the abnormal gland (figure 5.1). Where both techniques failed to identify the abnormal parathyroid tissue, gland size was smaller at (median, range) 170mg (50-2500mg) compared with where thallium/technetium subtraction scanning was positive (750mg, 150-6820mg; $p<0.03$), 10MHz ultrasound was positive (960mg, 100-6820mg; $p<0.03$) and both techniques combined were positive (980mg, 600-6820mg; $p=0.002$). While thallium/technetium subtraction scanning tended to be able to localise smaller glands than 10MHz ultrasound (and both techniques combined), this was not a statistically significant difference.
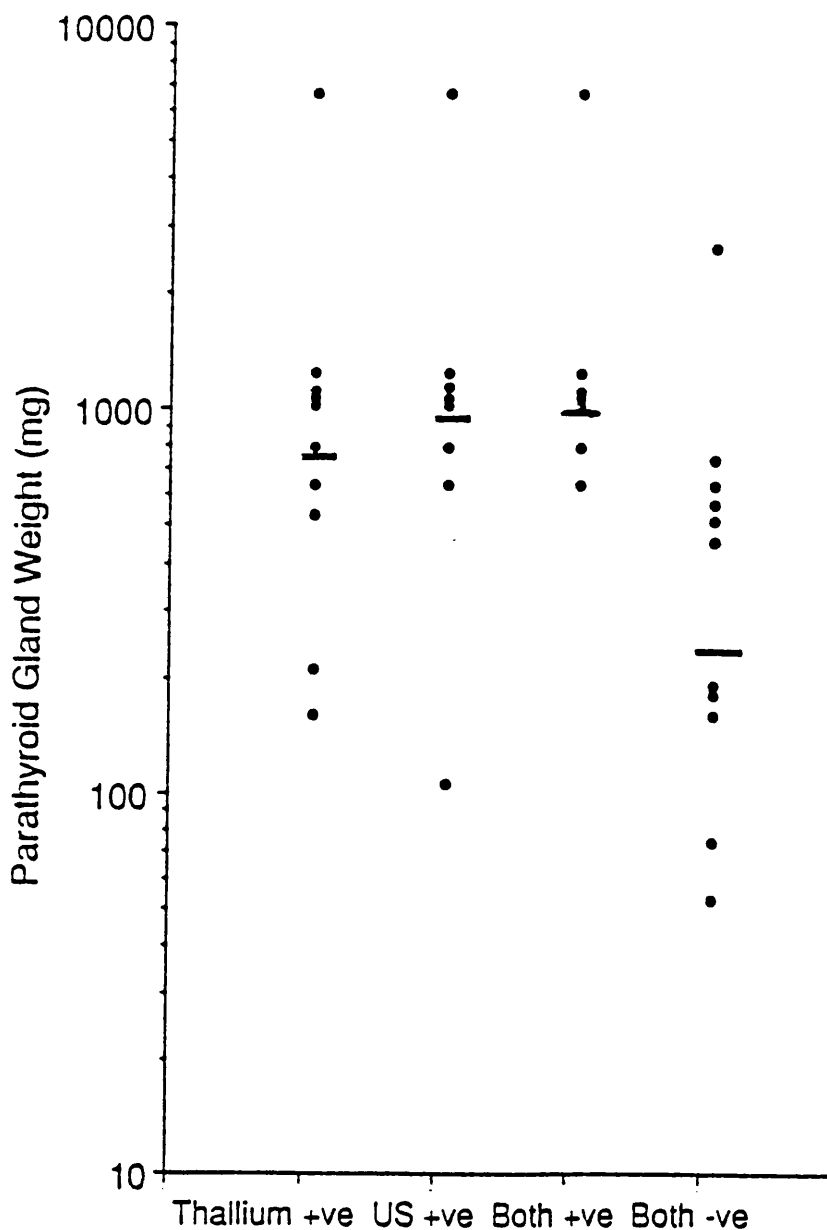
Figure 5.1

Parathyroid gland size in those patients with positive thallium/technetium (Tl/Tc) scans, positive 10MHz ultrasounds or both positive compared with those patients where both investigations were negative.

A significant positive correlation was evident between PTH concentration and the size of the parathyroid adenoma (r-squared = 0.78, p<0.0001) and PTH concentration and pre-operative serum calcium concentration (r-squared = 0.63, p<0.0001). A weaker, though again significant correlation, was evident between the pre-operative serum calcium concentration and the size of the parathyroid adenoma (r-squared = 0.52, p<0.0001).

5.1.4. Discussion

The role of parathyroid imaging as a routine procedure prior to parathyroidectomy remains controversial. A recent "Consensus" statement from the National Institutes of Health in the United States has suggested that parathyroid imaging should be reserved only for patients who have had a failed neck exploration (165). Some evidence exists, however, that the use of pre-operative parathyroid imaging may both reduce operating time and result in an increased number of successful operations (176).

Many different techniques have been used in an attempt to localise parathyroid adenomas. At the present time the most widely used techniques include thallium/technetium subtraction scanning, 10MHz ultrasound, computed tomographic (CT) scanning and magnetic resonance imaging (MRI) (451). Only one study to date has prospectively compared these 4 modalities (184). Specificities of all 4 of these techniques was generally high (87-95%), however sensitivities were not so good, with thallium/technetium scanning best at 73% and high resolution ultrasound poorest at 55%.

Direct comparison between thallium/technetium scanning and high resolution ultrasound generally shows both techniques are comparable with specificities very high and sensitivities in the range 74-80% (185,186). Roses et al.(453),

however, in a study of 36 patients with PHPT noted a sensitivity of only 49% for thallium/technetium subtraction scanning and 34% for ultrasound. Results were similar in this present study, with thallium/technetium subtraction scanning only having a sensitivity of 42% in localising parathyroid adenomas with the sensitivity of 10MHz ultrasound being even less good at 38%. These sensitivities are less good than the figures quoted in many of the initial studies using these modalities. Many of the initial studies with thallium/technetium scanning quoted sensitivities of the order of 65 to 90% (178,179,183). Similarly sensitivities of around 70 to 80% were quoted for 10MHz ultrasound (180). However, other more recent studies have not been able to achieve sensitivities as high as in these initial series (184,451,452). The reason for this discrepancy is not clear, however the most important variable in localising a parathyroid adenoma appears to be gland size (184). With respect to thallium/technetium subtraction scanning Gimlette et al.(454) noted the lower limit of detectability to correspond to a gland size of around 250mg. The smallest gland detectable in this present study was 150mg, though in keeping with Gimlette's data (454) the median gland size in those patients with positive thallium/technetium scans was significantly higher at 750mg. With the use now of multichannel serum calcium analysers, hypercalcaemia is now to be found commonly as a chance occurrence and the average degree of hypercalcaemia at presentation is less marked than was previously the case (47). Since, as has been demonstrated in this study, serum calcium concentration is related to gland size, it may be that the mean size of parathyroid adenomas being treated surgically today is less than was previously the case and therefore any imaging technique is likely now to be less effective. Another possible factor to be taken into account is the fact that parathyroid hyperplasia is less easy to localise on imaging (184) - possibly because the glands tend to be smaller than parathyroid adenomas (452) - and if a greater proportion of patients in any given group had primary hyperparathyroidism secondary to hyperplasia rather than a solitary adenoma, then the sensitivity of

the localising technique used may be less good. This could not account for the poor sensitivities seen in this study as parathyroid hyperplasia was only present in 1/25 patients.

Where both these techniques are carried out the overall sensitivity is higher than with either thallium/technetium scanning or ultrasound alone. Krubsack et al.(184) noted an overall sensitivity of 85% when thallium/technetium scanning and ultrasound were combined. Combining three or even four imaging techniques, however, did not add further to the sensitivity observed. Similarly Gooding et al.(186) found combining thallium/technetium scanning with ultrasound to be more sensitive than either technique alone and these authors recommended that both these techniques should be used together in the pre-operative assessment of the patient with primary hyperparathyroidism as both these techniques appear to be complementary. However, given the fact that ultrasound involves no exposure to radioactivity, the logical sequence of investigation would be to carry out high resolution ultrasound first. If this is positive, proceed to surgery. If this is negative a thallium/technetium scan should also then be carried out prior to surgery. While this sequence of investigation would appear attractive the fact that 44% of patients in this study had negative results for both techniques would appear to limit their usefulness even when combined.

This study has only investigated the use of imaging at the time of first neck exploration and furthermore only those patients with adenomas in the neck were studied. The effect of surgery in distorting tissue planes is such that thallium/technetium scanning is probably the investigation of choice in patients who have had a previous unsuccessful neck exploration (455). However, some evidence exists that in this situation an experienced operator may achieve similar results to Tl/Tc scanning with high resolution ultrasound (186). Furthermore

thallium/technetium scanning is probably also the technique of choice where a mediastinal adenoma is suspected (179). Advances are also taking place in attempting to improve the sensitivity of nuclear medicine imaging, particularly with the use of 99m-technetium-sestamibi in place of 201-thallium (456) - however, the place of this technique in routine imaging remains to be established.

While evidence exists that preoperative parathyroid localisation may be beneficial, results from this study suggests that neither thallium/technetium subtraction scanning nor high resolution ultrasound are sufficiently sensitive alone to be of value in the routine assessment of patients with primary hyperparathyroidism. This is particularly the case where hypercalcaemia is relatively mild. There may be a place, however, for combining these two techniques. Further studies are required to specifically address this issue.

5.2 **Augmentation of parathyroid 201-Thallium/99m-Technetium scanning by infusion of trisodium edetate.**

5.2.1 Introduction

The previous study illustrated that, in general, conventional imaging methods do not have the necessary sensitivity to be useful in the routine pre-operative assessment of primary hyperparathyroidism. In this study an attempt was made to increase the metabolic activity of the parathyroid glands in an attempt to stimulate increased parathyroid uptake of radiolabelled thallium.

5.2.2 Patients and methods

Six patients (four female) were studied (table 5.2). As before, the diagnosis of primary hyperparathyroidism was made by the finding of hypercalcaemia and a PTH concentration elevated or inappropriate to the prevailing serum calcium. All of the patients included in this study had had a negative standard 201-thallium/99m-technetium (Tl/Tc) subtraction scan carried out in the preceding 6 months, and thus acted as their own control.

Ninety minutes before the repeat scan was carried out these patients were commenced on an infusion of the calcium chelating agent, trisodium edetate at a dose of 24mg/kg/hour. This was administered in 500ml 0.9% saline along with 10ml 1% lignocaine to relieve any local arm discomfort which may be associated with this infusion. Blood samples were taken before and during this infusion from the opposite arm.

Table 5.2

Clinical and biochemical parameters of patients studied (values shown are means; calcium adjusted for serum albumin)

Age (years)	71.8
range	57-80
Serum calcium (mmol/l)	2.80
range	2.70-2.95
PTH (pmol/l)	6.0
range	3.5-9.0
PcAMP (nmol/l)	24.9
range	17-30.2

PTH - parathyroid hormone

PcAMP - plasma cyclic adenosine monophosphate

Statistical analyses of changes in calcium, PTH and plasma cAMP (PcAMP) before and during infusion of trisodium edetate was carried out using a paired Student's t-test.

5.2.3 Results

Following the repeat scan three patients had areas of discordant thallium uptake on the subtraction images (figures 5.2a&b, 5.3a&b and 5.4a&b), consistent with the presence of a parathyroid adenoma. The scans in the remaining three patients were again negative. Of the three patients with positive scans, two went on to have surgery and a parathyroid adenoma was found at the site corresponding to the scan appearances in both instances. It is not known whether the remaining patients had adenomas as the one further patient with a positive scan refused surgery and those with negative scans were managed conservatively.

During the infusion of trisodium edetate the mean fall in total serum calcium (±SEM) was 0.42mmol/l (±0.04, p<0.0001), the mean rise in PTH was 15.6pmol/l (±3.3, p<0.0005) and the mean rise in PcAMP was 2.44nmol/l (±1.50, p=NS). Interestingly PcAMP only rose in three patients and only one of those subsequently developed a positive scan.

There were no side-effects from the infusion of trisodium edetate and no patient was symptomatic from their relative hypocalcaemia.

5.2.4 Discussion

The study described in section 5.1 along with previous studies by other authors have suggested that parathyroid localisation by Tl/Tc subtraction scanning may be less good where primary hyperparathyroidism is mild and the adenomatous
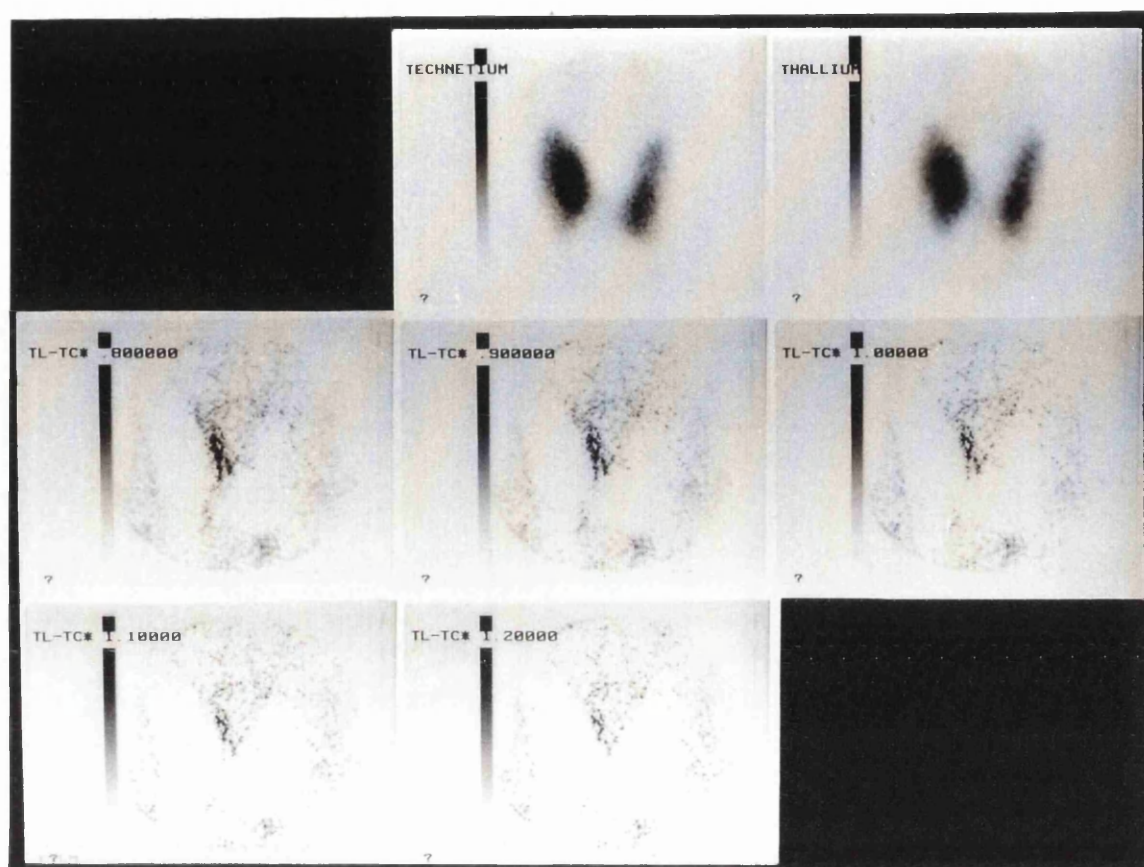
Figure 5-2a

Patient 1

Original 201-thallium/99m-technetium (Tl/Tc) subtraction scan carried out prior to trisodium edetate augmented scan.
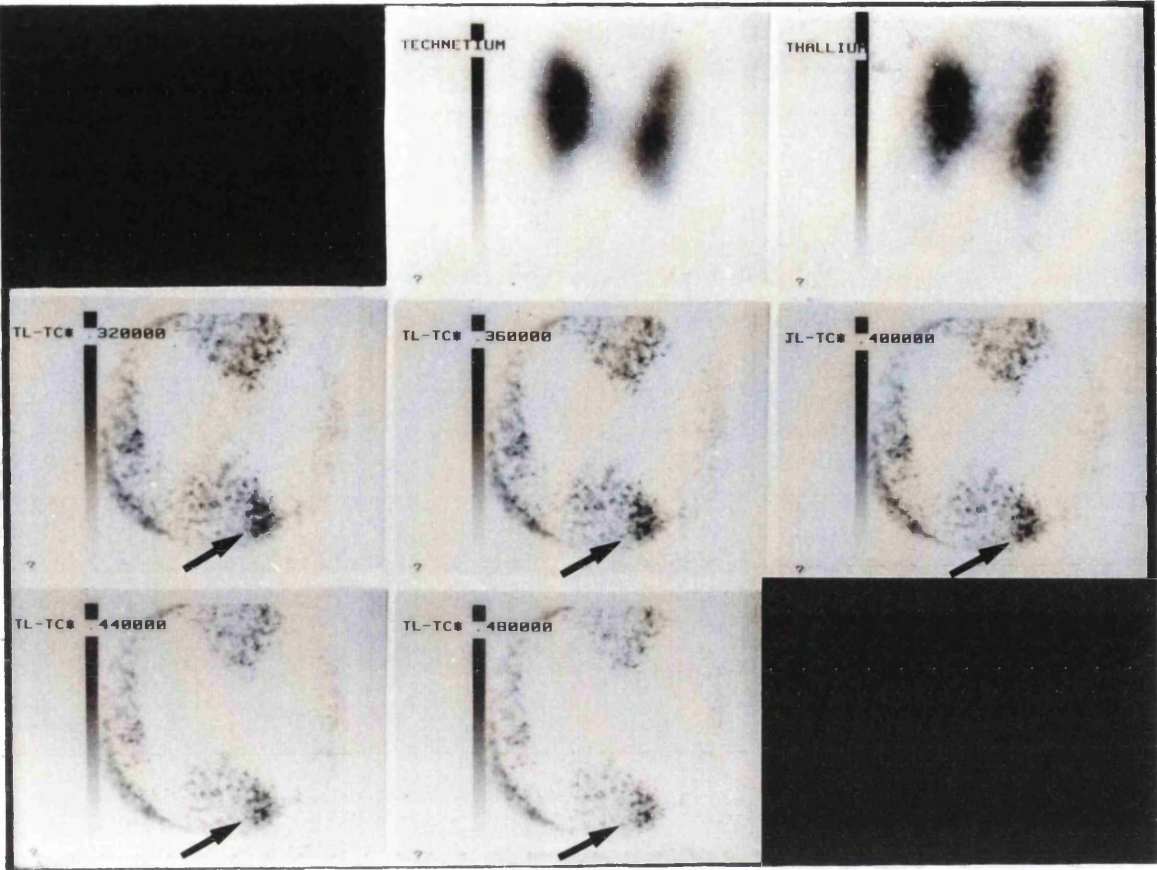
Figure 5-2b

Patient 1

201-thallium/99m-technetium (Tl/Tc) subtraction scan carried out after infusion of trisodium edetate. Arrow indicates area of discordant thallium uptake corresponding to site of parathyroid adenoma.
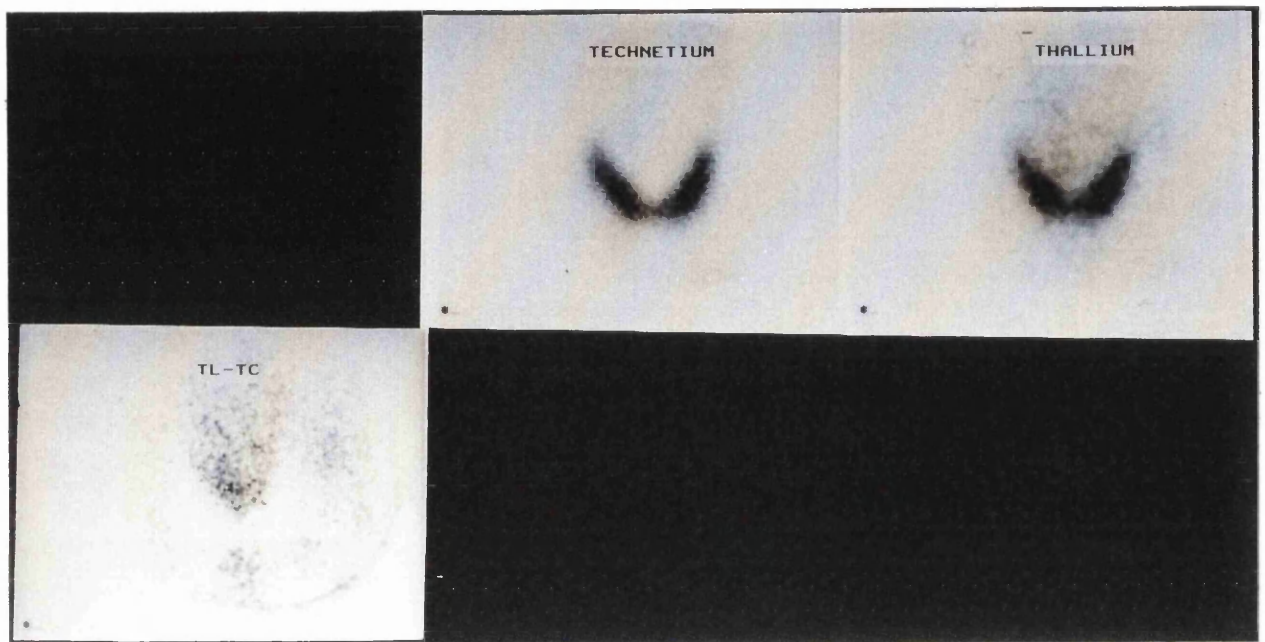
Figure 5-3a

Patient 2

Original 201-thallium/99m-technetium (Tl/Tc) subtraction scan carried out prior to trisodium edetate augmented scan.
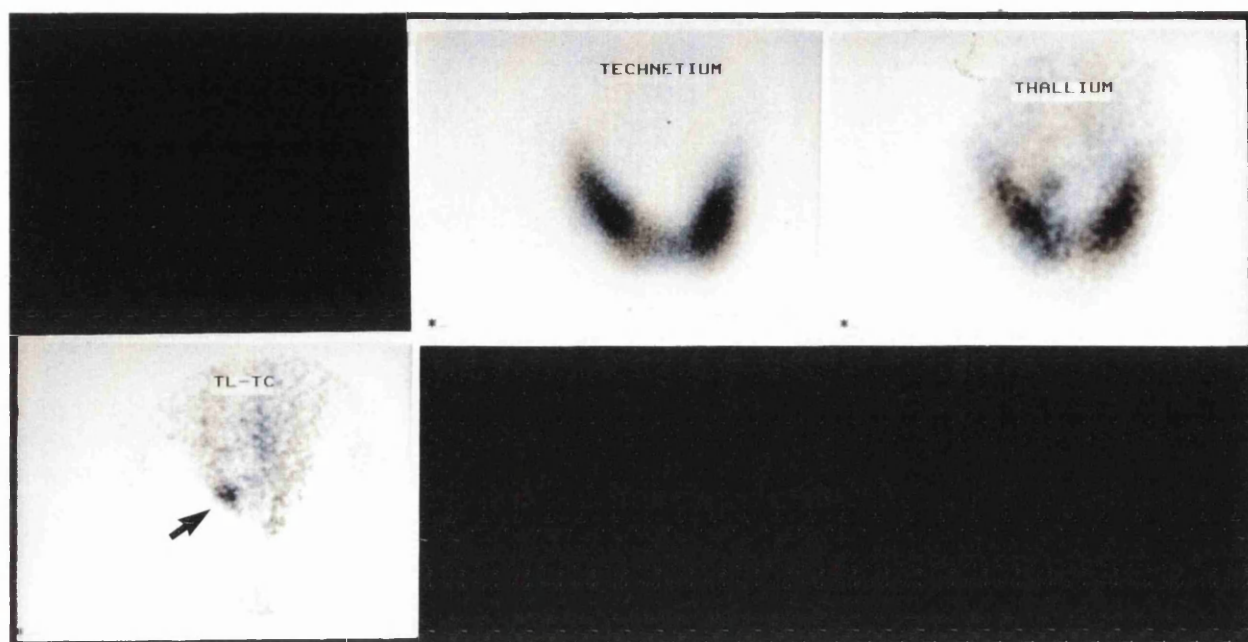
Figure 5-3b

Patient 2

201-thallium/99m-technetium (Tl/Tc) subtraction scan carried out after infusion of trisodium edetate. Arrow indicates area of discordant thallium uptake corresponding to site of parathyroid adenoma.
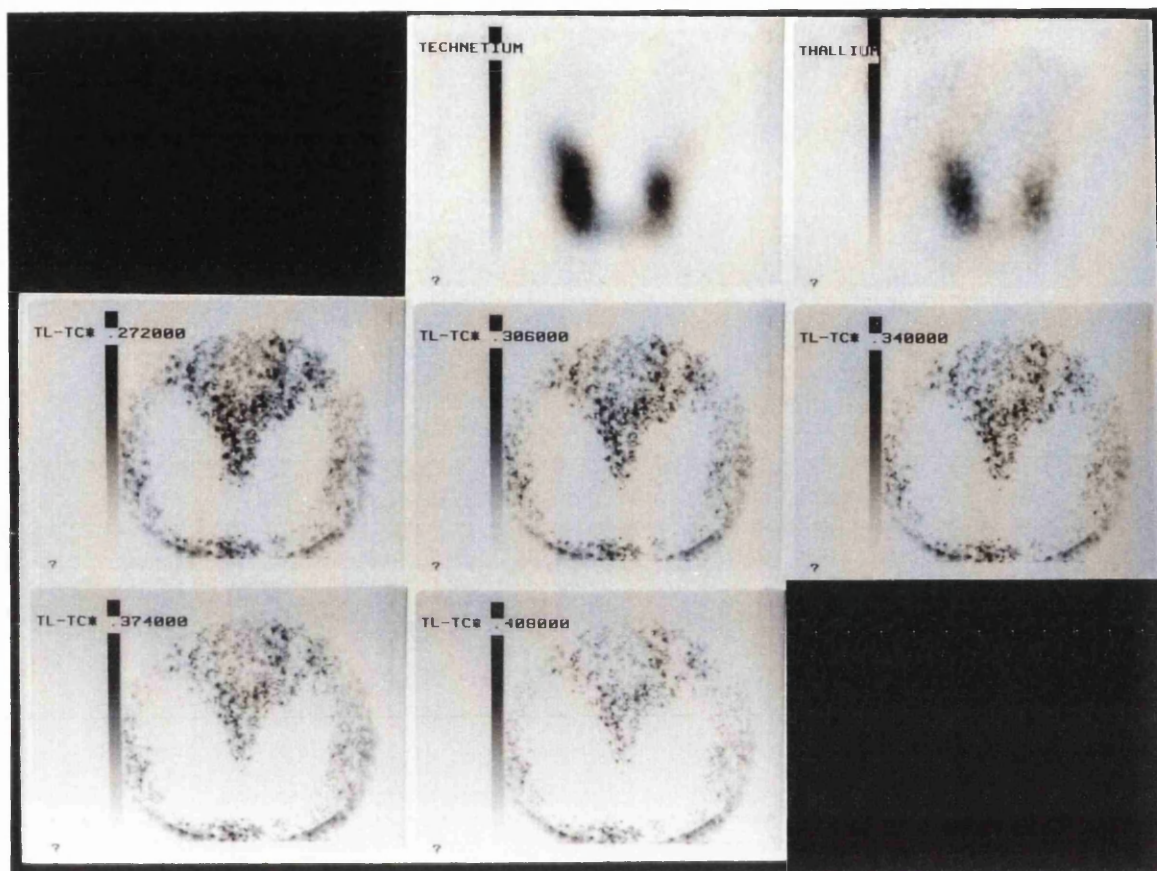
Figure 5-4a

Patient 3

Original 201-thallium/99m-technetium (Tl/Tc) subtraction scan carried out prior to trisodium edetate augmented scan.
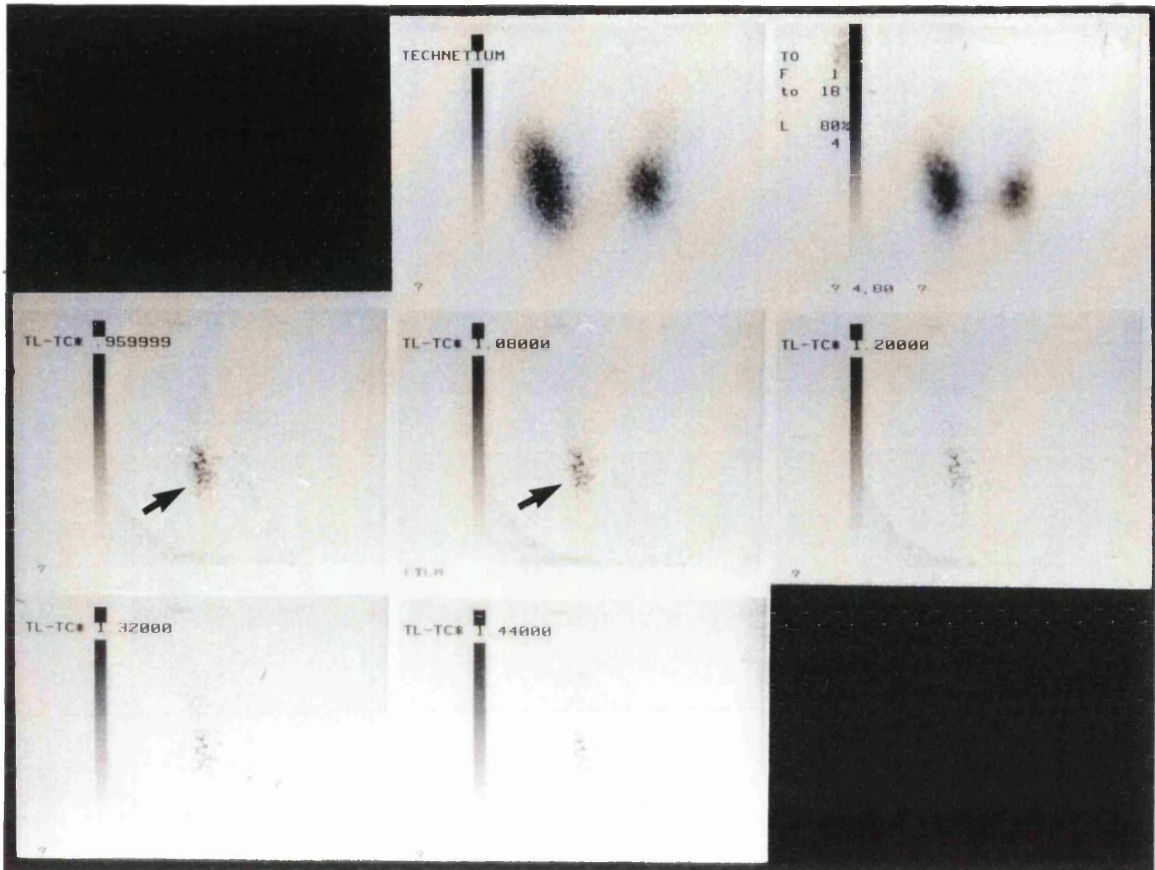
Figure 5-4b

Patient 3

201-thallium/99m-technetium (Tl/Tc) subtraction scan carried out after infusion of trisodium edetate. Arrow indicates area of discordant thallium uptake corresponding to site of parathyroid adenoma.

gland is small (184,457). The results in this study would suggest that this modified method may be useful for these patients should their initial scan be negative.

It has been previously noted that creating relative hypocalcaemia in patients with primary hyperparathyroidism (even though the albumin adjusted calcium remains above the reference range) will trigger PTH secretion (160). Thallium is thought to substitute for potassium in the ATPase dependent sodium/potassium pump (458). Thallium, therefore, acts as a specific marker of cellular activity and uptake of thallium is greater where cellular metabolic activity is increased. It may be that by triggering PTH secretion, the metabolic activity of the parathyroids has been increased to the extent that greater thallium uptake will ensue. In this context it is interesting and surprising that PcAMP, which is thought to be a marker of cell metabolic activity (459), was not raised in each case and in 2/3 instances where it was raised the repeat scan was negative. However, it is also known that changes in other hormones affect PcAMP including catecholamines and glucagon (459) and it is possible that changes in these hormones may be contributing to the variability seen with PcAMP.

The reason for some scans remaining negative is unclear, though it is possible that these patients either had very small adenomas or parathyroid hyperplasia which tends not to show well on 201Tl/99mTc subtraction scanning (184).

The infusion of trisodium edetate was well tolerated in all patients. The most common complaint with administration of this drug is local discomfort in the area of the vein into which the drug is being infused. This side-effect was virtually eliminated by adding a small amount of lignocaine to the infusion.

Although significant rises in PTH were seen, this was associated with only relatively modest changes in serum calcium which in all cases fell to levels within the normal reference range and therefore, as might have been expected, no patient developed symptoms of hypocalcaemia.

In summary it would appear that by creating relative hypocalcaemia and transiently increasing PTH secretion localisation of parathyroid adenomas may be improved on Tl/Tc subtraction scanning. Because of the additional complexity of administering an infusion of the chelating agent, this technique is probably best reserved for those patients who have had negative conventional Tl/Tc subtraction scans and/or unsuccessful surgery.

CHAPTER 6

PAMIDRONATE IN THE MANAGEMENT OF HYPERCALCAEMIA

6.1 **A comparison of low versus high dose pamidronate in cancer-associated hypercalcaemia**

6.1.1 Introduction

The previous chapters have illustrated the clinical application of currently available biochemical and imaging techniques in the understanding and management of various aspects of metabolic bone diseases. The next two chapters illustrate the use of the bisphosphonate, pamidronate in the treatment of various disorders of bone and calcium metabolism.

Bisphosphonates have been shown to be effective in the management of cancer-associated hypercalcaemia (130,134-140), and have now probably become the treatment of choice (134). The optimum dose of pamidronate, however, remains controversial (140,141-143). On the basis of data from Thiebaud et al.(141), it is currently recommended that the dose of pamidronate be titrated against the prevailing serum calcium, with 30mg suggested for mild hypercalcaemia (<3.00mmol/l) rising up to 90mg for severe hypercalcaemia (>4.00mmol/l). In previous studies, however, no evidence of any difference in calcium lowering response has been reported in the ranges 15-45mg (140) or 30-60mg (142).

In this study the effects of randomly allocating 30mg and 90mg doses was investigated in patients with varying degrees of cancer-associated hypercalcaemia.

6.1.2 Patients and methods

32 patients with cancer-associated hypercalcaemia were randomly allocated to receive either 30mg pamidronate intravenously in 500ml 0.9% saline over 4

hours (n=16) or 90mg pamidronate in 1 litre 0.9% saline given over 24 hours (n=16). These dilutions and infusion intervals were chosen in keeping with what is currently recommended by the manufacturers (Ciba Geigy Ltd, Horsham, UK). All treatments were given after 48 hours of rehydration with a minimum of 4 litres 0.9% saline. After pamidronate was given, saline rehydration was continued for a further 48 hours with each patient receiving a minimum of 2 litres saline per day.

None of the patients had received chemotherapy or radiotherapy in the 4 weeks up to, or for at least 10 days after pamidronate therapy. None had been previously treated with a bisphosphonate or other anti-hypercalcaemic drug. Chemotherapy was commenced in 1 patient with myeloma in the 30mg group and 2 patients with myeloma in the 90mg group at between 10 and 14 days post pamidronate therapy.

An assessment was made of the presence and extent of bony metastases on the basis of a radionuclide bone scan as described in section 3.1.

Measurement was made of serum calcium and urinary calcium/creatinine (Ca/Cr) ratio at days 0 (pre-treatment),1,2,3,6 and 9.

Differences between and within treatment groups was assessed by two-way analysis of variance (ANOVA) and Chi squared test. Results are described as means ±SEM.

6.1.3 Results

Relevant clinical and biochemical details for each group are shown in table 6.1. There was no significant difference between the groups in terms of serum

Table 6.1

Pre-treatment biochemical parameters, tumour types and bone metastases in the two treatment groups. (Values shown are means)

	30mg group	90mg group
Serum calcium(mmol/l) (+/-SEM,range)	3.23 (0.08,2.80-3.85). . . .	3.31 (0.07,2.90-3.75)
Serum creatinine(umol/l) (+/-SEM,range)	103 (15,55-150)	97 (13,60-150)
PTH(pmol/l)	<0.8 (14/16 patients)	<0.8 (14/16 patients)
NcAMP(nmol/l GF) (+/-SEM,range)	40.4 (12.7,1.8-182.8)	53.8 (9.3,6.3-118.4)
Urine Calcium/creatinine (+/-SEM,range)	2.12 (0.36,0.55-4.77)	1.65 (1.65,0.77-4.83)
TmPO4(mmol/l GF) (+/-SEM,range)	0.65 (0.08,0.34-1.10)	0.74 (0.08,0.31-1.40)
Tumour types: Bronchial Breast Others*	6 4 6	6 3 7
Bone metastases: None Light load Heavy load Unknown	2 12 2	2 10 3 1

*Other tumour types were:

30mg: myeloma (2), renal, tongue, bladder, anaplastic (unknown primary).

90mg: myeloma (2), renal, bladder (2), mouth, adenocarcinoma (unknown primary).

PTH - parathyroid hormone

NcAMP - nephrogenous cyclic adenosine monophosphate

TmPO4 - renal tubular threshold for phosphate reabsorption

calcium, creatinine, PTH, NcAMP, urinary Ca/Cr or TmPO4. PTH values were just detectable in 2 patients in each group (1.4,1.9pmol/l [30mg group] and 1.4,1.6pmol/l [90mg group]), though this was measured after 48 hours of saline rehydration in which time there was a slight fall in serum calcium. Tumour types were well matched in both groups with bronchial carcinoma being most common followed by breast carcinoma. Presence and extent of bone metastases was also similar in the two groups.

The response of serum calcium following treatment is shown in figure 6.1. It can be seen that serum calcium fell to a nadir of 2.48mmol/l (±0.06) at day 6 in the 30mg group and to 2.51mmol/l (±0.03) at day 9 in the 90mg group. Using analysis of variance there was no relationship between serum calcium and the dose of pamidronate given (F=2.90, df= 1/180, p=NS), though serum calcium fell significantly in both groups post-treatment (F=48.5, df= 5/180, p<0.01). At the mean serum calcium nadir, 10 patients in the 30mg group were normocalcaemic compared with 8 patients in the 90mg group (Chi squared = 0.31, p=NS). Even when the results for those patients with more severe pre-treatment hypercalcaemia (ie serum calcium >3.30mmol/l, n=7 in each group) (figure 6.2) were analysed, while again pamidronate was successful in restoring normocalcaemia (F=30.5, df= 5/72, p<0.01), there was no significant difference between the two groups (F=0.06, df= 1/72, p=NS). Furthermore, there were no major differences in the tumour types between these two subgroups (table 6.2). The nadir in serum calcium was again at day 6 in the 30mg group at 2.56mmol/l (±0.12) and at day 9 in the 90mg group at 2.57mmol/l (±0.09).

Though the nadir serum calcium was obtained at day 6 in the 30mg group and day 9 in the 90mg group, the differences between day 6 and 9 were marginal and not indicative of a significant difference in response.
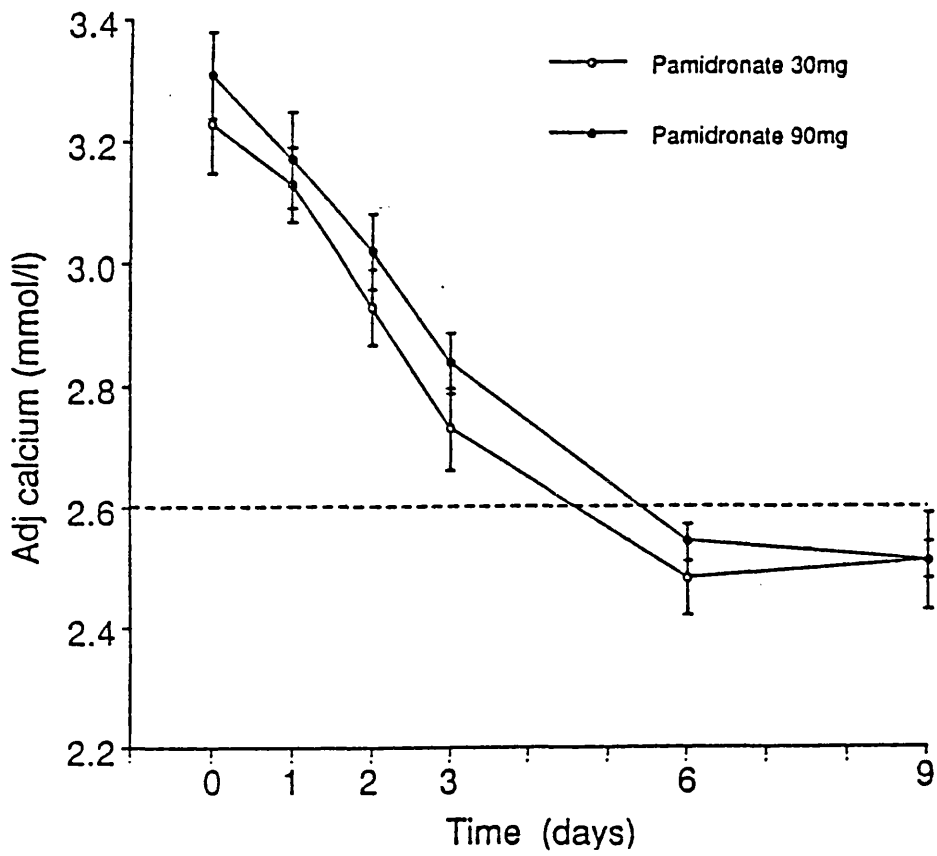
Figure 6.1

Changes in serum adjusted calcium in the two treatment groups. Points shown are means ± SEM. Pamidronate was given on day 0 (n=16 for each group).
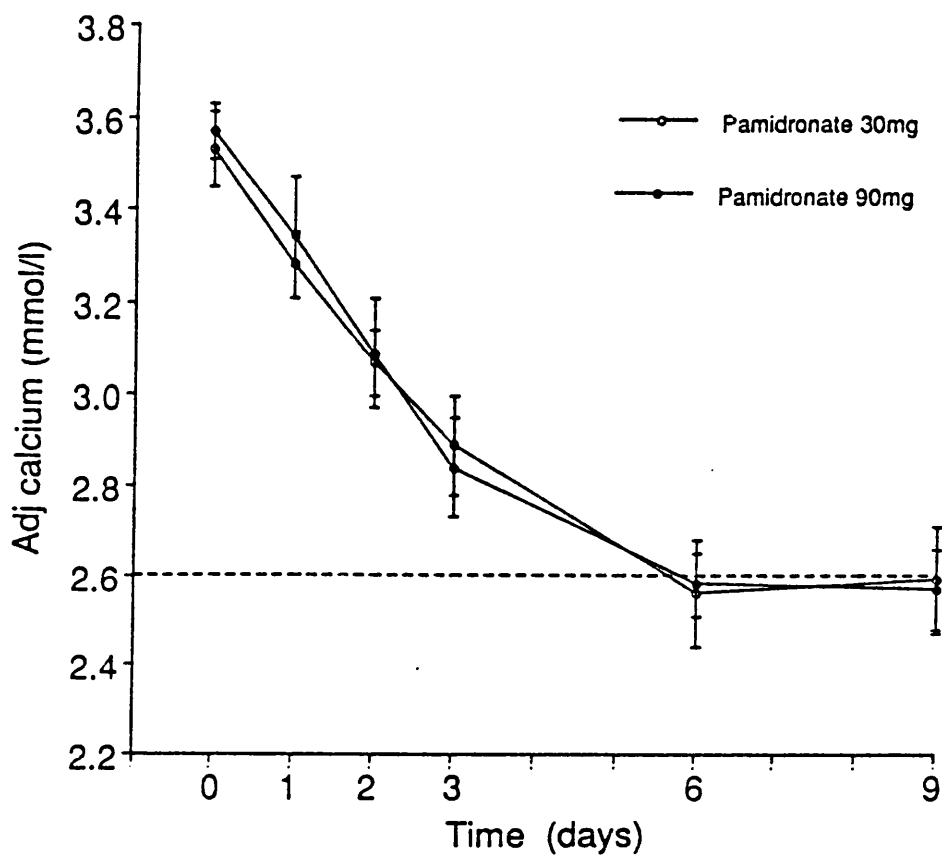
Figure 6.2

Changes in serum adjusted calcium following pamidronate treatment in the subgroup of patients with more severe hypercalcaemia (pre-treatment calcium >3.30mmol/l). Points shown are means ± SEM. Pamidronate was administered on day 0 (n=7 in each group).

Table 6.2

Tumour types in the subgroup with more severe hypercalcaemia (serum calcium >3.30mmol/l).

	30mg	90mg
	Bronchus (2)	Bronchus (2)
	Breast (2)	Breast (2)
	Tongue	Tongue
	Myeloma	Myeloma (2)
	Bladder	

Hypocalcaemia did not occur in either treatment group nor were there any significant side-effects associated with either treatment regimen other than asymptomatic post-treatment pyrexia in 2 patients in each group.

Similarly for urinary Ca/Cr (figure 6.3), the nadir in both groups was reached at day 6 of 0.33 ($\pm$0.05) in the 30mg group and 0.37 ($\pm$0.10) in the 90mg group (F=21.5, df= 5/180, p<0.01). Again no significant differences were evident between the two treatment groups (F=1.5, df= 1/180, p=NS). There was a suggestion of a slight rise in Ca/Cr in the 30mg group but this was marginal and not statistically significant.

Normocalcaemia was achieved in 11/11 patients where pre-treatment NcAMP was within the reference range as opposed to 7/17 patients where NcAMP was elevated (figure 6.4). Mean nadir of serum calcium was 2.48mmol/l (range 2.30-2.60) in the normal NcAMP group versus 2.67mmol/l (range 2.30-3.30) in the elevated NcAMP group (p=0.01, Mann-Whitney U-test).

Follow up values for serum calcium were available in 6 patients in the 30mg group and 8 patients in the 90mg group. Relapse in hypercalcaemia was defined as a rise in serum calcium to within 0.05mmol/l of the pre-treatment value (135). The mean number of days to relapse was 38 (range 18-90) in the 30mg group and 34 (range 11-105) in the 90mg group. Pre-treatment serum calcium was similar in both these subgroups (3.32mmol/l, range 2.75-3.75 [30mg group] and 3.41mmol/l, 2.90-3.75 [90mg group]). Tumour types in these two subgroups were similar (table 6.3), which excludes tumour variation as the reason behind the lack of a dose-response.
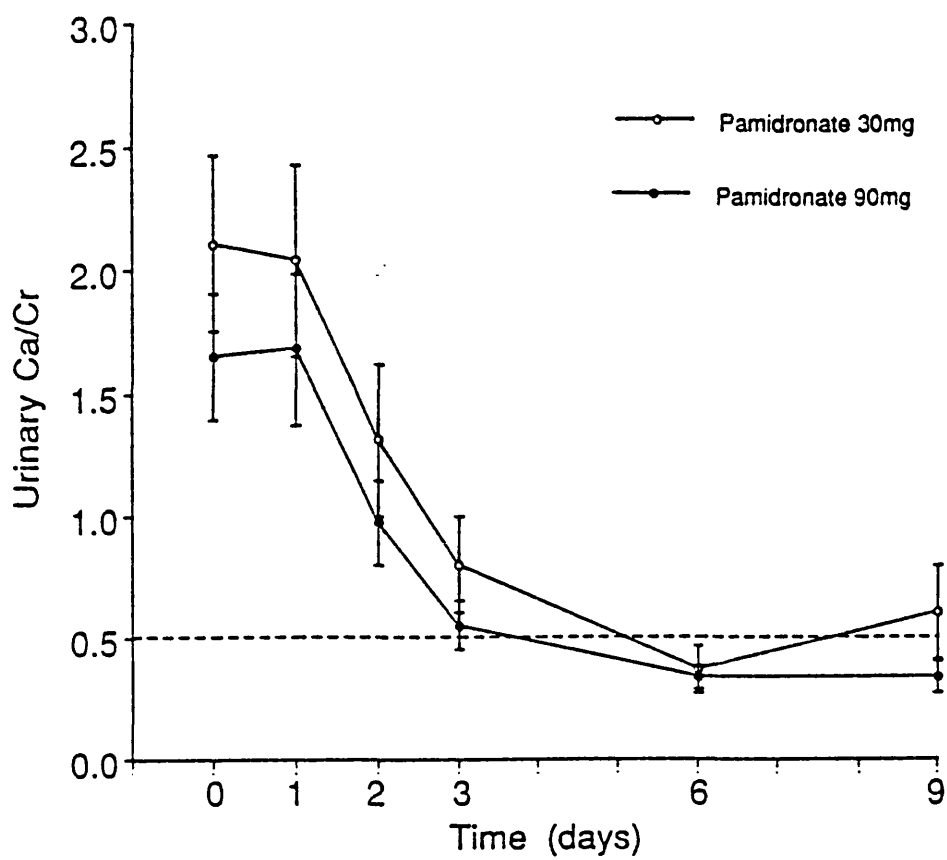
Figure 6.3

Changes in fasting urinary calcium/creatinine (Ca/Cr) ratio (mmol/mmol). Points shown are means ± SEM. Pamidronate was given on day 0. (n=16 for each group).

Figure 6.4

Nadir serum calcium obtained in patients with normal and elevated nephrogenous cyclic adenosine monophosphate (NcAMP) pre-treatment. Bars represent means.

Table 6.3

Tumour types in those patients who showed a relapse in hypercalcaemia after treatment

	30mg	90mg
	Bronchus(2)	Bronchus(3)
	Breast	Breast
	Tongue	Tongue
	Myeloma	Myeloma
	Bladder	Anaplastic carcinoma

6.1.4 <u>Discussion</u>

Both treatment regimens in this study were equally effective in both restoring serum calcium to normal and in maintaining normocalcaemia with no evidence of an improved rate of response with the higher dose.

While NcAMP and TmPO4 were a little higher and urinary Ca/Cr lower in the 90mg group pre-treatment, these differences were not statistically significant and are unlikely to account for the treatment effect observed.

The hypercalcaemia was due to a variety of tumours which were represented approximately equally in both treatment groups. Furthermore, tumour types were well matched in the subgroups described, and pre-treatment serum calcium levels were similar in those patients who relapsed. This would make it unlikely that the lack of a difference in response to the two different doses was due to differences in the spread of tumour types or serum calcium levels in the two groups. Similarly, the presence and extent of bone metastases were similar in both groups.

NcAMP values were generally raised in both groups; in the face of low or generally undetectable PTH levels this suggests that parathyroid hormone-related protein (PTHrP) was probably responsible for most instances of hypercalcaemia. It is postulated that bisphosphonates tend to be less effective where a PTHrP mediated mechanism of hypercalcaemia is thought to operate, as illustrated, for example, where TmPO4 is low (146). This would also appear to be the case in this study where those patients with elevated levels of NcAMP (and presumably also PTHrP) appear to respond less well to pamidronate than when NcAMP levels are lower. However, since pre-treatment NcAMP levels were similar in both groups this fact would not account for the treatment effect observed.

The calcium lowering effect of pamidronate is due to a combination of inhibition of osteoclastic recruitment and formation, which is seen with very low doses of pamidronate (460,461), and direct osteoclastic cytotoxicity (462). This former effect means that pamidronate may be effective even in very low doses. Previous work by Body et al.(143) has shown the presence of a dose response at very low doses (<0.05mg/kg body weight) and at standard doses, such as would be used therapeutically, there was no significant difference in efficacy. This has also been the experience in the clinical situation at Glasgow Royal Infirmary (140). Ralston et al.(140) previously reported that there is no difference either in calcium lowering effect or in duration of effect when pamidronate is used at doses of 15, 25 and 45mg nor does the duration of infusion create any difference. Only when doses as low as 5mg are used does the calcium lowering effect appear to diminish. Davis and Heath (142) have similarly shown no difference in calcium lowering action between 30mg and 60mg doses.

While Thiebaud et al.(141) have suggested that a dose response effect exists at all doses from 30mg through to 90mg, this study was not properly prospective nor were patients randomly allocated to treatment groups as those patients with modest hypercalcaemia were excluded from the 90mg group. Furthermore, patients in this study who were given higher doses of pamidronate tended to have poorer renal function than those treated with lower doses and this raises the possibility that the apparently greater calcium lowering effect at high dose may have come from an improvement in renal function rather than a greater effect on suppressing bone resorption.

While higher doses of pamidronate may obviously be indicated in certain cases of resistant hypercalcaemia (144,145), the data from this study would suggest that, in routine practice, on average there is little difference in the acute response to 30mg and 90mg pamidronate. In the first instance, therefore, a single infusion

of 30mg pamidronate in 500ml 0.9% saline infused over 4 hours is recommended

in the treatment of cancer-associated hypercalcaemia.

6.2 **Factors predicting the acute effect of pamidronate on serum calcium in cancer-associated hypercalcaemia.**

6.2.1 Introduction

In the previous study measurement of NcAMP pre-treatment seemed to be of some value in predicting the effect of pamidronate upon serum calcium levels. In this study a retrospective analysis was made between various pre-treatment biochemical and clinical variables and the acute serum calcium response to pamidronate in cancer-associated hypercalcaemia.

6.2.2 Patients and methods

Results from 35 patients with cancer-associated hypercalcaemia treated with different doses of pamidronate were reviewed. The dose of pamidronate that these patients had received had been determined randomly.

Thirteen patients received 30mg and nine 45mg pamidronate as a single intravenous infusion in 500ml 0.9% saline administered over 4 hours. A further 13 patients received 90mg pamidronate in 1 litre 0.9% saline administered over 4 hours. Five of the patients given 30mg and ten of those given 90mg had been included in the previous study.

Patient characteristics are shown in tables 6.4 and 6.5. Treatment was administered and all measurements were made after rehydration with a minimum of 4 litres 0.9% saline over 48 hours. No patient received any other treatment which would interfere with their calcium metabolism either during the period of study or for at least 1 month prior to study and no patient had been previously treated with a bisphosphonate.

Table 6.4

Pre-therapy biochemical parameters of the three treatment groups. Values shown are means.

	30mg	45mg	90mg
Serum calcium (mmol/l) (pre-treatment)	3.25	3.41	3.32
SEM	0.09	0.15	0.08
range	2.90-3.85	2.85-4.35	2.90-3.75
Serum calcium (mmol/l) (nadir)	2.62	2.56	2.63
SEM	0.08	0.07	0.04
range	2.30-3.25	2.20-2.75	2.35-2.85
PTH	generally undetectable (see text)		
	11/13 patients 7/8		11/13
NcAMP (nmol/l GF)	45.4	41.9	52.5
SEM	13.2	7.9	10.0
range	9.1-182.8	15.5-74.6	6.3-118.4
TmPO4 (mmol/l GF)	0.63	0.78	0.75
SEM	0.10	0.09	0.12
range	0.34-1.30	0.48-1.40	0.36-2.05
TmCa (mmol/l GF)	2.66	2.73	2.40
SEM	0.09	0.16	0.07
range	2.14-3.37	2.27-3.71	1.94-2.83
NaE (mmol/l GF)	4.3	3.9	3.5
SEM	1.1	1.7	0.9
range	1.5-11.2	2.2-5.5	0.3-11.0
CaE (umol/l GF)	197	224	151
SEM	43	65	21
range	46-461	42-574	29-362
Urine calcium/creatinine (mmol/mmol)	2.09	1.65	1.46
SEM	0.38	0.50	0.18
range	0.55-4.77	0.56-4.78	0.37-2.58

Table 6.5

Pre-therapy biochemical and clinical parameters of the subgroups of good and poor responders. Values shown are means. **p=0.004.

	Good Responders	Poor Responders
Serum calcium (mmol/l)	3.31	3.32
(SEM, range)	0.08,2.90-4.35	0.08,2.85-3.85
NcAMP (nmol/l GF)	29.6	65.0**
(SEM, range)	6.3,6.3-182.8	9.4,18.8-118.4
TmPO4 (mmol/l GF)	0.75	0.65
(SEM, range)	0.06,0.44-1.40	0.09,0.34-1.10
TmCa (mmol/l GF)	2.56	2.53
(SEM, range)	0.10,2.14-3.71	0.07,1.94-2.92
Dose pamidronate		
30mg	8	5
45mg	4	5
90mg	6	7
Tumour types		
Bronchial	6	6
Breast	3	6
Others*	9	5
Bone metastases		
None	2	3
Light	10	10
Heavy	3	2
Unknown	3	2

*Other tumour types were:-

Good Responders: myeloma (2), bladder (2), renal (3), tongue, neck.
Poor responders: bladder, renal adenocarcinoma (2), tongue, larynx.

The main parameters measured were NcAMP, TmPO4 and TmCa pre-treatment.
Furthermore, an assessment was made of the presence and extent of bone
metastases by review of standard radionuclide bone scan films as described in
section 3.1.

Analysis of the data was carried out by comparing the nadir of serum calcium
obtained in days 1-9 of treatment with the pre-treatment level of NcAMP,
TmPO4 and TmCa by Kendall Rank Correlations. Since we have already shown
in the previous study that the dose of bisphosphonate administered does not
influence the acute response of serum calcium, this was not considered further in
these correlations. Correlations between NcAMP and TmPO4 and NcAMP and
TmCa were also made using Kendall Rank Correlations. Differences between
groups were assessed using a Student's t-test.

6.2.3 Results

Patients were subdivided into two groups by the post-treatment serum calcium
values; those who were rendered normocalcaemic (adjusted calcium
<2.60mmol/l); "good responders") and those who remained hypercalcaemic
(adjusted calcium >2.60mmol/l); "poor responders").

It can be seen from table 6.4 that the three treatment groups were similar
biochemically. From table 6.5 it can be seen that there was no significant
difference in the dose of pamidronate given between the good and poor
responders, or in the pre-treatment serum calcium or TmCa. Furthermore, tumour
types and presence and extent of bone metastases were also similar in these two
groups. Pre-treatment NcAMP values were significantly higher in the poor
responders (mean±SEM: 65.0±9.4nmol/l GF versus 29.6±6.3nmol/l GF,
p=0.004). However, while pre-treatment TmPO4 values tended to be lower in

poor responders (mean±SEM: 0.65±0.09mmol/l GF versus 0.76±0.06mmol/l GF) this was not statistically significant (figures 6.5 and 6.6).

A significant correlation was present between pre-treatment NcAMP and nadir serum calcium (r=0.45, p=0.0001) (figure 6.7). A significant negative correlation was also present between pre-treatment TmPO4 and nadir serum calcium (r= -0.41, p=0.003) (figure 6.8). No significant correlation was found between TmCa and nadir serum calcium (r=0.11, p=NS) (figure 6.9). A significant negative correlation was evident between NcAMP and TmPO4 (r= -0.35, p=0.003), however, there was no significant correlation between NcAMP and TmCa or TmPO4 and TmCa.

The correlations do not hold in every case. For example, in figure 6.5, one patient has very high pre-treatment NcAMP (182.8nmol/l GF) but shows a good response in terms of serum calcium (nadir 2.40mmol/l). The reason for these discrepancies is not clear.

When the normal ranges for NcAMP, TmPO4 and serum calcium are considered (figures 6.6 and 6.7), 13/18 patients with NcAMP values <27nmol/l GF were rendered normocalcaemic by pamidronate whereas 16/17 patients with NcAMP values >27nmol/l GF remained hypercalcaemic. NcAMP as a predictor of the acute response of serum calcium to pamidronate, therefore, has a sensitivity of 83% and a specificity of 72%. Although 7/12 patients with pre-treatment TmPO4 values >0.70mmol/l GF were also rendered normocalcaemic, normocalcaemia was also achieved in 7/23 patients where TmPO4 was <0.70mmol/l GF. TmPO4, therefore, as a predictor of the acute response of serum calcium has a sensitivity of 66% and a specificity of 58%.
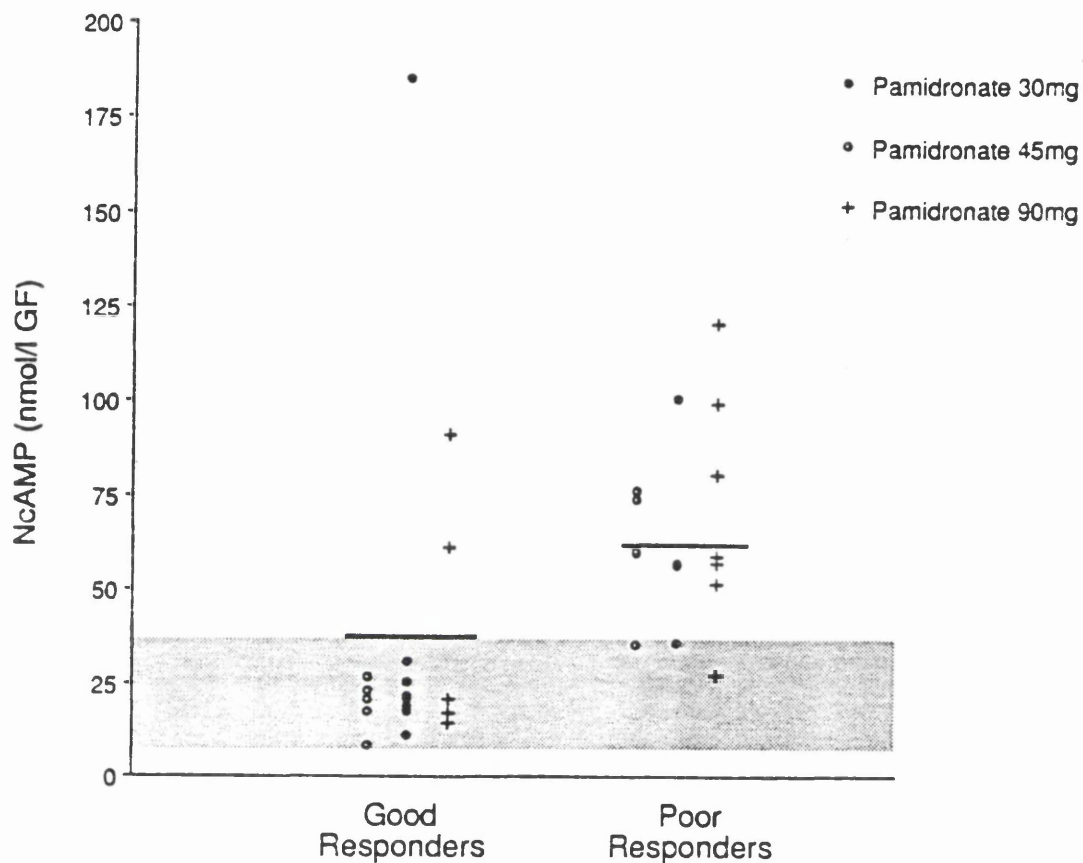
Figure 6.5

Pre-treatment levels of nephrogenous cyclic adenosine monophosphate
(NcAMP) shown against patients divided into good and poor responders (good
responders = nadir serum calcium within the reference range) (p=0.004). Bars
represent means. Dose of pamidronate administered is also shown.
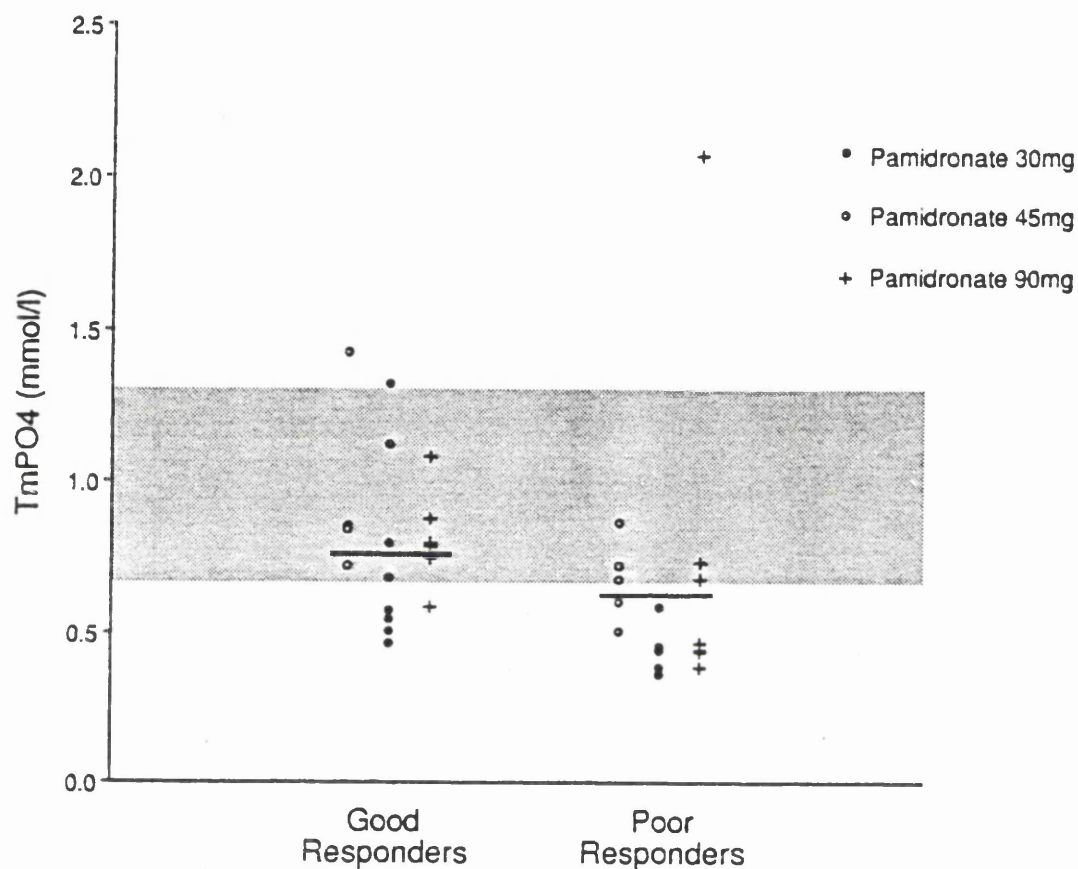
Figure 6.6

Pre-treatment levels of renal tubulular threshold for phosphate reabsorption (TmPO4) shown against patients divided into good and poor responders (good responders = nadir serum calcium within the reference range) (p=NS). Bars represent means. Dose of pamidronate administered is also shown.
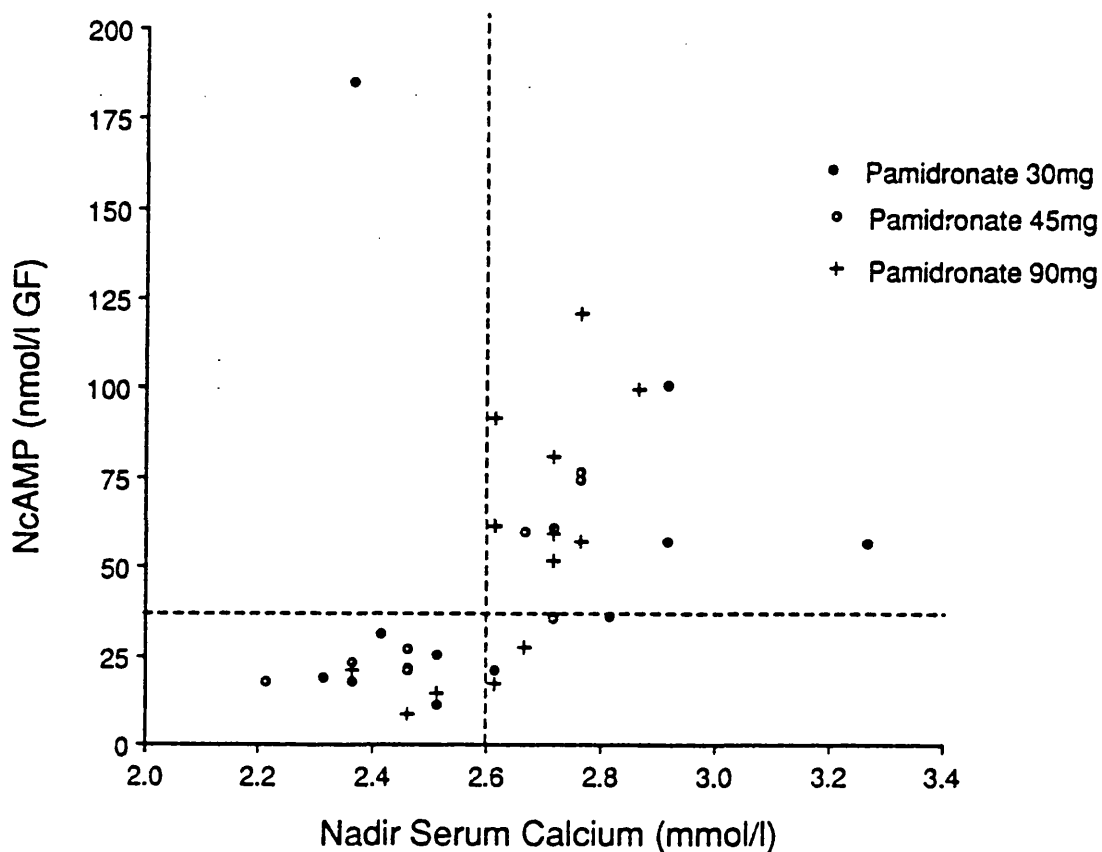
Figure 6.7

Level of pre-treatment nephrogenous cyclic adenosine monophosphate (NcAMP) expressed against nadir value of serum calcium obtained in the first 9 days of treatment (r=0.45, p=0.0001). Vertical dotted lines indicate the upper limit of the reference range for serum calcium and horizontal dotted lines the upper limit of the reference range for NcAMP.
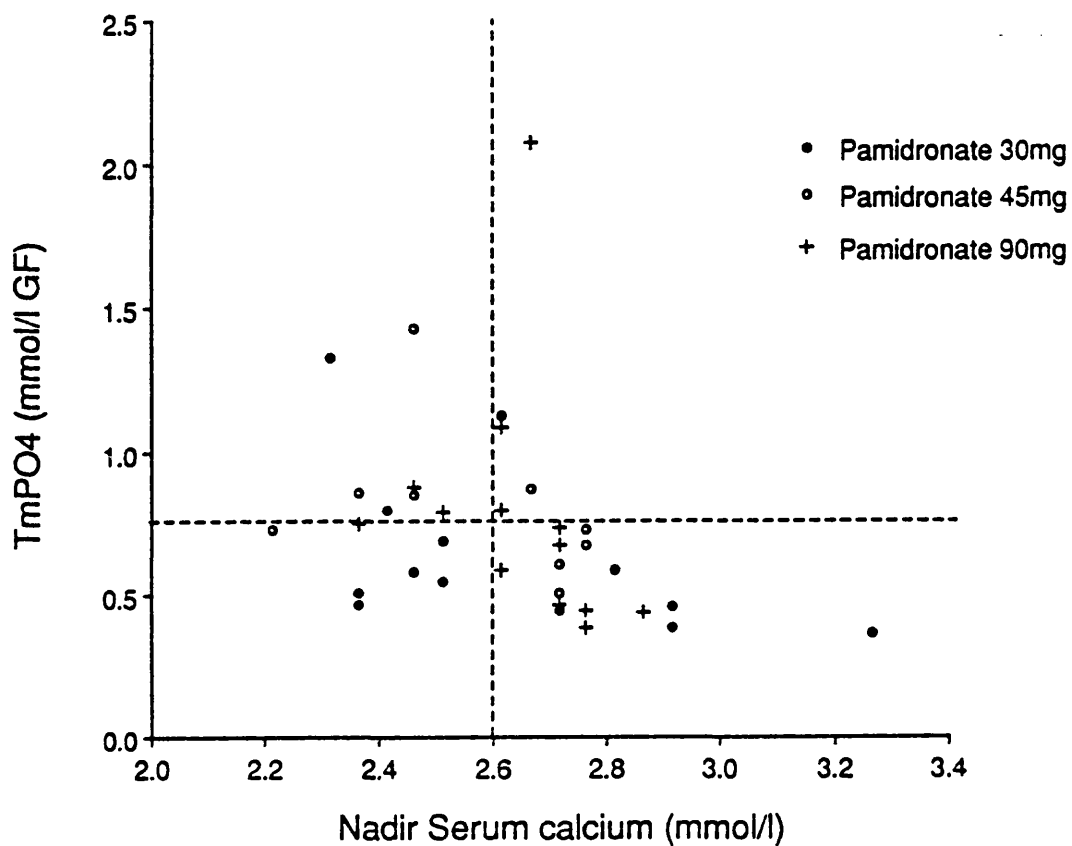
Figure 6.8

Level of pre-treatment renal tubular threshold for phosphate reabsorption (TmPO4) expressed against nadir serum calcium obtained in the first 9 days of treatment (r= -0.41, p=0.003). Vertical dotted lines indicate the upper limit of the reference range for serum calcium and horizontal dotted lines indicate the lower limit of the reference range for TmPO4.
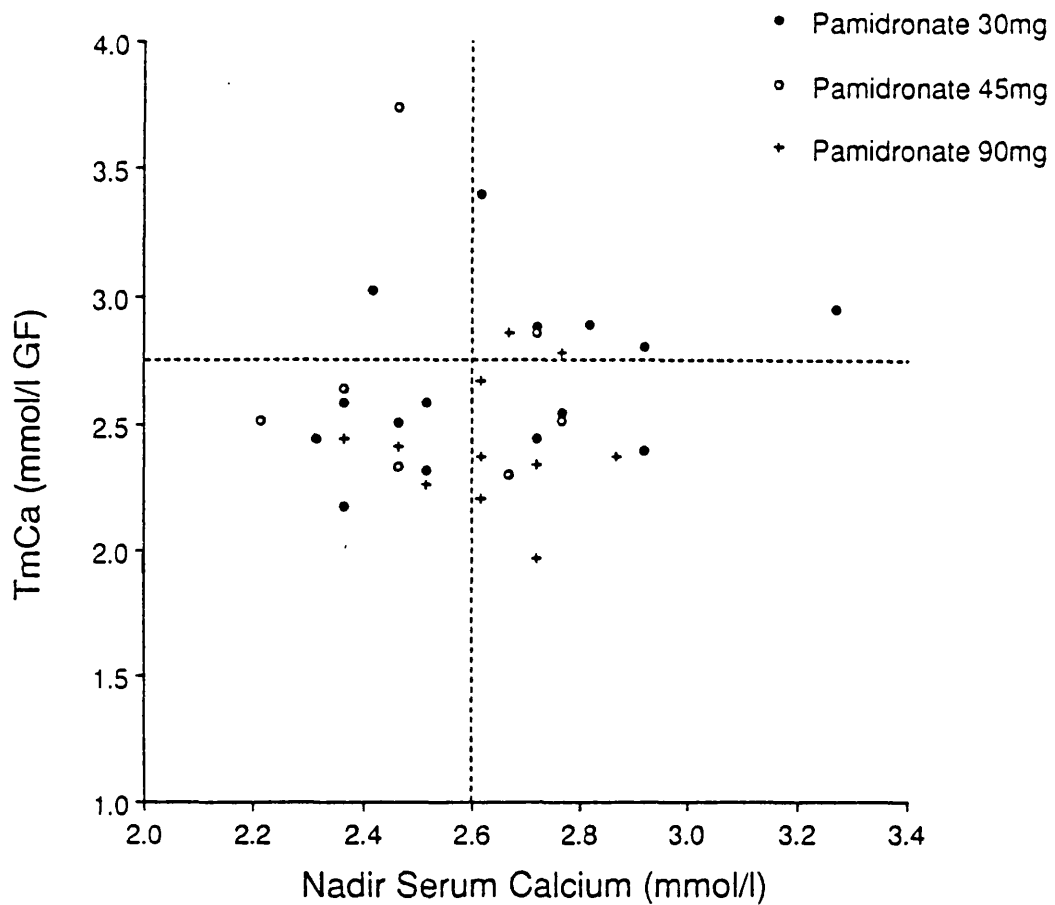
Figure 6.9

Level of pre-treatment renal tubular threshold for calcium reabsorption (TmCa) expressed against nadir serum calcium obtained in the first 9 days of treatment (r=0.11, p=NS). Vertical dotted lines indicate the upper limit of the reference range for serum calcium and the horizontal lines indicate the upper limit of the reference range for TmCa.

Only 5 of the 35 patients had detectable PTH, and in these patients the PTH concentrations remained at the lower limit of the reference range.

6.2.4 Discussion

In this study, of all the clinical and biochemical variables which were assessed, only NcAMP provided the necessary degree of sensitivity (93%) and specificity (72%), for the accurate prediction of response to pamidronate therapy in clinical practice.

As described previously, the presence of factors indicative of PTH-like activity in the absence of PTH, implies the presence of PTHrP (85,92). Although the presence of other factors such as interleukin-1 (IL-1) may be implicated to a greater or lesser extent in some instances of cancer-associated hypercalcaemia, the presence of these other factors would not cause a rise in NcAMP (463). PTHrP is thought to cause hypercalcaemia by increasing bone resorption and increasing the renal tubular reabsorption of calcium (83-85,88). Since pamidronate acts by inhibiting bone resorption (460,461,462) and does not have a direct renal effect, it is likely to be most effective where hypercalcaemia is due to increased bone resorption alone. When PTHrP levels are thought to be elevated, therefore, pamidronate is likely to be less effective. These data would be consistent with this theory.

These results differ from those of Gurney et al.(146), who looking at only 15 patients, suggested that those patients who had a lower pre-treatment TmPO4 responded less well to pamidronate. While TmPO4 may indeed tend to be lower in poor responders (as has been shown in this study), the spread of the data is such that TmPO4 cannot be used with any confidence for predictive purposes. Gurney et al.(146) failed to show any correlation between pre-treatment NcAMP

and serum calcium response. This was probably due to the small numbers of patients in their study where NcAMP data was available. However, in agreement with Gurney et al.(146), data from this present study showed that TmCa did not predict the response of serum calcium to pamidronate. This is perhaps because both an increase in bone resorption and an increase in PTH (and probably also PTHrP) mediated renal tubular calcium reabsorption are associated with an elevated TmCa (464), and this parameter cannot distinguish between humoral and non-humoral hypercalcaemia.

In summary, this study shows that pre-treatment levels of NcAMP but not TmPO4 or TmCa may predict the acute response of serum calcium to pamidronate. In clinical practice it appears there is a sub-group of patients who are resistant even to large doses and multiple infusions of pamidronate (144,145), and a single measurement of NcAMP - or probably also PTHrP - seems likely to predict these patients. Their treatment, however, remains problematical. Assuming that the hypercalcaemia is being driven by PTH-like factors, such as PTHrP, what is needed is a specific agent which will block the action of these tumour products at the site at which they act - the PTH receptor.

6.3 **The use of ionised calcium in monitoring the calcium lowering effect of pamidronate**

6.3.1 Introduction

In the previous two studies the measurement obtained of serum calcium was adjusted to take into account the prevailing serum albumin concentration. Since serum albumin levels are generally low in patients with cancer (150), it is important that these are taken into account in the assessment of hypercalcaemia. Failure to do so will result in the incidence of hypercalcaemia being significantly underestimated (147). Although calcium in serum is highly protein bound (20), it is also known that changes in this calcium/protein binding may occur in critically ill patients (465,466) and this may render the usual formulae for calculation of adjusted calcium less useful (148). Measurement of ionised calcium is useful in such situations (465). In order to determine whether measurement of adjusted calcium is a valid way of following the actions of a calcium lowering drug, serial measurements of ionised calcium were carried out in patients with cancer-associated hypercalcaemia before and after treatment with pamidronate.

6.3.2 Patients and methods

Eight consecutive patients with cancer-associated hypercalcaemia were studied and their details are shown in table 6.6. Following 48 hours of intravenous rehydration with a minimum of 4 litres 0.9% sodium chloride solution, these patients were treated with a single intravenous infusion of pamidronate of either 30mg or 90mg. Serum ionised calcium (ICa), adjusted calcium (AdjCa) and total calcium (TotCa) was then measured daily for 7 days. The dose of pamidronate given was chosen essentially randomly. Five of the patients included in this study had also been included in the study described in 6.1. No patients in this present

study were receiving any drugs thought likely to interfere with their calcium homoeostasis either during or for a minimum of 6 months prior to this study. Furthermore, no patients were given any drugs which may potentially alter their plasma protein binding and none required blood transfusion during the study period. All blood samples were obtained after overnight fast with the patient in recumbency. Prolonged venous stasis was avoided during venepuncture.

Statistical methods used were the Wilcoxon test for paired samples and Linear Regression Analysis.

6.3.3 Results

As expected mean serum calcium fell to within or just above the reference range irrespective of whether ionised calcium, adjusted calcium or total calcium was measured (figure 6.10). Nadir (mean±SEM) ICa was reached at day 7 (1.28mmol/l±0.04, p<0.001), as was nadir AdjCa (2.61mmol/l±0.04, p<0.001) and nadir TotCa (2.26mmol/l±0.05, p<0.001). TotCa, however, had been within the reference range for serum calcium from day 3 post-treatment.

The correlations between ICa and AdjCa and ICa and TotCa at each day of treatment are shown in table 6.7. It can be seen that there was a very close correlation between both AdjCa and ICa and TotCa and ICa pre-treatment. Post-treatment, however, the correlation between TotCa and ICa was inconsistent and at times no significant correlation was present between these two variables. This was particularly the case on days 1 and 2 and 6 and 7 post-treatment. In contrast, though the post-treatment correlation between AdjCa and ICa was also somewhat variable, this correlation did generally remain close enough to make measurement of adjusted calcium a reasonably accurate reflection of ionised calcium levels. The greatest discrepancy between ICa and AdjCa was seen at
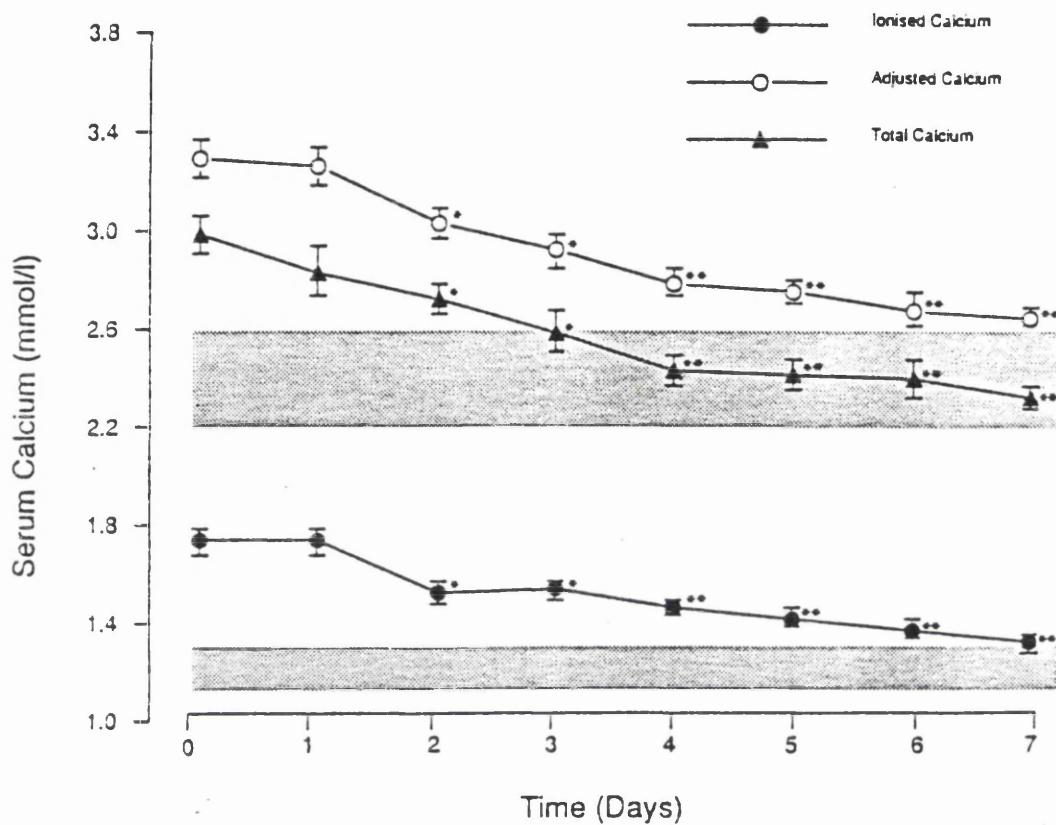
Figure 6.10

Changes in ionised, adjusted and total calcium after treatment. Pamidronate was administered on day 0. Points shown are means ± SEM. *p<0.01 **p<0.001

Table 6.7

Correlations between ionised calcium and adjusted and total calciums before and after treatment.

	Adjusted Calcium	p	Total Calcium	p
Day 0	r=0.95	0.0001	r=0.86	0.0001
Day 1	r=0.74	0.04	r= -0.14	NS(0.77)
Day 2	r=0.51	NS(0.26)	r=0.31	NS(0.68)
Day 3	r=0.91	0.0001	r=0.80	0.01
Day 4	r=0.80	0.05	r=0.82	NS(0.35)
Day 5	r=0.77	0.05	r=0.76	0.05
Day 6	r=0.71	0.04	r=0.15	NS(0.86)
Day 7	r=0.62	NS(0.09)	r=0.15	NS(0.77)

days 2 and 7 post-treatment. One patient with myeloma was included in this study and, even in this case, ICa and AdjCa correlated closely after treatment (r=0.73). Serum albumin levels pre-treatment were all generally low (mean 30g/l, range 22-35) and were <30g/l in 3/8 cases. Pre-treatment venous pH values were all within the reference range of 7.35-7.45. No significant change in pH was noted with treatment.

6.3.4 Discussion

The amount of "free" or ionised calcium in the circulation depends largely on the concentration of circulating albumin (467). Serum albumin levels are typically low in patients with cancer-associated hypercalcaemia (150) and furthermore, the binding affinity of calcium for albumin is not constant at albumin concentrations below 30g/l (150) - such as was the case for 3/8 of the patients in this study. However, results from this study show that the correlation between ionised calcium and adjusted calcium is very close and even after pamidronate therapy, while the correlation may be more variable, the discrepancy observed is, on average, not likely to be of major clinical relevance.

Correlation between ionised calcium and total calcium pre-treatment was also close, though after treatment this relationship tended to be less consistent. Furthermore if total calcium was used as a measure of the serum calcium response to treatment, many patients would be erroneously described as normocalcaemic. In this study total calcium was within the reference range from day 3 post-treatment, whereas ionised and adjusted calcium concentrations did not fall to the upper limit of the reference range until day 7. The greatest discrepancy between ICa and both TotCa and AdjCa was noted in the initial period just after pamidronate treatment and again around day 7 after treatment. One possible explanation for the discrepancy noted in the initial 48 hours after

therapy is that intravenous pamidronate may generate an acute phase response with pyrexia often prominent (468). A rise in interleukin-6 (IL-6) levels has been noted in association with this pyrexia (469) and this in turn may stimulate a rise in acute phase proteins (470) which may have a significant effect on calcium binding. A less likely explanation is that pamidronate itself is binding calcium and therefore reducing ionised calcium levels while at the same time leaving both total and adjusted calcium levels unchanged. While it is true that bisphosphonates will generally bind divalent cations strongly (471), these drugs in general, including pamidronate, have a very short plasma half-life being quickly excreted in urine or taken up into bone (472). Any direct effect on serum calcium levels are, therefore, likely to be very transient.

The reason why the correlation between ICa and TotCa and AdjCa is less good around 7 days after treatment is not clear. By this stage after treatment many patients are symptomatically improved (109) and may be more mobile than was the case before pamidronate therapy. This increase in mobility may alter plasma protein concentrations and when this alteration is not taken into account (as where total calcium is measured), the correlation with ICa becomes less reliable. This, of course, would not explain changes in the relationship between ICa and AdjCa. A second possible explanation is that changes may be occurring which affect the measurement of ICa itself. It is known that measurement of ICa depends to some extent on the prevailing serum albumin concentrations with ICa levels rising where albumin rises (151). In this present study, though no statistically significant changes in serum albumin were observed after treatment, the mean post-treatment albumin level was lower and it is possible that minor changes in serum albumin may have occurred and this in turn may have had a direct effect on ICa measurement. This would, therefore, affect the correlation both with TotCa and AdjCa.

It has been suggested that calcium binding to plasma proteins may be altered in certain disease states such as myeloma due to the presence of excess immunoglobulin. This might mean a normal ionised calcium concentration accompanying an increased adjusted calcium concentration. Two case reports have described this scenario (473,474). Myeloma was the diagnosis in one patient in this study, however this problem was not encountered. Ionised and adjusted calcium correlated closely.

Discrepancies between albumin adjusted calcium and ionised calcium concentrations have been described in critically ill patients where adjusted calcium concentrations may be low though ionised calcium normal (466). It has been suggested that this misclassification is due to the presence in the circulation of an (unidentified) factor which alters the binding of calcium to albumin (465). This problem was not apparent in the patients followed in this study. This may suggest that this putative factor is not present in patients with cancer. An alternative explanation would be that these patients were at an earlier stage in their illness and therefore less unwell than the "critically ill" patients (466) studied previously. This would seem unlikely as it has been shown that the median life expectancy for patients with cancer-associated hypercalcaemia is only 30 days (109).

In this study all ionised calcium measurements were corrected to pH 7.40 to correct for losses in carbon dioxide that may have occurred between the time of venepuncture and calcium measurement (375). It has been suggested that critically ill patients may have alterations in serum pH and that ionised calcium results should not, therefore, be pH adjusted (148). The data from this study show venous pH to be within normal limits and correction of ionised calcium concentrations to pH 7.40 is therefore both valid and desirable.

Though measurement of ionised calcium is certainly the "gold standard" use of this technique presents several practical problems compared with measuring total calcium then adjusting for the prevailing albumin concentration. In particular samples must be collected anaerobically and analysed as soon as possible, generally within 1 hour of collection. As a result few laboratories use ionised calcium as a routine measurement and only some studies use this method to monitor the effect of treatment after administration of a calcium lowering drug. In those studies where ICa, TotCa and AdjCa were measured simultaneously (475), formal correlations between these parameters were not made and it is therefore not possible to draw any conclusion about which method is preferable to use.

This study would suggest that while the relationship between ionised calcium and adjusted calcium may vary when pamidronate is given, this alteration is slight and on average, unlikely to be of any major clinical significance. Furthermore, measurement of total (unadjusted) calcium is potentially misleading. In routine circumstances, therefore, measurement of adjusted calcium is sufficient when monitoring the response to treatment in patients with cancer-associated hypercalcaemia.

6.4 Immobilisation-related hypercalcaemia: a possible novel mechanism and response to pamidronate

6.4.1 Introduction

Immobilisation-related hypercalcaemia is characterised by increased bone resorption and osteopenia. Increased osteoclastic activity is seen in bone biopsies of such patients (196) and biochemical studies have demonstrated increased urinary hydroxyproline excretion (197).

Pamidronate acts by inhibiting bone resorption and, as such, might be expected to be effective in the treatment of immobilisation-related hypercalcaemia. The studies described in sections 6.1 and 6.2 both suggested that pamidronate is less effective where parathyroid hormone-related protein (PTHrP) is thought to be operative in the pathogenesis of the hypercalcaemia. Since this is not thought to be the case in immobilisation-related hypercalcaemia, if this hypothesis is indeed correct, the calcium lowering effect of pamidronate may be more sustained, and effective at lower doses in patients with immobilisation-related hypercalcaemia than is usually the case in cancer-associated hypercalcaemia.

Immobilisation-related hypercalcaemia is usually associated with conditions where bone turnover is already increased such as in children, adolescents (191) and in patients with Paget's disease of bone (192). This study describes a series of patients where these predisposing factors were not present.

6.4.2 Patients and methods

The details of the patients studied are shown in table 6.8. All were normally hydrated both clinically and biochemically prior to treatment. All infusions of

Table 6.8

Clinical and biochemical details of patients with immobilisation-related hypercalcaemia

	Dose pamidronate (mg)	Age	Sex	Diagnosis	Duration of immobilisation before hypercalcaemia	adjust calcium	serum alb	serum creat	PTH	25D3	1,25D3
Patient 1	10	35	M	Abdominal fistulae and sepsis	4 months	3.20	30	60	<250	12	16
Patient 2	15	19	M	Septic hip	3 months	3.10	27	85	480	-	-
Patient 3	30	58	F	Pulmonary TB	5 weeks	3.15	31	65	<0.8	12	-
Patient 4	30	46	F	Crohn's - fistulae and sepsis	3 months	3.30	34	85	<1.0	13	10
Patient 5	45	62	F	Ruptured oesophagus and mediastinal sepsis	3 months	3.00	30	50	<0.8	71	-

calcium (mmol/l)   alb - albumin (g/l)   creat - creatinine (mmol/l)   PTH - parathyroid hormone (ng/l,
pmol/l)   25D3 - 25-hydroxyvitamin D3 (nmol/l)   1,25D3 - 1,25-dihydroxyvitamin D3 (pmol/l)

pamidronate were given intravenously in 500ml 0.9% saline over 4 hours. A

diagnosis of sepsis was made in each patient on the basis of elevated white cell

counts, erythrocyte sedimentation rates (ESR) and positive bacterial culture from

the site of infection, and in all cases (except patient 3) from blood cultures. At

the time of study all patients were receiving appropriate antimicrobial therapy.

None of these patients |was receiving any therapy thought likely to interfere in

their calcium homoeostasis except patient 4 who was receiving 50mg per day

prednisolone, though this had been commenced 4 months prior to the

development of hypercalcaemia.

In patients 1 and 2, PTH was measured by a double antibody radioimmunoassay

and patients 3,4 and 5 intact PTH(1-84) was measured (see 2.1.2).

Transiliac bone biopsies were carried out after tetracycline double labelling using

a Meunier trephine needle in patients 2 and 4 prior to treatment.

Statistical assessment of changes in serum calcium and urinary

calcium/creatinine were carried out using a Mann-Whitney U-test.

6.4.3 Results

The response of serum calcium and urinary calcium/creatinine ratio following

pamidronate treatment is shown in figures 6.11 and 6.12. It can be seen that all

patients were normocalcaemic by day 3 post-treatment. Patients 1-3 began to

mobilise soon after pamidronate treatment and remained normocalcaemic.

Patients 4 and 5 remained immobile and recurrence of hypercalcaemia occurred

at days 33 and 26 respectively. Calcium/creatinine ratios fell in all patients with a

nadir at day 3. Figure 6.13 shows the relationship between serum calcium and the

amount of calcium excreted per unit glomerular filtrate. Values tended to lie to
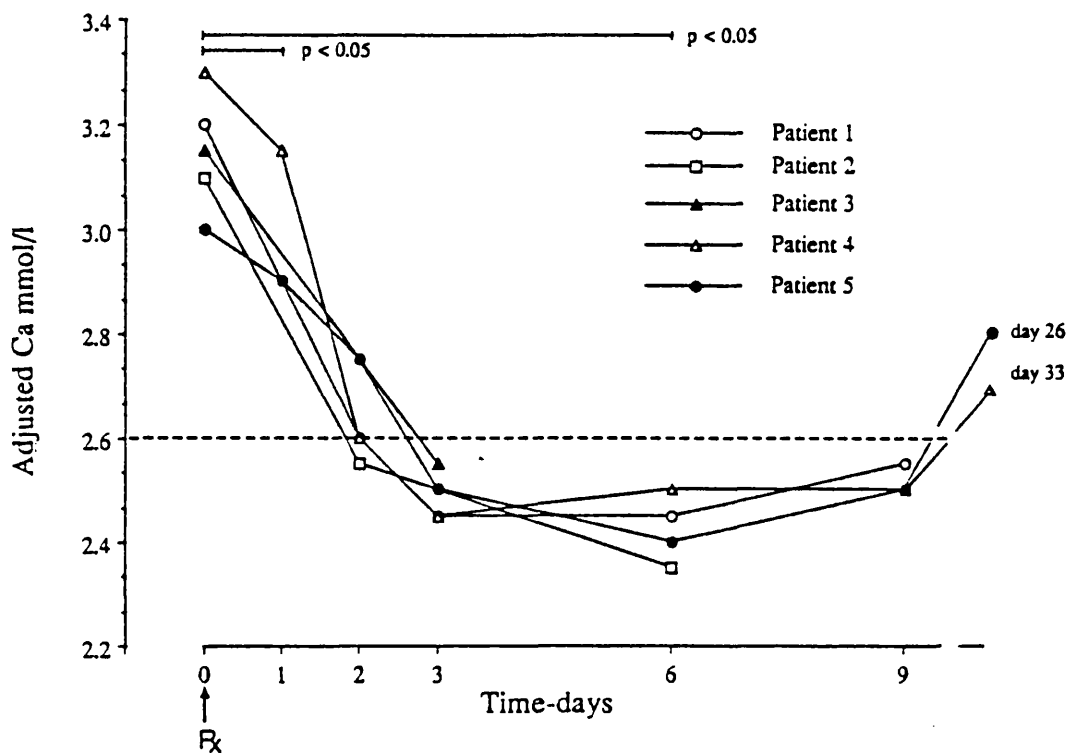
Figure 6.11

Changes in adjusted serum calcium after pamidronate therapy (dotted line indicates upper limit of the reference range).
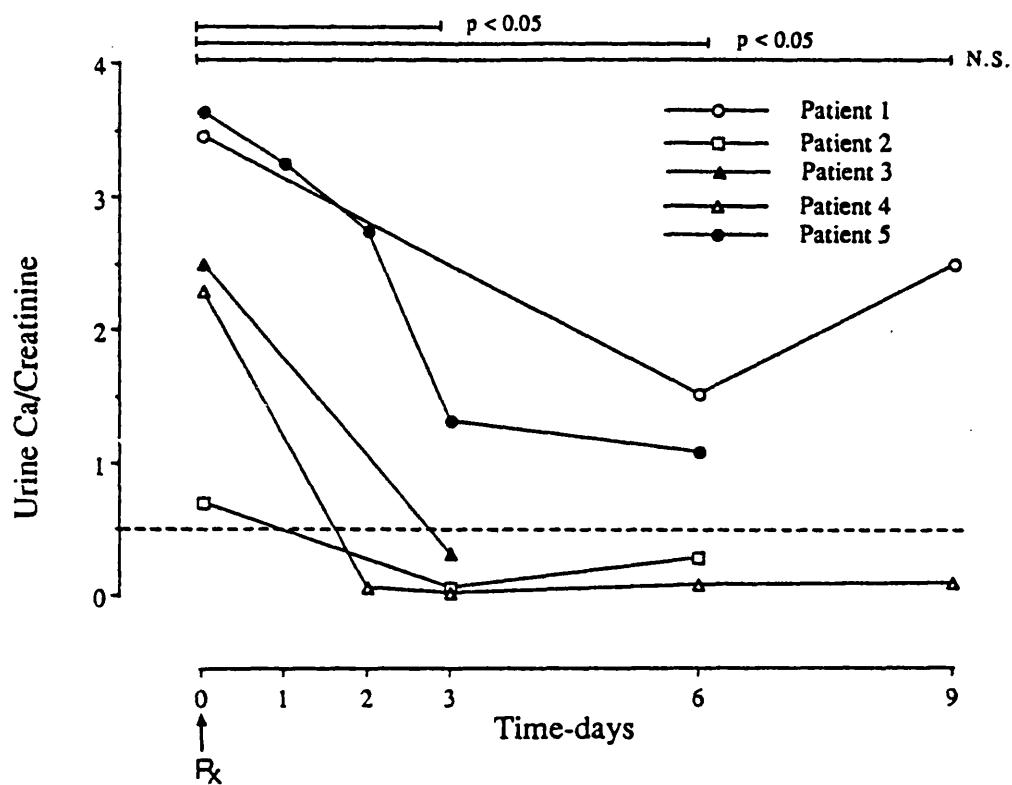
Figure 6.12

Changes in urinary calcium/creatinine ratio following pamidronate therapy (dotted lines indicate upper limit of the reference range).
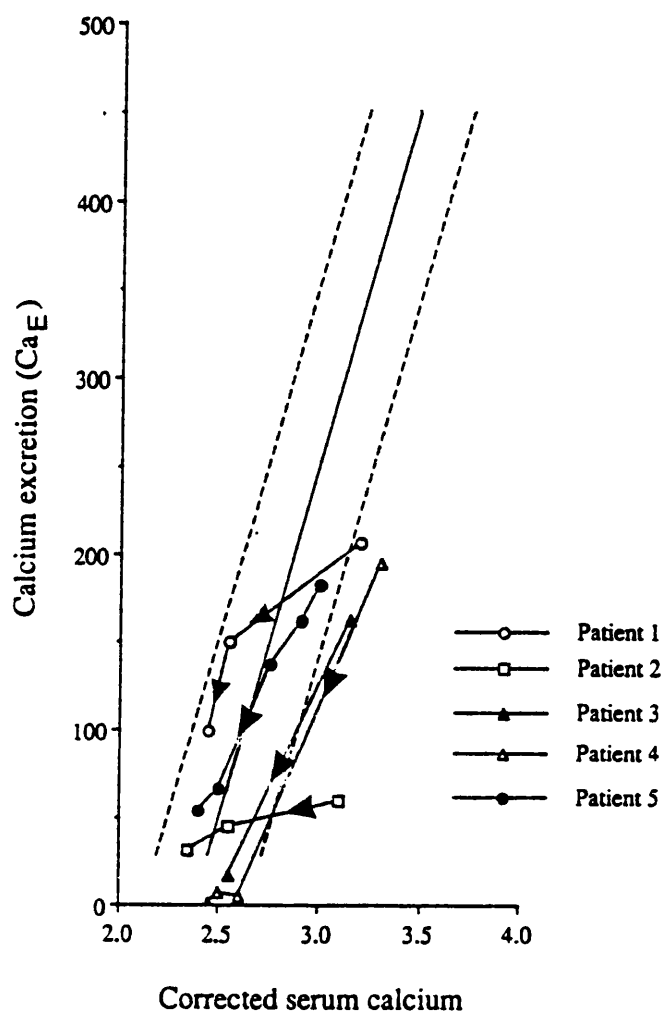
Figure 6.13

Relationship between adjusted serum calcium and calcium excretion (CaE).
Dotted lines indicated two standard deviations from the mean of the normal
relationship (from Peacock et al.(36)). Arrows indicate post-treatment changes in
CaE/serum calcium relationship.

the right of the normal reference range described by Peacock et al.(36), though this was marginal.

From table 6.8 it can be seen that serum PTH was undetectable in all patients except patient 2 where it was measured at the low end of the reference range. As might be expected 1,25(OH)2D3 levels (where measured) and 25(OH)D3 levels were low, possibly reflecting poor nutritional state, lack of sunlight exposure or general illness. In patient 5, 25(OH)D3 levels were normal, although this patient was receiving total parenteral nutrition which included 200 units per day of vitamin D. Hydroxyproline/creatinine (HP/Cr) ratios were markedly elevated in all patients pre-treatment. Median value was 0.101 (range 0.045-0.180). Similarly pre-treatment urinary calcium/creatinine ratios were markedly elevated. Median was 2.50 (range 0.69-3.63).

Marked osteopenia was present histologically in patients 2 and 4 with trabecular bone volumes of 21% and 16% respectively (normal >25%). Bone formation rates were normal with osteoid surfaces of 9% and 17% (normal <24%). Bone resorption, however, tended towards the upper limit of normal with resorption surfaces of 6.3% and 5.2% (normal <7.0%). There was no histological evidence of hyperparathyroidism and mineralisation was normal. The presence of osteopenia was later confirmed clinically in patient 4, as this patient sustained an atraumatic vertebral compression fracture.

Relapse was defined as a rise in serum calcium to 2.80mmol/l. In the two patients where this occurred a further infusion of pamidronate at the same dose as the initial infusion restored normocalcaemia. The duration of normocalcaemia varied after this second infusion from 14 days (patient 5) to 97 days (patient 4). This may have been because patient 4 became at least partly mobile. Both

patients were fully mobile after they had received a total of three infusions each and normocalcaemia was maintained thereafter.

6.4.4 Discussion

Immobilisation-related hypercalcaemia, though apparently uncommon, is important in that complications such as renal stones and osteoporosis may occur. In one series (476), over 50% of children immobilised due to spinal cord injury who were hypercalcaemic developed urolithiasis. The prolonged period of negative calcium balance which occurs as a result of the hypercalciuria predisposes to osteoporosis and fracture, an example of this was seen in patient 4 in this study.

Previous work using radiolabelled calcium (477) and the incorporation of tetracycline into bone (44) has suggested that the mechanism of the hypercalciuria and osteoporosis is due to increased bone resorption and decreased bone formation. The high urinary hydroxyproline/creatinine and calcium/creatinine ratios in this study would be consistent with this proposed increase in bone resorption. The data from the two bone biopsies in this study, however, suggest that bone formation rates are within normal limits.

The patients in this study were significantly older than would usually be the case in immobilisation-related hypercalcaemia as this condition usually occurs in children and/or adolescents where bone turnover is higher (191). Furthermore, there was a greater time interval between the onset of immobilisation and the development of hypercalcaemia than is usual. The main feature which all these cases had in common was major sepsis with elevated white cell counts in all cases. Cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factors alpha and beta (TNFα,TNFβ) and transforming growth factors

alpha and beta (TGFα,TGFβ) have all been demonstrated to be associated with

increased bone resorption and, in some instances, hypercalcaemia (106,107).

Furthermore, elevated levels of IL-1 have been described in patients with

infection post-surgery (478). Unfortunately at the time of this study it was not

possible to measure these cytokines. However, it may be postulated that the

presence of some or all of these factors was contributory to the pathogenesis of

the hypercalcaemia.

Although some calcium excretion (CaE) and associated serum calcium values

were, when plotted graphically, to the right of the line defining their normal

relationship (figure 6.13), these were borderline and not indicative of any major

increase in the renal tubular calcium reabsorption component of the raised serum

calcium. This feature may simply reflect the fact that these patients did not

receive intravenous saline rehydration prior to therapy.

Other workers have suggested that calcitonin (197), etidronate (131) and

clodronate (201) are of some value in the treatment of immobilisation-related

hypercalcaemia and hypercalciuria. A recent case report, concerning a single

patient, has also demonstrated that intravenous pamidronate may be useful in the

management of this condition (202).

The data presented in 6.1 and 6.2 has demonstrated that in patients with cancer,

PTHrP mediated hypercalcaemia may respond less well to pamidronate, and it

was suggested that larger doses of this drug be reserved for these patients. This

study shows that the converse is also true. Immobilisation-related

hypercalcaemia is not PTHrP mediated and in this study doses of as low as 10mg

pamidronate were effective and had a sustained calcium lowering effect.

In conclusion, therefore, pamidronate is both safe and effective as a treatment for the hypercalcaemia and hypercalciuria which may accompany immobilisation. Furthermore, it would appear that sepsis, possibly by way of cytokine release, may also contribute to the pathogenesis of this syndrome.

CHAPTER 7

THE USE OF PAMIDRONATE IN THE TREATMENT OF PAGET'S DISEASE OF BONE AND OSTEOPOROSIS

7.1 **The use of pamidronate in the management of Paget's disease of bone**

7.1.1 Introduction

The results from the previous chapter showed that pamidronate is generally effective in inhibiting the increased bone resorption often associated with hypercalcaemia. Increased bone resorption is one of the cardinal features of Paget's disease of bone. Calcitonin (222,224,232-236) and mithramycin (224) are potent inhibitors of bone resorption and have been shown to be effective in treating Paget's disease. Significant problems exist, however, with respect to the development of resistance to calcitonin (124,479) and the toxicity of mithramycin (118-120).

The bisphosphonates have also been shown to be effective in the management of Paget's disease (133). Recent work has suggested that pamidronate is both safe and effective in the management of this condition (251-257) however, optimum dosage regimens are yet to be defined. Furthermore, it is not yet established whether a dose/response effect exists with pamidronate in the management of Paget's disease. This study aimed to address these points.

7.1.2 Patients and Methods

Patients with symptomatic Paget's disease were recruited for this study. The diagnosis of Paget's disease was based on finding characteristic abnormalities in biochemistry, radiology and radionuclide bone scanning. Where possible, further confirmation was obtained by transiliac bone biopsy. A total of 39 patients were included (table 7.1) and the study consisted of two main treatment regimens.

Table 7.1

Profile of patients with Paget's disease before treatment

	Group 1 (n=15)	Group 1A (n=6)	Group 2 (n=24)
Sex(male/female)	7/8	3/3	10/14
Mean age (years)	70	67	66
range	41-87	41-75	56-85
Mean ALP (U/l)	770	1725	880
95% CI	210-5700	950-5700	175-5600
Mean number lesions	4	6	4
range	2-10	5-10	2-11
Mean WBR (%)	49.7	57.9	50.5
95% CI	35.6-57.7	45.6-73.6	34.8-58.4

ALP = serum alkaline phosphatase

95% CI = 95% confidence interval

WBR = whole body retention of radiolabelled bisphosphonate

Fifteen patients (group 1) received weekly infusions of 30mg pamidronate for 6 weeks. Severe Paget's disease was considered to be represented by a pretreatment serum alkaline phosphatase (ALP) of >1000U/l (i.e. four times the upper limit of the reference range). Six patients fell into this category and these 6 comprised a subgroup (group 1A) that received infusions of 60mg per week for three weeks in addition to the initial 6, 30mg infusions (table 7.1). Group 2 consisted of 24 patients who received infusions of 45mg pamidronate every three months for 1 year, irrespective of their pre-treatment ALP levels. The total dose of pamidronate given was the same in both groups, i.e 180mg, although group 1A received a total dose of 360mg. All infusions were given in 500ml 0.9% sodium chloride solution and infused intravenously over 4 hours. Five patients in group 1 and 2 patients in group 2 had previously been treated with oral etidronate. None, however, had received treatment in the year prior to this study and all were considered to have relapsed biochemically.

Symptomatic responses were assessed by visual analogue scores in 12 patients in group 1. A randomly selected group of 6 of these patients was given placebo infusions of 0.9% sodium chloride solution weekly for three weeks prior to commencing pamidronate. This phase of the study was carried out in a single-blind manner.

Biochemical variables were measured in group 1 patients after 1 month of treatment, at the end of treatment, at 6 months and at 1 year. In group 2 patients measurements were made at intervals of three months throughout the study.

As the data were skewed, they were logarithmically transformed and a Student's t-test was used to assess within the group. One way analysis of variance was used to assess changes between different treatment groups. Correlations were carried out using Spearman rank coefficients.

7.1.3 <u>Results</u>

The levels of serum ALP and urinary hydroxyproline/creatinine (HP/Cr) ratios
are expressed as means with 95% confidence intervals (95% CI).

Serum ALP decreased steadily throughout the year in the two treatment groups
(figure 7.1). At the end of 1 year mean ALP was 230U/l in group 1 (95% CI: 188
to 281, p<0.00001) and 297U/l (227 to 389, p<0.00001) in group 2. Figure 7.2
shows the decrease in serum ALP in both groups as their absolute values. In
group 1, the ALP level in most patients decreased to within or just above the
reference range, irrespective of the pretreatment level of ALP. In group 2,
however, although there was a significant decrease in ALP levels, this was less
marked in the more severely affected patients (i.e. those with a pretreatment ALP
>100U/l). In group 1A, 4 of the 6 patients with a pretreatment ALP >100U/l
achieved normal levels of ALP and in the remaining 2 the ALP level decreased
to less than 500U/l. In all 9 of the remaining patients in group 1, the ALP
decreased to within the normal reference range. In group 2, 10 patients had an
ALP level greater than 1000U/l. None of these patients achieved a normal level
of ALP. Of the remaining 13 patients in this group, all but 1 patient achieved a
normal ALP level and in this patient the level of ALP was just above the upper
limit of the reference range at 310U/l. Pretreatment values for these patients are
shown in table 7.1.

The maximum decrease in HP/Cr (figure 7.3) occurred at the end of treatment in
group 1, with a mean of 0.022 (0.015 to 0.033, p<0.001), and at 3 months after
the start of treatment in group 2, with a mean of 0.029 (0.022 to 0.037, p<0.003).
In 2 patients in group 1 and 5 patients in group 2, HP/Cr remained elevated.
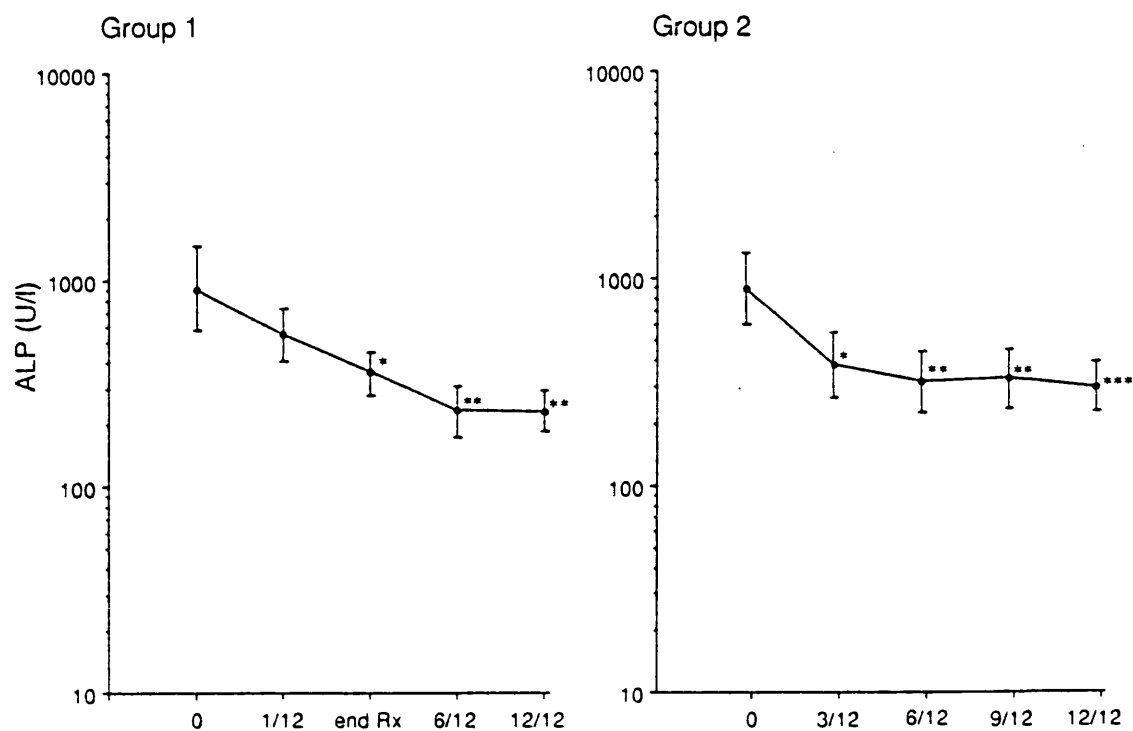
Figure 7.1

Changes in levels of serum alkaline phosphatase (ALP). Values shown are means with 95% confidence intervals on a log scale. *p<0.002, **p<0.0001, ***p<0.00001 (versus pre-treatment values).
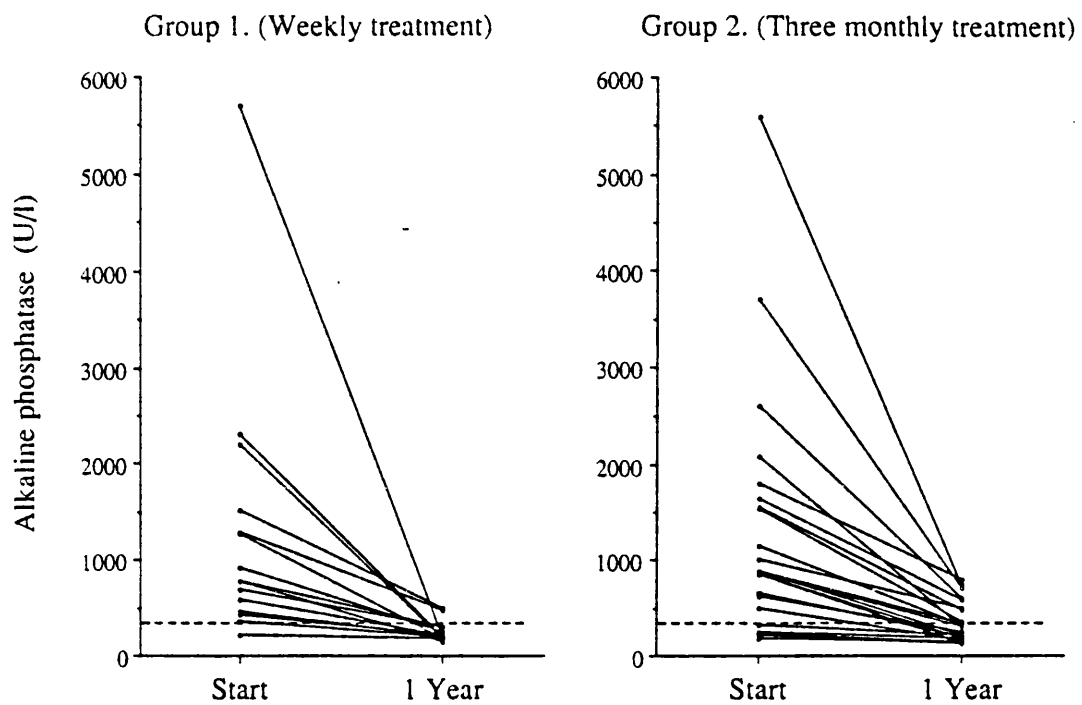
Figure 7.2

Changes in levels of serum alkaline phosphatase (actual values). Dotted line indicates upper limit of reference range.
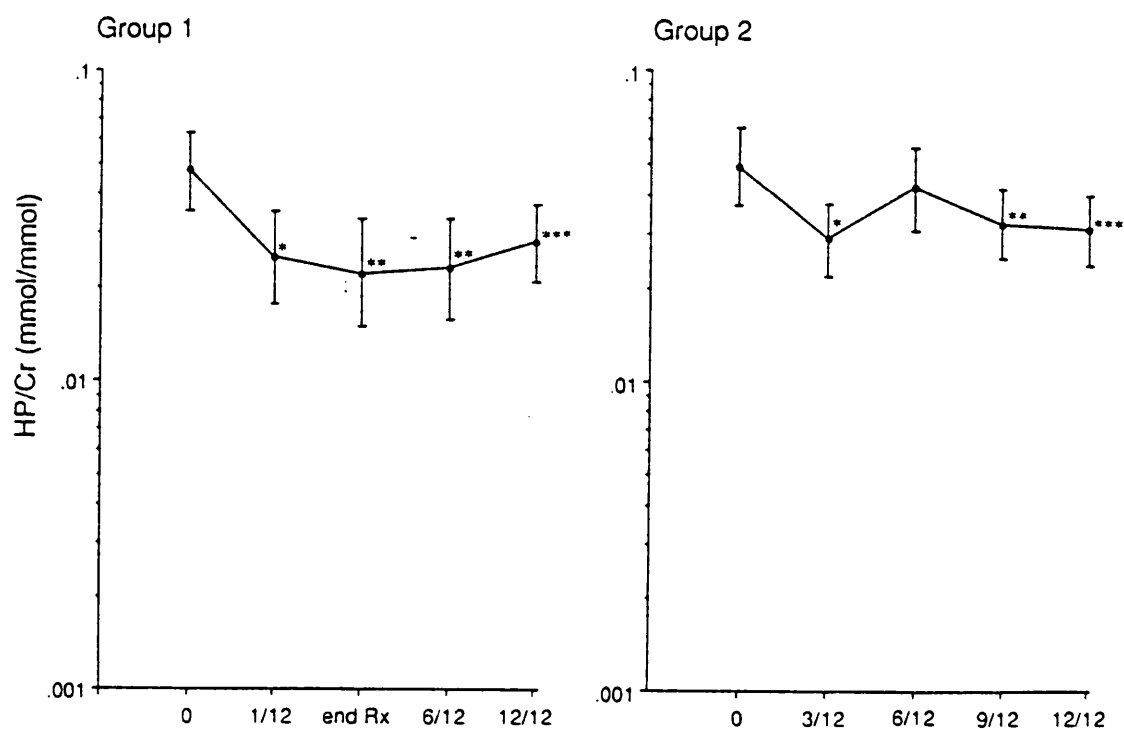
Figure 7.3

Changes in fasting urinary hydroxyproline/creatinine (HP/Cr) ratio. Values shown are means with 95% confidence intervals on a log scale. *p<0.003, **p<0.001, ***p<0.01 (versus pre-treatment values).

There was a significant positive correlation between serum ALP and HP/Cr both before (r=0.63, p=0.0002) and after treatment (r=0.41, p=0.01). No statistically significant difference was present between groups 1 and 2 in terms of serum ALP or HP/Cr.

A significant overall improvement in symptoms, assessed by visual analogue pain scores, was seen during treatment (figure 7.4). Where placebo infusions were given there was little change in pain scores, though these did subsequently improve (after 3 weeks) when these patients received the active drug.

There was a significant decrease in the whole body retention of radiolabelled bisphosphonate at 24 hours over the year of follow up from a mean of 49.3% to 41.0% (p<0.01).

Treatment was well tolerated by most patients, with asymptomatic pyrexia occurring in only 19%. This is a lower figure than might be expected, perhaps because the first 15 patients treated received pamidronate as outpatients and asymptomatic pyrexia would not have been noticed. Three patients in group 2 experienced mild rigors and influenza-like symptoms in the first 24 hours after the infusion. These symptoms did not recur with subsequent infusions.

7.1.4 Discussion

This study shows that pamidronate given at wide intervals over 1 year is as effective for the treatment of active Paget's disease as a short course of weekly treatment. The findings of this study also suggest that there is a dose/response relationship with pamidronate in the treatment of Paget's disease. The more severely affected patients had a less marked reduction in serum ALP when given the same dose of pamidronate as less severely affected patients. Also, the 6 most
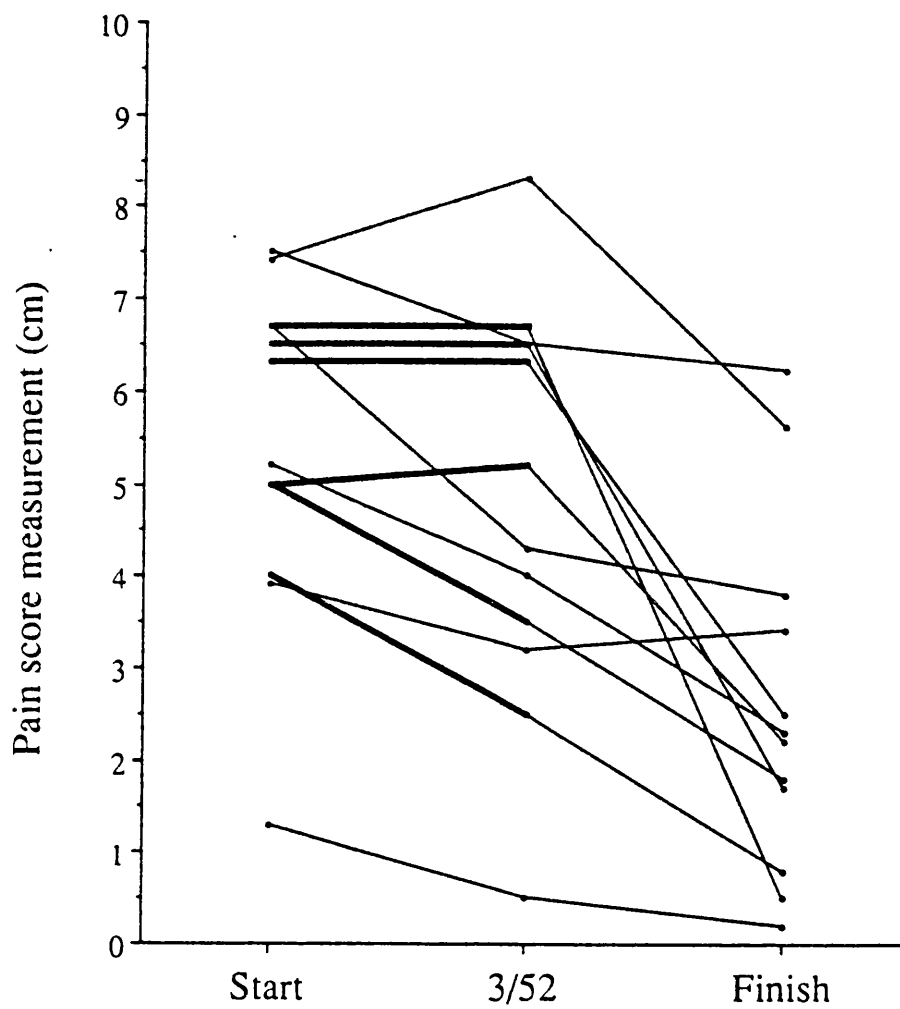
Figure 7.4

Pain score measurements for the 12 individual patients assessed. The bold lines indicate those patients who received placebo infusions and the pain score changes during this time. A fall in pain score measurement represents a symptomatic improvement.

severely affected patients who comprised group 1A, who received a higher total dose of pamidronate than the remaining 9 patients, subsequently had a better response. A possible explanation for this observation may be that in more severely affected patients, who generally have a greater number of active pagetic lesions, the drug is spread more widely and therefore is present at lower concentrations at the affected sites than in less severely affected patients. These observations are similar to those of Cantrill et al.(256) who found that the decrease in serum ALP was less marked where the initial ALP level was higher.

It is difficult to compare results between reported studies as Paget's disease may be of different severity in different groups. In addition, some patients might have been treated previously with bisphosphonates which remain in the skeleton for prolonged periods and can affect the response to further treatment (249). The decrease in ALP observed in this study, however, is similar to that noted by others with pamidronate whether given orally or intravenously (251).

The optimum length of treatment is controversial. Some workers have found that short courses of daily or weekly treatment lead to significant suppression of serum ALP that is still present 1 year after the start of treatment (251,256). In one study a single dose of 60mg of pamidronate was given intravenously once and this led to significant suppression of ALP to about 30% of the pretreatment level and this suppression was still evident after 1 year of follow up. All but one of these patients, however, had mild disease and the one patient present with severe disease responded poorly to this regime (257). In this present study, in group 1 patients who received 6 once weekly infusions, the ALP level continued to decrease after treatment was discontinued, and decreased at a similar rate to that observed in group 2 patients who received a single infusion every 3 months.

There are some features of this study which are interesting and unexpected. In a small number of patients the urinary excretion of hydroxyproline increased to greater than the pretreatment levels. In these patients, however, the levels of serum ALP fell with treatment. Although it is possible that this decrease in ALP may be caused by the relative inhibition of mineralisation caused by pamidronate, the concurrent decrease in the whole body retention of radiolabelled bisphosphonate suggests that it is more likely that bone turnover has been reduced and that these urinary hydroxyproline measurements obtained reflect the difficulty in obtaining true fasting urine samples from outpatients. This apparent variability in hydroxyproline excretion has been previously noted in Paget's disease treated with pamidronate (255). HP/Cr ratios began to increase after the end of treatment (group 1) or after 3 months (group 2). This is particularly surprising in group 2 as further treatment with pamidronate was being given to these patients. This may either be due to an "escape" phenomenon, or it may be that there is a subgroup of osteoclasts that are relatively resistant to pamidronate.

Although symptoms are notoriously difficult to assess, often because of associated osteoarthritis, there appears to have been a significant decrease in pain and discomfort after treatment. Side effects occurred rarely and were self-limiting.

In conclusion, pamidronate appears to be safe and effective in the treatment of Paget's disease. A dose/response effect appears to exist, with more severely affected patients requiring a higher total dose of pamidronate to achieve disease suppression. In the short term an effective and convenient dosage regimen for patients with levels of serum ALP up to four times the upper limit of the reference range is 45mg pamidronate by intravenous infusion every 3 months for

1 year. For those patients with ALP levels greater than this, larger doses may be more effective. Recommendations for the very long term remain conjectural.

7.2 Intravenous pamidronate in the treatment of osteoporosis associated with corticosteroid dependent lung disease

7.2.1 Introduction

Increased bone resorption is generally a prominent feature in most examples of osteoporosis (264). This is particularly true of corticosteroid-associated osteoporosis where this is one of the principal mechanisms involved in the pathogenesis of this condition (332,333). In view of this, considerable interest has concentrated on the therapeutic use of anti-resorptive agents. Some evidence exists for a role for calcitonin in the treatment of osteoporosis (299,300), however most interest has recently centred on the use of bisphosphonates - in particular cyclical etidronate/calcium regimens (303,304).

In the previous study intravenous pamidronate was shown to be effective at reducing the increased bone turnover associated with Paget's disease. In this study the use of intravenous pamidronate was investigated in the setting of corticosteroid-associated osteoporosis.

7.2.2 Patients and Methods

Patients were recruited for this study from the chest clinics at Glasgow Royal Infirmary and associated hospitals. These patients all presented consecutively for routine review. Patients were included who had been taking an average minimum of 7.5mg prednisolone (or equivalent) per day for a minimum of 2 years, irrespective of whether or not they were thought to have significant osteoporosis. No significant changes in oral corticosteroid dose administered to these patients was noted during the year of treatment. Patients were excluded if they were on any other therapy or were suffering from any other medical condition thought

likely to interfere with their bone metabolism. Twenty patients were recruited for
this study, however 1 patient died (from respiratory failure) having only had one
infusion of pamidronate, 1 moved away from Glasgow and 1 defaulted from
further follow-up after only 1 infusion. Results at 1 year of follow-up, therefore,
were available in 17 patients - 15 suffering from asthma and 2 from sarcoidosis.
The two patients with sarcoidosis had consistently normal 1,25 dihydroxyvitamin
D3 (1,25(OH)$_2$D$_3$) levels and were normocalcaemic. Patient details are shown in
table 7.2. Patients were seen at three monthly intervals. Patients were treated
with 30mg pamidronate given as an intravenous infusion in 500ml 0.9% sodium
chloride solution over 4 hours once every 3 months. The first infusion was given
as an in-patient in order to observe for side-effects. At the time of review each
patient had received 5 infusions (150mg pamidronate). An assessment of each
patients dietary calcium intake was made prior to treatment. In 5 patients this
was thought likely to be less than 1000mg per day and in each case, 1 Sandocal
400 tablet twice daily was prescribed, otherwise no formal calcium
supplementation was given.

Plain radiographs of dorso-lumbar spine were obtained pre-treatment and after 1
year of therapy. These were reported by an experienced radiologist and graded
into normal, wedge deformity, bi-concave deformity or compression deformity.
Each category was further classified as grade 1 or grade 2 depending upon the
degree of deformity present. The radiologist reviewing these X-rays was unaware
of when these X-rays were carried out in relation to the administration of
pamidronate.

Pre-treatment bone densities in these 17 patients was compared with a cohort of
100 normal age and sex matched volunteers. These subjects had all been
recruited into a longitudinal study monitoring long-term changes in bone density
following a local advertising campaign. This group comprised 80 females

Table 7.2

Patient's clinical and biochemical details pre-treatment (values shown are means)

Number of patients	17*
Age (years)	52.9
range	35-68
Steroid dose (prednisolone/day)	14mg
range	7.5-40mg
Duration of treatment (years)	14
range	3-30
Time post menopause (years)	15.5
range	1-24
Number with pre-treatment vertebral fractures	14
Pre-treatment PTH (pmol/l)	3.6
SEM, range (normal 1-5pmol/l)	0.5, 0.8-9.0
Pre-treatment 25(OH)D3 (nmol/l)	36
SEM, range (normal 15-100nmol/l)	5, 14-98)
Pre-treatment 1,25(OH)$_2$D$_3$ (pmol/l)	82
SEM, range (normal 20-120pmol/l)	8,22-185

*17 patients - 5 male, 3 premenopausal female, 9 postmenopausal female

PTH - parathyroid hormone
25(OH)D3 - 25-hydroxyvitamin D3
1,25(OH)$_2$D$_3$ - 1,25-dihydroxyvitamin D3

(median age 52 years, range 33-71) and 20 males (median age 38 years, range 29-57). These subjects were all apparently healthy and did not suffer from any condition or take any medication thought likely to interfere with their bone metabolism. X-rays were not carried out in this group.

Statistical comparison of pre and post-treatment data was carried out using a two-tailed paired Student's t-test. Comparison of pre-treatment bone density with normal controls was carried out using an unpaired t-test and correlations between serum alkaline phosphatase (ALP) and fasting urinary hydroxyproline/creatinine (HP/Cr) were carried out using Spearman rank correlations.

7.2.3 Results

82% of patients included in this study had previously sustained at least 1 vertebral compression fracture on the basis of plain radiology. Pre-treatment L2-4 and NOF bone density (mean±SEM) was significantly lower than in the group of age/sex matched controls (L2-4: 0.906g/cm2±0.050 [steroid patients] versus 1.142g/cm2±0.016 [controls]; p=0.0002, NOF: 0.793g/cm2±0.030 [steroid patients] versus 0.936±0.013 [controls]; p<0.00001). Thus these patients had evidence of significant osteoporosis.

After 1 year of treatment there was a significant rise in L2-4 density to 0.927g/cm2±0.047, p=0.031. This represents a mean increase of 3.5% (95% confidence interval: 1.1% to 5.2%) (figure 7.5). Post therapy NOF density was 0.748g/cm2±0.025; p=NS, which represents a mean change of 1.8% (95% CI: -2.0% to 2.3%) (figure 7.6). In three instances L2-4 density decreased over the year of treatment and in one case no change was noted. In each of these instances pre-treatment bone densities were relatively normal and were all greater than
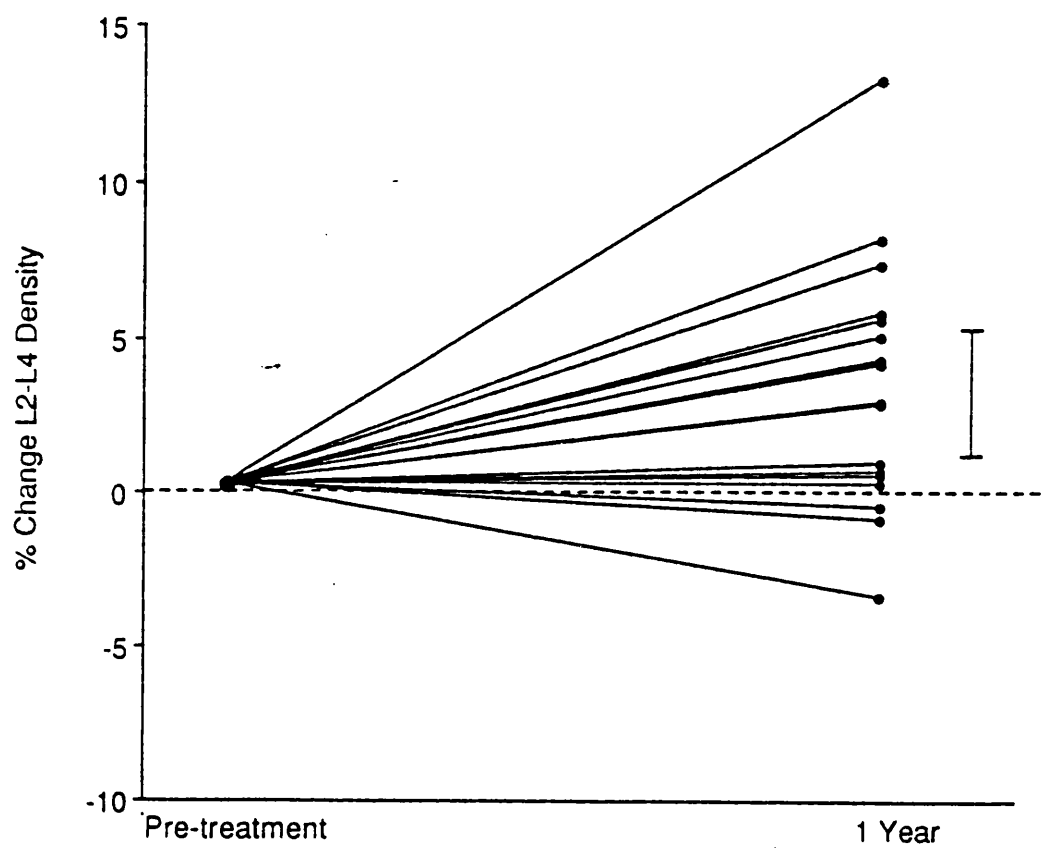
Figure 7.5

Changes in L2-4 spinal density following pamidronate treatment (expressed as a percentage of pre-treatment level). Vertical bar represents 95% confidence interval.
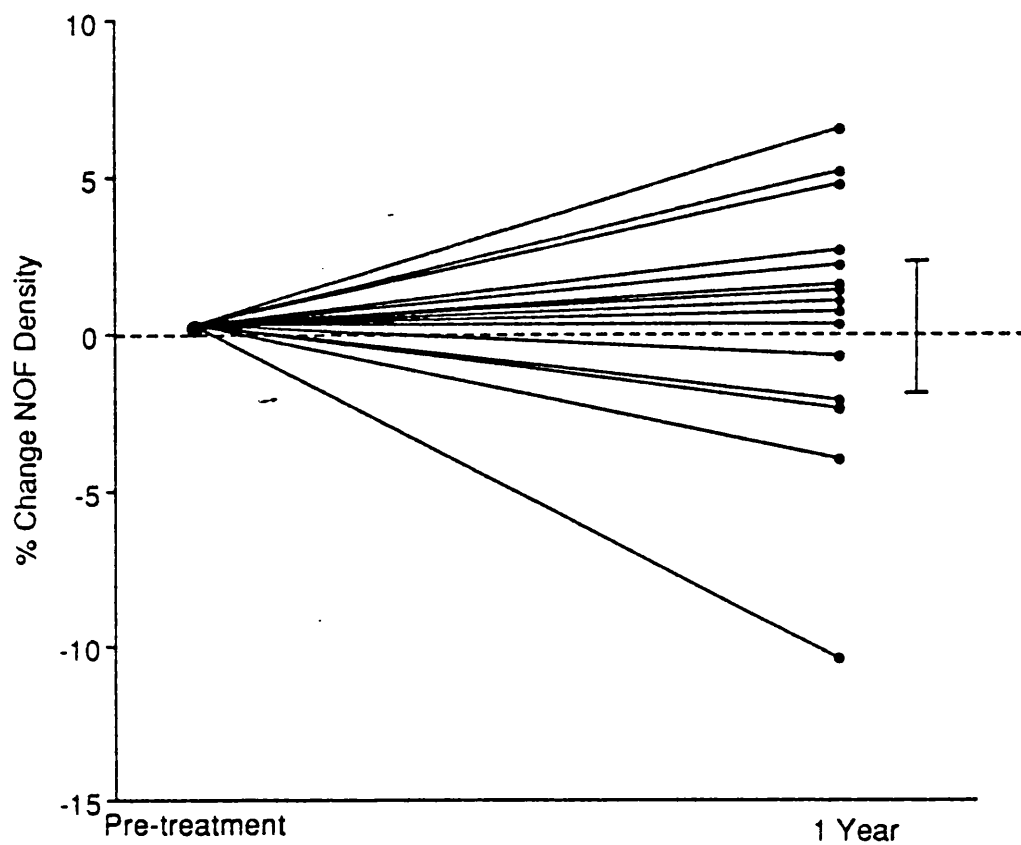
Figure 7.6

Changes in neck of femur (NOF) density following pamidronate treatment (expressed as a percentage of pre-treatment level). Vertical bar represents 95% confidence interval.

1.000g/cm2. Those patients who responded best to treatment tended to have the lowest pre-treatment bone densities.

Radiologically 1 patient suffered a new wedge fracture at D9 and 3 patients showed progression of wedging of 3 individual vertebrae.

Pre-treatment levels of PTH, 25(OH)D₃ and 1,25(OH)₂D₃ were generally within the reference range - 4 patients had elevated PTH values (5.8, 9.0, 8.3, 6.3pmol/l), none had subnormal 1,25(OH)₂D₃ levels. Mean ($\pm$SEM) pre-treatment HP/Cr ratio was elevated at 0.040 ± 0.006 indicative of increased bone resorption. Pre-treatment serum ALP was within the reference range. There was a significant fall both in serum ALP (figure 7.7) with treatment from (mean$\pm$SEM, 220U/l$\pm$16 to 174$\pm$9; $p<0.002$) and HP/Cr (0.040 ± 0.006 to 0.024 ± 0.003; $p<0.01$) (figure 7.8). No significant correlation was evident either pre or post-treatment between ALP and HP/Cr.

Five patients developed pyrexia (range 37.5°C - 38.8°C) following treatment, which was asymptomatic in 4 instances. This commenced approximately 24 hours after the infusion finished, lasted 12-18 hours and resolved spontaneously. This side-effect is well recognised after intravenous pamidronate (468) and only occurred after the first infusion. No change was noted in peak expiratory flow rate after infusion.

7.2.4 Discussion

On the basis of their pre-treatment bone densities and the presence of fractures these patients would appear to have significant osteoporosis. Furthermore, intravenous pamidronate would appear to be effective in both reducing bone
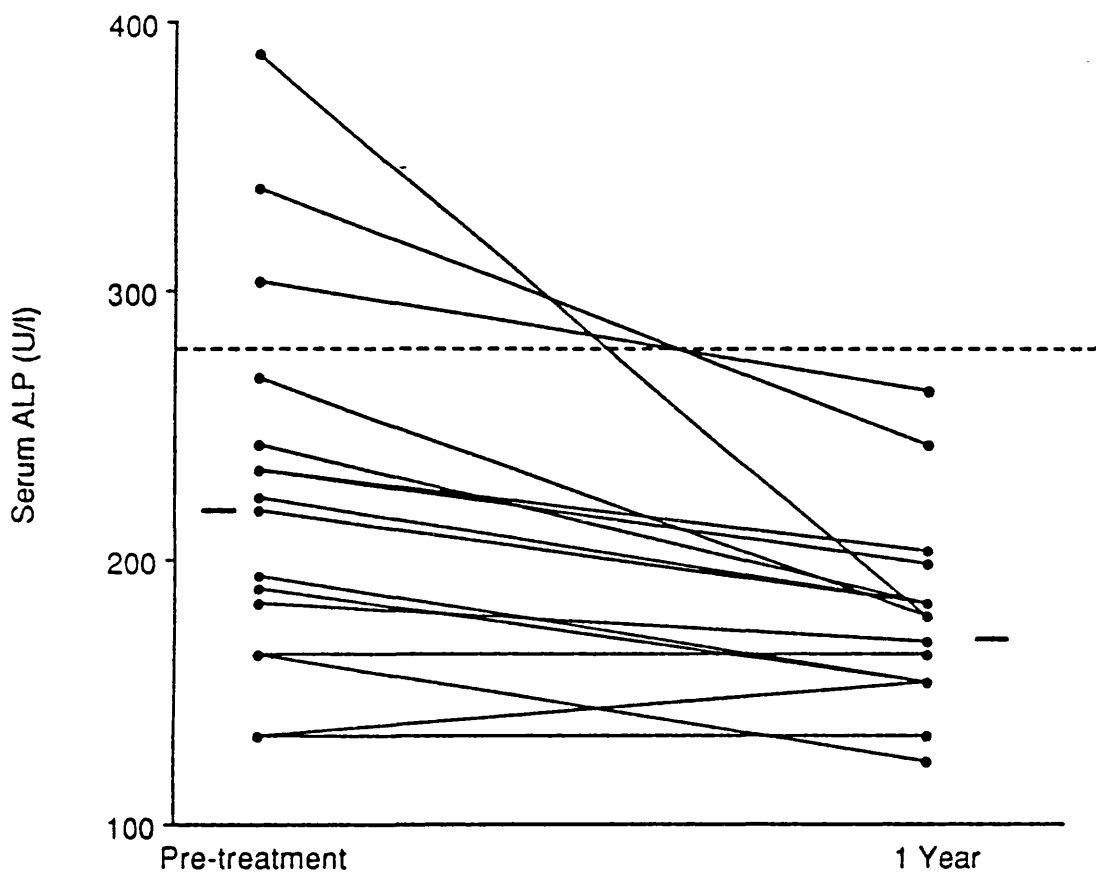
Figure 7.7

Changes in serum alkaline phosphatase (ALP) with treatment for each individual patient. Dotted line indicates upper limit of the reference range. Horizontal bars represent pre and post-treatment means.
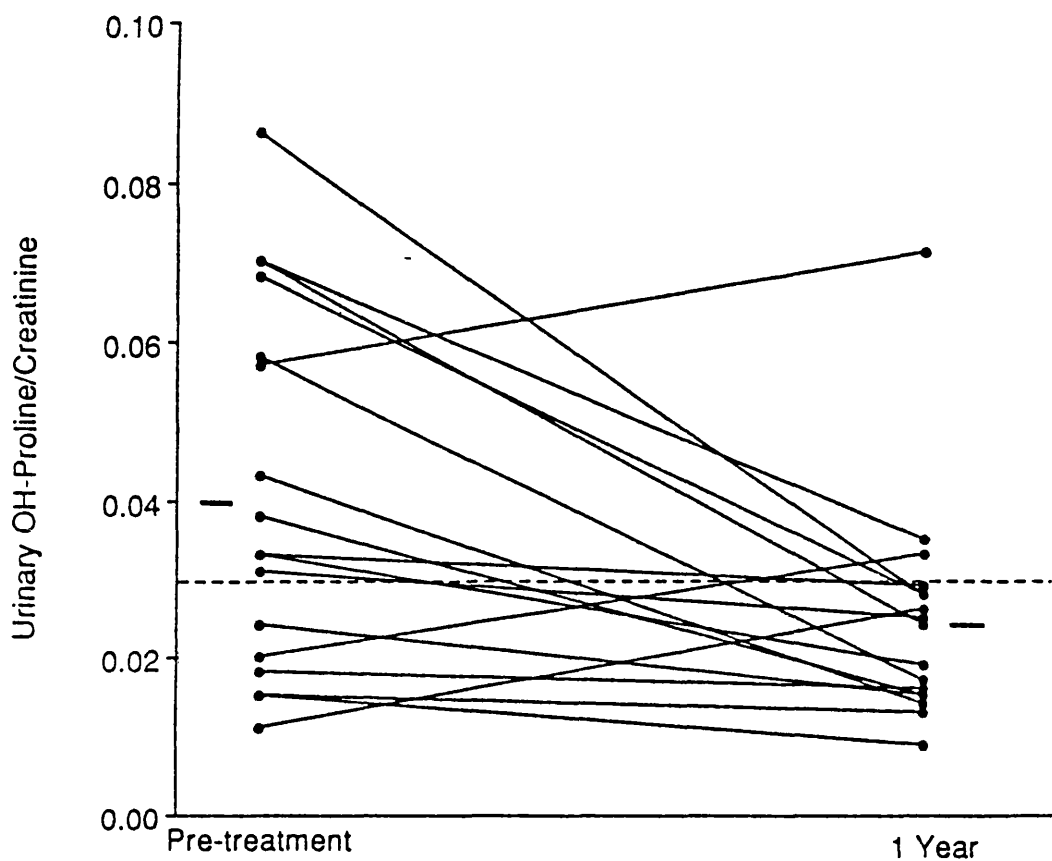
Figure 7.8

Changes in fasting urinary hydroxyproline/creatinine ratio following pamidronate treatment for each individual patient. Dotted line indicates upper limit of reference range. Horizontal bars represent pre and post-treatment means.

turnover and increasing bone density in these patients with asthma and
sarcoidosis.

Though data from Gennari (480) suggest that bone loss with corticosteroids
occurs only in the first 6 months of therapy, it is likely that bone loss continues
both with age and continued corticosteroid usage (336). This view is supported
by the data from this study showing increased urinary HP/Cr (and therefore
increased bone resorption) prior to therapy.

In the preceding study, intermittent infusions of pamidronate were shown to be
effective in the management of Paget's disease of bone. Although the dose of
pamidronate administered in this study was lower, bone turnover in
corticosteroid-associated osteoporosis is likely to be increased to a lesser extent
than in Paget's disease. Furthermore, the data presented in Chapter 6 showed
that, in the setting of cancer-associated hypercalcaemia, 30mg pamidronate is
generally as effective in inhibiting osteoclastic bone resorption as higher doses.

To date, most studies using bisphosphonates for the treatment of osteoporosis
have used oral therapies. Intravenous preparations have some theoretical
advantages in that they overcome the problem of poor absorption of these drugs
(237,470) and they ensure that patient compliance with therapy can be accurately
assessed. There has been considerable recent interest in the use of etidronate
given in a cyclical manner for two weeks at a time alternating with 11 weeks of
calcium supplementation, in the treatment of postmenopausal osteoporosis
(303,304). The use of intermittent infusions, as in this study, would be analogous
to these cyclical oral regimens. As bisphosphonates, and pamidronate in
particular, bind avidly to hydroxyapatite (129) and therefore have a prolonged
biological half-life, the cyclical nature of the treatment regimen may not be
necessary but rather the dose of bisphosphonate used should be low enough so as

not to inhibit mineralisation - a known side-effect of bisphosphonate therapy (153). With intravenous therapy, however, an intermittent treatment regimen is convenient both for the patient and the attending physician.

Little information is available concerning the use of bisphosphonates in corticosteroid-associated osteoporosis. However, a previous study by Reid et al.(347) has suggested that oral pamidronate administered continuously is effective in increasing bone density in corticosteroid-associated osteoporosis and this increase in bone density can be maintained for at least 2 years (348).

The mean increase in L2-4 density of 3.4% is of a similar order of magnitude to that noted after etidronate at 1 year in postmenopausal osteoporosis (303,304). The fact that there was no significant change in NOF density would suggest that the increase in L2-4 density was not achieved at the expense of worsening of osteoporosis at the hip. This lack of effect on NOF density was also noted in the study using etidronate in postmenopausal osteoporosis by Watts et al.(304). The data from this present study and from those using cyclical etidronate/calcium (303,304) would suggest that this treatment is likely to be most effective where osteopenia is more severe to begin with. Intermittent intravenous pamidronate therapy, therefore, should probably be reserved for those patients who have already sustained an osteoporosis related fracture or who have densitometric evidence of osteopenia.

While increases in bone density are important, the main end-point in studies of this kind must be fracture rates. The fact that fractures appear to have continued to occur is not surprising as, although these patients show an increase in bone density, they still probably lie below the fracture threshold. Obviously, in the absence of a control group, it is not possible to be certain of the significance of these further fractures. Certainly in the study of Storm et al.(303), no significant

change in vertebral fracture rate was seen until at least 12 months into the study. This is also likely to be the case with pamidronate. However, further studies with larger patient numbers and longer term follow-up are required to address this issue.

Mean HP/Cr pre-treatment was elevated implying increased rates of on-going bone resorption. Following treatment bone resorption is reduced and bone formation would also appear to be reduced (as illustrated by the fall in serum ALP) while bone density increases. A possible explanation for this paradoxical effect is that resorption is inhibited and there then follows a delay before local coupling mechanisms result in a decrease in bone formation and, therefore, bone formation goes unopposed for some weeks (481).

Several studies have suggested that vitamin D levels are low in corticosteroid-associated osteoporosis (199,330). This was not the case in this study, where in agreement with Hahn et al.(482) 1,25(OH)$_2$D$_3$ levels were normal or high. Similarly, biochemical secondary hyperparathyroidism was found in only 4 of our patients, a lower number than might be expected (331). These results would imply that the most important mechanism in the pathogenesis of this condition is the direct effect of corticosteroids upon bone resorption and formation.

In summary it would appear that intermittent intravenous infusions of pamidronate are effective at reducing the increased bone turnover of corticosteroid-associated osteoporosis. Furthermore, this regimen can significantly increase bone density which ultimately should lead to a decrease in the rate of new fractures. In addition this regimen would appear to be well tolerated and acceptable both to the patient and the attending physician. Long term controlled studies with this therapy are now indicated.

CHAPTER 8

CONCLUDING DISCUSSION AND AREAS FOR FUTURE RESEARCH

8.1 <u>Concluding discussion and areas for future research</u>

The work presented in this thesis illustrates the potential application of currently available investigative techniques in the diagnosis and management of various disorders of bone and calcium homoeostasis and illustrates the potential therapeutic value of the bisphosphonate, pamidronate.

Many examples of the application of biochemical techniques exist. One of the most fascinating is the unravelling of the pathogenesis of cancer-associated hypercalcaemia. In many instances in the past this was thought to be due to the excessive release of skeletal calcium by accelerated osteoclastic bone resorption associated with metastatic bone disease. However, the use of biochemical markers of PTH activity, such as NcAMP and TmPO4, along with accurate direct measurement of PTH, led to the insight that in many cases, cancer-associated hypercalcaemia is due to elaboration of PTHrP by the tumour itself (73,77-81,83-85). It is likely that both PTHrP and osteolysis secondary to metastatic bone disease vary in relative importance as the cause of the hypercalcaemia in different primary cancers. Chapter 3.1 illustrates the presence of a PTH-like factor (presumably PTHrP) in 45-60% patients with breast cancer-associated hypercalcaemia depending upon whether urinary cAMP excretion (UcAMP) or TmPO4 are measured. Since this work was carried out, assays for the direct measurement PTHrP have become available. Bundred et al.(483) found detectable concentrations of PTHrP in 12/13 (92%) of hypercalcaemic breast cancer patients with bone metastases compared with 10 of 28 (36%) of normocalcaemic patients with bone metastases. Although it would appear that PTHrP plays an important role in the pathogenesis of breast cancer-associated hypercalcaemia, the natural history of this condition has yet to be completely mapped out. In particular, in normocalcaemic patients, the significance of both an elevated NcAMP (as noted in chapter 3.1) or PTHrP (483) is not clear. It is

certainly possible that these patients may be at risk of developing hypercalcaemia and perhaps should be targeted for prophylactic bisphosphonate therapy. Certainly, it would appear that measurement of both PTH and PTHrP is likely to be of value in the diagnosis and monitoring of cancer-associated hypercalcaemia (484).

The value of direct measurement of PTHrP is further illustrated in chapter 3.2. In this study, where changes in plasma PTHrP through normal pregnancy were studied, these concentrations were generally within the reference range. In this context it is unlikely that indirect markers of PTH-like activity are of sufficient sensitivity to monitor changes in plasma PTHrP. This study would appear to confirm the potential role for PTHrP in both maternal-fetal calcium transport and breast-breast milk calcium transport. Whether or not PTHrP in this setting has an endocrine role, or merely reflects spill over into the circulation from areas of high local PTHrP concentrations remains unclear. Further human studies investigating the localisation of PTHrP in the placenta are now indicated. Furthermore, it is possible that PTHrP might be added to the list of biochemical markers of placental function.

Biochemical investigation has allowed greater understanding of areas of bone and calcium homoeostasis other than just the pathogenesis of hypercalcaemia and the discovery of PTHrP. It is now apparent that PTH and $1,25(OH)_2D_3$ are of considerable importance in maintaining both normocalcaemia and normal bone turnover (1,412). Up until very recently PTH has not been available for use therapeutically. Some recent evidence, however, has suggested that synthetic PTH may be useful in the treatment of osteoporosis (313). These data, though exciting, remain preliminary. $1,25(OH)_2D_3$ and its pharmacological precursor, $1\alpha(OH)D3$ (alfacalcidol), have been available for therapeutic usage for some time. These preparations have been shown to be useful in the treatment of

hypocalcaemia, for example that secondary to hypoparathyroidism, and

osteomalacia. Since 1,25(OH)2D3, physiologically, has an inhibitory effect on

PTH secretion, this preparation is potentially useful in the treatment of primary

or secondary hyperparathyroidism (31,416,418,421-424). Studies have, however,

shown it to be of limited value in the treatment of primary hyperparathyroidism,

though it is certainly of some value in the management of secondary

hyperparathyroidism, such as is associated with chronic renal failure (416,423).

Unfortunately oral 1,25(OH)2D3 and alfacalcidol tend to be associated with

hypercalciuria and ultimately hypercalcaemia which limits their usefulness (30).

Recent evidence has shown that hypercalcaemia does not occur when

1,25(OH)2D3 or alfacalcidol are administered intravenously (417,419,424,425).

The effect of intravenous alfacalcidol on normal subjects and patients with

osteoporosis and primary hyperparathyroidism is described in chapter 3.3. This

shows that peak levels of 1,25(OH)2D3 are seen 2-3 hours after administration of

alfacalcidol, however PTH suppression (or osteocalcin stimulation) are not seen.

Further studies are required to look at the effects of longer term administration of

this drug.

Another important advance seen in the last decade in the study of disorders of

bone and calcium metabolism was the introduction of technologies which would

allow accurate and precise measurement of bone density. One of the most useful

methods currently available is dual-energy X-ray absorptiometry (DXA) which

allows direct measurement of bone density in those areas where fractures are

most important in terms of their associated morbidity and mortality i.e. the neck

of femur and spine. DXA is also a safe technique with minimal exposure to

ionising radiation. It is also relatively quick and therefore bone density can be

potentially measured in a large number of patients (274). There is little evidence

at present to support the use of this technology to carry out widespread

population screening for osteoporosis (485), though a recent Consensus

conference recommended its "routine" use in certain "at risk" patient groups (286). As well as its ability to measure bone density in large numbers of patients, DXA allows detailed assessment of bone density in specific sub-populations such as patients with diabetes mellitus as described in chapter 4.1 and haemophilia (chapter 4.2).

The existence of a possible association between diabetes mellitus and osteoporosis has been discussed for many years (349). Most studies of this subject to date have yielded conflicting results, probably because patient groups studied have been very heterogeneous. The work described in chapter 4.1 suggests that osteopenia may not be associated with type 1 diabetes, but rather bone density may even be slightly increased. Further work is required to investigate this further, in particular the possible role of insulin-like growth factor 1 (IGF-1) in stimulating this increase in bone density. Furthermore the effects of improving glycaemic control upon bone density and turnover requires further study. As suggested above part of the reason for the conflicting results in studies of diabetes related osteopenia is the heterogeneous nature of the patient groups studied. It is possible, therefore, that osteopenia may be present in a sub-group of the diabetic population. Given that data presented in this thesis suggests that diabetes is associated with a high bone turnover state, it is very likely that in situations where bone resorption is increased such as after the menopause, bone loss will be significant and result in osteoporosis. This is an area that deserves further study.

The association between haemophilia and osteopenia described in this thesis (chapter 4.2) is novel and warrants further investigation. Preliminary investigations described would suggest that these patients have relative hypogonadism and further work is required to look at the role of androgen supplementation in these patients. If the prime problem in these patients is

indeed hypogonadism then it might be expected that bone formation be reduced and bone resorption increased. Since invasive histomorphometric bone turnover assessment is impractical in this situation, further investigation of biochemical indices of bone turnover are indicated. Future studies should also be directed at whether these bone density changes are restricted to those patients with severe haemophilia or if they correlate with the factor VIIIc level present.

As well as being able to define the presence of absence of osteopenia in population groups, the precision of DXA allows longitudinal studies of bone density. This is illustrated in chapter 7.2 where intermittent intravenous infusions of the bisphosphonate, pamidronate, were shown to result in a significant in crease in lumbar spine bone density.

Although DXA is undoubtedly a very useful tool, the capital expenditure on purchase of the machine is high as is the cost of a dedicated technician to run it. It is unlikely that these machines will be available widespread throughout the UK and other methodologies will have to be used to screen populations, where this is thought to be of benefit. This could be done by use of single photon absorptiometry (SPA) of the forearm (271) or by use of broad band ultrasound of the os calcis (486).

The introduction of multichannel biochemical analysers along with the availability of precise measurement of PTH has meant an increase in the frequency of diagnosis of primary hyperparathyroidism (46). In addition advances have been made in the technologies available for pre-operative parathyroid imaging. There is now good evidence that knowledge of the site of the putative parathyroid adenoma prior to surgery both lessens the chance of a negative neck exploration and shortens anaesthetic time (176). In chapter 5.1 the two methods of parathyroid imaging most widely available in the UK - 10MHz

ultrasound and thallium/technetium subtraction scanning - were compared. This study showed that though specificities of these techniques are high, sensitivities are low and neither of these techniques can be recommended for routine pre-operative use. There was a suggestion in this study that more useful information might be obtained where both these techniques are used together, though further studies are required to address this issue. In chapter 5.2 an attempt was made to improve the sensitivity of thallium/technetium subtraction scanning by transiently lowering serum calcium at the time of the scan. This preliminary study showed that this technique could be used to stimulate parathyroid thallium uptake, however, the technique itself is rather cumbersome and not likely to be of routine use. Much interest currently lies in imaging with 99m-Technetium-sestamibi which would appear to be preferentially taken up by parathyroid tissue (453). Use of this technique appears to result in improved localisation sensitivity for both parathyroid adenomas and hyperplasia.

Considerable advances have also been made in the pharmacological treatment of many disorders of bone and calcium metabolism in the past decade.

One of the most significant therapeutic advances has been the introduction of the bisphosphonates into clinical practice. These drugs are structurally similar to pyrophosphate with the exception of a carbon atom substituting for oxygen. This renders the bisphosphonates resistant to pyrophosphatases (127) and as they are actively taken up into bone, they may have very long biological half-lives (127). These drugs act by inhibiting osteoclastic bone resorption (129), usually by a direct toxic effect upon the osteoclast. The aminobisphosphonates, of which pamidronate is an example, may also have an effect on inhibiting osteoclast formation from monocyte precursors (458). Bisphosphonates are particularly effective in situations where bone resorption is high such as hypercalcaemia associated with cancer (130), immobilisation (131) and hyperthyroidism (132),

Paget's disease of bone (133) and post-menopausal, senile and corticosteroid-associated osteoporosis (303,304,347).

Pamidronate is currently licensed for use in the treatment of cancer-associated hypercalcaemia in the UK. It is recommended that the dose of this drug be titrated against the prevailing serum calcium i.e. the higher the calcium, the higher the dose of pamidronate should be. The evidence for this assertion is weak. Chapter 6.1 shows that, on average, there is no difference between 30mg and 90mg pamidronate in terms of its calcium lowering effect (these two doses are the highest and lowest recommended). There are, however, undoubtedly some patients who require a larger dose of pamidronate. The reasons for this are explored further in chapter 6.2. This study shows that the calcium lowering effect of pamidronate is less pronounced where there is evidence of PTHrP-mediated hypercalcaemia. The study described in chapter 6.2 was carried out before measurement of PTHrP was generally available and, therefore NcAMP, TmPO4 and TmCa are used as markers of PTH-like activity. Further studies are indicated comparing the calcium lowering effect of bisphosphonates to the pretreatment concentration of PTHrP. In the absence of a measurement for PTHrP or NcAMP or TmPO4, the dose of pamidronate should be higher (60-90mg) in the presence of squamous cancer or breast cancer as it is likely that PTHrP is involved in the pathogenesis of the hypercalcaemia in a large proportion of cases with these tumour types and the dose should be lower (30-45mg) in association with, for example haematological malignancy, where PTHrP is likely to be absent or low.

A further example of non-PTHrP driven hypercalcaemia is immobilisation-related hypercalcaemia. From the evidence presented in chapters 6.1 and 6.2 it might be expected that patients with immobilisation-related hypercalcaemia would be particularly sensitive to bisphosphonate therapy. This, indeed, appears to be the case (chapter 6.4). The cases presented in chapter 6.4 further raise the

question as to whether this hypercalcaemia may be cytokine driven. Certainly, interleukin-1 has been shown in the rat model to increase bone resorption and cause hypercalcaemia (106). Further studies are required to investigate this hypothesis further in man.

Bone resorption is generally also considerably increased in Paget's disease of bone. Bisphosphonates are therefore effective in this condition. It would appear that a dose/response effect exists (chapter 7.1) and that an intermittent treatment regimen with intravenous pamidronate is effective and can result in effective biochemical disease suppression. Intravenous therapy is, in general terms, likely to be both uncomfortable and inconvenient. There is now, therefore, considerable interest in the newer oral bisphosphonates such as risedronate, tiludronate and alendronate which are considerably more potent than even pamidronate and are likely to be effective in the treatment of Paget's disease.

Bone resorption is usually increased in corticosteroid-associated osteoporosis. The data presented in chapter 7.2 would suggest that intermittent infusions of intravenous pamidronate can both reduce this bone resorption and also stimulate an increase in bone density at least at the lumbar spine. Although there is a large volume of literature concerning the treatment of post-menopausal osteoporosis, there is relatively little on the treatment of corticosteroid-associated osteoporosis. The data presented in this thesis would suggest that pamidronate is likely to be of benefit, however longer term controlled trials are required to address the question of fracture risk. A large multi-centre study has been set up in the UK by the British Thoracic Society which will look at the effect of oral etidronate on bone density and fracture risk in patients with corticosteroid dependent lung disease.

The work described in this thesis describes how various investigative techniques that are available today can be applied to clinical situations. It is likely with the

development of sensitive assays for PTHrP that more will be learned about the physiological role of this hormone. In addition considerable advances are being made in the biochemical assessment of bone turnover with indices such as measurement of urinary excretion of the collagen cross-links, pyridinoline and deoxypyridinoline, likely to supersede urinary hydroxyproline excretion as markers of bone resorption (319,487) and measurement of serum procollagen peptide III likely to supersede serum alkaline phosphatase and osteocalcin as a marker of bone formation (488). These measurements will allow more information to be obtained on bone turnover in various physiological and pathological states, making recourse to invasive histological assessment of bone turnover less necessary. Techniques such as those described above and those used in this thesis can also be applied to the monitoring of various treatment strategies, including the use of pamidronate as described here. It is likely that the future of bisphosphonate therapy will lie with the newer potent oral formulations which are now entering clinical trial. Hope now exists for the effective treatment of conditions such as Paget's disease of bone and osteoporosis which until recently have been considered by many to be untreatable.

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