Parathyroid Hormone Sensitivity, Parathyroid Hormone Circadian Rhythm and Phospho-calcium Metabolism in the Development of Osteoporosis - The Effects of Age, Bone Mineral Density, Gender and Growth Hormone

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of

Doctor of Medicine

By

Franklin Joseph MBBS MRCP

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Department of Diabetes & Endocrinology

Royal Liverpool University Hospital

Liverpool

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Declaration

This thesis is a result of work performed whilst registered as a candidate for the degree of Doctor of Medicine at the University of Liverpool. The experimental work described in this thesis was performed in the Departments of Diabetes/Endocrinology and Clinical Biochemistry at The Royal Liverpool University Hospital, Liverpool between 2003 and 2011. I was involved in the design, performance and analysis of results of all the work presented in this thesis. I declare that no portion of the original work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

Franklin Joseph

List of Abbreviations

Adjusted Serum Calcium	ACa
Adult Growth Hormone Deficiency	AGHD
Alkaline Phosphatase	ALP
Analysis of variance	ANOVA
Bone Mineral Content	BMC
Bone Mineral Density	BMD
Calcium	Ca
Coefficient of Variation	CV
Creatinine Clearance	CCr
Cyclic Adenosine Monophosphate	cAMP
Diacylglycerol	DAG
Electrochemiluminesscence immunoassays	ECLIA
Extracellular Signal-Regulated Kinase	ERK
Femoral Neck	FN
Fibrobalst Growth Factor-23	FGF-23
Fibrobalst Growth Factor Receptor	FGFR
General linear model analysis of variance	GLM ANOVA
Glomerular Filtration Rate	GFR
Growth Hormone	GH
Growth Hormone Deficiency	GHD
Growth Hormone Replacement	GHR
Guanine Nucleotide-Binding Regulatory Protein	G protein
Immunoradiometric assay	IRMA
Inositol 1,4,5-trisphosphate	IP3

Insulin-like Growth Factor-1	IGF-1
Insulin-like Growth Factor-1 Receptor	IGF-1R
Insulin-like Growth Factor-1 Standard Deviation Score	IGF-SDS
Insulin-like Growth Factor-2	IGF-2
Insulin-like Growth Factor Binding Protein	IGF-BP
Insulin-like Growth Factor Binding Protein 3	IGF-BP3
Insulin-like Growth Factor Binding Protein 5	IGF-BP5
Janus tyrosine kinase 2	JAK 2
Lumbar Spine	LS
Mitogen Activated Protein Kinases	MAPK
Maximum Tubular Phosphate Reabsorption	$TmPO_4/GFR$
Midline Estimate Statistic of Rhythm	MESOR
Nephrogenous cyclic Adenosine Monophosphate	NcAMP
National Institutes of Health	NIH
Osteoprotegerin	OPG
Parathyroid Hormone	PTH
Parathyroid Hormone-Related Protein	PTHrP
Phosphate	PO_4
Phospholipase C	PLC
Plasma cyclic Adenosine Monophosphate	PcAMP
Procollagen type-I amino-terminal propeptide	PINP
Procollagen type I carboxyterminal propeptide	PICP
Radioimmunoassay	RIA
Receptor Activator of Nuclear Factor-Kappa B	RANK
Receptor Activator of Nuclear Factor-Kappa B Ligand	RANKL

Recombinant human Growth Hormone	rhGH
Signal Transducers and Activators of Transcription	STAT
Standard deviation	SD
Stadard deviation score	SDS
Type-I collagen C-telopeptide	CTX
Type-I collagen β C-telopeptide	βCTX
Type I collagen cross-linked N-telopeptide	NTX
Type I collagen cross-linked N-telopeptide Creatinine Ratio	NTX/Cr
Urine Calcium	UCa
Urine Calcium Creatinine Ratio	UCa/Cr
Urine Calcium Excretion per Liter of Creatinine Clearance	UCaE
Urine cyclic Adenosine Monophosphate	UcAMP
Urine Phosphate	UPO_4
Urine Phosphate Creatinine Ratio	UPO_4/Cr
Phosphate Excretion per Liter of Creatinine Clearance	UPO_4E
World Health Organisation	WHO
1,25-dihydroxyvitamin D	$1,25(OH)_2D$
24,25-dihydroxyvitamin D	$24,25(OH)_2D$
25 hydroxyvitamin D	25(OH)D

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PTH, largely regulates ionised Ca levels directly through its actions on PTH receptors expressed abundantly on the kidney and bone, which are the primary target organs for PTH action (99-102). PTH-dependent regulation of mineral ion homeostasis also occurs indirectly through the stimulation of $1,25(OH)_2D$ and its action on the intestine (95-97).

(a) IGF-1: lower concentrations in postmenopausal women, (b) PTH: higher concentrations in postmenopausal women, (c) NcAMP: lower concentrations in postmenopausal women, (d) ACa: no difference, (e) PO_4: higher concnenttations in postmenopausal women with normal BMD and lower concentrations in women with low BMD, (f) 25(OH)D: no difference, (g) 1,25(OH)2D: no difference, (h) UCaE: increased in postmenopausal women, (i) TmPO4/GFR: trend toward a lower level in postmenopausal women with low BMD, (j) UPO_4E: higher in postmenopausal women with normal BMD and lower in women with low BMD, (k) βCTX: trend toward higher levels in postmenopausal women with normal BMD and significantly higher in women with low BMD, (l) PINP: trend toward higher levels in postmenopausal women with normal BMD and significantly higher in women with low BMD.

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Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs for each group and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated for all 3 groups with differences in MESOR, acrophase and amplitude. The MESOR was highest in postmenopausal women with low BMD with alterations to the rhythm that may be contributory to bone loss.

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(a) IGF-1: lower concentrations in older men, (b) PTH: higher concentrations in older men, (c) NcAMP: lower concentrations in older men, (d) ACa: no difference, (e) PO_4: lower concnentrations in older men, (f) 25(OH)D: no difference, (g) 1,25(OH)2D: no difference, (h) UCaE: no difference, (i) TmPO4/GFR: lower level in older men, (j) UPO_4E: no difference, (k) βCTX: lower levels in older men with normal BMD and higher in older men with low BMD, (l) PINP: lower levels in older men both with normal and low BMD, (m) total testosterone: no difference.

Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs for each group and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated for all 3 groups with differences in MESOR, acrophase and amplitude. The MESOR was highest in men with low BMD with alterations to the rhythm that may be contributory to bone loss.

Differences were demonstrated in (a) IGF-1, (b) PTH, (c) NcAMP, (d) ACa, (e) PO_4, (f) 25(OH)D, (g) 1,25(OH)2D, (h) UCaE, (i) TmPO4/GFR, (j) UPO_4E, (k) βCTX, (l) PINP between men and women in the 3 different groups.

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Figure 5.2. Figure showing differences in serum and urine biochemistry in young healthy premenopausal women compared to older postmenopausal women with low BMD at baseline and changes in biochemical parameters 1,3,6 and 12 months following GH administration in the postmenopausal women with low BMD. **146**

(a) PTH: higher concentrations in patients compared to controls with decreasing levels following GH administration up to 6 months and levels remaining below baseline at 12 months, (b) NcAMP: lower concentrations in patients compared to controls with increasing levels following GH administration up to 6 months with levels no different from baseline at 12 months, (c) ACa: no difference in patients compared to controls with higher levels at 1 and 3 months settling to levels similar to baseline at 6 and 12 months following GH administration, (d) PO_4: lower concentrations in patients compared to controls with concentrations increasing upto 12 months following GH administraion, (e) 1,25(OH)2D: no difference in patients compared to controls with levels increased by 3 months and maintained

at 6 and 12 months following GH administration, (f) UCaE: higher in patients compared to controls with UCaE increasing by 3 and 6 months and no significant change compared to baseline, (g) UPO_4E: no difference between patients compared to controls and UPO_4E increasing up to 6 month with levels no different from baseline at 12 months, (h) TmPO4/GFR: no difference between patients and controls with higher TmPO4/GFR at 1,3,6 and 12 months following GH administraion, (i) βCTX: higher levels in postmenopausal women with low BMD and increasing levels following GH administration, (j) PINP: higher levels in postmenopausal women with low BMD compared to controls with levels increasing following GH administration.

Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs for each group and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated for for both groups with differences in MESOR, acrophase and amplitude. The MESOR was highest in the postmenopausal women with alterations to the rhythm that may be contributory to bone loss.

Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated at both time points with a decrease in MESOR following GH administration.

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Figure 6.2. Cross-correlation analysis of PTH, OPG and βCTX in healthy older men, premenopausal women and postmenopausal women. Graphs represent the time lag between changes in one analyte in relation to another. **172**

Highest r values representing strength of correlation with time lag in hours is represented in each graph. Relationship between PTH and OPG as well as relationship between OPG and βCTX were altered in postmenopausal women when compared with older men and premenopausal women.

Abstract

Background and Aim: Abnormalities in target organ sensitivity to PTH, PTH circadian rhythm and phospho-calcium metabolism contribute to the development of osteoporosis in adult growth hormone deficiency (AGHD) and growth hormone (GH) replacement therapy increases bone turnover and BMD by restoring PTH sensitivity and circadian rhythm. As AGHD has phenotypical features similar to those of advancing age, the studies in this thesis aimed to investigate PTH sensitivity and rhythm in men and women of different age groups and bone mineral densities (BMD) to understand the possible impact of these mechanisms on age related osteoporosis. The effects of GH administration on PTH sensitivity, PTH rhythm and bone mineral metabolism were also studied in older postmenopausal women with osteoporosis. The relationship between the circadian rhythm of PTH, osteoprotegerin (OPG), a regulator of osteoclast activity, and type I collagen C-telopeptide (βCTX), a marker of bone resorption, was also studied.

Methods: Subjects were hospitalized for 25 h during study visits and half-hourly blood and 3-h urine samples were collected. PTH, calcium, phosphate, nephrogenous cyclic AMP, βCTX, procollagen type I amino-terminal propeptide, and 1,25-dihydroxyvitamin D were measured. Circadian rhythm analysis was performed using Chronolab 3.0 and Student's t-test and general linear model ANOVAs for repeated measures were used where appropriate.

Results: Postmenopausal women demonstrate decreased target-organ sensitivity to PTH and abnormal PTH circadian rhythm when compared with younger premenopausal women with more pronounced abnormality in postmenopausal women with low BMD. Older men with normal and low BMD demonstrated decreased PTH sensitivity compared to younger men but PTH rhythm abnormality was only observed in older men with low BMD. IGF-1 concentrations were lower in older postmenopausal with low BMD and GH administration resulted in an increase in IGF-1 with improvement in PTH sensitivity, restoration of PTH circadian rhythm and improved bone mineral metabolism. Significant circadian rhythms were observed for PTH, OPG and βCTX. There was loss of the normal PTH rhythm with a sustained increase in PTH concentration overnight in postmenopausal women. There was a corresponding, greater percent decrease in OPG secretion in the postmenopausal women and a corresponding, higher nocturnal increase in βCTX.

Conclusions: Abnormalities in PTH sensitivity and rhythm are present in older men and women with low BMD. These abnormalities are related to the age related decline in IGF-1 and can be corrected by the admisnitration of GH. OPG has a circadian rhythm and the catabolic effects of the abnormal PTH rhythm in older postmenopausal women may be mediated by corresponding abnormalities in OPG rhythm. The findings add a further dimension to the complex regulation of bone mineral metabolism and the changes that occur with age and also elucidate the mechanisms by which GH exerts its previously demonstrated anabolic effects on bone.

Chapter 1

Introduction and Literature Review

1.1. Osteoporosis

1.1.1. Introduction

In 1994, the World Health Organisation (WHO) Working Group defined osteoporosis based on measurement of BMD (BMD) using dual-energy X-ray absorptiometry (DXA). For epidemiological purposes severity of bone loss was reported using T and Z-scores (1). However, bone strength is affected, not just by bone density but by bone quality as well and osteoporosis is characterised by both low bone mass affecting bone density as well as micro-architectural deterioration of bone tissue affecting bone quality. The consequence of these changes is an increase in bone fragility and susceptibility to fracture. In 2000, the National Institutes of Health (NIH) Consensus Development Panel developed a clinical definition of osteoporosis, defining osteoporosis as a progressive, systemic, skeletal disorder characterised by compromised bone strength predisposing a person to an increased risk of fracture (2). This definition took into consideration bone density, bone quality and concomitant risk factors that contribute to fracture risk. Thus, over the years, the focus of diagnosis, risk stratification, prophylaxis and treatment of osteoporosis has shifted. The focus is no longer limited to bone density alone. Based on more in-depth understanding of bone cellular and bone metabolic changes, this focus now encompasses other factors that underlie and contribute to the development of osteoporosis and fracture risk in different groups of individuals.

1.1.2. Aetiology and Risk Factors

The aetiology of osteoporosis is multi-factorial with multiple risk factors having been identified. These include age, muscle mass, body weight, the level of peak bone mass prior to the onset of age-related bone loss, behavioural factors such as decreased physical activity, smoking, alcohol consumption, low intake of important nutrients, consumption of various drugs, diseases such as malabsorption, anorexia nervosa and renal hypercalciuria. The FRAX® tool, developed by the WHO to evaluate fracture risk of patients is based on individual patient models that integrate the risks associated with clinical risk factors as well as BMD (BMD) at the femoral neck (FN). The FRAX® models have been developed from studying population-based cohorts from Europe, North America, Asia and Australia. The FRAX® algorithms give the 10-year probability of hip fracture and the 10-year probability of a major osteoporotic fracture (clinical spine, forearm, hip or shoulder fracture). The risk factors identified as being important to risk contribution, excluding secondary causes of osteoporosis, include age, gender, body mass index, a prior fragility fracture, parental history of hip fracture, current tobacco smoking and alcohol consumption of 3 or more units per day. The FRAX® tool is however limited by the lack of adequate international data on other factors that may be contributory to risk (e.g. biochemical markers of bone turnover, growth factors), the complexity of practically quantifying certain risk factors (e.g. physical exercise). Endocrine factors including sex steroids, parathyroid hormone (PTH), GH and IGF-1 have, in epidemiological studies, been related with fracture risk and do contribute significantly to the development of osteoporosis via different, complex and often interlinked mechanisms at different stages of bone loss that are still not fully understood and warrant further investigation.

1.1.3. Types of Osteoporosis

Primary osteoporosis is the most common form of osteoporosis and is diagnosed when other disorders known to cause osteoporosis are not present. Primary osteoporosis has been classified according to age groups as juvenile osteoporosis when it affects prepubescent boys and girls, idiopathic osteoporosis in young adults when the cause is not related to another disease, postmenopausal osteoporosis when it occurs in women within 15-20 years after menopause and age-related, or senile, osteoporosis in the elderly. Primary or involutional osteoporosis in these individuals of different ages, in which no underlying cause can be identified, is believed to develop as a result of excessive bone loss caused by varying but more marked expression of what would be considered age-related changes in bone. Secondary osteoporosis, on the other hand is defined by an identified underlying cause.

Others have suggested that two subtypes of primary osteoporosis can be distinguished in aging women: (i) postmenopausal osteoporosis and (ii) age-related or senile osteoporosis (3). Arbitrarily, postmenopausal osteoporosis is said to affect women who are postmenopausal but younger than 70 years. These women are said to have type I or postmenopausal osteoporosis if excessive bone loss that meets WHO criteria occurs within 15-20 years after menopause. This type I osteoporosis is characterized by increased bone resorption due to osteoclastic activity and is generally believed to be related to oestrogen deficiency and is associated with preferential loss of trabecular bone making them prone to vertebral crush fractures and fractures of the distal radius. Age-related osteoporosis, also called senile or type II osteoporosis occurs when there is excessive bone loss manifested after age 70 years in both women and men. Type II osteoporosis results from normal aging and is associated with a steady loss of both cortical and trabecular bone mass each year. Age-related bone loss begins

at age 35-40 years when the balance shifts to favour resorption and the skeleton begins to lose bone mass. Hip and vertebral fractures are most common in this type of osteoporosis. In addition to vertebral crush fractures, vertebral wedge fractures with gradual deformation of the spine are also seen.

1.2. Bone Loss

1.2.1. Patterns of Bone Loss

The prevalence of osteoporosis increases with age in both men and women as bone mineral loss is a predictable accompaniment of aging in both women and men. After closure of the endochondral growth plate, bone mass increases by radial growth until peak skeletal mass is achieved between 20 and 25 years of age as reported in the majority of studies. Studies also highlight that the age of attainment of peak bone mass is bone site specific (4-9).

Several anatomical studies (10, 11) and most cross-sectional densitometric studies (12-14) have demonstrated that after a transient period of stability, the slow phase of age related bone loss begins. This slow phase involves a linear, continued, steady, age-related annual loss of skeletal mass of about 0.5-1.0% in women and 0.3% in men that continues into old age (10, 11, 15). Latterly, however, the pattern of age related bone loss varies between genders and a biphasic pattern of bone loss has been identified in women, who undergo an accelerated transient phase of bone loss superimposed on the slow continuous phase, whereas men undergo only the protracted slow continuous phase (3, 16). It is this slow pattern of bone loss that results in what is called by some as type 2 or senile osteoporosis. The accelerated phase in women begins at menopause, involves predominantly cancellous bone loss and has been

attributed to the cessation of ovarian function. Oestrogen restrains bone turnover and when this restraint is lost at menopause, overall bone turnover increases and resorption increases more than formation. As the rapid bone loss phase subsides, there is an absolute increase in bone resorption and a relative decrease in bone formation involving equal losses of cortical and cancellous bone. The accelerated phase of bone loss decreases over 5-10 years to merge again with the slow phase that continues indefinitely. A decrease in BMD into the osteoporotic range during the immediate postmenopausal period as a result of the increased bone resorption is called by some as type 1 osteoporosis.

It has also been suggested that even within a population of postmenopausal women with increased bone resorption there are "fast" losers and "slow" losers of bone. Some studies have suggested that the "fast" loss of bone is only transient but most agree that a higher bone resorption rate in the "fast" losers increases their risk of fractures independent of BMD. The reasons why some women lose bone faster than others is still unexplained. The difference in the rate of loss may be the reason why some postmenopausal women develop osteoporosis and others don't. Also, "fast" losers benefit more from antiresorptive therapy than "slow" losers. Thus an understanding of the pattern of bone loss in individuals is of importance from a clinical perspective and the easier assessment of this "fast" loss with reliable biochemical markers of bone resorption allows risk stratification and choice of appropriate therapy.

1.2.2. Mechanisms of Bone Loss

The adult skeleton is in a dynamic state, continually being broken down and reformed by the coordinated actions of osteoclasts and osteoblasts on bone surfaces and in haversian systems. This turnover or remodeling of bone occurs in focal and discrete packets or units, known as bone remodeling units or bone multicellular units, throughout the skeleton. The sequence of bone turnover involves activation of osteoclast precursors, and then osteoclastic bone resorption, followed by osteoblastic bone formation to repair the defect, known as the "coupling phenomenon". In the steady state, this coupling of bone resorption and formation maintains bone mass.

The rate of bone remodeling is to a large extent dependent on the frequency of osteoclast activation which is the initial step in the remodeling sequence and occurs in specific focal sites by mechanisms that are still not understood. One possibility is that osteoclast precursors recognise a change in the mechanical properties of aging bone, which requires replacement with new bone for optimal structural integrity. This theoretical possibility could occur because other cells, such as immune cells or osteocytes, recognise a change in the bone surface and send signals to osteoclasts to activate them. However, the initial trigger for such activation of immune cells is unknown. The resorptive phase of the remodeling process has been estimated to last 10 days. This is followed by repair of the resorption pit by osteoblasts, a process that takes approximately 3 months resulting in new bone formation. Following osteoclast formation and activation, the specific cellular events that occur at sites of osteoclast resorption include osteoclast apoptosis. This is then followed by a series of sequential changes in cells in the osteoblast lineage, including osteoblast chemotaxis,

proliferation and differentiation, which in turn are followed by formation of mineralised bone and cessation of osteoblast activity.

In normal young adults, the resorption and formation phases are tightly coupled and bone mass is maintained. Bone loss implies an uncoupling of the phases of bone remodeling with a relative or absolute increase in resorption over formation. When this uncoupling occurs, an increase in bone turnover leads to increased bone loss. The slow and accelerated phases of bone loss are associated with two different abnormalities of bone remodeling (15, 17). The slow age-dependent phase results mainly from impaired bone formation; osteoclasts construct resorption cavities of normal depth, or even decreased depth (18), but osteoblasts fail to refill them completely. It has been suggested that this is a combination of various factors including (i) decreased osteoblast and osteoclast numbers resulting in a decreased bone formation rate; (ii) increased reactive oxygen species resulting in decreased glutathione reductase activity, a corresponding increase in the phosphorylation of p53 and p66(shc) (two key components of a signaling cascade that are activated by reactive oxygen species and influences apoptosis and life span) and a consequent increase in osteoblast and osteocyte apoptosis (19, 20). The accelerated phase of bone loss occurring in women soon after the menopause is associated with a high rate of bone turnover and increased numbers of osteoclasts, each creating a deeper resorption cavity (18, 21).

The cellular and humoral mechanisms responsible for mediating bone turnover and the coupling process are still not completely clear and neither are the mechanisms responsible for the altered bone turnover and the uncoupling that occurs in age-related bone loss contributing possibly to the pathophysiology of the development of age-related osteoporosis. Many

hormones and factors with differing mechanisms of action have been shown to stimulate osteoclast activity. PTH stimulates differentiation of committed progenitors to fuse to form mature multinucleated osteoclasts and also activates preformed osteoclasts to resorb bone. $1,25(OH)_2D$ is a potent stimulator of osteoclastic bone resorption and like PTH stimulates osteoclast progenitors to differentiate and fuse (22). It also activates mature preformed osteoclasts, possibly by a mechanism similar to that of PTH. It had also previously been proposed that activation of osteoclasts may also be indirect and mediated through cells in the osteoblast lineage (23) as osteoblasts express receptors for various growth factors, cytokines, oestrogen, PTH and vitamin D. Bone is also a major reservoir of IGF-1 and in vitro studies have suggested that IGF-1 secreted by local stromal cells such as osteoblasts, may mediate GH effect on osteoclast activity (24, 25). The predominant messenger by which osteoblasts talk to osteoclasts in response to various stimuli has been identified as receptor activator of nuclear factor-kappa B ligand (RANKL), which is produced by osteoblasts and exerts its effects through binding to its receptor (RANK) on osteoclast precursor cells. The binding results in activation of osteoclasts. Osteoblasts also produce osteoprotegerin (OPG), a potent inhibitor of osteoclast formation and a decoy receptor for RANK. The relative ratio of OPG and RANK ligand in the bone marrow microenvironment determines the number of active osteoclasts, bone resorption rate, and bone mass. IGF-1 has been shown to alter OPG production, suggesting a role in maintaining the cross talk between osteoblasts and osteoclasts and the coupling phenomenon (26). Recently, the involvement of osteocytes in osteoclastogenesis and bone resorption was reported using conditional knockout mice of β-catenin by Dmp1-Cre, which resulted in enhanced bone resorption, and conditional knockout mice of receptor activator of NF-κB ligand (Rankl) by Dmp1-Cre, which resulted in osteopetrosis. Using a newly established method for the isolation of high purity osteocytes from bone matrix, it has also been demonstrated that osteocytes express a much higher

amount of RANKL and have a much greater capacity to support osteoclastogenesis than osteoblasts and bone marrow stromal cells, thus suggesting that osteocytes may be the more relevant cell at the initiation of the bone remodeling through regulation of osteoclastogenesis (27-31).

Several theories have also been proposed to account for coupling of resorption to formation. Many workers have favoured the notion that coupling is humorally mediated and that an osteoblast-stimulating factor, such as GH, IGF-1, transforming growth factor-β, skeletal growth factor, bone-derived growth factor or macrophage derived growth factor is released from bone matrix during the process of osteoclastic bone resorption and the stimulation of osteoblast activity leads to new bone formation (32). Systemic or local age related decline or defects in GH, IGF-1 and these other growth factors may result in impaired osteoblast activity leading to uncoupling of bone formation from resorption resulting in age related bone loss. A variation in this humoral concept is that the factor that stimulates resorption also acts directly on osteoblasts to cause their activation and subsequent new bone formation (33). This notion suggests that coupling does not involve sequential signals released during the process of bone remodeling, but rather the factors that work through the GP-130 signal-transduction mechanism are responsible for simultaneous stimulation of osteoclast and osteoblast lineages. An alternative to the humoral hypothesis for coupling is that because osteoblasts normally line bone surfaces, once the phase of osteoclastic resorption is over and the osteoclasts disappear from the resorption site, osteoblasts and their precursors repopulate the resorption site and merely reline the bone surface, thus, repairing the resorption defect without the involvement of a humoral mediator.

1.3. Bone Metabolism

1.3.1. PTH and Bone

PTH plays an important role in bone remodeling (34) and has complex but only partially understood actions on bone that requires the presence of and often direct contact with several different specialised cell types (35). PTH has also been shown to have both catabolic and anabolic effects on bone (36, 37).

1.3.1.1. End organ effects of PTH

The effects of PTH on bone are mediated via direct effects on PTH receptors in bone and indirectly through regulation of the vitamin-D and phospho-calcium metabolism via receptors in the kidney (38). The PTH receptors in bone mediate the regulation of chondrocyte and osteoclast proliferation and differentiation (39), whereas, in the kidneys activate the mitochondrial vitamin D 1- α hydroxylase leading to increased serum $1,25(OH)_2D$, which in turn is an inducer of intestinal Ca absorption as well as bone resorption (40).

PTH acts through guanine nucleotide-binding regulatory protein (G protein) receptors that are present on the cells of the target organs, and results in the stimulation of at least 2 different intracellular signal transduction pathways (41). Activation of the G_S component of the receptor results in stimulation of the adenylate cyclase / cyclic adenosine monophosphate (cAMP) pathway. Activation of the G_Q component leads to the production of phospholipase C (PLC). Binding of PTH to its receptor leads to activation of PLC with the subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) and

diacylglycerol (DAG). IP3 generation leads to the release of intracellular Ca stores, which produces an increase in the intracellular Ca concentration (41, 42). The cellular functions regulated by each pathway after PTH receptor binding, together with the inter-pathway synergy, are not fully explained.

The second messenger cAMP mediates the action of PTH at target organs, including the bone turnover in the skeleton and regulation of Ca and phosphate (PO_4) excretion in the kidney (43-46). Plasma cAMP (PcAMP) can be quantified but is a poor measure of PTH activity as cAMP also acts as a second messenger for other systems (47). PcAMP is excreted by a process of simple glomerular filtration; to this filtered load of the nucleotide is added a quantity of cAMP formed *de novo* in the kidney, nephrogenous cAMP (NcAMP). NcAMP is the only pool of the nucleotide easily quantified *in vivo* and can be calculated from measurement of plasma and urine cAMP (UcAMP), after adjustment has been made for glomerular filtration (47, 48). NcAMP almost exclusively reflects the effects of circulating PTH in normal individuals (45, 46, 49) and appears to be added directly to the tubular urine, thus accounting for the magnitude, rapidity and sensitivity of the PTH-induced changes in cAMP excretion. The determination of NcAMP, therefore, provides a sensitive and specific assessment of parathyroid function and a reliable index of the circulating activity of PTH on kidney and bone in both physiological and pathophysiological conditions (47, 50). Simultaneous measurement of PTH and NcAMP has recently been used to demonstrate the phenomenon of target organ insensitivity to PTH in patients with AGHD (51). Ca infusion studies have also demonstrated insensitivity at the level of the parathyroids themselves in these patients (52). It has previously also been suggested that renal sensitivity to the effects of PTH is decreased in untreated women with osteoporosis who demonstrate a less pronounced renal cAMP response to changes in PTH (53, 54). Also post-menopausal women with

osteoporosis have shown less suppression of PTH (1-84) in response to PTH (1-34) infusion than normal postmenopausal women at various Ca concentrations, suggesting parathyroid gland insensitivity to changes in Ca concentration in this group of patients (55). It is possible, therefore that alterations in the end-organ responsiveness to the effects of PTH and changes in parathyroid gland sensitivity may contribute to the skeletal changes leading to the development of age related osteoporosis in a manner similar to that observed in AGHD.

1.3.1.2. Circadian Rhythm of PTH

True endogenous PTH circadian rhythmicity is well established in healthy individuals (56-58) and there is increasing evidence that fluctuations in PTH secretion may have an important effect on bone remodeling (59-66). The circadian rhythm of PTH is characterised by biphasic peaks of PTH concentration occurring in the early evening and at night (56-58). The nocturnal peak in PTH concentration is larger than the early evening peak and appears to have an important effect on bone remodeling (66, 67). Temporal fluctuations in PTH are necessary for its biological activity, a fact that is well demonstrated by primary hyperparathyroidism which is associated with a loss of PTH circadian rhythmicity (60), an increase in osteoclast cell number and activity (68) with the release of Ca, PO_4 and matrix components of collagen from bone leading to the subsequent development of osteoporosis (59, 68).

The importance of fluctuation in PTH concentration, for biological effect, is further reinforced by studies in which intermittent and continuous PTH have been administered (62-65). Intermittent PTH administration by subcutaneous injection is associated with increased indices of bone formation including increased osteoblast number, osteocalcin, osteoid surface

and cancellous bone volume, as well as increased indices of bone resorption such as increased osteoclast number and resorption surface (69) but with a net anabolic effect. Continuous PTH infusion is associated only with increased markers of bone resorption and favours bone resorption with a catabolic effect (64).

The factors that initiate and regulate the circadian rhythm of PTH are as yet not fully understood. The nocturnal rise in PTH has previously been related to the circadian rhythm, of prolactin and sleep cycles (57, 70) suggesting an element of neuroendocrine control, but acute shifts in sleep timing did not alter the timing of PTH nocturnal rise arguing against such a relationship (71). Although the majority of the PTH component has been shown to be truly endogenous, there is evidence that other factors, especially ones that cause acute shifts in Ca and PO_4, may contribute in maintaining the PTH secretory pattern (58).

Circadian rhythms in the serum concentrations of ionised Ca, total Ca and PO_4 themselves have also been demonstrated in healthy subjects (72-75). There are, however, inconsistencies in the reported patterns of ionised and total Ca circadian rhythms with the nadir in total Ca reported between 2400 and 0400 h, while that of ionised Ca in the late afternoon and evening (74-79). In some studies no circadian rhythmicity has been observed for ionised and total Ca in women (66). In contrast, the PO_4 pattern has been the most highly reproducible of the mineral rhythms (74-79).

The biological origin and significance of these temporal variations in mineral concentrations are not well defined and the relationship between the circadian rhythm of ionised Ca and PTH has been inconsistent (58, 66, 67, 75, 79, 80). There is a negative feedback system for the regulation of Ca in extra-cellular fluid which involves PTH acting directly on the kidney

and bone and indirectly on the intestine. Although correlation analysis has suggested that changes in serum Ca precede that of PTH, it is difficult to explain the nocturnal rise in PTH occurring when ionised Ca levels are already increasing (75). PTH circadian rhythm also persists in subjects infused with Ca to maintain a steady serum Ca level (72) making ionised Ca an unlikely candidate to regulate PTH.

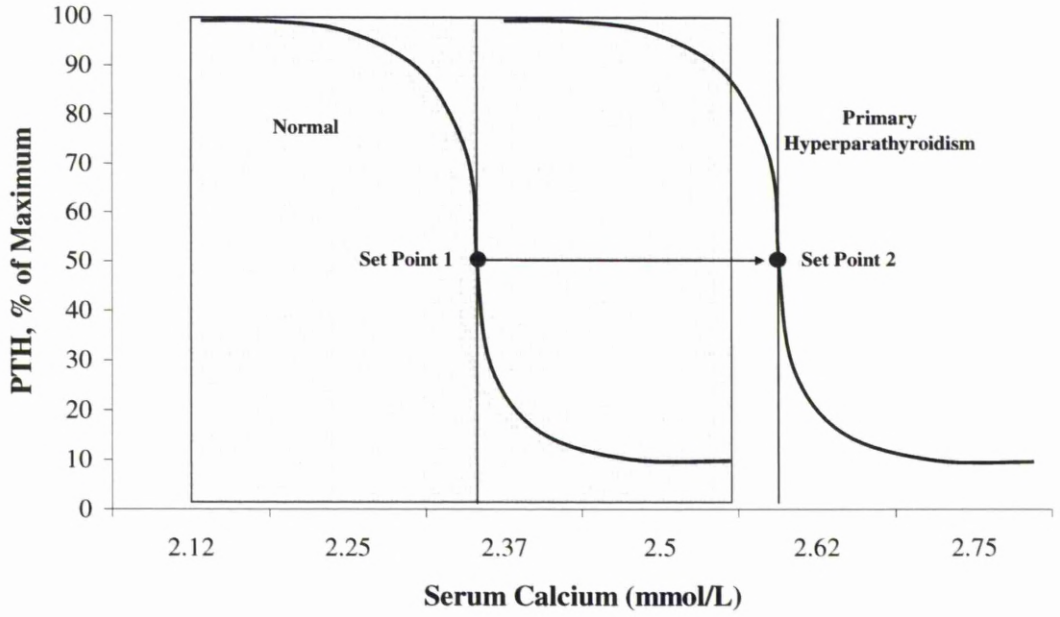
Both, acute (81-83) and chronic oral PO_4 (84) intake increases circulating serum PO_4 and PTH. In such circumstances a transient decrease in ionised Ca may contribute to PTH release but PO_4 has been shown to stimulate PTH secretion independent of the concentration of ionised Ca (85, 86). A strong correlation exists between circulating serum PO_4 and PTH and it has been suggested that PO_4 has a direct interaction with PTH (87) and may regulate PTH circadian secretion (72, 81, 88) independent of Ca and $1,25(OH)_2D$ (89, 90). Cross correlation analysis has shown that the maximal relationship between the circadian rhythms of PTH and PO4 exists when PTH follows PO_4 by one and a half hours (51).

Various pathological conditions are associated with abnormal PTH rhythm and treatment of the underlying pathology has restorative effects on the rhythm. The loss of PTH circadian rhythm observed in primary hyperparathyroidism returns following parathyroid surgery (59, 60). AGHD patients have also been shown to have clear differences in PTH circadian rhythm as compared to normal controls, possibly related to the rhythm of circulating PO_4 (51). GH replacement therapy in these patients has been shown to have beneficial effects on restoring the circadian rhythm of PTH (91-94). PTH circadian rhythm is also altered in postmenopausal women both with and without osteoporosis and may have a role in the development of age-related osteoporosis (61, 66).

1.3.2. Calcium and Phosphate Metabolism

The maintenance of serum Ca within normal limits is accomplished through the complex interplay of various hormones including PTH and vitamin D, acting on various organs via a number of different receptors (95-97). The calcium-sensing receptor on the parathyroid gland cells is highly sensitive to very small changes in Ca concentration, and feedback from circulating Ca completes the feedback loop (98). An inverse sigmoidal relationship exists between PTH secretion and extra-cellular Ca concentration (98) and the sigmoid curve is described by the maximal PTH secretory rate, the slope of the curve at the point where PTH secretion is at half its maximal rate, the Ca set point (the Ca concentration at which the rate of PTH secretion is half its maximal value) and the minimal PTH secretory rate (Figure 1.1.).

Figure 1.1. Schematic Representation of Calcium Set Point



PTH, largely regulates ionised Ca levels directly through its actions on PTH receptors expressed abundantly on the kidney and bone, which are the primary target organs for PTH action (99-102). PTH-dependent regulation of mineral ion homeostasis also occurs indirectly through the stimulation of $1,25(OH)_2D$ and its action on the intestine (95-97).

In the kidney, PTH regulates Ca homeostasis by stimulating the reabsorption of Ca, inhibiting reabsorption of PO_4 and enhancing the synthesis of $1,25(OH)_2D$. The majority of glomerular filtered Ca is passively reabsorbed in the proximal tubule (103-105), but approximately 5% of Ca reabsorption is PTH dependent and occurs in the distal tubule by means of a combination of sodium-calcium channels and an ATP-driven Ca pump (104-106). Urine calcium (UCa) excretion reflects all the tubular reabsorption processes and the filtered load of Ca. PTH dependent renal reabsorption of Ca increases with increasing PTH concentration but the absolute amount of Ca excreted in the urine increases as the circulating Ca concentration increases. This increase in UCa excretion is especially seen when the circulating concentration of PTH is chronically increased and results in hypercalcaemia; such as in patients with primary hyperparathyroidism. This increase in UCa excretion is caused by the substantial increase in the filtered load of Ca (107, 108). The calcium-sensing receptor plays, independent of PTH, an important role in directly regulating renal Ca reabsorption by inhibiting tubular reabsorption of Ca when the level of peritubular Ca increases (98).

PTH also stimulates microsomal vitamin D 1 α-hydroxylase in proximal tubular cells that leads to an increase in serum $1,25(OH)_2$ D concentration (40, 109), which in turn, is a potent inducer of both intestinal Ca absorption as well as of bone resorption. This effect of PTH is not immediate, because the stimulation of $1,25(OH)_2D$ production occurs over several hours and requires the synthesis of new protein (110, 111) and therefore the consequent rise in Ca

occurs more slowly than that achieved by the direct action of PTH on bone and renal Ca reabsorption. In addition to PTH, hypophosphataemia is a potent stimulator of 1α-hydroxylase activity whereas, Ca and $1,25(OH)_2D$ itself suppress 1α-hydroxylase as part of normal homeostasis (112, 113).

25-hydroxy vitamin D [25(OH)D] also undergoes hydroxylation to 24,25-dyhydroxy vitamin D [$24,25(OH)_2D$] (114). Hydroxylation to $24,25(OH)_2D$ reduces the availability of 25(OH)D for hydroxylation to $1,25(OH)_2D$ (115). PTH suppresses vitamin D 24-hydroxylase activity in the kidney, thus increasing substrate available for the production of $1,25(OH)_2D$ (112).

PTH effects on bone results in increased bone resorption within 1-2 h either as result of the direct effect of PTH activation of mature osteoclasts or via an initial activation of osteoblasts (116), which then through cellular and humoral paracrine mediators stimulate osteoclasts (96, 97). Increased resorption results in breakdown of the calcium-phosphate matrix product and Ca release from bone stores into circulation. The homeostatic system has negative feedback elements, and restoration of serum Ca towards normal directly inhibits PTH synthesis while $1,25(OH)_2D$ provides an additional feedback loop (117-119). Hypercalcaemia acutely inhibits PTH release and more prolonged elevations result in suppression of PTH gene expression (120). Hypercalcaemia, generated by the sustained increases in PTH, suppresses the 1 α-hydroxylase activity and thus limits the production of $1,25(OH)_2D$ in a homeostatic manner (112, 113). It was initially thought that there was a linear relationship between serum Ca and PTH (121). Subsequently, several groups have shown a sigmoidal relationship between serum PTH and Ca (122-125).

The Ca set-point (the Ca concentration at which the rate of PTH secretion is one half of its maximal value) (126) of the parathyroid gland plays a key role in "setting" the level of serum Ca (127) and not surprisingly, changes in the set-point of the parathyroid gland produce major changes in PTH secretion at any given level of serum Ca (123) and in turn, the steady state level of the serum Ca concentration. Increase in the set-point are typically seen in hyperparathyroid (123) and in familial hypocalciuric hypercalcaemic states (128, 129), implying varying degrees of Ca resistance of the parathyroid/Ca sensing mechanism in these conditions. It is, therefore, possible that qualitative changes in Ca-regulated PTH release may play an important role in the secretory control of normal as well as abnormal parathyroid tissue.

The kidney plays the dominant role in systemic phosphorus homeostasis and PO_4 enters the renal tubules after glomerular filtration and is reabsorbed in both the proximal and distal renal tubules. The rate of reabsorption is dependent upon the renal threshold for PO_4 excretion, also known as the maximal tubular phosphate reabsorption rate ($TmPO_4$/GFR; (GFR = glomerular filtration rate)) and the serum phosphorous concentration is maintained by the kidney at a value very close to the $TmPO_4$/GFR (130). The systemic supply of phosphorus is very rarely a limiting factor and most disorders in humans relating to PO_4 homeostasis result from either intrinsic or extrinsic alterations in $TmPO_4$/GFR. $TmPO_4$/GFR is affected by various factors including PTH, vitamin D and GH (131-136). PO_4 reabsorption is inhibited by PTH (107, 108) producing phosphaturia via inhibition of sodium-dependent PO_4 transport (137). The PTH receptors in the proximal tubule that mediate the regulation of $TmPO_4$/GFR and those in the distal nephron that regulate Ca reabsorption are coupled to different intracellular signal-transduction systems. The sequence of events in the face of a hypophosphataemic challenge includes stimulation of $1,25(OH)_2D$ synthesis in the kidney, enhanced mobilisation of

phosphorus and Ca from bone and a hypophosphataemia-induced increase in $TmPO_4/GFR$, the exact mechanism of which is unknown. The increased circulating concentration of $1,25(OH)_2$ D leads to increase in phosphorus and Ca absorption in the intestine and provides an additional stimulus for phosphorus and Ca mobilisation from bone. Thus there is an increase in flow of Ca from bone and intestine resulting in inhibition of PTH secretion. The inhibition of PTH diverts the systemic flow of Ca into the urine and further increases $TmPO_4/GFR$. The net result of this sequence of adjustments is a return of serum PO_4 concentration to normal without change in the serum Ca concentration (138).

More recently, distinct tubular PO_4 transporters and a group of PO_4 regulating peptides called phosphatonins that also regulate the $TmPO_4/GFR$ have been identified and have increased the understanding of the mechanisms involved in renal PO_4 handling (139-142). Fibroblast growth factor-23 (FGF-23) is a phosphatonin (30 Kda, 251 amino acids) (143, 144) that has been shown to have a physiological role in maintaining PO_4 concentrations within a defined range as well as a role in pathological conditions (145-149). FGF-23 in turn is regulated by changes in PO_4 (145, 150). FGF-23 decreases extracellular fluid PO_4 concentrations by directly reducing renal PO_4 reabsorption and suppressing $1,25(OH)_2D$ formation independent of PTH (146-149). In vitro, FGF-23 binds to known FGF receptors (FGFR) and it is assumed that FGF-23 mediates its effects in vivo through a known FGFR. FGF-23 regulates PO_4 metabolism by impairing PO_4 reabsorption, at least in part, via a PTH independent decrease in the NaPi IIa (specific sodium/inorganic PO_4co-transporter) protein in the brush border membrane of renal proximal tubules (146, 148). It also has an NaPi IIa independent mechanism of action possibly mediated via the recently identified NaPi IIc dependent renal reabsorption pathway (151, 152) or via the type III sodium PO_4 cotransporter, pit-1, which regulates PO_4 transport in bone (153). FGF-23 mRNA expression demonstrated in kidney

also raises the possibility of an autocrine or paracrine role for FGF-23 in regulating renal transport (150).

1.3.3. The GH / IGF-1 Axis and Bone

GH and IGF-I are important regulators of bone homeostasis throughout life (154). Their effects on longitudinal bone growth, skeletal maturation and acquisition of peak adult bone mass are well established (155, 156). It is also proposed that GH and IGF-1 are important for the maintenance of bone architecture and mass in adulthood but their roles following complete maturation of the skeleton and attainment of peak bone mass is less clear (157, 158). Changes in bone structure and increased bone mass in patients with GH excess and acromegaly are well documented, but it is not until relatively recently that advances in laboratory techniques have, together with the increased availability of recombinant human GH (rhGH), allowed further understanding of the role of GH in bone remodeling. GH stimulates bone turnover, beginning with an increase in bone resorption. The initial increase in bone resorption results in an increased number of bone multicellular units and the production of unmineralised bone, that leads to an apparent lower or unchanged bone mass on measurements of BMD. Bone resorption is then coupled to bone formation and as the effect of GH on osteoblasts is more pronounced and prolonged than on osteoclasts the rate of bone formation exceeds that of bone resorption resulting in an increase in bone mass (91).

The complex effects of GH on bone are direct as well as indirect and have been elucidated through studies in animals and different groups of human subjects including patients with AGHD and patients with age-related or postmenopausal osteoporosis.

1.3.3.1. Direct Effects of GH

The effect of GH on bone appears to occur through a combination of direct and indirect actions. The direct effects of GH include stimulating the proliferation of cells of the osteoblast lineage (159, 160) and inhibiting osteoblast apoptosis (161). GH affects the fate of mesenchymal precursors directly, opposing adipogenesis and favoring osteoblastogenesis as well as chondrogenesis (162). It stimulates bone formation by expressing bone morphogenetic proteins, important for the differentiation of osteoblasts (163-165) and also stimulates the differentiated function of mature osteoblasts. GH also stimulates the carboxylation of osteocalcin as well as the synthesis of type 1 collagen and alkaline phosphatase (ALP) which are markers of osteoblastic function (166-168). GH exerts its direct effects on osteoblasts by binding to single-chain transmembrane glycoprotein receptors that belong to the cytokine/haemopoetic growth factor receptor family and have been identified on human and murine osteoblasts (169). The GH receptor consists of an extracellular, a transmembrane and an intracellular domain. Activation of the receptor occurs by ligand induced dimerization and internalization of the receptor to initiate signaling, primarily by the activation of the Janus tyrosine kinase 2 (JAK 2) in the cytoplasm (170). This leads to auto-phosphorylation and to phosphorylation of the internalized GH receptor and recruitment and activation of intracellular proteins of which the signal transducers and activators of transcription (STAT) are the most important (171-174). GH also signals through extracellular signal-regulated kinases (ERK) 1 and 2, mitogen activated protein kinases (MAPK) that regulate osteoblastic cell growth (175-178). Acting through STATs and ERKs, GH may modulate the activity of runt related transcription factor-2 (runx-2), which is an intracellular protein required for osteoblast cell differentiation (179, 180).

Although a GH receptor is yet to be identified on osteoclasts (181), in vitro studies have shown that GH is able to directly stimulate the proliferation and differentiation of osteoclasts. GH is also thought to be directly involved in the coupling process of bone resorption and formation, perhaps through the release of IL-6 (157).

1.3.3.2. Indirect Effects of GH: IGF-1

The indirect action of GH occurs through stimulation of systemic IGF-1 production from the liver as well as IGF-1 expressed locally in bone. The physiology of IGF-1 is complex as it acts as a circulating hormone and as a local growth factor that can act in a paracrine as well as autocrine fashion in a variety of tissues including cartilage and bone. IGF-1 produced locally in the bone appears to be important for the development and function of osteoblasts and osteoclasts, probably at a point further along the line of differentiation than the steps requiring direct GH action (182, 183). IGF-I has modest effects on the proliferation of cells of the osteoblastic lineage, and although IGF-I does not direct the differentiation of undifferentiated stromal cells toward cells of the osteoblastic lineage, IGF-I enhances the function of the mature osteoblast (184, 185). Thus the fundamental role of IGF-I is the stimulation of osteoblastic function and bone formation. IGF-1 signals through the IGF-1 receptor (IGF-1R), a transmembrane glycoprotein tetramer with ligand activated tyrosine kinase activity. Upon ligand binding, the IGF-1R dimerizes and undergoes autophosphorylation, leading to the activation of the insulin receptor substrate (IRS)-1 and IRS-2 (186). IRS-1 and -2 mediate the effects of IGF-I in osteoblasts.

IGF-1R expression has been demonstrated on human preosteoclasts (187). In vitro studies suggest that GH enhances osteoclast formation and activates mature osteoclasts indirectly via IGF-1 secreted by local stromal cells of osteoblast lineage (24, 25, 188, 189) thus acting as a coupling factor between bone resorption and formation (190). IGFs previously embedded in newly formed bone by osteoblasts are released when bone is resorbed, thus enabling the stimulation of further new bone formation.

1.3.3.3. Indirect Effects of GH: Phospho-calcium Metabolism

GH also influences bone metabolism indirectly by the modulation of phospho-calcium metabolism and by interaction with hormones including PTH and $1,25(OH)_2D$ (191). The ability of GH to cause retention of Ca in man was first described by Beck et al. (192) and subsequently by Henneman et al. (193). It has been suggested that GH and IGF-1 may have a permissive role in the stimulation of vitamin D 1α-hydroxylase (135) whilst suppressing the 24-hydroxylase (194) thus increasing the production of the active $1,25(OH)_2D$, and consequently resulting in an increase in intestinal Ca and PO_4 absorption. Sensitization of gut epithelium to $1,25(OH)_2D$ requires the presence of GH as well (195). This has been demonstrated by the reduction in levels of vitamin D dependent Ca binding protein in the intestine of hypophysectomised rats that increase following GH treatment (196, 197).

GH also increases serum PO_4 concentration by potentiating renal PO_4 reabsorption by increasing the renal tubular threshold for PO_4 excretion also known as the $TmPO_4/GFR$ (131, 198). Children and adolescents have been shown to have a higher $TmPO_4/GFR$ and serum PO_4 concentrations than adults and this is likely to be an effect of the higher GH levels associated with growth and puberty (199, 200). The effect of GH on $TmPO_4/GFR$ is mediated

by locally produced renal IGF-1, and appears to be independent of PTH and vitamin D (131-136, 201). Thus, GH has an antiphosphaturic effect and supports PO_4 retention. These mechanisms may contribute to an increase in extracellular calcium-phosphate product, and possibly bone mineralization.

1.3.3.4. Indirect Effects of GH: PTH

Studies have shown that GH and IGF-1 influence bone metabolism indirectly by affecting the end-organ effects of PTH, the anabolic actions of PTH on bone (91, 93, 94, 202-204) and by modulating PTH secretion and its circadian levels (91, 93, 94, 205). This effect is mediated in part by changes in serum PO_4 levels (51, 58) as the serum PTH peaks around 0500 h coinciding with the serum PO_4 peak (89, 206, 207).

1.4. Biochemical Markers of Bone Turnover

Osteoid matrix consists principally of collagen, of which the main structural protein is type 1 collagen. All collagens contain molecular domains of triple helical conformation which require the presence of repeating amino acid sequences. Proline and hydroxyproline feature prominently within the sequences. The collagen propeptide has amino (N) and carboxyl (C) terminal extensions which are cleaved by specific peptidases and released into the circulation, prior to the cross-linking of collagen molecules (208). The type 1 procollagen peptides, procollagen type 1 carboxyterminal propeptide (PICP) and procollagen type 1 aminoterminal propeptide (PINP) can be measured in serum as markers of bone formation. Other markers of

bone formation which are released into the circulation as a by-product of osteoblast activity include alkaline phosphatase and osteocalcin.

Bone resorption occurs as a result of osteoclast secretion of acid and neutral proteases which act sequentially to degrade the collagen fibrils into molecular fragments. The circulating products range in size from free amino acids to segments of cross-linked N-telopeptide and C-telopeptide domains. The fragments are metabolised by the liver and kidney until all fragments are sufficiently small to be excreted in the urine. The products of bone resorption which include hydroxyproline, C-telopeptide (CTX) and N-telopeptide (NTX) can be measured in serum or urine and used as markers of bone resorption. Acid phosphatase is a product of osteoclasts which may also be used for the assessment of bone resorption.

1.4.1. Assessment of Bone Turnover

Ideally, methods used to assess bone turnover should be minimally invasive and reproducible, allowing for easy and frequent measurement over time without undue risk or discomfort to the patient. Bone remodeling can be assessed most accurately using radiolabelled Ca kinetic studies, however the technique is expensive and time-consuming, and therefore generally limited to use as a research tool (209). Dynamic histomorphometry of bone biopsy samples may also be used to assess bone status, but obtaining bone samples is invasive and the results from a single core biopsy may not apply to all skeleton sites (209). Any technique used to assess bone remodeling should correlate with standard methods of assessing bone remodeling. In addition, for validation, the technique should correlate to changes in bone mass and respond appropriately after treatment in pathologies known to affect bone resorption or formation.

Recent advances in the development of sensitive assays for urine and serum markers of bone turnover has greatly increased the ability to assess bone remodeling in both research and clinical settings (209-211). The measured markers of bone turnover are either the products of bone collagen synthesis or catabolism, or the products of osteoblast or osteoclast cell activity; thus concentrations reflect collective remodeling throughout the entire skeleton and not just activity at a specific site.

The earliest markers of bone turnover to be used were ALP and urine hydroxyproline (211-215). Although both correlate well with Ca kinetic studies, they both lack specificity (215). Hydroxyproline is present in collagen which is ubiquitous to all tissues of the body and therefore changes in excretion of hydroxyproline do not necessarily equate to differences in bone resorption (209). ALP is secreted by several other tissues, predominantly liver, kidney and spleen and therefore measurements of total ALP may not accurately represent changes in bone formation. Assays for bone-specific ALP do exist and correlate well with Ca kinetic studies in Paget's disease and osteomalacia but less well in primary osteoporosis (216).

The newer markers of bone turnover, N- and C-propeptides and cross-linked N- and C-telopeptides of type 1 collagen, have the advantage that they are specific to bone metabolism. PINP and PICP are amongst the most sensitive markers available of bone formation and have been extensively used and validated in the assessment of bone formation in health and disease (216-222). Comparative studies have shown that PINP is more sensitive and reliable in predicting bone formation compared to PICP (216, 217, 221, 222).

CTX and NTX have been shown to be more sensitive to changes in bone resorption occurring at the menopause and during treatment with antiresorptive agents, than other available markers of bone turnover (211, 223-225). The measurement of urine cross-linked telopeptides, however, does have a number of disadvantages including collection of timed samples and the need to correct for renal function (226). More recently, the development of assays to measure serum type-I collagen β C-telopeptide (βCTX) has further improved the assessment of bone resorption (211, 223-225). Serum βCTX has been shown to be a specific and sensitive index of bone resorption, in both healthy controls and states of bone pathology (211, 223-225).

Markers of bone formation and resorption are secreted in circadian rhythms, with peaks occurring during the night and nadirs at approximately 10 am (67, 227, 228). It has been postulated that the circadian rhythm of bone turnover markers probably has an endogenous component and can also be affected by exogenous factors such as endocrine hormones including cortisol, PTH and calcitonin that have circadian rhythms and can modulate bone turnover. PTH has been shown to be important in the regulation of the circadian rhythm of bone turnover, with changes in PTH preceding those in osteocalcin by 5 hours (229-231). The physiological implications of the circadian rhythmicity of bone turnover are unclear, but there are suggestions that it may be important in the regulation of Ca homeostasis (67). In patients with postmenopausal osteoporosis, a blunted nocturnal PTH concentration is associated with increased renal Ca loss and exaggerated nocturnal peak in markers of bone resorption. The increased nocturnal bone resorption occurring in osteoporosis may be a compensatory mechanism to maintain serum Ca concentration (67), as evidenced by the suppression of a nocturnal rise in bone resorption following evening supplementation with Ca (232).

1.5. GH, IGF-1 and the Development of Osteoporosis – The AGHD Model

1.5.1. The GH/IGF-1 Axis and AGHD related Osteoporosis

AGHD in addition to its other characteristic features is associated with an increased risk of osteoporosis and osteoporotic fractures (231, 233-238) thus providing a model to study the effects of the GH/IGF-1 axis on bone. Initial studies performed in adults with childhood-onset GHD concluded that the reduction in BMD detected in adulthood occurred as a result of failure to attain peak bone mass during development (239-241). However, subsequent reports have confirmed an increased prevalence of osteoporosis in AGHD compared with a matched, healthy population where the study populations have been made up exclusively of adult-onset GHD patients (231, 233-238). Decreased BMD was also specifically observed in patients who were estimated to have developed AGHD after the age of 30 years, and therefore, after the attainment of peak bone mass (234-238) providing further evidence that GH is required not only for the acquisition but also the maintenance of bone mineral mass. Furthermore, the severity of AGHD, as defined by stimulated peak GH, has been correlated with bone loss, and FN and lumbar spine (LS) T-scores are significantly lower in those with severest impairment of GH secretion (242).

The pituitary aetiology and therefore high prevalence of abnormalities in sex steroid deficiency in patients with AGHD had led to the suggestion that it may have been oestrogen deficiency rather than GH deficiency that was responsible for the abnormalities observed in BMD (157). However, reduced BMD has been demonstrated in AGHD patients who have

normal gonadal function (233) and no difference in BMD was observed when patients who had isolated GHD were compared with patients who had co-existent gonadotrophin deficiency (235). There have however been gender based differences in the responses of patients with AGHD to GH replacement therapy and women have been found to be less responsive to the effects of GH (93).

Most studies have shown that bone formation is reduced in AGHD (51, 243-245). Bone resorption appears not to be significantly different (246-248) or reduced (51) in AGHD patients compared with healthy controls. The negative equilibrium between formation and resorption, and reduced bone remodeling are therefore likely to underlie the development of osteoporosis in AGHD.

1.5.2. AGHD and Bone Metabolic Parameters

1.5.2.1. AGHD and PTH

Studies that have looked at PTH changes in AGHD have been associated with increased, decreased or unchanged concentrations of PTH (249-251). The reason for such conflicting reports is likely to be an effect of the single time point measurements performed in the methodology of these studies (51). PTH is secreted in a circadian rhythm (56-58, 252) and, as a result shows significant day-night variability. Therefore, the timing of venesection for PTH analysis is critical to avoid misleading results. Ahmad et al recently demonstrated that a variability in sampling timing of as little as 2 hours may result in significant differences in PTH concentration (51). The only study to date to determine the effect of AGHD on PTH concentration whilst excluding the confounding sampling time variable was also performed

by Ahmad et al. (51). The authors took blood samples every 30 minutes from 14 AGHD patients and 14 matched healthy controls for a 24 hour period. They conclusively found that the mean 24-hour PTH concentration and the PTH concentration at each individual time point was significantly higher in the AGHD patients compared with the healthy controls, therefore suggesting that AGHD is associated with a relatively increased PTH concentration.

The determination of NcAMP has been shown to provide a sensitive and specific assessment of parathyroid function and is a reliable index of the circulating activity of PTH on the kidney and bone in both physiological and pathophysiological states (47, 50). Early reports suggested a reduction in NcAMP in patients with AGHD (253), which has been confirmed more recently in a cross-sectional study comparing AGHD patients with healthy controls (51). In this latter study, the reduced NcAMP occurred in association with an increase in mean 24-hour PTH concentration and reduction in serum adjusted calcium (ACa) and markers of bone turnover, suggesting that AGHD is associated with a reduction in the sensitivity of the target organs to the effect of PTH. The authors concluded that reduced PTH target-organ sensitivity may underlie the pathogenesis of reduced bone turnover and development of osteoporosis in AGHD.

Infusion of PTH into AGHD patients is associated with a delayed and reduced calcaemic response (52) and supports the hypothesis of reduced sensitivity of target organs to the effects of PTH. AGHD is also associated with a reduction in the responsiveness of the calcium-sensing receptor to both hypo- and hypercalcaemic stimuli with a reduction in the Ca set point (52). Both factors are thought to be important in the development of abnormalities in Ca metabolism seen in AGHD, and thereby in the contribution to a reduction in bone mass.

Circadian variability and temporal fluctuations in PTH are necessary for its biological activity (59-66). Abnormalities in the circadian, biphasic rhythm of PTH contribute to bone loss in various conditions (59, 66, 67). Patients with AGHD have been shown to exhibit significant PTH circadian rhythmicity but with a blunted nocturnal rise and a sustained early evening rise in PTH concentration (51); changes which may contribute to the pathogenesis of osteoporosis in AGHD.

1.5.2.2. AGHD and Calcium Metabolism

Limited published data exists, however, on the effect of GH deficiency on circulating Ca. An earlier study, investigating young adults with childhood-onset GHD, found no difference in serum Ca compared with healthy controls (241). No difference in PO_4 concentrations, PTH concentrations, intestinal Ca absorption or Ca and PO_4 excretion were found either. However, all patients studied had previously received GH prior to the study and single time point measurements were used. These findings may thus have been inaccurate and a more recent study did show that serum ACa concentration was significantly lower in AGHD patients compared with controls (51). In this study, blood was taken every 30 minutes for a 24 hour period in truly GH naïve patients and healthy controls. The 24-hour mean serum ACa concentration was calculated and compared between the groups and therefore gives a more accurate reflection of serum Ca concentration than the earlier study. The reduction in serum ACa seen in AGHD patients was explained by a reduction in renal sensitivity to PTH and so resultant decreased renal Ca reabsorption, together with a reduction in PTH mediated 1α-hydroxylase activity.

1.5.2.3. AGHD and Phosphate Metabolism

GHD is associated with a relative phosphate deficiency which occurs through a combination of reduction in GH-mediated $TmPO_4$/GFR resulting in phosphaturia and reduced intestinal PO_4 absorption (51, 191, 254). The absorption of PO_4 from the small intestine is decreased as a consequence of GH-dependent desensitisation of the intestine to $1,25(OH)_2D$ and reduction in $1,25(OH)_2D$ concentration occurring as a result of decreased PTH-mediated renal vitamin D 1α-hydroxylase activity (51, 157, 195). Although the reduced PTH target-organ sensitivity associated with AGHD would result in some renal PO_4 retention, the direct effect of the GH/IGF1 axis on $TmPO_4$/GFR and vitamin D metabolism appear to be greater and result in a net PO_4 loss (51).

1.5.3. AGHD and Growth Hormone Replacement

1.5.3.1. Bone Mineral Density

Growth Hormone Replacement (GHR) in AGHD has been associated with a reduction in BMD during the initial 3 months of treatment (255-257). This initial decrease may be explained by the increased remodeling activity occurring with GHR, that results in increased remodeling space and an increase in new unmineralised bone. BMD then increases as early as 6 months (243, 246, 255, 256, 258-263), and thereafter, bone mass continues to increase as long as GH is replaced and has been reported to continue increasing even as long as 10 years (264, 265). Discontinuation of GHR is not associated with immediate bone loss and BMD may actually continue to increase for at least 12 months after discontinuation of GH, suggesting a prolonged effect of GH on bone metabolism (266). Response to GHR appears to

depend on factors such as age, gender and body mass index (93, 94, 267, 268). At present there are not any studies which have investigated the effect of GHR on reduction in risk of fractures. BMD has been shown to correlate with risk of osteoporotic fracture, which may suggest that GHR could reduce fracture risk in the long term.

1.5.3.2. Bone Turnover

Data from several placebo-controlled studies have reported that GHR is consistently associated with a significant increase in all markers of bone resorption and bone formation (248, 269, 270). An increase in bone turnover has been confirmed by histomorphometric assessment (271). Increases in bone resorption and formation appear to occur as early as 1 and 3 months, respectively, following the initiation of GHR (91). The increase in markers of bone resorption, followed closely, temporally, by an increase in markers of bone formation supports the concept of coupling of bone turnover where resorption precedes formation, ultimately resulting in increased bone mass (91). Increases in markers of bone turnover may be sustained for at least 2 years after the initiation of GHR, suggesting that the effect of GH on bone remodeling is persistent, a concept supported by the progressive increase in BMD with discontinuation of GHR (258, 264, 267, 268, 272). Even after short term treatment with GH, markers of bone turnover remain elevated for several weeks indicating that GH has a prolonged effect on bone remodeling, in keeping with the sustained increase in bone mass observed following discontinuation of GHR (266). The mechanism of this "catch-up" is not readily apparent, although there are some interesting possibilities. First, it has long been recognized that GH, IGF-I, and PTH all stimulate bone resorption while activating bone formation. IGF-I stimulates RANKL expression in a dose dependent manner and down regulates OPG in ST-2 cells. These findings suggest that bone turnover with anabolic agents

is accelerated by targeting stromal cells, which in turn generate not only growth factors that increase bone formation, but also cytokines that are necessary for osteoclast recruitment and differentiation. Thus, discontinuation of rhGH might lead to an immediate cessation in IGF-I–mediated RANKL expression, while at the same time continuing the stimulatory actions on osteoblasts initiated earlier during treatment.

1.5.3.3. PTH

The results of earlier reports investigating the effect of GHR on PTH had been conflicting with increased, decreased or no change in concentration detected (91, 196, 249, 250, 253). The different outcomes observed may have been due to single time point sampling not accounting for the circadian variability of circulating PTH (51). In a more recent study, PTH was measured on blood samples that were taken every 30 minutes over a 24 hour period thus eliminating any error associated with single-time point sampling methodology (91). PTH decreased significantly following 1 month of GHR, with maximal changes occurring after 6 months. In the same study, NcAMP increased significantly after 1 month of treatment, suggesting that GH resulted in an increase in renal sensitivity to the effects of PTH. PTH (1-34) infusion is associated with larger and earlier increases in serum ACa concentration in AGHD patients after GHR than prior to GHR which supports the concept of an increase in PTH target-organ sensitivity (52). An increase in parathyroid gland sensitivity to smaller changes in serum Ca concentration was also observed following GHR, with a lowering of the Ca set point (52). With the 24 hour sampling the effects of GHR on PTH circadian rhythm were also studied and GHR was associated with an increase in the nocturnal PTH peak and a restorative effect on the PTH circadian rhythm (91).

1.5.3.4. Calcium

The majority of studies investigating the effect of GHR in AGHD have shown that GHR is associated with an increase in serum Ca concentration (52, 91, 249, 262, 273). This may occur as a result of increased renal sensitivity to PTH, as evidenced by a simultaneous reduction in PTH concentration and increase in NcAMP (91), thus leading to increased renal tubular Ca reabsorption. Increased renal PTH sensitivity following GHR would also increase PTH mediated 1α-hydroxylase activity and thus $1,25(OH)_2D$ concentration, thereby increasing serum Ca concentration further. Finally, GH sensitizes the small intestine to $1,25(OH)_2D$ potentiating the increase in intestinal Ca absorption (195).

1.5.3.5. Phosphate

GHR in AGHD is associated with an increase in serum PO_4 concentration (91, 249, 262, 273). The interplay of several mechanisms contributes to the increase of PO_4 following GHR. The dominant effect of GHR on PO_4 is an increase in $TmPO_4/GFR$ (198, 254) which promotes an antiphosphaturic effect, and a reduction in 24-hour urine PO_4 excretion (91). The effect of GHR on $TmPO_4/GFR$ appears to be the result of a direct GH effect mediated through locally produced IGF-1 and occurring independently of PTH , UcAMP or $1,25(OH)_2D$ (131-135). The increase in $1,25(OH)_2D$ and GH-induced increased gut sensitization to $1,25(OH)_2D$ observed following GHR contributes also to the increase in serum PO_4 through an augmentation of intestinal PO_4 absorption (91, 195). In addition, it was previously proposed that the increase in serum Ca concentration that occured in response to GHR resulted in suppression of PTH, thereby decreasing PTH-mediated phosphaturia. In contrast, the more recent demonstration of increased renal PTH activity, as defined by an

increase in NcAMP observed following GHR, would promote renal PO_4 excretion (91). Therefore, the positive PO_4 balance occurring following GHR is likely to represent the net positive effect of a greater, direct GH-mediated increase in $TmPO_4$/GFR, increased intestinal PO_4 absorption and a PTH-mediated phosphaturia of lesser magnitude.

1.6. GH, IGF-1 and the Development of Age-related Osteoporosis

1.6.1. Age-related Changes in the GH/IGF-1 Axis

The complex role of the GH axis in bone metabolism is illustrated by the direct and indirect mechanisms of GH studied in various groups of subjects including patients with AGHD as described above. Our understanding of the role of GH on bone must also take into consideration the age-dependent decline in GH secretion during an individual's lifetime. This decline has been ascribed to changes occurring at the hypothalamic level, without a known cause, resulting in a decrease in GH releasing hormone, decreased central cholinergic tone and an increase in somatostatin secretion (274, 275). The decline in the production of sex steroids, physical activity, and the presence of aberrant sleep patterns also may contribute to the decline in GH levels during aging (276).

Serum levels of GH and IGF-1 decrease with advancing age reaching a nadir at the sixth decade (274, 277, 278). Variable reductions (15-70%) in 24-hour GH secretion have been demonstrated with the GH production rate decreasing by 14% with each advancing decade after puberty (277, 279-283). GH production decreases twice as rapidly in men than in women so that GH release remains higher in women than in men after the age of 50 (281,

284). The changes in GH and IGF-I secretion that occur with aging are paralleled by a progressive loss of lean muscle mass and strength, a decline in physical performance, a decrease in quality of life, an increase in body fat and a decrease in BMD (BMD) (285-289), clinical features also seen in the syndrome of AGHD (290). It has been hypothesized, therefore, that the ageing process and particularly age related osteoporosis, previously referred to in the literature as type 2 osteoporosis, may be due to a relative GH deficient state (12, 290-292). A relative GH deficiency has also been implicated in the development of postmenopausal osteoporosis, previously referred to in the literature as type 1 osteoporosis (290), as oestrogen is required for the secretion of GH (293). Thus GH and IGF-1 may have a role to play in the continuous slow phase bone loss with advancing age and a possible additional role in the superimposed rapid phase bone loss immediately after the menopause.

1.6.2. Age-related Osteoporosis

Although pituitary GH content is normal in patients with type 1 and type 2 osteoporosis, the total 24-hour, peak and stimulated GH secretion are reduced compared with age-matched controls (294) thus supporting the concept of GH involvement in the pathogenesis of primary osteoporosis. IGF-1 and integrated GH secretion demonstrate strong correlation with BMD and have been reported to be significantly lower in women as well as men with osteoporosis (295, 296) and IGF-1 is an independent predictor of bone mineral content (BMC) even in healthy elderly women (297-299). Decreased IGF -1 concentration is associated with an increased risk of fractures (296) and patients with a recent hip fracture have decreased concentrations of GH and IGF-1 (300). In addition to lower IGF-1 concentration, post menopausal women with osteoporosis also have lower serum levels of insulin like growth factor 2 (IGF-2) and insulin like growth factor binding protein 3 (IGFBP-3) (301). Low

circulating IGF-1 and IGFBP-3 are associated with low rates of bone turnover as well as reduced BMD in the spine of men with idiopathic osteoporosis (299, 302, 303). IGF-1, IGF-2, IGFBP-3 and insulin like growth factor binding protein 5 (IGFBP-5) concentrations within femoral cortical and trabecular bone microenvironment are also lower and decrease with advancing age in men with osteoporosis (278, 296, 304).

Thus GH and IGF-1 are involved in bone metabolism and the maintenance of bone mass in adults through the complex interaction of systemic circulating GH, IGFs, IGFBPs, locally produced IGFs and IGFBPs (154, 157, 158, 305, 306); the age related decline in GH and IGF-1 may contribute to the pathogenesis of age related osteoporosis (307-309).

1.6.3. Changes in Bone Metabolism with Age

1.6.3.1. Aging and PTH

PTH concentration increases with age (310-312) and is also higher in women with primary osteoporosis (311, 313-315). The increase has variously been attributed to decreases in renal function, residual oestrogen levels, Ca absorption, levels of and response to 25(OH)D as well as to aging itself (316-323). It has been a popular concept that the alterations in PTH may be causally related to bone loss in primary osteoporosis (313) and that the age related increase in PTH may contribute to the age related increase in bone resorption (324-326) .

The rapid bone loss phase in the immediate postmenopausal period is associated with a high rate of bone resorption. It is proposed that the increased resorption increases skeletal Ca outflow into the extracellular pool, leading to a partial suppression of PTH secretion, a

compensatory increase in UCa excretion (16) and decreased intestinal Ca absorption (327) preventing hypercalcemia. It has also been observed that bone responsiveness to infused PTH is enhanced during the immediate postmenopausal rapid phase of bone loss (328) but this has been attributed to the overall increase in bone remodeling units seen in this phase (329).

As the rapid bone loss phase subsides, serum levels of PTH increase progressively throughout the remainder of life with an absolute increase in bone resorption and a relative decrease in bone formation involving equal losses of cortical and cancellous bone (330). The increases in PTH correlate with the increases of the biochemical markers of bone turnover (16). 24 hour infusion of Ca to postmenopausal women with elevated PTH and bone turnover markers was shown to decrease both PTH and bone markers suggesting that the increased PTH may be the cause of the increased bone turnover (324). Men do not have an equivalent of the rapid phase of bone loss that women experience following the menopause and after accounting for the absence of this phase, the patterns of late bone loss and of the increases in serum PTH and bone resorption markers in ageing men are virtually super-imposable upon those occurring in women (16).

The increase in PTH during the slow phase is also not associated with an increase in ionised Ca. This was considered indicative of secondary hyperparathyroidism as a result of age-related abnormalities in extra-skeletal Ca homeostasis. Studies demonstrating impaired intestinal Ca absorption and impaired renal Ca reabsorption with advancing age resulting in Ca loss would support this supposition (323, 324, 331-334). Decreasing serum concentrations of the vitamin D metabolites, 25(OH)D (335-337), $1,25(OH)_2D$ (320, 332, 336, 338-343) and the renal enzyme 25(OH)D 1α-hydroxylase (343) have also been demonstrated with ageing, and may contribute to the increasing PTH.

The nocturnal rise in PTH secretion is absent in women with primary osteoporosis and there is blunting of the rhythm in postmenopausal women compared with premenopausal women even without a demonstrated decrease in BMD (66). Abnormalities in circadian rhythms of bone resorption and renal Ca conservation in postmenopausal osteoporosis have been shown to be associated with the blunting of the nocturnal rise in PTH secretion (67). Thus, changes in the circadian rhythm of PTH may have a role to play in the development of age related osteoporosis, but the factors influencing PTH rhythm as well as mechanisms by which the abnormal PTH rhythm may affect bone are unclear.

1.6.3.2. Aging and Calcium Metabolism

Serum total Ca has been reported to decrease progressively from the age of 20 upto the age of 80 in men, but in women, some studies have reported no change in Ca concentration with age whilst some have suggested a minimal increase in serum Ca after the menopause. Aging and the menopause are however associated with a negative shift in Ca balance (344, 345). Thus, a number of different age-related observations have been reported on the complicated and multi-factorial regulation of Ca metabolism with various hypotheses proposed to explain these observations.

Aging is associated with changes in vitamin D metabolism which would impact on Ca homeostasis. It is reported that 25(OH)D, which is an indicator of vitamin D nutrition status, decreases by 30-60% in both genders with advancing age, probably as a result of poor nutrition, decreased intestinal absorption and inadequate exposure to sunlight. $1,25(OH)_2D$

has been reported to decrease by about 50% with aging and levels are lower in patients with osteoporosis as compared to age matched controls (336). The serum levels of calcitriol, however, overlap to a significant degree in young adults, in the elderly or elderly adults with osteoporosis and not all investigators have demonstrated these age related differences. The decrease in activated vitamin D is probably due to decreased renal production. The normal increase in $1,25(OH)_2D$ in response to infusions of PTH are blunted in the elderly as compared to young adults (343) and a primary impairment in the renal enzyme 24(OH)D 1α-hydroxylase, responsible for the conversion of 25(OH)D to $1,25(OH)_2D$ has been demonstrated directly in kidney slices of aging rats. Aging may result in primary deficiency of 24(OH)D 1α-hydroxylase or a decrease in the renal sensitivity to PTH. Low 1 α-hydroxylase activity may be due, indirectly, to the absence of the permissive action of GH/IGF-I axis on the enzyme, as the GH/IGF-I axis may mediate the hypophosphataemic stimulus on renal 1 α-hydroxylase (135, 346, 347).

It is well established that intestinal Ca absorption efficiency declines with age as well as following the menopause and is lower in elderly people with osteoporotic fractures than in subjects of the same age without osteoporosis (336, 348-351). The decrease in intestinal absorption contributes to the negative shift in Ca balance (333, 336, 352-354) and is only partly attributed to the decrease in serum levels of calcitriol. Some reports however suggest that the decrease in Ca absorption begins about age 50 to 60 years, preceding the decrease in $1,25(OH)_2D$ and may even be due partly to decreased sensitivity of the intestine to the action of $1,25(OH)_2D$ (332) but this has not been observed in all studies (355). The decrease in oestrogen following the menopause (356, 357) as well as an age related decrease in the active intestinal transport mechanism (356) also possibly contribute to the decrease in Ca absorption.

The negative Ca balance is also partly explained by increased UCa excretion which is greater in postmenopausal women than in premenopausal women and has been shown to be even greater in postmenopausal women with osteoporosis when compared with postmenopausal women with a normal BMD (358). Explanations proposed to account for the decrease in renal tubular Ca transport have included a direct effect of lower oestrogen on renal Ca absorption as well as an indirect effect of oestrogen via increased bone resorption leading to Ca release, ensuing PTH suppression and a resultant decrease in PTH effect on renal Ca reabsorption. The indirect effect is thought to be the predominant mechanism in the early postmenopausal period and the direct effect in later stages.

The increase in UCa excretion in women with osteoporosis has been shown to be maximum at night time and it has been suggested that this increased nocturnal loss may contribute to the blunting of the nocturnal PTH rhythm as well as to an increased in nocturnal bone resorption for maintenance of circulating ionised Ca concentrations overnight (67, 358). It has also been suggested that the decrease in Ca reabsorption may also be a result of decreased renal sensitivity to the action of normal serum levels of PTH (51, 359).

1.6.3.2.1. The Sequence of Metabolic Events

Various hypotheses have been proposed to account for the sequence of the complex metabolic events involving PTH, vitamin D and Ca that lead to bone loss.

(i) Decreased renal production of calcitriol, brought on by age-related decreases in renal function, deficiencies of gonadal steroids, or other factors, may be primary. Malabsorption of

Ca follows and the reduced flow of Ca into the blood stimulates a reactive increase in PTH secretion, which in turn increases renal secretion of calcitriol. While this response may maintain serum levels of calcitriol, the elevated levels of PTH produce bone resorption and eventual osteoporosis. Under this hypothesis, age-related bone loss occurs as a form of mild calcitriol deficiency with mild compensatory parathyroid-mediated bone loss.

(ii) Under another hypothesis the primary abnormality may be an imbalance between bone resorption and bone formation, brought on either by reduced physical activity, age, deficiency, or other factors. Because Ca released from the skeleton exceeds Ca taken up by the skeleton, PTH secretion is suppressed, with consequent reduction of calcitriol synthesis. Reduced efficiency of intestinal Ca absorption would thus constitute an appropriate adaptation to net flux of Ca from bone, exactly analogous to the adaptations which take place in response to the resorptive excess of bed rest.

Both hypotheses have, in common, decreased efficiency of intestinal Ca absorption, decreased levels of calcitriol in the serum, net bone resorption, and osteoporosis. However, the causal sequences are directly opposite, and the PTH levels differ under the two hypotheses. In discussing the complicated and often discrepant evidence for and against these hypotheses, it is important to bear in mind that both may be correct, but in different individuals and at different stages of bone loss with different patterns of bone loss. The former sequence of events would be in keeping with changes in the late slow bone loss phase whilst the latter hypothesis would be in keeping with the rapid bone loss phase in the immediate postmenopausal period.

The development of osteoporosis in AGHD is also associated with the same spectrum of abnormalities in Ca, vitamin D and bone turnover. PTH concentrations are however higher in patients with AGHD than in healthy controls. Thus the overall biochemical picture is in keeping with the events in hypothesis (i) and age-related slow bone loss. The findings of Ahmad et al. however suggest that the primary biochemical abnormality in AGHD is PTH resistance as a result of low GH and IGF-1 concentrations. This results in the increased PTH concentration and simultaneous abnormality in Ca and vitamin D metabolism (51). This provides a third hypothesis: the age related decline in GH and IGF-1 may contribute to age related bone loss as result of PTH resistance as the first step in the sequence of metabolic events.

1.6.3.3. Aging and Phosphate Metabolism

Serum PO_4 levels have been shown to decrease gradually with age in adult men in an almost linear pattern (360-363). The decline in serum PO_4 levels in women under the age of 45 years is similar to that in men. Serum PO_4 then increases between the ages of 45 and 54 years or until after the menopause. Following this, levels progressively decline (360). Some studies have demonstrated a negative relationship between increased PO_4 and decreased vitamin D (364, 365).

A greater phosphaturic effect as a result of higher PTH concentrations (107, 108) with increasing age may account for the decreasing PO_4. One would also expect an increase in $1,25(OH)_2D$ as a result of the higher PTH. Increased vitamin D should in turn increase circulating PO_4. This is however not the case as $1,25(OH)_2D$ concentrations decrease with age (336). Thus current hypotheses do not provide a unifying mechanism of events for PO_4 changes. PO_4 metabolism is affected by the GH and IGF-1 axis. GH and IGF-1 independently

increase $TmPO_4/GFR$. Theoretically, the age related decline in GH and IGF-1 may also affect PO_4 metabolism by decreasing $TmPO_4/GFR$ and increasing phosphaturia.

In patients with AGHD, PTH concentrations are increased in association with low GH and IGF-1 and $1,25(OH)_2D$. Circulating PO_4 concentrations are low and urine PO_4 excretion is high with a low $TmPO_4/GFR$ (51). The increase in circulating PTH is attributed to PTH resistance as a result of the low GH and IGF-1. Renal resistance to the effect of PTH also accounts for the low $1,25(OH)_2D$. The low $1,25(OH)_2D$ in combination with the low $TmPO_4/GFR$ (as a direct result of low GH and IGF-1) would account for the phosphaturia and lower circulating PO_4. Thus, a unifying hypothesis applicable to age related changes in PO_4 metabolism may be derived from the mechanisms elucidated in patients with AGHD.

1.6.3.4. Aging and Markers of Bone Turnover

Biochemical markers of bone turnover have been studied in several groups of women. Levels of both bone formation and resorption have been shown in some studies to increase during the perimenopausal and postmenopausal period but with a relatively greater increase in bone resorption markers as compared to formation (215). Some studies have shown an increase in resorption markers with no change in formation markers (366). In postmenopausal women with osteoporosis the markers of bone resorption are significantly higher when compared to women with out demonstrated bone loss, but with a relatively smaller increase in bone formation markers (215).

Biochemical markers of bone turnover are less well studied in men than in women. Levels of both markers of formation and resorption are high in young men and decrease with advancing age (367-370). In elderly men, bone resorption increases with age more than bone formation. In elderly men, levels correlate negatively with BMD suggesting that accelerated bone turnover or the imbalance of a greater increase in bone resorption compared to bone formation may underlie age-related bone loss (367-373).

1.6.4. Age-related Osteoporosis and GH Administration

The effects of GH therapy on bone mineralization and bone turnover has been assessed in several studies involving subjects with primary osteoporosis. Many of these studies were of short duration, studied only a small number of patients and also involved co-administration of other bone active substances (374-377). GH therapy has been shown consistently to stimulate biochemical markers of bone turnover in models of primary osteoporosis, with increases in IGF-1 and IGF-BPs (258, 264, 267, 268, 272, 378-387). These initial studies were however, disappointing as a result of dose related side effects and limited benefit on BMD. In a 2-year study in older women with low bone mass rhGH increased bone density in the spine by 3% and lesser changes were observed in the hip. Radial and lumbar BMD increased in 8 elderly postmenopausal osteoporotic women 1 year after discontinuation of GH (380). GH treatment for 6 months increased lumbar BMD by 1.6% in elderly men (309).

Encouragingly, a more recent randomized placebo controlled study performed by Landin-Wilhelmsen et al. followed patients for 5 years and showed GH to be well tolerated and beneficial with a delayed, extended and dose dependent effect resulting in almost a 15% increase in femoral and lumbar BMD (BMD) (388). All the patients in this study were given

oestrogen replacement therapy, Ca and vitamin D. They were then randomly assigned to receive either placebo or GH at a dose of 1U (0.3mg) /day or 2.5U (0.8mg) /day. Maximum benefit was observed in the group administered the higher dose. A "catch up" effect, with an increase in BMD, 2 years after the cessation of GH was observed as in previous studies (380). In contrast to previous studies in postmenopausal women, benefit was observed when GH, an anabolic agent, was combined with oestrogen, an antiresorptive agent (374).

Over and above the assessment of increases in bone turnover and BMD the underlying mechanisms by which GH effects these observed changes are less studied and even less well understood. Henneman et al. reported that GH administration for 1 day to 24 months produced a positive Ca balance and in a double-blind placebo-controlled study, daily high dose GHR given to 10 healthy individuals for 7 days resulted in a significant increase in serum Ca compared to placebo (389). In contrast GH treatment for 12 months in osteoporotic patients had no significant effect on total body Ca as assessed by neutron activation. No change in ionized Ca concentrations were observed in 8 postmenopausal women following short term (5 days) and sustained (5 weeks) rhGH administration. Studies have reported either a decrease or no change in PTH concentrations with differences dependent on GH dose. Similarly serum PO_4 has been shown to increase, with an increase in $TmPO_4$/GFR by some but not others. Enhanced effect on PTH action with regard to renal 1α- hydroxylase activity with increasing $1,25(OH)_2D$ have been reported but so have unaltered $1,25(OH)_2$ D and unaltered 24(OH)D (389). Limitations, including small numbers, single time point sampling methodology and co-administration of other agents with GH in these studies may be responsible for some of these discrepancies.

The renewed interest in the use of GH in the treatment of osteoporosis with the demonstration of increased BMD with its long term use should allow a greater understanding into the role of GH in the pathophysiology of bone metabolism looking at effects on and interactions with PTH and phospho-calcium metabolism. The more widespread use of GH as replacement therapy in patients with AGHD and the recent effects of GH on PTH sensitivity and circadian rhythm in AGHD patients has also provided further insight into the mechanistic interactions between GH, PTH and phospho-calcium metabolism but whether these mechanisms are involved in the context of the physiological age related decline in GH and IGF-1concentrations and age related bone loss needs further elucidation. To date there are no studies reporting the effects of GH on PTH secretory pattern, circadian rhythmicity and end-organ sensitivity in patients with age-related osteoporosis.

Chapter 2

Study Aims

The aims of the studies in this dissertation were to provide a greater understanding of the underlying pathogenesis of age-related osteoporosis. The studies were designed to examine differences in bone mineral metabolism between older men and women with osteoporosis and control subjects; particularly changes in the GH/IGF-1 axis, PTH sensitivity, PTH circadian rhythm, phospho-calcium metabolism and biochemical markers of bone turnover. The effect of GH administration on bone metabolism in postmenopausal women with osteoporosis was also studied.

The study objectives were to determine:

1. Serum IGF-1 concentration, PTH sensitivity, PTH circadian rhythm, phospho-calcium metabolism and bone turnover in men and women in different age groups and with normal and low BMD.

2. The effects of GH administration on PTH sensitivity, PTH circadian rhythm, phospho-calcium metabolism and bone turnover in postmenopausal women with osteoporosis.

3. The relationship between PTH, OPG and βCTX over a 24-hour period in premenopausal women and elderly postmenopausal women.

Chapter 3

Methods

3.1. Subjects

3.1.1. Recruitment

Patients with low BMD and older control subjects with normal BMD were identified via a community osteoporosis screening program run by the Royal Liverpool University Hospital. The younger control men and premenopausal women were recruited from hospital personnel and from a database of volunteers willing to participate in medical research.

3.1.2. Patients and Controls

6 groups of subjects were recruited for cross-sectional comparison as detailed in Chapter 4. These included postmenopausal women and older men with low BMD as well as normal BMD. Younger men and premenopausal women were also recruited as healthy controls. Postmenopausal women with osteoporosis, who participated in the cross-sectional studies, were invited to participate in the longitudinal study detailed in Chapter 5.

3.1.3. Exclusion Criteria

The exclusion criteria are detailed in Table 3.1. Postmenopausal status was confirmed by serum FSH concentrations above 40U/L and absence of menstruation for at least 12 months.

Table 3.1 Exclusion Criteria

Exclusion Crieria:
Diabetes
Severe cardiac, liver or renal disease
Primary hyperparathyroidism
Vertebral fracture
Men with hypogonadism
Prescription of Bisphosphonate therapy prior to recruitment
Prescription of HRT or had been prescribed HRT in the year prior to start of the study
Prescription of calcium and vitamin D
Prescription of diuretics
Prescription of corticosteroids
Pregnancy

3.2. Biochemistry

3.2.1. Insulin-like Growth Factor 1

IGF-1 was measured with a specific radioimmunoassay (RIA) in the presence of a large excess of insulin like growth factor 2 (IGF-2) (Mediagnost, Tübingen, Germany) to block the interference of IGF-binding proteins (390). Intra- and interassay coefficients of variation

(CVs) were 1.6 and 6.4% respectively. The samples for IGF-1 were sent to Germany and the assays were performed at the lab designated to run the IGF-1 assays for the hypopituitary control and complications study (HYPOCCS). Due to the overlap of IGF-1 concentrations across age groups, the IGF-1 concentrations were measured at the speciliast lab so as to provide mean concentrations with standard deviation scores (SDS) for age, based on normal ranges that were established with a cohort of 450 healthy adults (age 18-80 years; number of men = 225). The distribution of the values obtained was log normal, and consequently the measured values were log transformed before further calculations. Means and standard deviation (SD) of the log IGF-1 values were calculated for age intervals (10 years). Best fit regression curves were then derived for the means and mean minus SD that were separate for men and women under 28 years (polynomial 2^{nd} degree) but since there was no significant difference in the measured variable after the age of 28 years, the values obtained for men and women were combined for 28 to 66.8 years and for >66.8 years (polynomial of 3^{rd} degree). Standard deviation scores (SDS) were then calculated by: log (IGF-I) – mean/ SD. The mean was calculated as a function of age from the corresponding polynomial and SD was calculated by mean minus the value of the polynomial for the curve.

3.2.2. Calcium/ Phosphate/ Creatinine/ Albumin

Serum Ca, PO_4, creatinine (Cr) and albumin were measured on all samples by the standard auto-analyser method (Roche Modular Systems (Incorporating the ISE1800, D2400, P800 and E170), Roche Diagnostics, Lewes, U.K.). Serum Ca was adjusted for albumin (391). Serum ACa has been shown to strongly correlate with ionized Ca and has been found to be precise in subjects with Ca and albumin within the reference range (391, 392).

Urine Ca, PO_4 and Cr were analysed on all samples using standard laboratory methods (Roche Diagnostics, Lewes, UK). Urine values were expressed as molar ratios to creatinine (UUCa/Cr, UPO_4/Cr) and as excretion per liter of creatinine clearance (CCr) by multiplying the urinary ratios and the serum creatinine to yield the urine calcium excretion (UCaE), urine phosphate excretion (UPO_4E) respectively. The $TmPO_4/GFR$ (mmol/L of GFR) was derived from the nomogram by Walton and Bijvoet (393).

3.2.3. Vitamin D

For the $1,25(OH)_2D$ assay, serum was treated with acetonitrile and the supernatant purified through C18-OH reverse phase column to obtain the fraction containing $1,25(OH)_2D$ which after evaporation was measured by radioimmunoassay (RIA) (Immune Diagnostic Systems (IDS) Ltd. Tyne and Wear, U.K.). Each sample contained tritiated $1,25(OH)_2D$ to act as a recovery. The intraassay CV was <9% and the interassay CV was <12% across the working range, with a detection limit of 15pmol/L. Serum 25(OH)D was measured using an RIA kit (DiaSorin, Inc., Stillwater,MN) after acetonitrile extraction. The intraassay CV was <8%, and the interassay CV was <11% across the working range, with a detection limit of 4nmol/L.

3.2.4. Parathyroid Hormone

Serum PTH (1-84) was measured on all samples using a commercial immunoradiometric assay (IRMA) (Nichols Institute, San Juan Capistrano, USA), with a detection limit of 0.5pmol/L and intra- and inter-assay CVs of <7% across the working range.

3.2.5. Plasma and Urine cyclic AMP

PcAMP was measured by RIA (BIOTRAC cAMP, Amersham Pharmacia Biotech, Little Chalfont, UK). The intraassay CV was <8%, the interassay CV was <10% across the working range, with a detection limit of 5nmol/L.

UcAMP was measured by in-house RIA methods as previously described (51, 394). The intra- and interassay CVs across the assay working range were <8% and 10% respectively, with a detection limit of 0.2μmol/L.

NcAMP, which is a reliable index of PTH activity at the level of the kidney (47), was determined from the formula:

$$NcAMP = (SCr \times UcAMP/UCr) - PcAMP$$

NcAMP is expressed as nmol/L GFR, with serum creatinine (SCr) in μmol/L, UcAMP in μmol/L, urine creatinine (UCr) in mmol/L and PcAMP in nmol/L.

3.2.6. Markers of Bone Turnover

Serum concentration of βCTX, a marker of bone resorption, and PINP, a marker of bone formation, were measured using electrochemiluminesscence immunoassays (ECLIA) (ELECSYS, Roche Diagnostics, Lewes, UK). The intra- and inter-assay CVs for βCTX were <4% and <5% respectively across the working range, with a detection limit of 0.01ug/L and

the intra- and inter-assay CVs for PINP, were <2% and <2.5% respectively across the working range, with a detection limit of 4μg/L.

3.2.7. Osteoprotegerin

OPG was measured by a direct sandwich-type enzyme linked immunoassay (Biomedica, Oxford Biosystems, UK) with a detection limit of 0.14 pmol/L and inter- and intra-assay CVs of less than 10% across the working range (0.14 pmol/L to 30 pmol/L).

3.3. Statistical Analysis

Statistical programs SPSS version 11.0 and CHRONOLAB 3.0 (Universdade de Vigo, Vigo, Spain) were used for analysis of the data. The numbers of patients required for the studies were calculated using two-sided significance levels of 0.05 and a power of 90% to detect a difference of 1.0pmol/l in PTH concentration, before and after GHR, with a standard deviation of 0.8.

3.3.1. GLM ANOVA for Repeated Measures

General linear model analysis of variance (GLM ANOVA) for repeated measures was used to analyse the data. Repeated measures analysis of variance (ANOVA) assumes normally distributed errors, equal variances and sphericity. The Kolmogorov-Smirnov test was used to confirm normal distribution and Levenes's test for equality of variances. Mauchly's test was

used to confirm the sphericity assumption. If the assumption was violated, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity.

3.3.2. Student's t-test

Comparisons of normally distributed, paired and unpaired data were performed using student's t-test. In all cases where this test was applied, a two-tailed hypothesis was used. Bonferroni's correction method was applied to allow for multiple comparisons between visits before and after GH treatment.

3.3.3. Cosinor Rhythmometry

Individual and population mean cosinor analysis was used first to confirm circadian rhythmicity and determine the circadian rhythm parameters for PTH using CHRONOLAB 3.0 (Universdade de Vigo, Vigo, Spain), a software package for analyzing biological time series by least squares estimation (51, 91, 395). The package has previously been well validated and used to analyse PTH and bone marker circadian rhythms in various groups of patients (51, 66, 91). The software thus provides the following circadian parameters: 1) midline estimate statistic of rhythm (MESOR), defined as the rhythm-adjusted mean or the average value of the rhythmic function fitted to the data; 2) amplitude, defined as half the extent of rhythmic change in a cycle approximated by the fitted cosine curve (difference between the maximum and MESOR of the fitted curve); and 3) acrophase, defined as the lag between a defined reference time (1400 h of the first day in our study when the fitted period is 24 h) and time of peak value of the crest time in the cosine curve fitted to the data. A p value for the rejection of the zero-amplitude (no rhythm) assumption is also determined for

each individual series and for the group. The method used by the program allows analysis of hybrid data (time series sampled from a group of subjects, each represented by an individual series). Given k individual series, the program fits the same linear model with m different frequencies (harmonics or not from one fundamental period) to each series. This fit provides estimations for 2m + 1 parameters, namely, the amplitude and acrophase of each component, as well as the rhythm-adjusted mean. The population parameter estimates are based on the means of estimates obtained from individuals in the sample. The confidence intervals depend on the variability among individual parameter estimates. The variance-covariance matrix is then estimated on the basis of the sample covariances. Confidence intervals for the rhythm-adjusted mean, as well as for the amplitude-acrophase pair, of each component is then computed using the estimated covariance matrix. The p-values for testing the zero-amplitude assumption for each component, as well as for the global model is finally derived using those confidence intervals and the t and F distributions (396). Bingham's test, developed for testing cosinor parameters and part of CHRONOLAB 3.0 software, was used to determine the significance of the differences of cosinor-derived circadian rhythm parameters between subjects.

Following the confirmation of concerted circadian rhythms further analysis of the more extensively studied PTH rhythm was performed as the next step. Over and above the diurnal variation, the PTH rhythm has previously been demonstrated to have bimodal peaks in healthy individuals (early evening and nocturnal) and previous studies in pathological conditions have demonstrated alterations during the time periods of these peaks (51, 66, 91, 94). Based on these previous data, time points for further analysis were selected for the individual peaks and using previously accepted techniques, we further analysed our data set. Based on recent work by Luboshitzky et al. the time of onset was defined as the time of first

occurrence of at least 3 consecutive samples exceeding the mean levels of PTH obtained between 0800 h and 1400 h by more than 1 SD (397).

3.3.4. Cross-correlation Analysis

Cross-correlation analysis was performed to determine the relationships between the 24 h profiles of PTH, OPG and βCTX in Chapter 6. Cross-correlation analysis determines the correlation between two time series of equal length that have been paired, data point by data point, and then one of the time series is shifted by one or more time points (lag time) and the correlation process is repeated. This process can be repeated with the time series shifted backward and forward, as many times as there are time points minus 1. PTH, OPG and βCTX time series for the group were derived by calculating the mean value at each time point for all subjects (51, 74, 75, 91). Thus, 25 means were determined for PTH, OPG and βCTX. To determine whether one time series led another, for instance whether changes in serum PTH preceded changes in OPG, we computed the cross-correlation functions at 12 lag time points (up to 12 h) (398). Previous studies using half hourly sampling have revealed significant interactions between PTH and other bone related metabolites using a 6 hour lag (51, 75, 91). To allow for the hourly sampling frequency and the lack of prior documentation of the circadian pattern of OPG we utilized a 12 hour lag time for cross correlation analysis.

Cross correlation with log transformed values and Monte Carlo simulation were performed to establish statistical replicability of the cross-correlation analysis between variables. In Monte Carlo simulation, statistical procedure was based on simulated samples of varying sizes (12, 24 and 48, multiple of number of patients and controls) repeated 100 times. The type I error was estimated by samples consisting of time series representing healthy controls. For a type I error of 0.05, cut-off point of 5% was selected. To determine whether the time or lag

difference between the original and simulated data was similar, z-score-transformed r values were obtained from each simulated sample. Significant cross-correlation values at any particular lag were then tested against the null hypothesis of purely random associations applied to the z-score-transformed r values, assuming that uncorrelated data show a unit normal z-score distribution with 0 mean unit-variances.

3.4. Measurement of Bone Mineral Density

BMD of the lumbar vertebrae (L2-4) and the FN was measured by DXA, using a Prodigy Oracle Fan-Beam bone densitometer (GE Medical Systems). DXA is the most widely used procedure for measuring bone mass and has been accepted as the gold standard (399). The measurements of BMD made by DXA are accurate and reproducible. The variability of repeat readings is <1% for the LS and <2% for the femur. Radiation exposure for DXA as minimal (<10mrem) and scanning time is short (5-20 minutes). T-scores were calculated against a reference population of UK subjects 20-39 years of age.

Experimental Work

Chapter 4

The Effect of Age, Bone Mineral Density and Gender on Parathyroid Hormone Sensitivity, Parathyroid Hormone Circadian Rhythm, Phospho-calcium Metabolism and Bone Turnover

4.1 Introduction

The process of aging is associated with bone loss and an increasing incidence of osteoporosis. Peak skeletal mass is achieved between 20 and 25 years of age as reported in the majority of studies. (4-9)

Several anatomical studies (10, 11) and most cross-sectional densitometric studies (12-14) have demonstrated that after a transient period of stability, a slow phase of age related bone loss begins. This slow phase involves a linear, continued, steady, age-related annual loss of skeletal mass of about 0.5-1.0% in women and 0.3% in men that continues into old age (10, 11, 15). The pattern of age related bone loss varies between genders and a biphasic pattern of bone loss has been identified in women, who undergo an accelerated transient phase of bone loss superimposed on the slow continuous phase, whereas men undergo only the protracted slow continuous phase (3, 16). It is this slow pattern of bone loss that results in what is referred to by some as type 2 or senile osteoporosis. The accelerated phase in women begins at the menopause and has been attributed to the cessation of ovarian function. This accelerated phase decreases over 5-10 years to merge again with the slow phase that continues indefinitely. A decrease in BMD into the osteoporotic range during the immediate postmenopausal period as a result of the increased bone resorption of the accelerated phase is referred to by some as type 1 osteoporosis.

The slow, linear, age-related decline has been attributed to alteration of various age related factors including GH and IGF-1. Serum concentrations of GH and IGF-1 decrease with advancing age (274, 277, 278). This age related decrease is paralleled by a progressive loss of lean muscle mass and strength, a decline in physical performance, a decrease in quality of

life, an increase in body fat and a decrease in BMD (285-289). These clinical features are also seen in AGHD(290). It has been hypothesized, therefore, that the ageing process and particularly age related or type 2 osteoporosis may be due to a relative GH deficient state (12, 290-292). A relative GH deficiency has also been implicated in the development of postmenopausal or type 1 osteoporosis (290), as oestrogen is required for the secretion of GH (293). Thus GH and IGF-1 may have a role to play in the continuous slow phase bone loss with advancing age and a possible additional role in the superimposed rapid phase bone loss immediately after the menopause. The development of osteoporosis in men is less well studied than in women, especially in the absence of an andropause, equivalent to the menopause in women. In the majority of studies secondary causes for osteoporosis have been described and the causes of primary idiopathic osteoporosis in men and whether it really exists is still debated. The GH and IGF-1 axis (233, 400-403), PTH (52, 91), testosterone and oestrogen (303, 404-406) all contribute to the regulation of bone metabolism and maintenance of BMD in men.

Target organ insensitivity to the effects of PTH, altered PTH circadian rhythm and altered phospho-calcium metabolism are mechanisms that contribute to the development of osteoporosis in AGHD. These abnormalities are a consequence of the GH and IGF-1 deficiency in patients with AGHD and can be reversed by GHR therapy. Given that the age related decline in GH and IGF-1 has been implicated in postmenopausal and age related bone loss, PTH insensitivity, altered PTH rhythm and abnormal phospho-calcium metabolism may also have a contributory role in these clinical settings. Thus, we investigated the differences in PTH sensitivity, PTH rhythm and phospho-calcium metabolism in men and women in different age groups with normal as well as low BMD.

4.2. Subjects and Methods

4.2.1. Patient and Control Groups

We studied premenopausal women, postmenopausal women with normal BMD and postmenopausal women with low BMD. We also studied 3 groups of men referred to as younger men, older men with normal BMD and older men with low BMD. Number of subjects, mean age, LS (L2 – L4) and FN T-score are summarized in Table 4.1.

The younger men and premenopausal women were recruited from hospital personnel and from a database of volunteers willing to participate in medical research. Subjects with low BMD were newly diagnosed and identified via a community osteoporosis screening program. The study was approved by the Royal Liverpool University Hospital Ethics Committee and written informed consent was obtained from each patient prior to recruitment.

4.2.2. Bone Mineral Density

All subjects underwent bone densitometric evaluationas described in section 3.4. .

4.2.3. Methods

All subjects were admitted to the Metabolic Bone Unit of the Royal Liverpool University Hospital at 1300h for a period of 25 hours. An indwelling venous cannula was inserted in the antecubital fossa of each patient at the time of admission, and blood samples were collected every half hour from 1400h on the day of admission to 1400h the following day. Samples were centrifuged immediately at -4^0C, and serum/plasma was separated to be frozen at -70^0C for later analysis. Subjects were provided with one and a half litres of water and encouraged to drink at fairly frequent intervals to maintain hydration and urine samples were collected at 3-hourly intervals between 1400-2300h and 0800-1400h, and after estimating the volume of urine passed aliquots of these samples were stored at -20^0C for later analysis. A 24-hour urine volume estimation was made to ensure fluid balance was maintained and no significant variability was observed in the hydration of individuals. Subjects remained recumbent during 2300-0800h and slept during this period. Each patient was served with standardised hospital meals at 0800, 1200, 1800 and 2200h. The serving sizes and combinations of foods contained recommended daily allowances of all nutrients including Ca and PO_4.

4.2.4. Biochemistry

Analytes were measured using methods as described in section 3.2. Serum PTH, Ca, PO_4 and albumin, concentration were measured on all 49 blood samples obtained from each individual. Serum IGF-1, 25(OH)D, $1,25(OH)_2D$, βCTX and PINP concentration were measured on single time point 0900 samples. Urine calcium, phosphate and creatinine were measured on all urine samples. PcAMP was measured on blood samples corresponding to the times of urine samples. NcAMP was calculated as described in section 3.2.5. The

$TmPO_4/GFR$ was calculated from the normogram derived by Walton and Bijvoet as described in section 3.2.2.

4.2.5. Statistical Analysis

Student's t-test for unpaired data and GLM ANOVA for repeated measures were used, as described in section 3.3, wherever appropriate. PTH circadian rhythms were assessed as described in section 3.3.3. Values are expressed as means ± standard error of mean (SEM). $P < 0.05$ was considered significant.

Table 4.1. Demographic characteristics [Mean(SEM)]

Group	n	Age	T-scores	
			Femoral Neck	Lumbar Spine
Premenopausal women	14	35.4(1.7)	0.58(0.24)	0.31(0.28)
Postmenopausal women with normal BMD	10	66.4(1.9) ***	0.45(0.29)	1.01(0.34)
Postmenopausal women with low BMD	12	67.5(1.8) ***	-1.91(0.26) ***	-3.34(0.31) ***
Younger men	9	27.1(1.4)	-0.24(0.30)	0.14(0.43)
Older men with normal BMD	12	68.4(1.7)+++	-0.46(0.30)	0.77(0.43)
Older men with low BMD	9	70.8(1.4) +++	-2.03(0.26) +++	-1.64(0.37) +++

*** $p<0.001$ compared to premenopausal women
+++ $p<0.001$ compared to younger men

4.3. Results

4.3.1. Age and BMD Dependent Differences in Women

4.3.1.1. IGF-1

IGF-1 concentration (Figure 4.1a) was significantly lower in the postmenopausal women with normal BMD (83.1±11.9μg/L, $p<0.001$) and low BMD (95.1±10.7μg/L, $p<0.001$) as compared to the younger women (137.9±9.4μg/L), with no significant difference between the 2 groups of postmenopausal women ($p=0.5$).

4.3.1.2. PTH and NcAMP

24-h mean PTH concentration (Figure 4.1b) was higher in the postmenopausal women with normal BMD (4.7±0.1pmol/L, $p<0.001$) and highest in the women with low BMD (5.4±0.1pmol/L, $p<0.001$) when compared with the premenopausal women (4.4±0.1pmol/L). The percentage increase in PTH concentration from premenopausal women to postmenopausal women with normal BMD was 6.8% and from premenopausal women to postmenopausal women with low BMD was 14.8%. The higher PTH in the postmenopausal women with normal BMD was associated with a trend toward a lower 24-h mean NcAMP concentration as compared to the premenopausal women (22.8±1.6 nmol/L GFR versus 18.4±3.1 nmol/L GFR, $p=0.08$, Figure 4.1c). The PTH concentration which was highest in the postmenopausal women with low BMD was associated with an even lower concentration of NcAMP (16.3±1.3 nmol/L GFR, $p<0.05$) which was significantly lower compared to the

premenopausal women but not significantly different compared to the postmenopausal women with normal BMD.

4.3.1.3. PTH Circadian Rhythm

Individual and population cosinor analyses for circulating PTH (Figure 4.2) demonstrated significant circadian rhythms for all subjects in all 3 groups of women ($p<0.001$) but with subtle differences in the early evening and nocturnal peaks. The mean PTH MESOR was significantly higher in the postmenopausal women with low BMD than in the premenopausal women (5.4±0.4pmol/L versus 4.4±0.3pmol/L, $p<0.05$) with an intermediate MESOR value in the postmenopausal women with normal BMD which was not significantly different from the premenopausal women or the postmenopausal women with low BMD (Table 4.2). There was no significant difference in the amplitude or acrophase in all 3 groups (Table 4.2).

Circulating PTH demonstrated distinct early evening (time of onset 1400h) and nocturnal peaks (time of onset 2330h) in the premenopausal women. A more sustained increase in PTH concentration between 1400h and 2300h and a less pronounced nocturnal increase between 2330h-0800h was observed in the postmenopausal women with normal as well as low BMD. The mean percentage change in PTH concentration between 1400h and 2330h [(value at each time point – 1400h value)/ 1400h value x 100] was significantly higher in the postmenopausal women with both normal BMD and low BMD when compared with premenopausal women ($p<0.05$) with no significant difference between the 2 groups of postmenopausal women confirming a more prolonged early evening rise. The nocturnal/early morning rise in PTH concentration (or maximum percentage increase in PTH concentration between 2330 and 0800h [(maximum value between 2330-0800h – 2330h value)/2330h value x 100]) was

significantly lower in both groups of postmenopausal women as compared to the premenopausal women ($p<0.01$) as was the mean percentage change in PTH concentration between 2330 and 0800h [(value at each time point – 2330h value)/ 2330h value x 100] ($p<0.01$) representing a less marked nocturnal peak. No significant difference was seen between the 2 groups of postmenopausal women with respect to the nocturnal peak.

4.3.1.4. Serum Adjusted Calcium

No significant difference in the 24-h mean ACa was observed in the three groups (premenopausal women, 2.34±0.003mmol/L; postmenopausal women with normal BMD, 2.35±0.003mmol/L; postmenopausal women with low BMD, 2.35±0.003mmol/L; p=0.9; Figure 4.1d)

4.3.1.5. Serum Phosphate

24 hour mean serum PO_4 concentration (Figure 4.1e) was significantly lower in the postmenopausal women with low BMD (1.09±0.01mmol/L, $p<0.01$) compared to the postmenopausal women with normal BMD (1.19±0.01mmol/L) and the premenopausal women (1.15±0.01mmol/L).

4.3.1.6. Vitamin D

25(OH)D (premenopausal women, 46±8nmol/L; postmenopausal women with normal BMD, 45±8nmol/L; postmenopausal women with low BMD, 51±7nmol/L; p=0.5; Figure 4.1f) and

$1,25(OH)_2D$ concentrations (premenopausal women, 75±10pmol/L; postmenopausal women with normal BMD, 89±12pmol/L; postmenopausal women with low BMD, 78±12pmol/L; p=0.4; Figure 4.1g) were not significantly different in all 3 groups of women.

4.3.1.7. Urine Calcium Excretion

UCa/Cr (0.6±0.03) and UCaE (Figure 4.1h) (0.04±0.003 mmol/L CCr) in the postmenopausal women with normal BMD and UCa/Cr (0.5±0.03) and UCaE (0.04±0.002 mmol/L CCr) in the postmenopausal women with low BMD were not significantly different (p=0.4) but were both significantly higher than in the premenopausal women (UCa/Cr 0.4±0.03; UCaE 0.03±0.002 mmol/L CCr; $p < 0.01$).

4.3.1.8. Urine Phosphate Excretion

UPO_4/Cr (3.0±0.14; $p < 0.01$) and UPO_4E (Figure 4.1j) (0.24±0.010 mmol/L CCr; $p < 0.01$) were higher in the postmenopausal women with normal BMD when compared with premenopausal women (UPO_4/Cr 2.5±0.12; UPO_4E 0.19±0.009 mmol/L CCr) and postmenopausal women with low BMD (UPO_4/Cr 2.2±0.13; UPO_4E 0.17±0.010 mmol/L CCr) with no significant difference between the latter 2 groups. No significant difference in the mean $TmPO_4/GFR$ (Figure 4.1i) was observed within the 3 groups and was 0.98±0.02 mmol/L GFR in the premenopausal women, 0.96±0.02 mmol/L GFR in the postmenopausal women with normal BMD and 0.95±0.02 mmol/L GFR in the postmenopausal women with low BMD but there was a trend towards a lower $TmPO_4/GFR$ in the women with low BMD (p=0.06).

4.3.1.9. Markers of Bone Turnover

There was a trend towards a higher βCTX (Figure 4.1k) in the postmenopausal women with normal BMD (0.33±0.08 ug/L, p=0.08) when compared with the premenopausal women (0.20±0.06 ug/L) with a further significantly higher βCTX concentration in the postmenopausal with low BMD (βCTX 0.71±0.07 ug/L) when compared to the postmenopausal women with normal BMD ($p<0.05$). A trend towards a higher PINP (Figure 4.1l) concentration was also observed when the postmenopausal women with normal BMD (48.8±6.0μg/L, p=0.07) were compared to the premenopausal women (35.2±4.8μg/L) and the PINP concentration was significantly higher in the postmenopausal women with low BMD (57.3±5.2μg/L, $p<0.05$) when compared to the premenopausal women. There was however no significant increase when compared to postmenopausal women with normal BMD (p= 0.3). The ratio of circulating βCTX to PINP was not significantly different in the postmenopausal women with normal BMD when compared to the premenopausal women but was significantly higher in the women with low BMD when compared to both the other groups ($p<0.05$).

Figure 4.1. Figure showing differences in serum and urine biochemistry in young healthy premenopausal women, older postmenopausal women with normal BMD and older postmenopausal women with low BMD

(a) IGF-1: lower concentrations in postmenopausal women, (b) PTH: higher concentrations in postmenopausal women, (c) NcAMP: lower concentrations in postmenopausal women, (d) ACa: no difference, (e) PO_4: higher concnenttations in postmenopausal women with normal BMD and lower concentrations in women with low BMD, (f) 25(OH)D: no difference, (g) 1,25(OH)2D: no difference, (h) UCaE: increased in postmenopausal women, (i) TmPO4/GFR: trend toward a lower level in postmenopausal women with low BMD, (j) UPO_4E: higher in postmenopausal women with normal BMD and lower in women with low BMD, (k) βCTX: trend toward higher levels in postmenopausal women with normal BMD and significantly higher in women with low BMD, (l) PINP: trend toward higher levels in postmenopausal women with normal BMD and significantly higher in women with low BMD.

NS^{*} not significant compared to premenopausal women

* $p<0.05$ compared to premenopausal women

** $p<0.01$ compared to premenopausal women

*** $p<0.001$ compared to premenopausal women

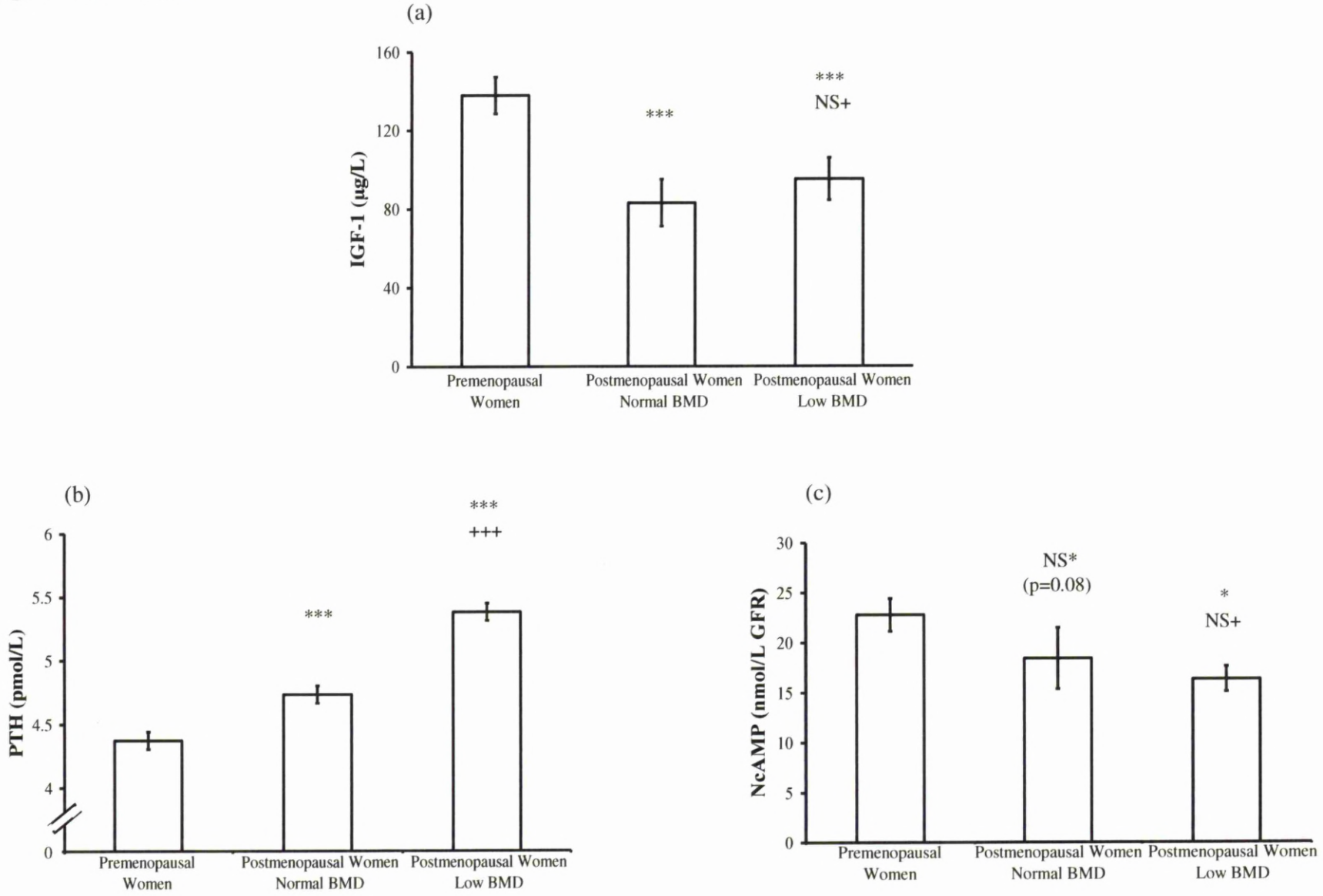
NS^{+} not significant compared to postmenopausal women with normal BMD

$^{+}$ $p<0.05$ compared to postmenopausal women with normal BMD

$^{++}$ $p<0.01$ compared to postmenopausal women with normal BMD

$^{+++}$ $p<0.001$ compared to postmenopausal women with normal BMD

Figure 4.1. Contd



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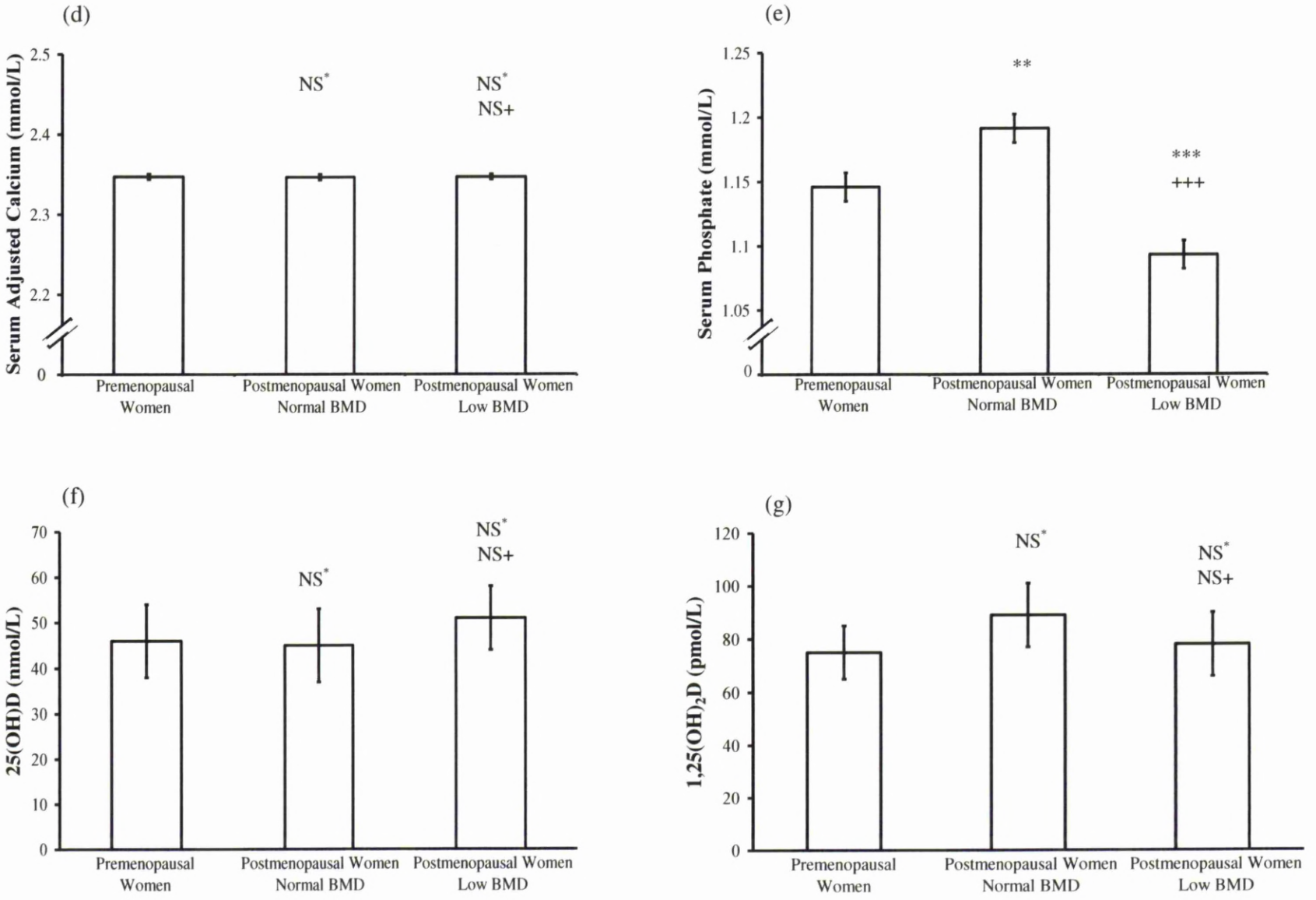
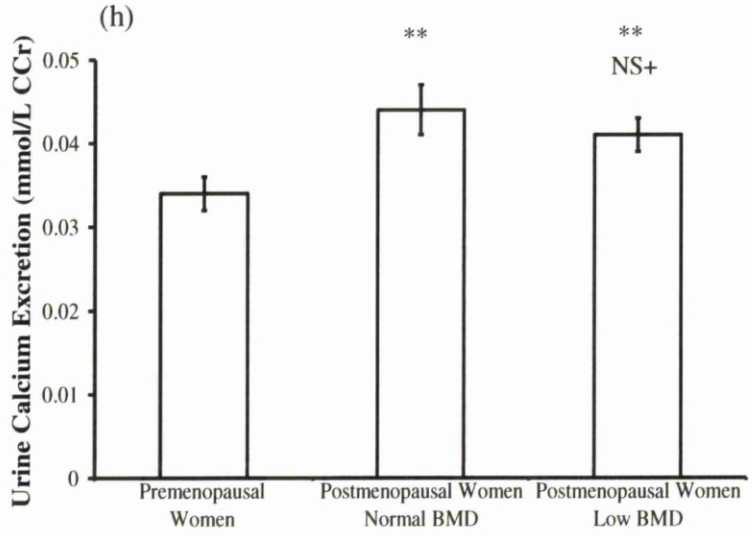
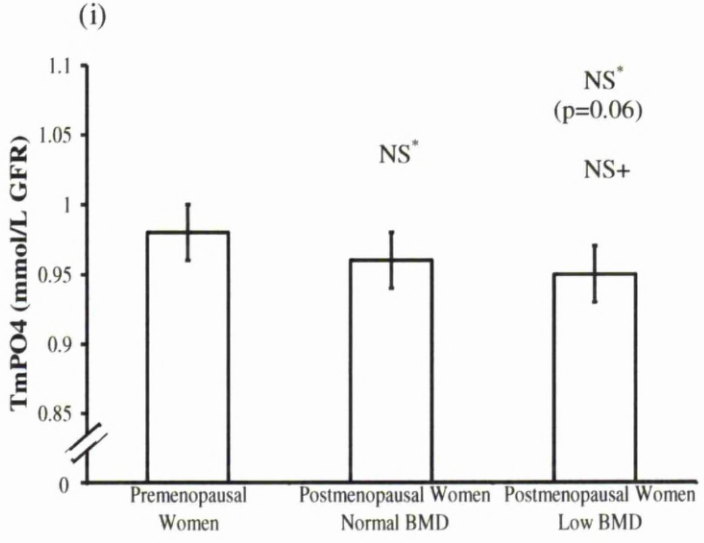


Figure 4.1. Contd.





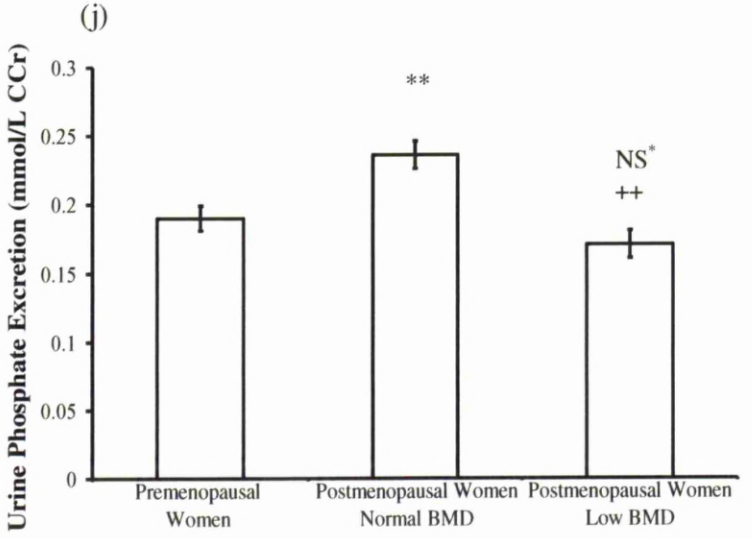
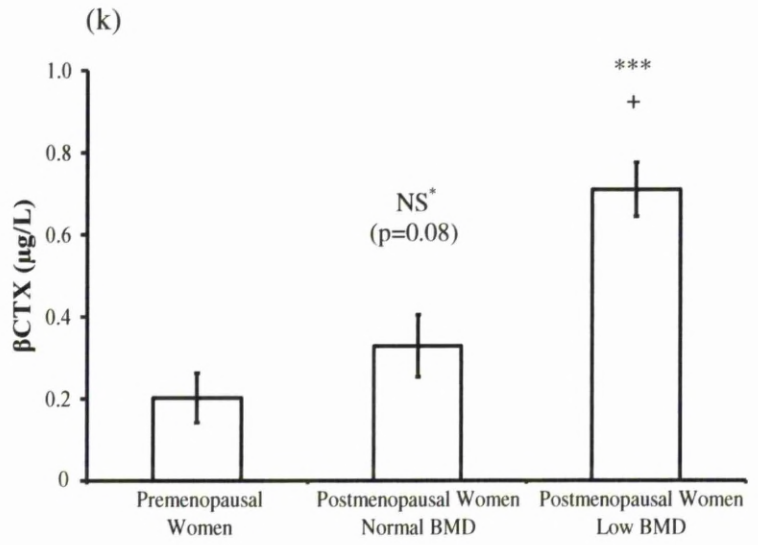


Figure 4.1. Contd.



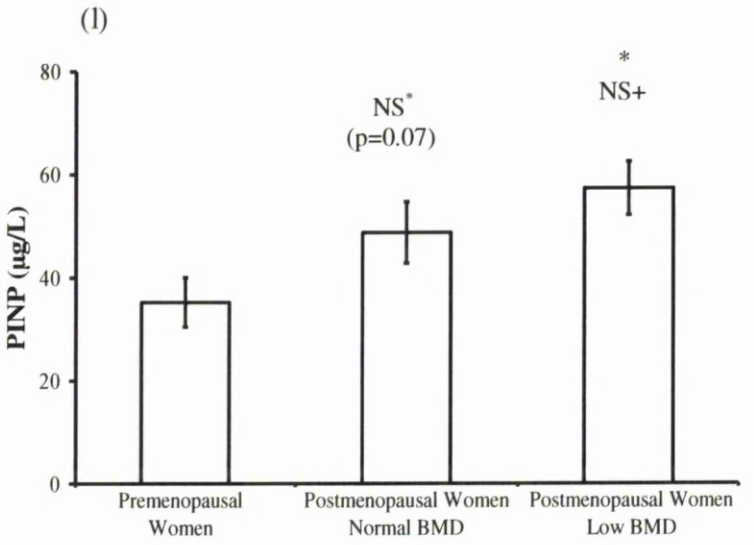
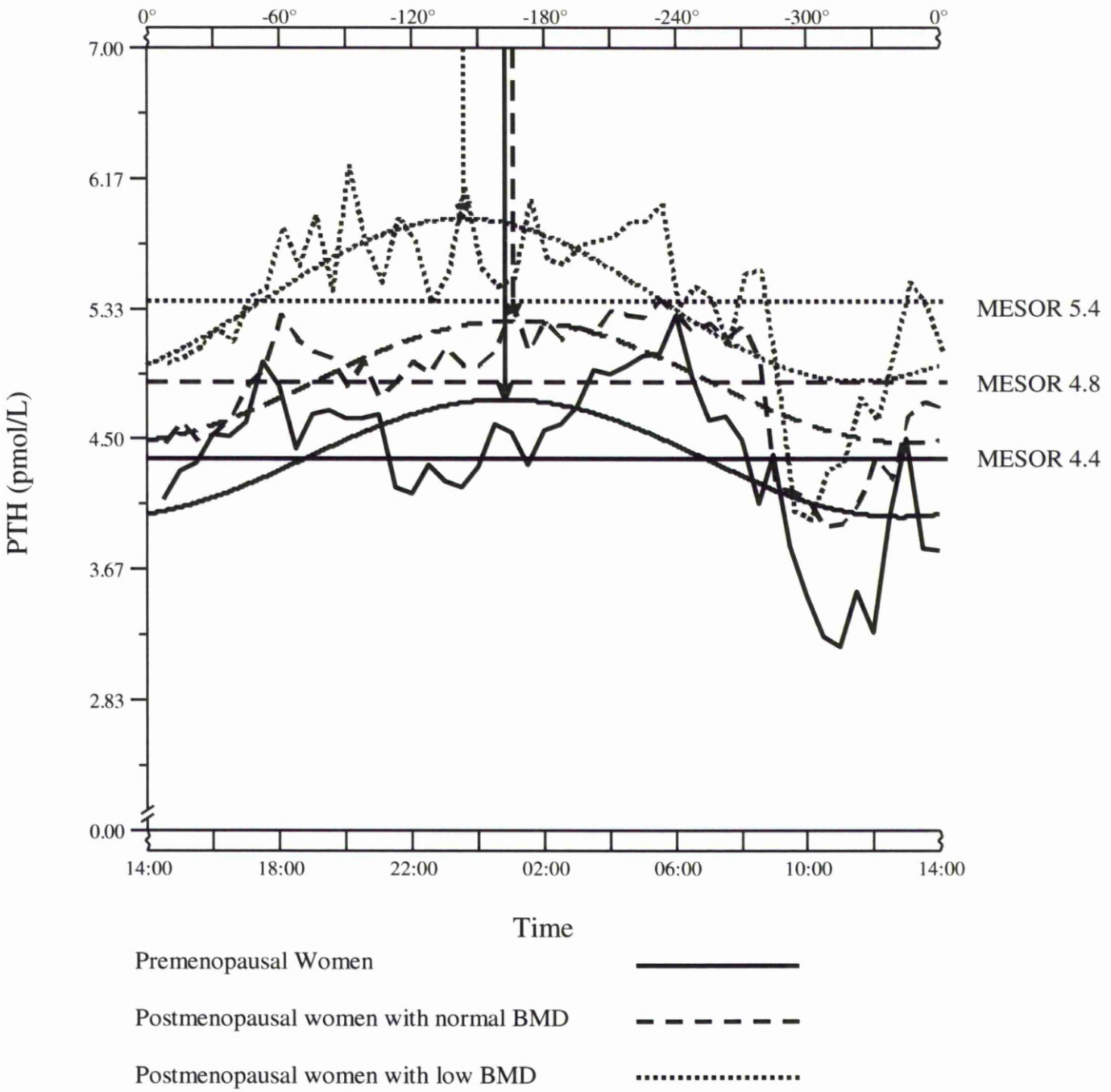


Figure 4.2. Cosinor-derived PTH circadian rhythms in young healthy premenopausal women, older postmenopausal women with normal BMD and older postmenopausal women with low BMD.

Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs for each group and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated for all 3 groups with differences in MESOR, acrophase and amplitude. The MESOR was highest in postmenopausal women with low BMD with alterations to the rhythm that may be contributory to bone loss.



4.3.2. Age and BMD Dependent Differences in Men

4.3.2.1. IGF-1

IGF-1 concentration (Figure 4.3a) was significantly lower in the older men with normal BMD (111.8±11.1μg/L, $p<0.001$) and even lower in the older men with low BMD (76.8±12.8μg/L, $p<0.001$) as compared to the younger men (160.7±12.8μg/L), with the difference between the 2 groups of older men approaching significance ($p=0.055$).

4.3.2.2. PTH and NcAMP

24-h mean PTH concentration (Figure 4.3b) was higher in the older men with normal BMD (5.1±0.07pmol/L, $p<0.001$) and highest in the men with low BMD (5.4±0.07pmol/L, $p<0.001$) when compared with the younger men (4.0±0.07pmol/L). The percentage increase in PTH concentration from younger men to older men with normal BMD was 27.5% and from younger men to older men with low BMD was 35.2%. The highest PTH concentration in the older men with low BMD was associated with a lower 24-h mean NcAMP concentration compared to younger men (25.7±3.4nmol/L GFR versus 18.4±2.1nmol/L GFR, $p<0.05$, Figure 4.3c). The older men with normal BMD with higher PTH concentration demonstrated an intermediate concentration of NcAMP (22.4±2.6nmol/L GFR, $p=0.4$) but with no statistically significant difference observed when compared with either the younger men or older men with low BMD.

4.3.2.3. PTH Circadian Rhythm

Individual and population cosinor analyses for circulating PTH (Figure 4.4) demonstrated significant circadian rhythms for all subjects in all 3 groups of men ($p<0.001$) but with subtle differences in the early evening and nocturnal peaks. The mean PTH MESOR was significantly higher in the older men with low BMD than in the younger men (5.5±0.4 pmol/L versus 4.0±0.5 pmol/L, $p<0.05$) with an intermediate MESOR value in the older men with normal BMD (5.1±0.5 pmol/L) which was not statistically different from the younger men or the older men with low BMD. There was no significant difference in the amplitude or acrophase in all 3 groups (Table 4.2).

Circulating PTH demonstrated distinct early evening (time of onset 1400h) and nocturnal peaks (time of onset 2330h) in the younger men and older men with normal BMD similar to that observed in the premenopausal women. A more sustained increase in PTH concentration between 1400h and 2300h and a less pronounced nocturnal increase between 2330h-0800h was observed in the older men with low BMD similar to that seen in the postmenopausal women. The mean percentage change in PTH concentration between 1400h and 2330h [(value at each time point – 1400h value)/ 1400h value x 100] was significantly higher in the older men with low BMD when compared with the younger men and older men with normal BMD ($p<0.05$) with no significant difference between the latter 2 groups. The nocturnal/early morning rise in PTH concentration (or maximum percentage increase in PTH concentration between 2330 and 0800h [(maximum value between 2330-0800h – 2330h value)/2330h value x 100]) was significantly lower in older men with low BMD compared to the younger men and older men with normal BMD ($p<0.01$). Similar differences in the mean percentage change in PTH concentration between 2330 and 0800h [(value at each time point – 2330h

value)/ 2330h value x 100] were observed when the older men with low BMD were compared with the other 2 groups ($p<0.01$). These changes represented a less marked nocturnal peak. No significant difference was seen between the younger men and older men with normal BMD with respect to the nocturnal peak.

4.3.2.4. Serum Adjusted Calcium

24-h mean ACa was not significantly different in the 3 groups of men (younger men, 2.35±0.003mmol/L; older men with normal BMD, 2.35±0.003mmol/L; older men with low BMD, 2.35±0.003mmol/L; Figure 4.3d).

4.3.2.5. Serum Phosphate

24 hour mean serum PO_4 concentration (Figure 4.3e) was lower in the older men with normal BMD (1.08±0.02mmol/L, $p<0.01$) and in the older men with low BMD (1.07±0.02mmol/L, $p<0.01$) when compared with the younger men (1.19±0.02mmol/L) with no difference observed between the 2 groups of older men.

4.3.2.6. Vitamin D

25(OH)D (younger men 36±4 nmol/L; older men with normal BMD 36±4 nmol/L; older men with low BMD 42±5 nmol/L; $p=0.5$; Figure 4.3f) and $1,25(OH)_2D$ (younger men 90±11pmol/L; older men with normal BMD 83±11pmol/L; older men with low BMD 98±10pmol/L; $p=0.4$; Figure 4.3g) concentrations were not significantly different among the 3 groups.

4.3.2.7. Urine Calcium Excretion

No significant difference was observed in the UCa/Cr (younger men 0.5±0.07, older men with normal BMD 0.5±0.07, older men with low BMD 0.5±0.06) or UCaE (Figure 4.3h) (younger men 0.04±0.006 mmol/L CCr, older men with normal BMD 0.04±0.006 mmol/L CCr, older men with low BMD 0.05±0.005 mmol/L CCr) in the 3 groups.

4.3.2.8. Urine Phosphate Excretion and $TmPO_4/GFR$

No significant difference in UPO_4/Cr (younger men 2.4±0.15, older men with normal BMD 2.1±0.15, older men with low BMD 2.4±0.13) and UPO_4E (Figure 4.3j) was observed (younger men 0.21±0.02 mmol/L CCr, older men with normal BMD 0.20±0.02 mmol/L CCr, older men with low BMD 0.24±0.01 mmol/L CCr) was observed. $TmPO_4/GFR$ (Figure 4.3i) was significantly different in all 3 groups and was lower in the older men with normal BMD (0.91±0.03 mmol/L GFR) and lowest in the older men with low BMD (0.82±0.02 mmol/L GFR, $p<0.01$) compared to the younger men (0.98±0.03 mmol/L GFR).

4.3.2.9. Markers of Bone Turnover

Both βCTX (Figure 4.3k) as well as PINP (Figure 4.3l) concentrations were lower in the older men with normal BMD when compared with the younger men (βCTX, 0.34±0.07 μg/L versus 0.55±0.07 μg/L, $p<0.05$; PINP, 44.3±7.6μg/L versus 75.1±7.6μg/L, $p<0.05$) but the ratio of βCTX to PINP however was not significantly different in the 2 groups. The PINP

concentration was also lower in the older men with low BMD (53.5±6.6μg/L, $p<0.01$) when compared to younger men but levels were similar to those in the older men with normal BMD. However the βCTX concentration in the older men with low BMD (0.63±0.06 μg/L) was higher than that observed in the older men with normal BMD ($p<0.05$) but similar to that in the younger men ($p=0.6$). The resulting ratio of βCTX to PINP was significantly higher in the older men with low BMD as compared to younger men and older men with normal BMD ($p<0.05$).

Figure 4.3. Figure showing differences in serum and urine biochemistry in young healthy premenopausal women, older postmenopausal women with normal BMD and older postmenopausal women with low BMD

(a) IGF-1: lower concentrations in older men, (b) PTH: higher concentrations in older men, (c) NcAMP: lower concentrations in older men, (d) ACa: no difference, (e) PO_4: lower concnentrations in older men, (f) 25(OH)D: no difference, (g) 1,25(OH)2D: no difference, (h) UCaE: no difference, (i) TmPO4/GFR: lower level in older men, (j) UPO_4E: no difference, (k) βCTX: lower levels in older men with normal BMD and higher in older men with low BMD, (l) PINP: lower levels in older men both with normal and low BMD, (m) total testosterone: no difference.

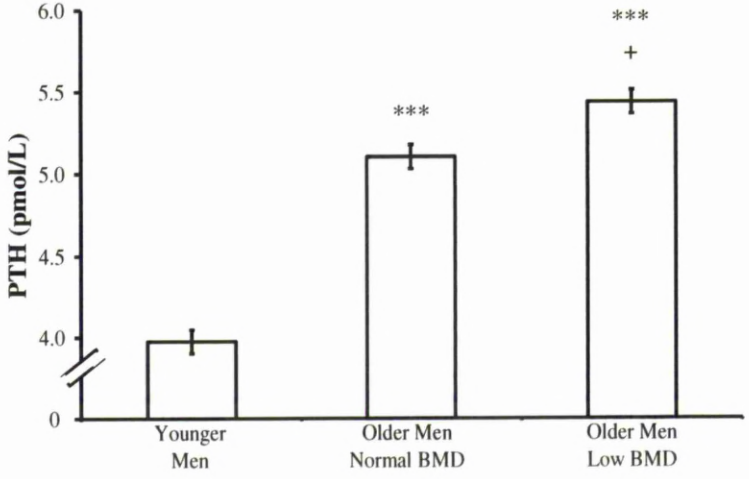
NS^*	not significant compared to younger men
*	p<0.05 compared to younger men
**	p<0.01 compared to younger men
***	p<0.001 compared to younger men

NS^+	not significant compared to older men with normal BMD
+	p<0.05 compared to older men with normal BMD
++	p<0.01 compared to older men with normal BMD
+++	p<0.001 compared to older men with normal BMD

2(a)


IGF-1 (µg/L)
200
160
120
80
40
0
***
***
NS+
(p=0.055)
Younger Men
Older Men Normal BMD
Older Men Low BMD

2(b)


PTH (pmol/L)
6.0
5.5
5.0
4.5
4.0
0
***
***
+
Younger Men
Older Men Normal BMD
Older Men Low BMD

2(c)

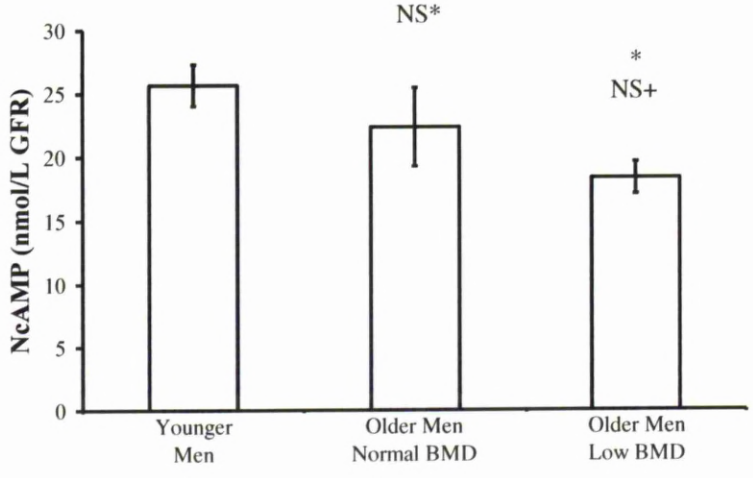
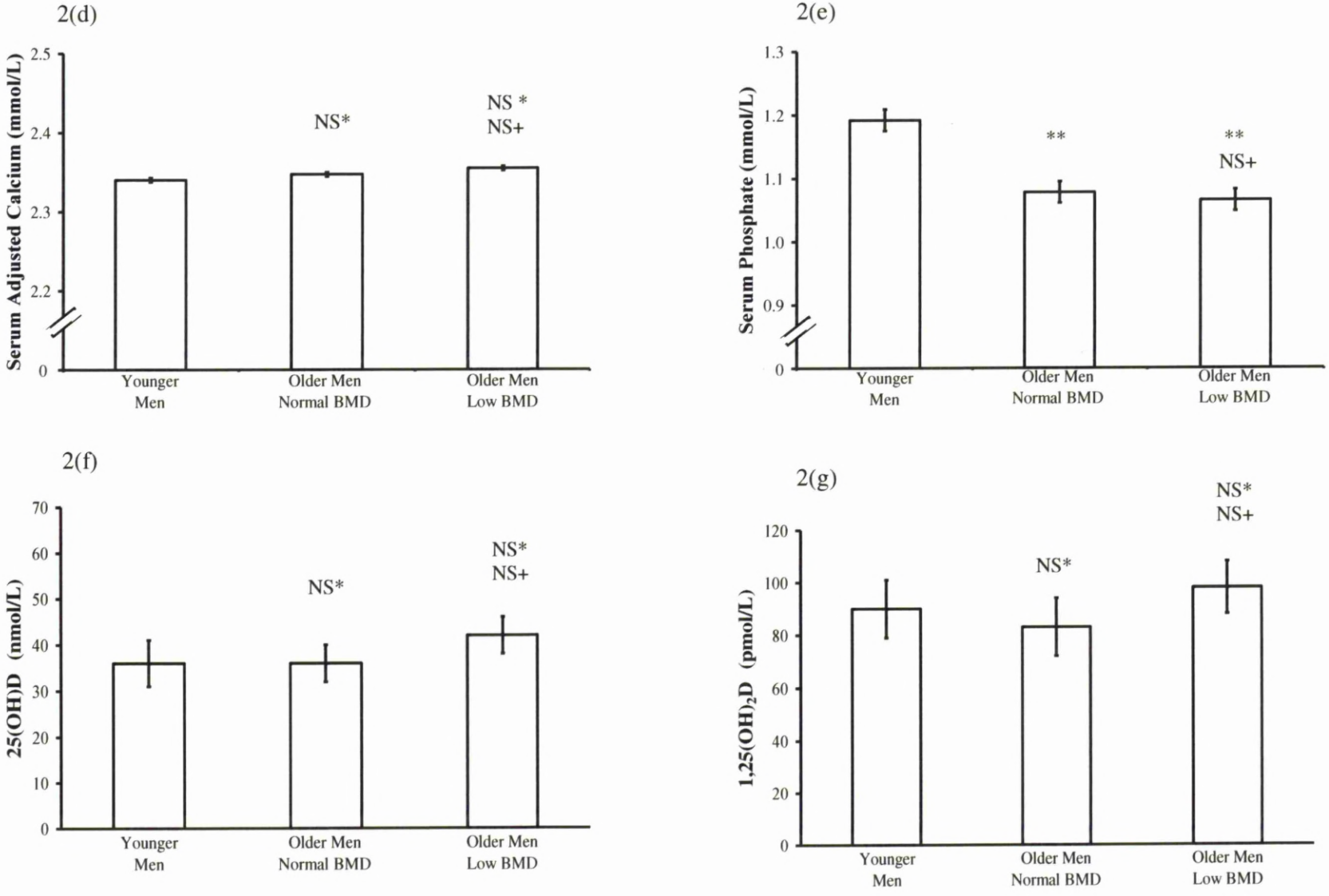

NcAMP (nmol/L GFR)
30
25
20
15
10
5
0
NS*
*
NS+
Younger Men
Older Men Normal BMD
Older Men Low BMD

Figure 4.3. Contd.



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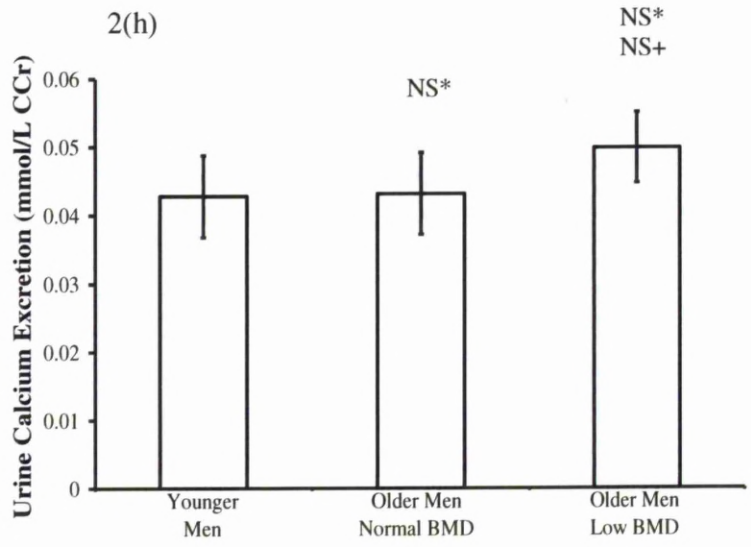
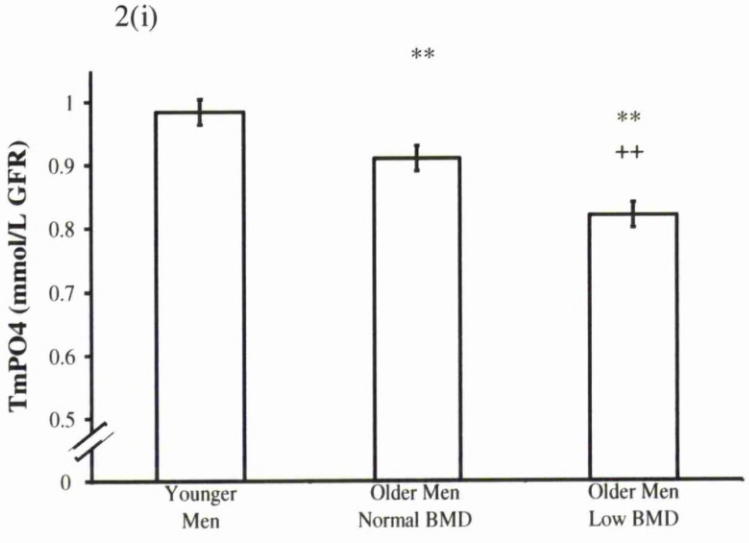




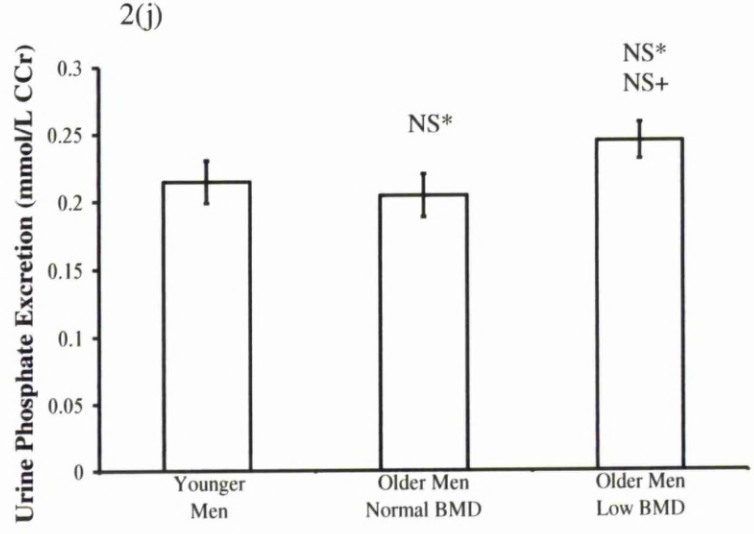
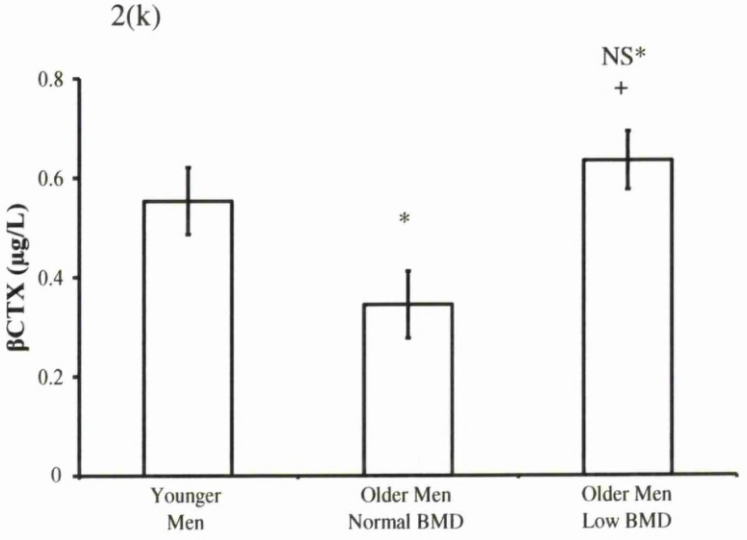
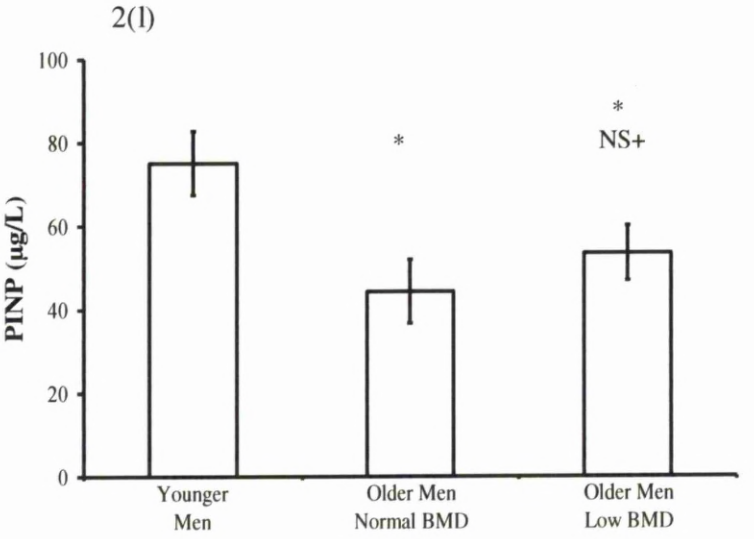


Figure 4.3. Contd.





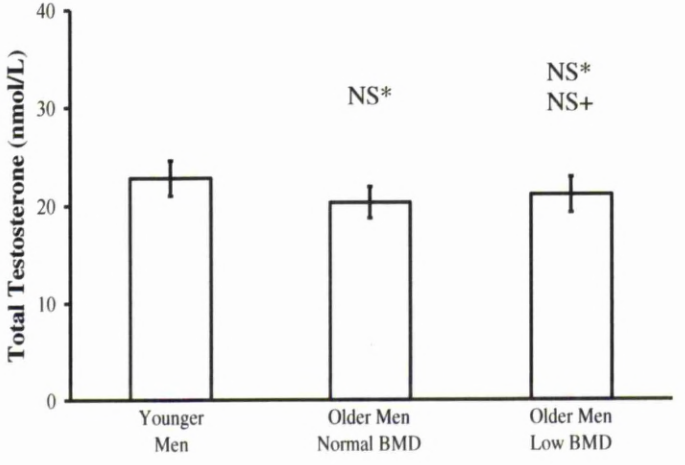


Figure 4.4. Cosinor-derived PTH circadian rhythms in young healthy men, older men with normal BMD and older men with low BMD.

Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs for each group and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated for all 3 groups with differences in MESOR, acrophase and amplitude. The MESOR was highest in men with low BMD with alterations to the rhythm that may be contributory to bone loss.

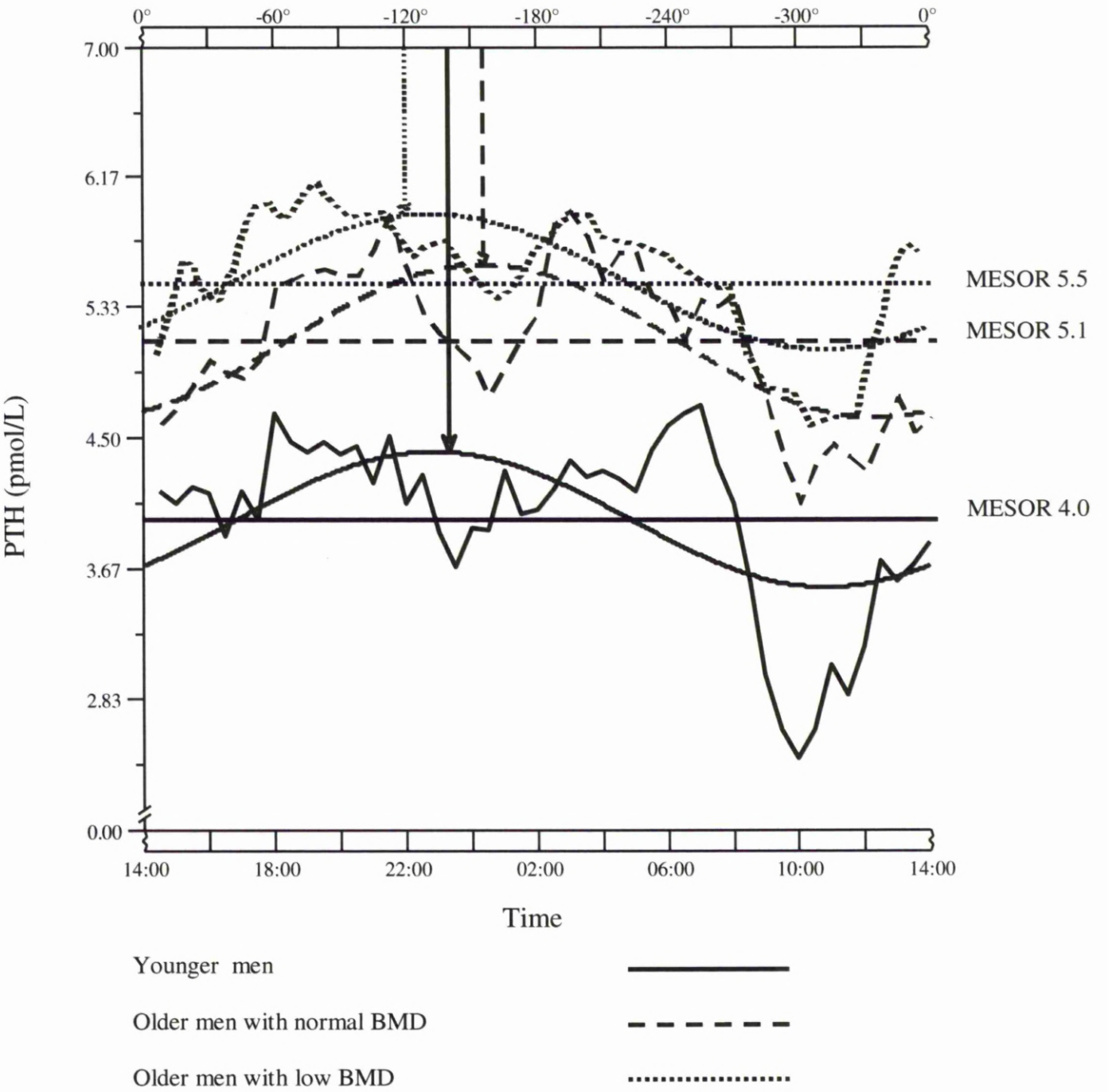


Table 4.2. PTH Circadian Rhythm Parameters [Mean(SEM)]

	MESOR (pmol/L)	Ampitude (pmol/L)	Time of Peak (h)
Premenopausal Women	4.4(0.34)	0.5(0.10)	0106 (2404-0208)
Postmenopausal women with normal BMD	4.8(0.40)	0.5(0.11)	0110 (2357-0223)
Postmenopausal women with low BMD	5.4(0.37)*	0.6(0.10)	2344 (2239-2449)
Younger men	4.0(0.47)	0.6(0.12)	2307 (2213-2401)
Older men with normal BMD	5.1(0.47)	0.6(0.12)	2418 (2324-0112)
Older men with low BMD	5.5(0.41)+	0.7(0.10)	2203 (2117-2249)

* $p<0.05$ compared to premenopausal women
\+ $p<0.05$ compared to younger men

4.3.3. Gender Dependent Differences

4.3.3.1. Younger Men versus Premenopausal Women

24-h mean PTH concentration was lower in the younger men when compared with the premenopausal women ($p<0.001$; Figure 4.4b) with no significant difference in NcAMP concentration between the 2 groups ($p=0.3$; Figure 4.4c). Both βCTX and PINP were significantly higher in younger men compared to premenopausal women ($p<0.01$; Figure 4.4k and Figure 4.4l) but with no difference in the ratio of βCTX to PINP. No significant difference was observed in any of the other parameters except a trend towards a slightly higher serum PO_4 concentration in men ($p=0.08$; Figure 4.4e).

4.3.3.2. Older Men with Normal BMD versus Postmenoapusal Women with Normal BMD

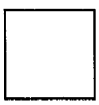
As opposed to the pattern seen in the comparison between younger men and premenopausal women, 24-h mean PTH concentration was found to be higher in the postmenopausal women with normal BMD as compared to the older men with normal BMD ($p<0.05$; Figure 4.4b) with no significant difference in NcAMP concentration between the 2 groups ($p=0.6$; Figure 4.4c). Serum PO_4 was also significantly higher in the postmenopausal women with normal BMD compared to the older men with normal BMD ($p<0.001$; Figure 4.4e) with no difference in other parameters except a trend towards higher $TmPO_4/GFR$ ($p=0.07$) in the women compared to the men. No significant difference was observed in bone turnover markers between these 2 groups.

4.3.3.3. Older Men with Low BMD versus Postmenoapusal Women with Low BMD

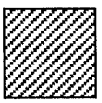
No difference in PTH, NcAMP, ACa, serum PO_4, vitamin D metabolites and UCaE were observed between these 2 groups. There was a trend toward a higher UPO_4E in the older men low BMD compared to the postmenopausal women with low BMD ($p=0.07$) with a significantly lower $TmPO_4/GFR$ in the men compared to the women ($p<0.01$). No significant difference in bone markers was seen between these 2 groups.

Figure 4.5. Gender Dependent Differences in Serum and Urine Biochemistry in younger subjects (premenopausal women versus younger men), older subjects with normal BMD (postmenopausal women with normal BMD versus older men with normal BMD)

Differences were demonstrated in (a) IGF-1, (b) PTH, (c) NcAMP, (d) ACa, (e) PO_4, (f) 25(OH)D, (g) 1,25(OH)2D, (h) UCaE, (i) TmPO4/GFR, (j) UPO_4E, (k) βCTX, (l) PINP between men and women in the 3 different groups.

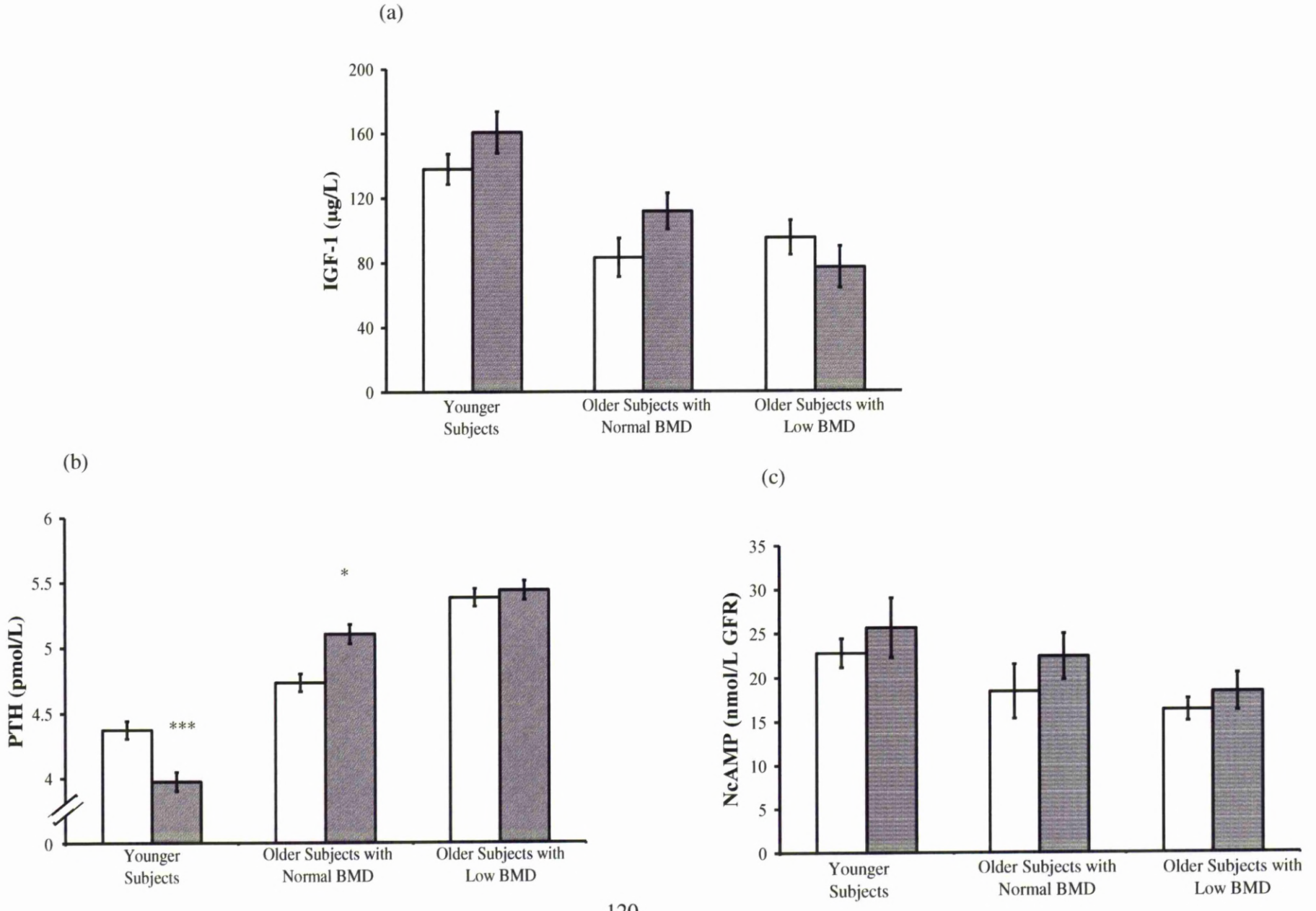


Women



Men

Figure 4.5. Contd.



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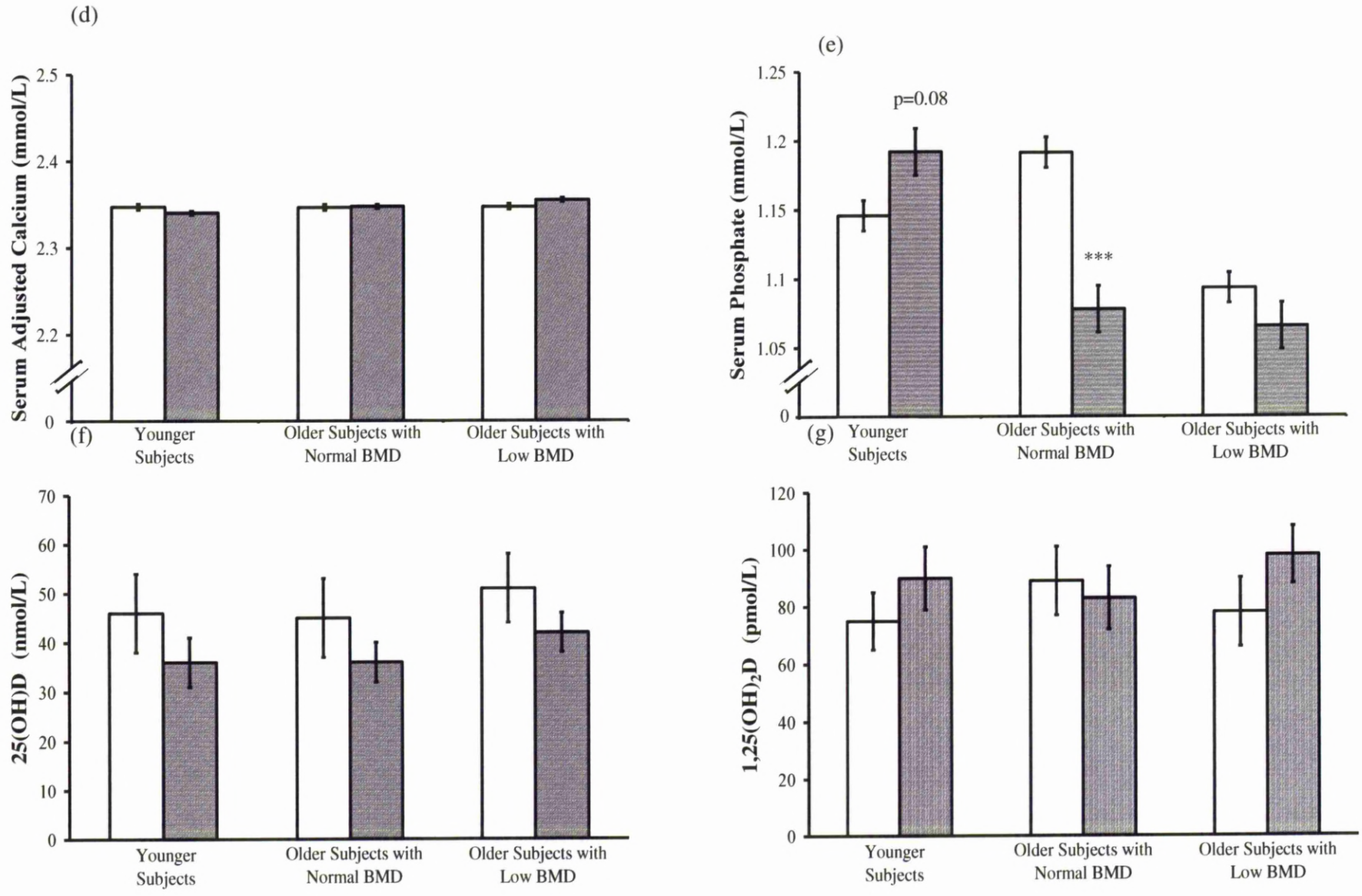
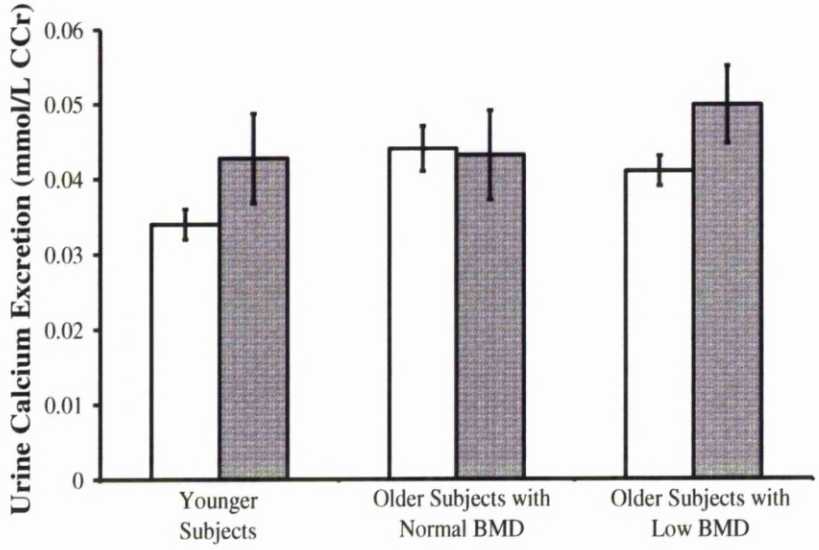
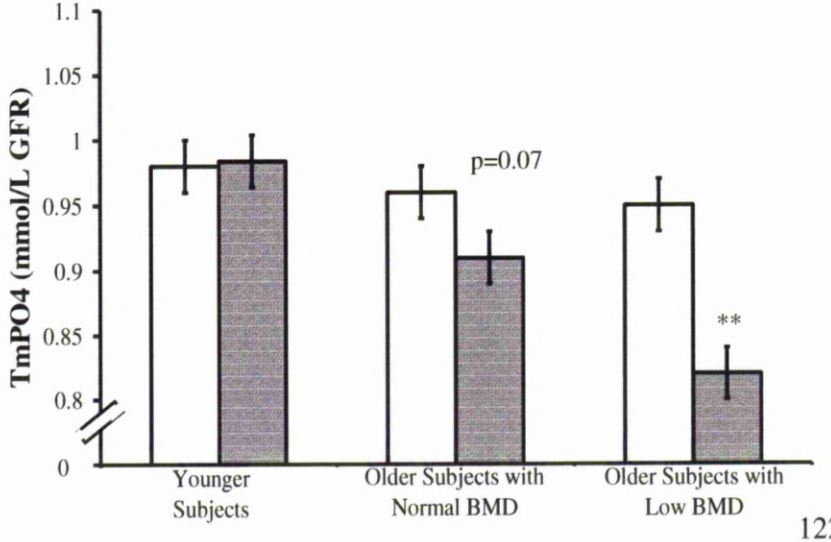


Figure 4.5. Contd.

(h)



(i)



(j)

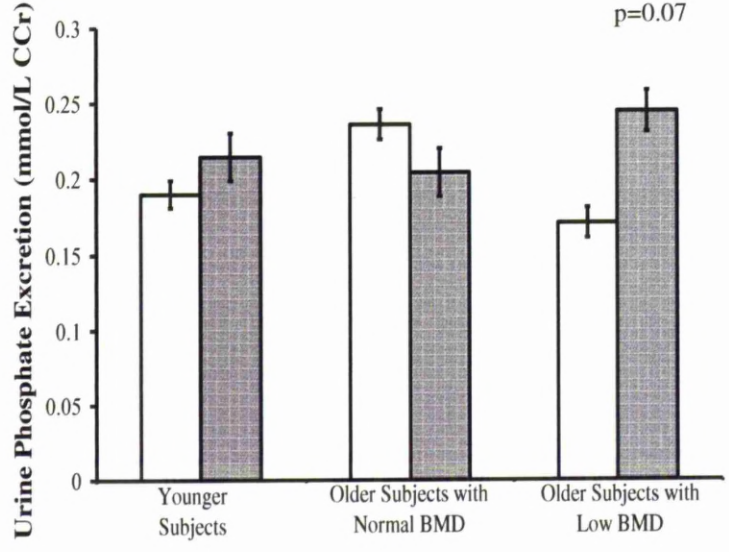
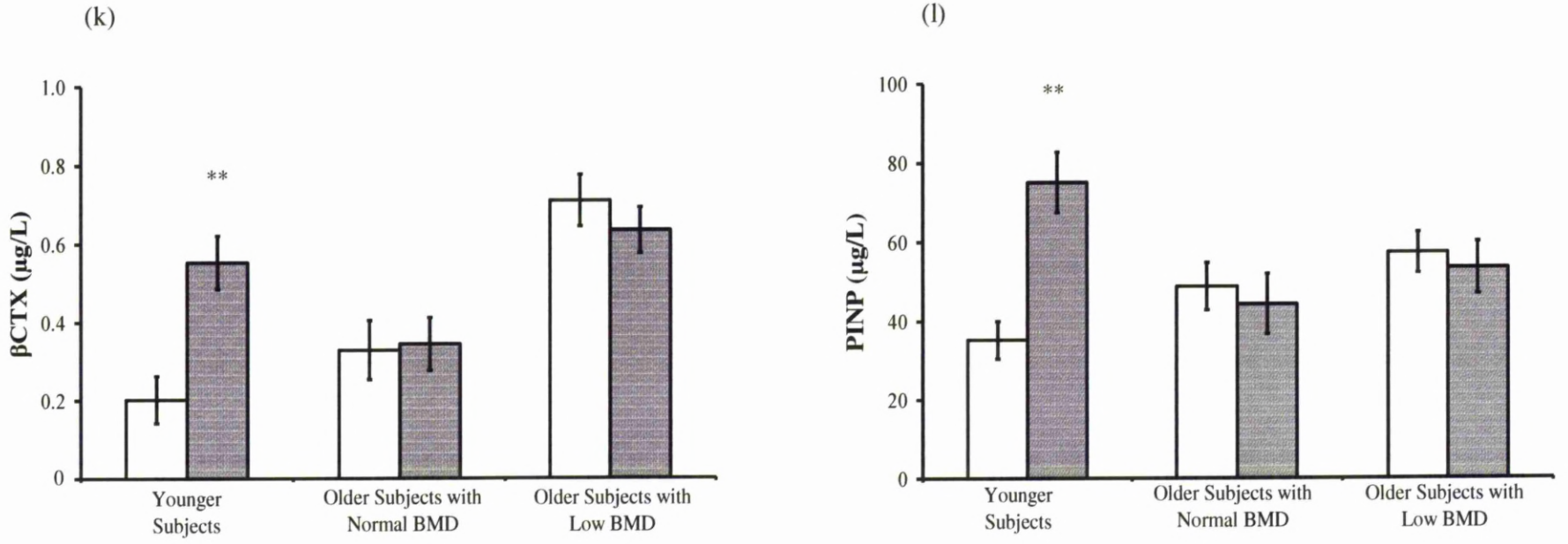


Figure 4.5. Contd.



4.4. Discussion

The results of this study demonstrate that abnormalities in PTH sensitivity and circadian rhythm occur in postmenopausal women with low BMD as well as in postmenopausal women with normal BMD. These abnormalities are, however, more pronounced in postmenopausal women with low BMD than in postmenopausal women of a similar age with normal BMD. These abnormalities result in increased bone turnover and net bone loss. Abnormal PTH sensitivity and rhythm are therefore, part of the altered age related biochemical milieu in postmenopausal women and may contribute to the development of osteoporosis in postmenopausal women. We also observed decreased PTH sensitivity and altered PTH rhythm in older men with normal BMD as well as in older men with low BMD when compared with younger healthy control men. The degree of these abnormalities was greater in the older men with low BMD when compared to the older men with normal BMD suggesting that these abnormalities may contribute to the development of age related osteoporosis in men as well.

Various factors have been proposed as being determinants of an individual's susceptibility to developing osteoporosis. Our findings suggest that differences in GH, IGF-1, PTH sensitivity and PTH circadian rhythm contribute to the differences in bone turnover in individuals. The bone turnover in individuals is also dependent on the the direct effect of gonadal hormones on bone as well as the changes that may occur in gonadal hormones with age. Gonadal hormones and age related changes in gonadal hormones may also affect bone indirectly by altering the effects of GH, IGF-1 and PTH on bone. As a consequence, these differences may determine an individual's bone turnover and hence, risk of developing osteoporosis. Progressively lower circulating IGF-1 concentrations with

significantly greater degrees of PTH insensitivity and rhythm abnormality amongst the groups studied would support the hypothesis that GH and IGF-1 contributes to PTH sensitivity and rhythm. We were able to demonstrate a significant difference in IGF-1 concentration between the younger and older subjects. We were, however, unable to demonstrate a significant difference in IGF-1 between the postmenopausal women with low BMD and those with normal BMD. The difference in IGF-1 in the older men with normal and low BMD only approached significance. As this was a study involving circadian rhythm analysis the number of subjects in each group was limited. Circulating IGF-1 concentrations were compared using single time point measurements and the comparison was subject to type 2 error. IGF-1 concentrations have, however, been shown in larger population studies to be lower in individuals with low BMD compared to age matched controls with normal BMD (407-410). Furthermore, the measurement of IGF-1 in peripheral circulation may also not directly reflect the concentration of IGF-1 and other growth factors in the bone microenvironment which may also account for some of the differences observed between individuals with normal and low BMD of the same gender and similar age. Lower concentrations of IGF-1 and other growth factors in the bone microenvironment may be responsible for the uncoupling of turnover in individuals with low BMD. Other factors including bio-available estradiol, cytokines such as OPG, RANKL, peak bone mass achieved in adult life, preexisting life style factors or a genetic predisposition may all contribute, individually or more likely in combination, to determining which individuals develop abnormal bone turnover and lower BMD.

Gender is, inherently, a determinant of the risk of developing osteoporosis; as men and women differ fundamentally in the aging process due to the occurrence of the menopause in women with no natural male equivalent. Loss of oestrogen restraint of osteoclasts,

following the menopause, results in an increase in bone resorptive activity. This should be followed by an increase in osteoblast activity due to the coupling of resorption to formation. Our data confirms that bone turnover is higher in postmenopausal as compared to premenopausal women. The increase in bone turnover was, however, of a lesser magnitude in postmenopausal women with normal BMD compared to those with low BMD. This lesser increase in bone turnover occurred such that the ratio of resorption to formation markers was not statistically different from that observed in the premenopausal women. This would suggest that despite an increased activation frequency, resorption may have, at least at the time of being studied, remained sufficiently coupled to formation. This could explain the absence of a reduction in BMD in these postmenopausal women. In the women with low BMD, however, the greater increase in bone turnover was associated with an increase in the ratio of resorption to formation markers suggesting an element of uncoupling of bone turnover which would contribute to bone loss and a low BMD.

Previous studies using various techniques, including histomorphometry and 3-dimensional micro-computed tomography, have suggested that even without demonstrated decreases in BMD postmenopausal women do demonstrate bone loss at a micro architectural level (411, 412). We utilized biochemical markers of bone turnover from peripheral circulation to measure bone turnover. It is possible that the lesser increase in turnover markers in the postmenopausal women with normal BMD was not of sufficient magnitude to allow us to appreciate a change in the ratio of bone resorption to formation markers as was observed in the postmenopausal women with low BMD. This may have been a consequence of type 2 error. It is also possible that the women with normal BMD may have been studied at a stage following the onset of menopause when bone turnover may have increased but bone loss may not have become evident. In time, these women may develop changes similar to

that seen in the women with low BMD and eventually demonstrate a reduction in BMD themselves.

The men we studied demonstrated a distinctly different pattern to the women. The bone turnover in the older men with normal BMD was lower than in the younger men (369, 370). The pattern is in keeping with previous studies in men that have shown a decrease in biochemical bone markers until the age of 60 (369). We also observed no difference in the resoprtion to formation ratio, suggesting that despite a low bone turnover state in the older men with normal BMD, coupling may be maintained. This may account for the preserved BMD in these older men. After the age of 60 years, markers of bone formation remain stable while resorption markers show a variable increase with aging and there is an inverse relationship between bone turnover and BMD (369). We observed a significant increase in the resorption to formation ratio in the men with low BMD, suggesting uncoupling of bone turnover that would contribute to the development of a low BMD in these men.

We, thus, observed distinct differences in the pattern of change in bone turnover with age and BMD between men and women. Our findings also suggest that the increase in bone turnover, degree of uncoupling and development of low BMD are not uniform or predictable consequences of advancing age. Individuals of a similar age over 60 demonstrate differences in bone turnover and BMD that are the result of differences in various contributory factors including GH, IGF-1, PTH sensitivity and PTH rhythm.

Altered PTH sensitivity contributes to the development of osteoporosis in patients with AGHD and GHR therapy improves PTH sensitivity and has a restorative effect on PTH rhythm (91). There is, however, a gender based difference in the benefit derived from GHR

therapy. Women with AGHD who are receiving oestrogen replacement demonstrate a smaller increase in PTH sensitivity and a less marked effect on PTH rhythm following GH replacement therapy as compared to men with AGHD receiving testosterone replacement therapy (93). It is well established that gonadal steroids modify the metabolic effects of GH and whilst androgens potentiate the effects of GH at a peripheral level, oestrogens antagonize the effects (276, 281, 413, 414). This would suggest that younger premenopausal women who are oestrogen replete would be more PTH resistant than younger men who are testosterone replete in the presence of similar GH and IGF-1 concentrations. Our observation of a higher PTH concentration in the younger premenopausal women when compared to the younger men with similar NcAMP concentrations would be in keeping with this.

The pattern of decline in gonadal steroids with age differs in men and women. In women, the acute decline in oestrogen concentrations following the menopause would theoretically be paralleled by an acute decrease in the antagonistic effect of oestrogen on GH and IGF-1 action. The gradual decline in testosterone in men, however, would be paralleled by a gradual decrease in the potentiating effect of testosterone on GH and IGF-1 action. We observed a smaller percentage increase in PTH concentration from younger premenopausal women to older postmenopausal women possibly as a result of the acute decline in oestrogen related antagonism of GH and IGF-1 in the postmenopausal women resulting in less PTH resistance. A greater percentage increase in PTH concentration from younger men to older men may be the result of the continuous decline in potentiation of GH and IGF-1 and hence relatively more marked increase in PTH resistance.

A further sequence of events may also be postulated. Initially, the acute decline in oestrogen in postmenopausal women would result in two simultaneous changes: an increase in osteoclast resorption and a decrease in antagonism of GH and IGF-1. This may allow GH and IGF-1 to act on osteoblasts to increase bone formation in an attempt to meet the demands of increased resorption. This would result in the increased bone turnover seen in postmenopausal women. In men, on the other hand, the steady decline in bio-available testosterone with age should result in a steady decline in the anabolic effect of GH and IGF-1. With no acute catabolic stimulus, like the oestrogen deficiency in postmenopausal women, one would expect a gradual decrease in bone turnover as was observed in the older men with normal BMD.

In the men with low BMD however, we did see an increase in bone resorption with a lesser increase in bone formation. This pattern was similar to that seen in postmenopausal women suggesting that bone turnover in these individuals was uncoupled. In postmenopausal women oestrogen withdrawal is the most obvious catabolic stimulus resulting in increased resorption but the catabolic stimulus in men is still unclear. Age related decline in both testosterone as well as oestrogen can directly result in increased resorption and have both been implicated in the development of osteoporosis in men (406). It has also been proposed that lower IGF-1 may potentially increase SHBG, result in lower bio-available sex steroid concentrations and therefore indirectly increase bone resorption (406). As bio-available testosterone dwindles and the balance shifts towards oestrogen this could also result in decreased potentiation and increased antagonism of the anabolic effect of GH and IGF-1 resulting in uncoupling. We analysed total testosterone concentrations in the older men we studied. All the men had concentrations that were in the normal reference range.

However, his does not account for individual differences in bioavailability of androgens or bio-available oestrogen either.

The effect of PTH rhythm on bone turnover is well established (64, 65, 415). PTH rhythm was abnormal in the older men with low BMD but remained intact in the men with normal BMD. PTH rhythm was also abnormal in the postmenopausal women with normal BMD and the postmenopausal women with low BMD. Thus abnormal PTH rhythms were observed in the same 3 groups that also had increased resorption markers. This would suggest that the abnormal secretory pattern of PTH may very well be a contributory catabolic stimulus contributing to the increased resorption in these groups. The loss of oestrogen is the predominant stimulus for increased resorption in postmenopausal women but abnormal PTH rhythmicity may also contribute. The mechanisms for bone loss in men are less well understood and our findings suggest that abnormal PTH rhythmicity may be involved.

PTH has a phosphaturic effect and renal insensitivity to the effects of PTH would result in an attempt to retain PO_4. GH modulates renal PO_4 handling via IGF-1, independently of PTH or $1,25(OH)_2D$ (131, 135) and a low IGF-1 results in a decrease in $TmPO_4/GFR$ and hence increased phosphaturia (131, 416). Gender dependent differences in renal PO_4 handling have also been suggested in patients with AGHD (93). PO_4 concentrations and $TmPO_4/GFR$ were low in both groups of older men as compared to younger men, whereas low PO_4 concentration was observed only in the women with low BMD with the difference in $TmPO_4/GFR$ approaching significance. Thus, there was a decrease in serum PO_4 and $TmPO_4/GFR$ in the older individuals despite increasing PTH resistance in these individuals. This would suggest that the effect of the lower IGF-1 concentrations on renal

PO_4 handling outweighs the effect of PTH in these individuals. The effects on PO_4 handling with age were more marked in the men than in the women. This may have been a reflection of the greater decrease in IGF-1 concentration in the men as compared to women. It could also have been the greater manifestation of the effects of the declining GH and IGF-1 in men as described earlier with respect to the pattern of decline in gonadal hormones.

A defect in renal Ca conservation has also previously been observed in postmenopausal women with osteoporosis and has been attributed to the lack of oestrogen following the menopause (67). It was postulated that the defect in renal Ca conservation was a primary catabolic stimulus. The increase in bone turnover in postmenopausal women was considered a mechanism to compensate for the renal Ca loss by releasing Ca from the skeletal reservoir. The postmenopausal women we studied also had a higher Ca excretion compared to the premenopausal women. This abnormality in renal Ca conservation could also be explained, at least in part, by renal insensitivity to the Ca conserving effects of PTH in these women. Alternatively a primary increase in bone resorption and Ca release from bone may result in an increased filtered load and subsequent increased urine excretion.

Despite a greater increase in PTH resistance in the older men when compared to the postmenopausal women, Ca excretion was no different in the older men when compared to the younger men. It is possible that oestrogen deficiency does have a role in regulating renal Ca conservation and hence men are less affected. The increase in bone turnover in older men was less than in the postmenopausal women. If the increase in bone turnover was the primary event then there would have been no increase in the filtered load and hence no increase in UCa excretion. Although the concentrations of 25(OH)D and

$1,25(OH)_2D$ were similar in all groups studied, they were in the low normal range and this may also have affected Ca and PO_4 metabolism in addition to effects on individual differences in PTH, bone turnover and BMD.

In conclusion, bone loss in older men and women is associated with target organ insensitivity to PTH, abnormal PTH circadian rhythm and abnormal bone mineral metabolism. There are distinct differences between men and women as consequence of the differential effects of oestrogen and testoseterone. There are also differences because men and women differ distinctly in the way that gonadal hormones change with age. Abnormal PTH sensitivity and rhythm were associated with declining IGF-1 concentrations in postmenopausal women and older men. Abnormal PTH sensitivity and rhythm may be consequences of the low IGF-1 in these patients as in patients with AGHD. We propose that our observations add a further component to the understanding of the extremely complex interplay of biochemical and hormonal factors that regulate bone metabolism in postmenopausal women and older men.

Chapter 5

The Effect of Growth Hormone on Parathyroid Hormone Sensitivity, Parathyroid Hormone Circadian Rhythmicity, Phospho-calcium Metabolism and Bone Turnover in Postmenopausal Women with Osteoporosis

5.1. Introduction

Bone loss and the increasing incidence of osteoporosis is an accompaniment of aging. Women undergo two phases of bone loss – a slow phase with a linear decrease in bone, continuing into old age and a superimposed, accelerated transient phase beginning at menopause caused by oestrogen deficiency (11, 14, 417). The slow phase in the development of osteoporosis has been attributed to alteration of age related factors resulting in impaired osteoblast function and bone formation. These include GH and IGF-1, both major determinants of adult bone mass (418, 419), that decrease with advancing age (277-283) and are lower in women with established osteoporosis (407).

The beneficial effects of GH on bone metabolism and BMD have been demonstrated in AGHD patients (91, 420). Target organ insensitivity to the effects of PTH resulting in increased circulating PTH and abnormal PTH secretion (51) contributes to the development of osteoporosis in AGHD. GHR in AGHD patients has been shown to increase bone and renal PTH receptor or target cell sensitivity to the effects of PTH and simultaneously restore PTH secretory rhythm, increase bone turnover markers, $1,25(OH)_2D$ concentration and Ca absorption/reabsorption, thus contributing to the positive effects of GH on bone (91).

Postmenopausal women with osteoporosis have high circulating PTH concentrations with abnormal PTH circadian rhythm (66, 67) and may consequently be insensitive to the effects of PTH (53-55). The decline in GH/IGF-1 with aging may contribute to these PTH related abnormalities via mechanisms similar to that observed in untreated AGHD (51, 91). GH has been previously administered to healthy elderly women and women with

postmenopausal osteoporosis and increases in bone turnover and bone density have been demonstrated (378, 385, 388). However, the mechanisms by which GH exerts its beneficial effects on bone in established postmenopausal osteoporosis remain unexplained. We therefore investigated the effects of 12 months of GH administration on PTH secretory pattern, PTH sensitivity and bone mineral metabolism in postmenopausal women with osteoporosis.

5.2. Subjects and Methods

5.2.1. Patients and Controls

Fourteen postmenopausal women (mean ± SEM: 63.4±2.1 years, range 52-79 years), newly diagnosed with osteoporosis, were recruited from a community osteoporosis screening program. The mean BMD T score ± SEM in the (LS (L2-L4) and FN was -3.3±0.2 and -2.0±0.2 respectively. For baseline comparison 14 healthy premenopausal control women (33.9 ± 2.2 years, range 25 – 39 years) with normal BMD (LS and FN T score was 0.3 ± 0.3 and 0.6 ± 0.2 respectively) were recruited from a database of volunteers willing to participate in medical research.

Table 5.1. Demographic Characteristics of Patients and Controls [Mean(SEM)]

	Osteoporotic women	Control subjects
n	14	14
Age (years)	63.4(2.0)**	33.9(2.2)
Height (m)	1.60(0.02)	1.63(0.02)
Weight (kg)	63.2(3.0)	70.7(3.1)
BMI (kg/m^2)	24.9(1.2)	26.8(1.2)
Waist/hip ratio	0.84(0.02)	0.85(0.02)
Fat Mass (kg)	21.6 (2.3)	25.6 (2.5)
Fat Percentage (%)	33.26 (2.37)	34.32 (2.56)
IGF-I (ng/ml)	102.8(10.0) *	140.9(10.8)
IGF-I SDS	-1.21(0.26)	-1.19(0.28)
BMD (T – score)		
Lumbar spine	-3.3(0.2) ***	0.3(0.3)
Femoral neck	-2.0(0.2) ***	0.6(0.2)

* p<0.05 osteoporotic women compared to control subjects
** p<0.01 osteoporotic women compared to control subjects
*** p<0.001 osteoporotic women compared to control subjects

5.2.2. Methods

Study visits occurred as documented in section 4.2.3. Baseline samples were collected in all controls and subjects with osteoporosis at the initial study visit, following which GH (Humatrope, Eli Lilly & Co., Basingstoke, Hampshire, UK) was commenced at a standard daily dose of 0.2mg, self-injected using an automated pen device (Humatrope-Pen II, Eli Lilly & Co., Basingstoke, Hampshire, UK) at 2200h every night only in the subjects with osteoporosis. GH was initiated at 0.2 mg/day for four weeks and then titrated by increments of 0.1mg/day every 2 weeks, according to insulin-like growth factor 1 (IGF-1) concentration. We aimed for a target IGF-1 concentration within ±1 SD of the median IGF-1 for a woman age 45 years. Study visits were repeated 1,3,6 and 12 months after the initiation of GH and all patients completed the 12 month study. The local ethics committee approved the study, and written informed consent was obtained from each patient before recruitment.

5.2.3. Bone Mineral Density

All subjects underwent bone densitometric evaluation as described in section 3.4.

5.2.4. Biochemistry

Analytes were measured using methods as described in section 3.2. Serum PTH, Ca, PO_4 and albumin, concentration were measured on all 49 blood samples obtained from each individual at every visit. Serum IGF-1, 25(OH)D, $1,25(OH)_2D$, βCTX and PINP concentration were measured on single time point 0900 samples at every visit. Urine calcium, phosphate and creatinine were measured on all urine samples at every visit.

PcAMP was measured on blood samples corresponding to the times of urine samples. NcAMP was calculated as described in section 3.2.5. The $TmPO_4/GFR$ was calculated from the normogram derived by Walton and Bijvoet as described in section 3.2.2.

5.2.5. Statistical Analysis

General linear model analysis of variance (GLM ANOVA) for repeated measures was used to analyse the data as described in section 3.3.1. Mauchly's test indicated that the sphericity assumption was violated and degrees of freedom were corrected using Greenhouse-Geisser estimate of sphericity (ε=0.53). This method has been validated for similar comparisons (51, 93). Student's t-test for unpaired data was used to determine the significance of the differences between premenopausal and postmenopausal women. PTH circadian rhythms were assessed as described in section 3.3.3. Values are expressed as the mean ± standard error of mean (SEM).

5.3. Results

5.3.1. GH Dose and IGF-1

IGF-I concentration (Figure 5.1a) was significantly lower in the women with osteoporosis compared to the controls (101.5±8.9μg/L versus 140.9±10.8μg/L; $p<0.05$). In the treated patients the mean GH dose (Figure 5.1b) was 0.2±0.01 mg/day at 1 month and increasing to 0.39±0.01 mg/day at 3 months ($p<0.001$) and further titrated to 0.56±0.04 mg/day at 6 months ($p<0.001$ compared to baseline; $p<0.01$ compared to 3 months). Maximum dose was achieved at 6 months in all patients and further increases were not tolerated. 3 patients had dose reductions from 6 months to 12 months due to increased musculo-skeletal pains.

The mean GH dose was 0.49±0.05 mg/day at 12 months ($p<0.001$ compared to baseline; $p<0.05$ compared to 3 months; no significant difference compared to 6 months). Mean serum IGF-I increased significantly from 101.5±8.9μg/L to 128.3±12.1μg/L by 1 month ($p<0.001$), 157.2±15.8μg/L at 3 months ($p<0.001$), 172.9±13.6μg/L at 6 months ($p<0.001$, compared to baseline; $p=0.06$, compared to 3 months) and 166.9±14.8μg/L at 12 months ($p<0.001$, compared to baseline; p=NS, compared to 3 and 6 months). Similarly IGF-I SDS increased from -1.26 ± 0.27 at baseline to -0.62± 0.29 at 1 month ($p<0.001$), -0.03± 0.26 at 3 months ($p<0.001$) 0.28± 0.26 at 6 months ($p<0.001$, compared to baseline; $p=0.07$, compared to 3 months) and 0.19 ± 0.26 at 12 months ($p<0.001$, compared to baseline; p=NS, compared to 3 and 6 months).

Figure 5.1. IGF-1 concentration in younger, healthy, premenopausal controls (shaded bar) compared to older, postmenopausal patients with low BMD at baseline (0 months) and IGF-1 concentrations at 1,3,6 and 12 months following GH administration with mean GH doses administered to patients at 1,3,6 and 12 months.

Figure 5.1 (a) demonstrates higher IGF-1 concentrations in controls compared to patients at baseline with IGF-1 concentrations increasing up to 6 months and maintained at 12 months following GH administration. Figure 5.1(b) demonstrates GH doses which increased up to 6 months and were maintained at 12 months.

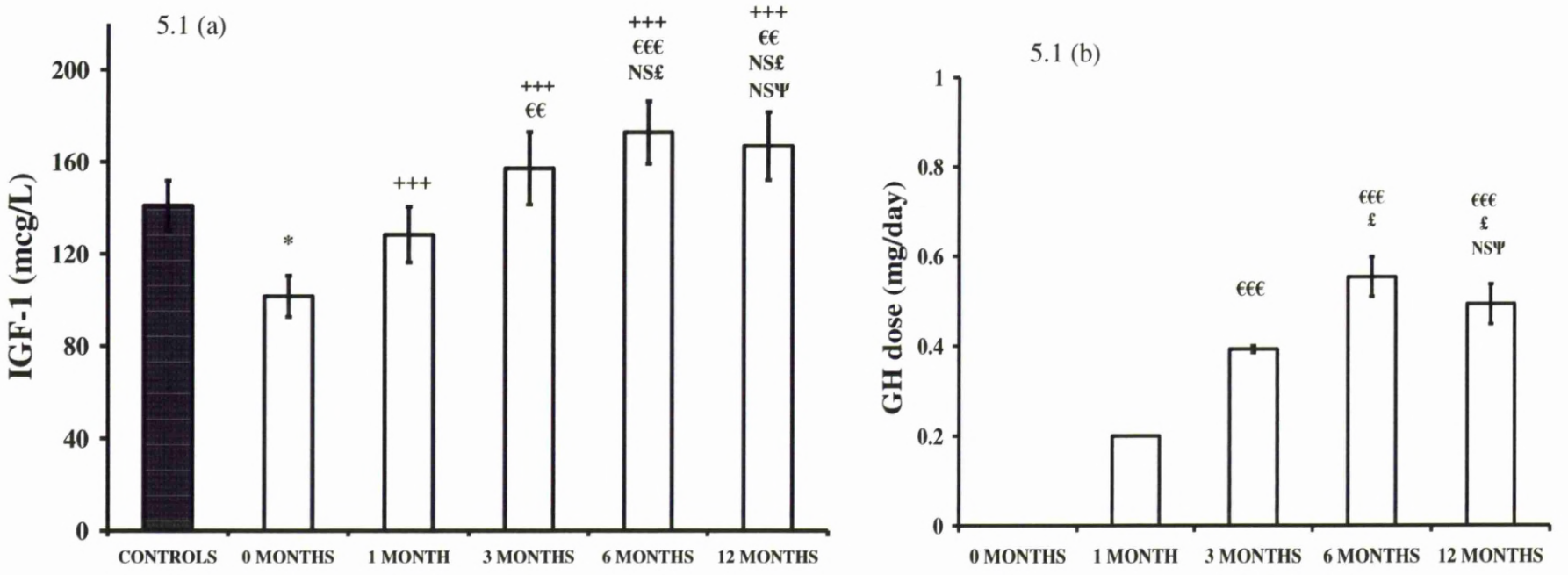


Figure 5.1. Contd.

Premenopaausal Women

Postmenopaausal Women with Osteoporosis Receiving GH

NS*	no significant difference, osteoporotic women compared to control subjects
*	p<0.05 osteoporotic women compared to control subjects
**	p<0.01 osteoporotic women compared to control subjects
***	p<0.001 osteoporotic women compared to control subjects
NS+	no significant difference compared to baseline
+	p<0.05 compared to baseline
++	p<0.01 compared to baseline
+++	p<0.001 compared to baseline
NS€	no significant difference compared to 1 month
€	p<0.05 compared to 1month
€€	p<0.01 compared to 1month
€€€	p<0.001 compared to 1month
NS£	no significant difference compared to 3 months
£	p<0.05 compared to 3months
££	p<0.01 compared to 3months
£££	p<0.001 compared to 3months
NSψ	no significant difference compared to 6 month
ψ	p<0.05 compared to 6months
ψψ	p<0.01 compared to 6months
ψψψ	p<0.001 compared to 6months

5.3.2. PTH

24-h mean PTH concentration (Figure 5.2a) was higher in the osteoporotic women (5.4 ± 0.1 pmol/L) than in healthy controls (4.4± 0.1 pmol/L, $p<0.001$). Following GH administration 24-h mean PTH concentration decreased progressively from baseline (5.4 ± 0.1 pmol/L) to 1 (5.2 ± 0.1 pmol/L, $p<0.001$), 3 (5.0 ± 0.1 pmol/L, $p<0.001$) and 6 months (4.7 ± 0.1 pmol/L, $p<0.001$) with maximum reduction in PTH concentration at 6 months. PTH concentrations then increased significantly by 12 months (4.9 ± 0.1 pmol/L, $p<0.05$ compared to 6 months) but remained below baseline concentrations.

5.3.3. NcAMP

NcAMP (Figure 5.2b) was significantly lower in osteoporotic women (17.2 ± 1.2 nmol/L GFR) as compare to controls (21.4 ± 1.4 nmol/L GFR, $p<0.05$). NcAMP increased following 1 month of GH administration (24.2 ± 2.5 nmol/L GFR, $p<0.05$) and remained elevated at 3 months (27.3 ± 1.5 nmol/L GFR, $p<0.001$) and 6 months (32.4 ± 2.5 nmol/L GFR, $p<0.001$) compared to baseline (17.2 ± 1.2 nmol/L GFR) and returned to levels not significantly different to baseline at 12 months (14.8 ± 1.6 nmol/L GFR).

5.3.4. Serum Adjusted Calcium

24-h mean ACa concentration (Figure 5.2c) was not significantly different in the osteoporotic women (2.36 ± 0.004 mmol/L, $p<0.05$) as compared to controls (2.35 ± 0.004 mmol/L). The 24-h mean ACa concentration increased progressively following 1 month (2.40 ± 0.002 mmol/L, $p<0.001$) and 3 months (2.38 ± 0.004 mmol/L, $p<0.001$) of GH

compared to baseline (2.36 ± 0.004 mmol/L) but returned to concentrations that were not significantly different from baseline at 6 months (2.35 ± 0.002 mmol/L) and 12 months (2.34 ± 0.002 mmol/L).

5.3.5. Serum Phosphate

24-h mean PO_4 concentration (Figure 5.2d) was lower in osteoporotic women (1.11 ± 0.01 mmol/L, $p<0.05$) as compared to controls (1.15 ± 0.01 mmol/L). 24-h mean PO_4 concentration increased progressively after 1 (1.18 ± 0.01 mmol/L), 3 (1.23 ± 0.01 mmol/L) and 6 months (1.27± 0.01 mmol/L) and remained elevated with no further increase at 12 months (1.28± 0.01 mmol/L) of GH administration compared to baseline (1.11 ± 0.01 mmol/L, $p<0.001$).

5.3.6. Vitamin D

No significant difference in serum 25(OH)D concentration was observed between controls (42.0 ± 6.1 nmol/L) and osteoporotic women (47.6 ± 6.1 nmol/L, $p=0.53$). 25(OH)D concentrations were not significantly different following 1 (45.1 ± 4.2 nmol/L), 3 (49.9 ± 5.3 nmol/L) and 12 (42.2 ± 4.8 nmol/L) months of GH administration. An increase was observed at 6 months (54.5 ± 4.5 nmol/L, $p<0.05$) as compared to baseline (47.6 ± 3.6 nmol/L). No significant difference in serum $1,25(OH)_2D$ concentration was observed between controls (74.0 ± 8.2 pmol/L) and osteoporotic women (78.1 ± 8.2 pmol/L, $p=0.73$) (Figure 5.2e). $1,25(OH)_2D$ concentrations increased by 3 months (99.4 ± 10.0 pmol/L,

p<0.001) and were maintained at 6 (95.7 ± 9.5 pmol/L, p<0.05) and 12 months (99.9 ±11.6 pmol/L, p<0.01) (Figure 5.2e).

5.3.7. Urine Calcium Excretion

UCa/Cr (0.6±0.03 versus 0.4±0.03; p < 0.001) and UCaE (Figure 5.2f) (0.05±0.002 versus 0.03±0.002 mmol/L CCr; p < 0.001) were higher in the osteoporotic women compared to controls. UCa/Cr increased following 3 (0.8±0.04; p < 0.001) and 6 (0.9±0.04; p < 0.001) months of GH and was not significantly different at 1 (0.7±0.04; p = 0.1) and 12 months (0.7±0.04; p = 0.2) as compared to baseline (0.6±0.03). UCaE also increased similarly (Baseline 0.05±0.002 mmol/L CCr; 1 month 0.06±0.003 mmol/L CCr, p=NS; 3 months 0.07±0.003 mmol/L CCr, p<0.001; 6 months 0.08±0.003 mmol/L CCr, p<0.001; 12 months 0.06±0.003 mmol/L CCr, p=NS)

5.3.8. Urine Phosphate Excretion and $TmPO_4/GFR$

UPO_4/Cr (2.2±0.12 versus 2.5±0.12; p = 0.1) and UPO_4E (Figure 5.2g) (0.18±0.009 mmol/L CCr versus 0.19±0.009 mmol/L CCr; p = 0.3) were not significantly different in the 2 groups. UPO_4/Cr increased significantly following 1(2.8±0.15; p < 0.001), 3 (3.2±0.16; p < 0.001), 6 (3.3±0.17; p < 0.001) and 12 months (2.7±0.13; p < 0.05) of GH as compared to baseline (2.2±0.12) with similar increases in UPO_4E (Baseline 0.18±0.009 mmol/L CCr; 1 month 0.22±0.012 mmol/L CCr, p<0.001; 3 months 0.25±0.012 mmol/L CCr, p<0.001; 6 months 0.28±0.014 mmol/L CCr, p<0.001; 12 months 0.21±0.012 mmol/L CCr, p<0.05).

$TmPO_4$/GFR (Figure 5.2h) was not significantly different between controls (0.98 ± 0.02 mmol/L GFR) and osteoporotic women (0.96 ± 0.02 mmol/L GFR, p=0.6). $TmPO_4$/GFR increased following GH administration for 1 month (0.99 ± 0.02 mmol/L GFR, p<0.05) and remained elevated at 3 (0.99 ± 0.02 mmol/L GFR, p<0.05), 6 (1.01 ± 0.02 mmol/L GFR, p<0.001) and 12 (1.08 ± 0.02 mmol/L GFR, p<0.001) months compared to baseline.

5.3.9. Markers of Bone Turnover

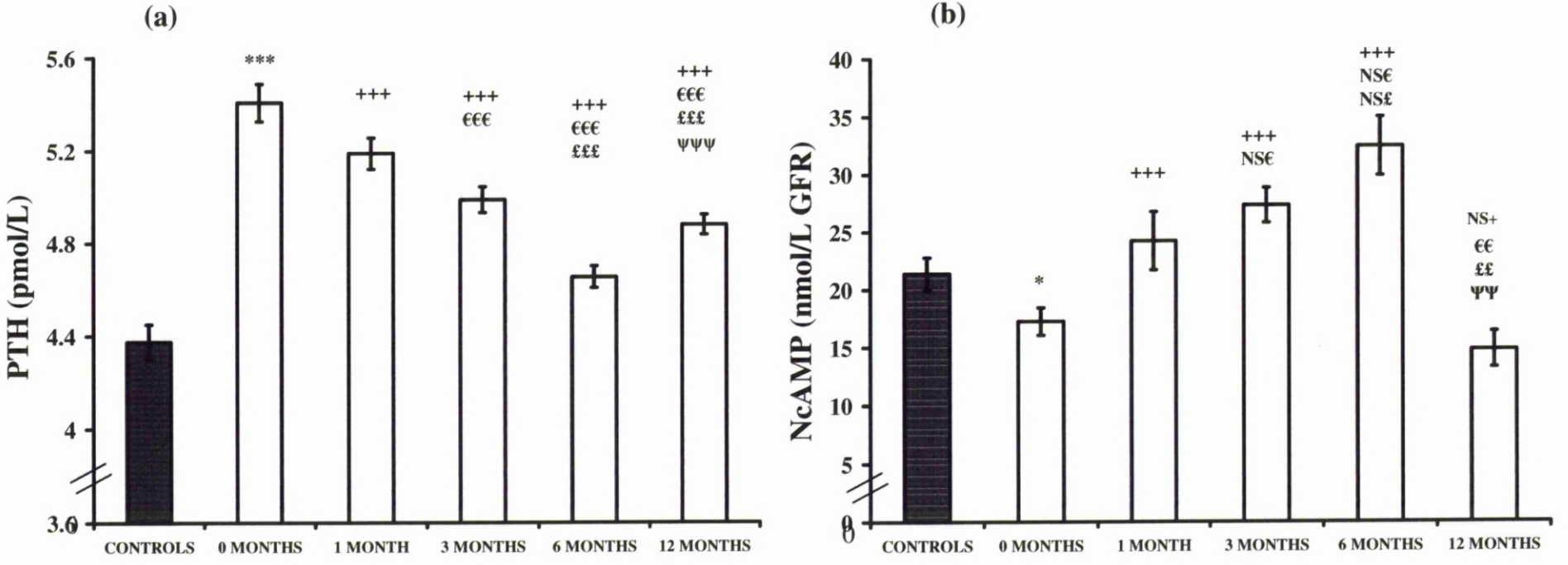
βCTX concentrations (Figure 5.2i) were significantly higher in osteoporotic women (0.74± 0.07μg/L) as compared to controls (0.20± 0.07μg/L, p<0.001). Following GH administration βCTX concentrations increased progressively from baseline (0.74± 0.07μg/L) to 1 (0.83± 0.07μg/L, p<0.05) and 3 months (1.07± 0.09μg/L, p<0.001) with no further increase seen at 6 (1.18± 0.10μg/L, p<0.001 compared to baseline) and 12 months (1.08± 0.12μg/L, p<0.001 compared to baseline).

PINP (Figure 5.2j) concentrations were significantly higher in osteoporotic women (60±5 μg/L) as compared to controls (35±5 μg/L, p<0.01). PINP concentrations increased progressively from baseline (60±5 μg/L) to 1 (69±5 μg/L, p<0.001), 3 (93±6μg/L, p<0.001) and 6 months (126±11 μg/L, p<0.001). The increase was maintained following 12 months (122±14 μg/L, p<0.001) of GH administration.

The percentage increase in PINP concentration was significantly higher than βCTX following 6 (76±25% vs. 142±25%, p<0.05) and 12 months (61±25% vs. 133±25%, p<0.05) of GH administration.

Figure 5.2. Figure showing differences in serum and urine biochemistry in young healthy premenopausal women compared to older postmenopausal women with low BMD at baseline and changes in biochemical parameters 1,3,6 and 12 months following GH administration in the postmenopausal women with low BMD.

(a) PTH: higher concentrations in patients compared to controls with decreasing levels following GH administration up to 6 months and levels remaining below baseline at 12 months, (b) NcAMP: lower concentrations in patients compared to controls with increasing levels following GH administration up to 6 months with levels no different from baseline at 12 months, (c) ACa: no difference in patients compared to controls with higher levels at 1 and 3 months settling to levels similar to baseline at 6 and 12 months following GH administration, (d) PO_4: lower concentrations in patients compared to controls with concentrations increasing upto 12 months following GH administraion, (e) 1,25(OH)2D: no difference in patients compared to controls with levels increased by 3 months and maintained at 6 and 12 months following GH administration, (f) UCaE: higher in patients compared to controls with UCaE increasing by 3 and 6 months and no significant change compared to baseline, (g) UPO_4E: no difference between patients compared to controls and UPO_4E increasing up to 6 month with levels no different from baseline at 12 months, (h) TmPO4/GFR: no difference between patients and controls with higher TmPO4/GFR at 1,3,6 and 12 months following GH administraion, (i) βCTX: higher levels in postmenopausal women with low BMD and increasing levels following GH administration, (j) PINP: higher levels in postmenopausal women with low BMD compared to controls with levels increasing following GH administration.


(a)
PTH (pmol/L)
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4.8
4.4
4
3.6
***
+++
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ΨΨΨ
CONTROLS
0 MONTHS
1 MONTH
3 MONTHS
6 MONTHS
12 MONTHS
(b)
NcAMP (nmol/L GFR)
40
35
30
25
20
15
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*
+++
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NS€
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CONTROLS
0 MONTHS
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12 MONTHS

Figure 5.2. Contd.

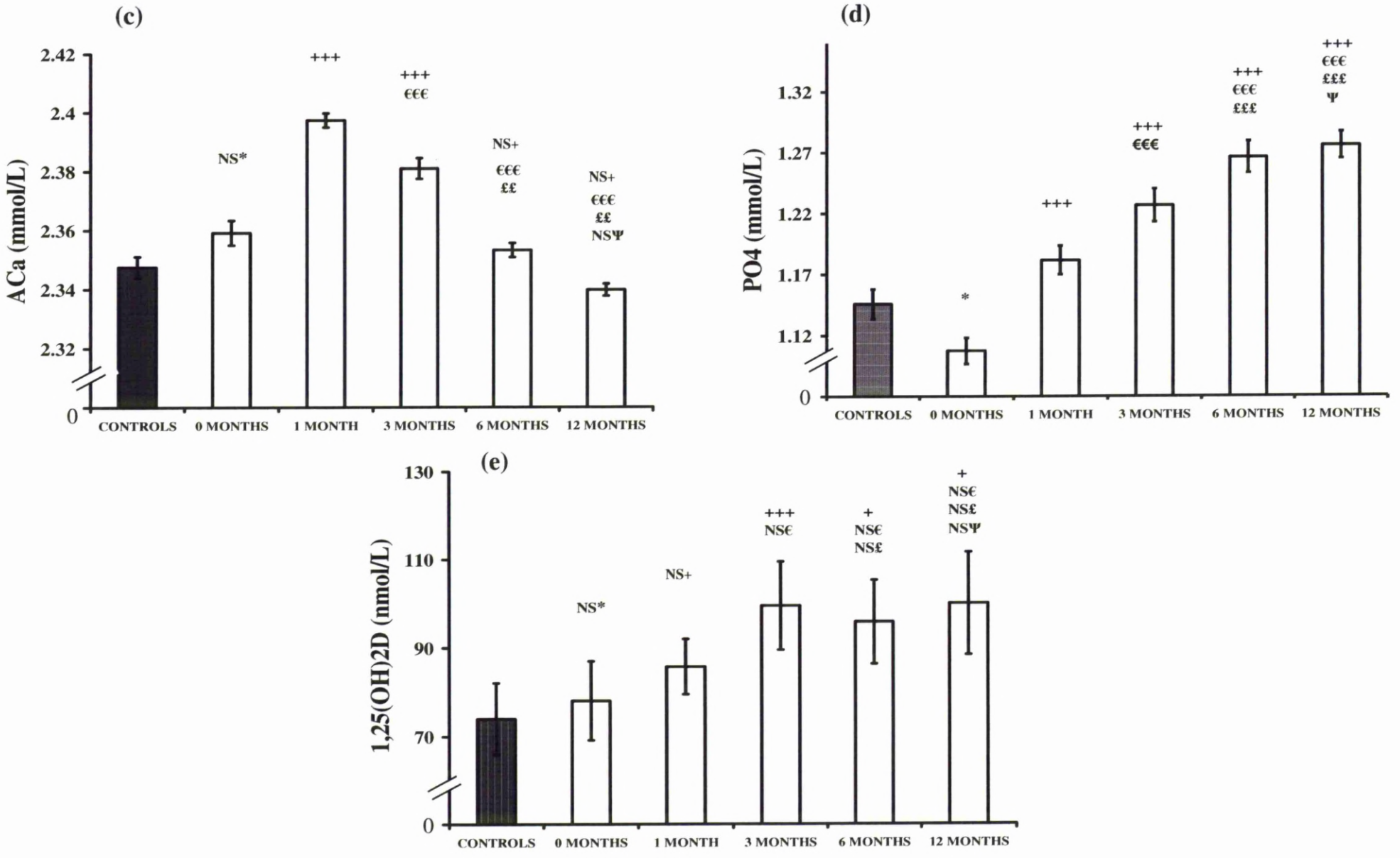


Figure 5.2. Contd.

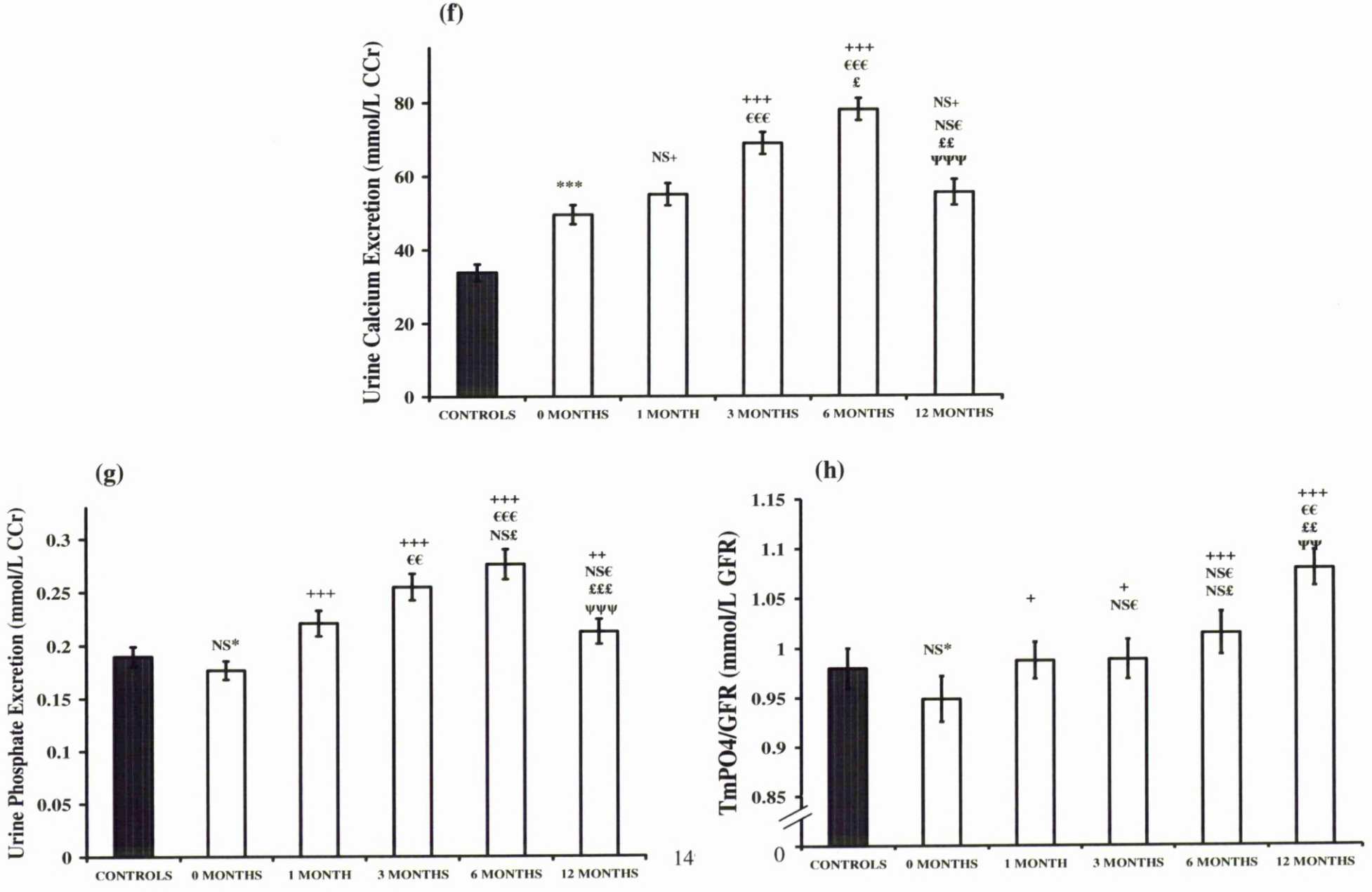
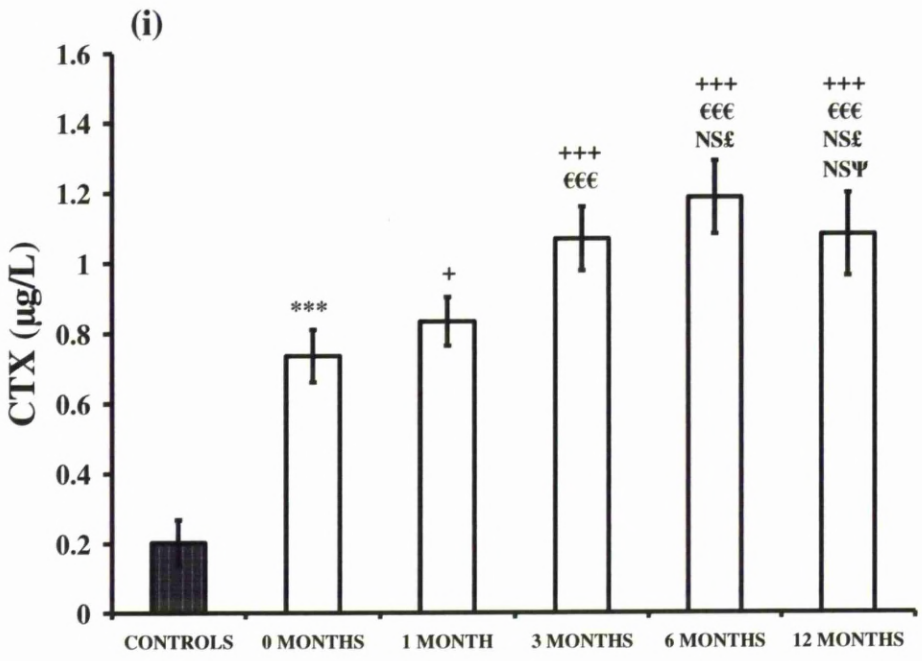
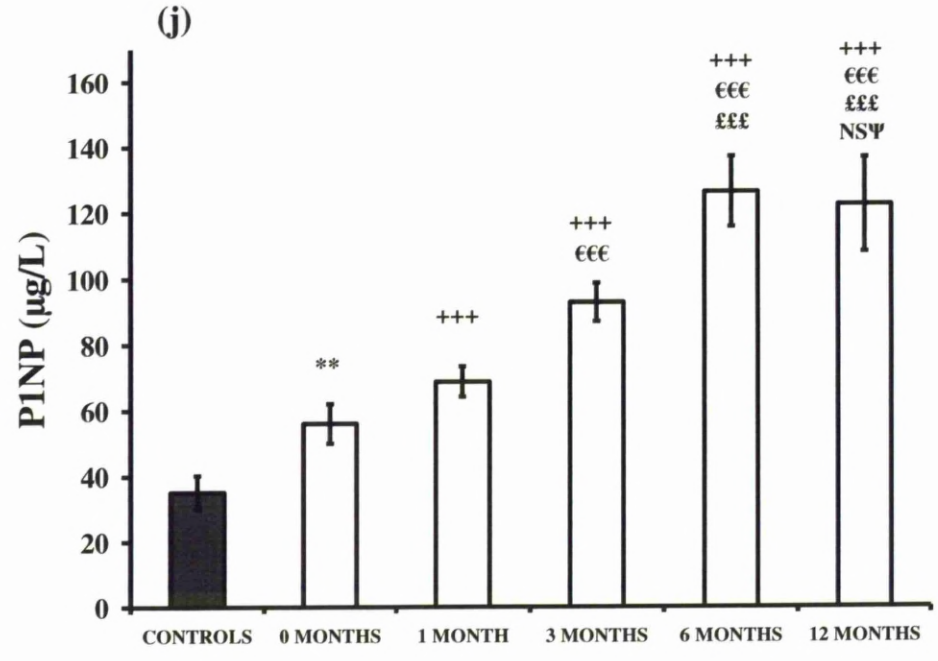


Figure 5.2. Contd.





Premenopaausal Women

Postmenopaausal Women with Osteoporosis Receiving GH

NS*	no significant difference, osteoporotic women compared to control subjects
*	p<0.05 osteoporotic women compared to control subjects
**	p<0.01 osteoporotic women compared to control subjects
***	p<0.001 osteoporotic women compared to control subjects

NS+	no significant difference compared to baseline
+	p<0.05 compared to baseline
++	p<0.01 compared to baseline
+++	p<0.001 compared to baseline

NS€	no significant difference compared to 1 month
€	p<0.05 compared to 1month
€€	p<0.01 compared to 1month
€€€	p<0.001 compared to 1month

NS£	no significant difference compared to 3 months
£	p<0.05 compared to 3months
££	p<0.01 compared to 3months
£££	p<0.001 compared to 3months

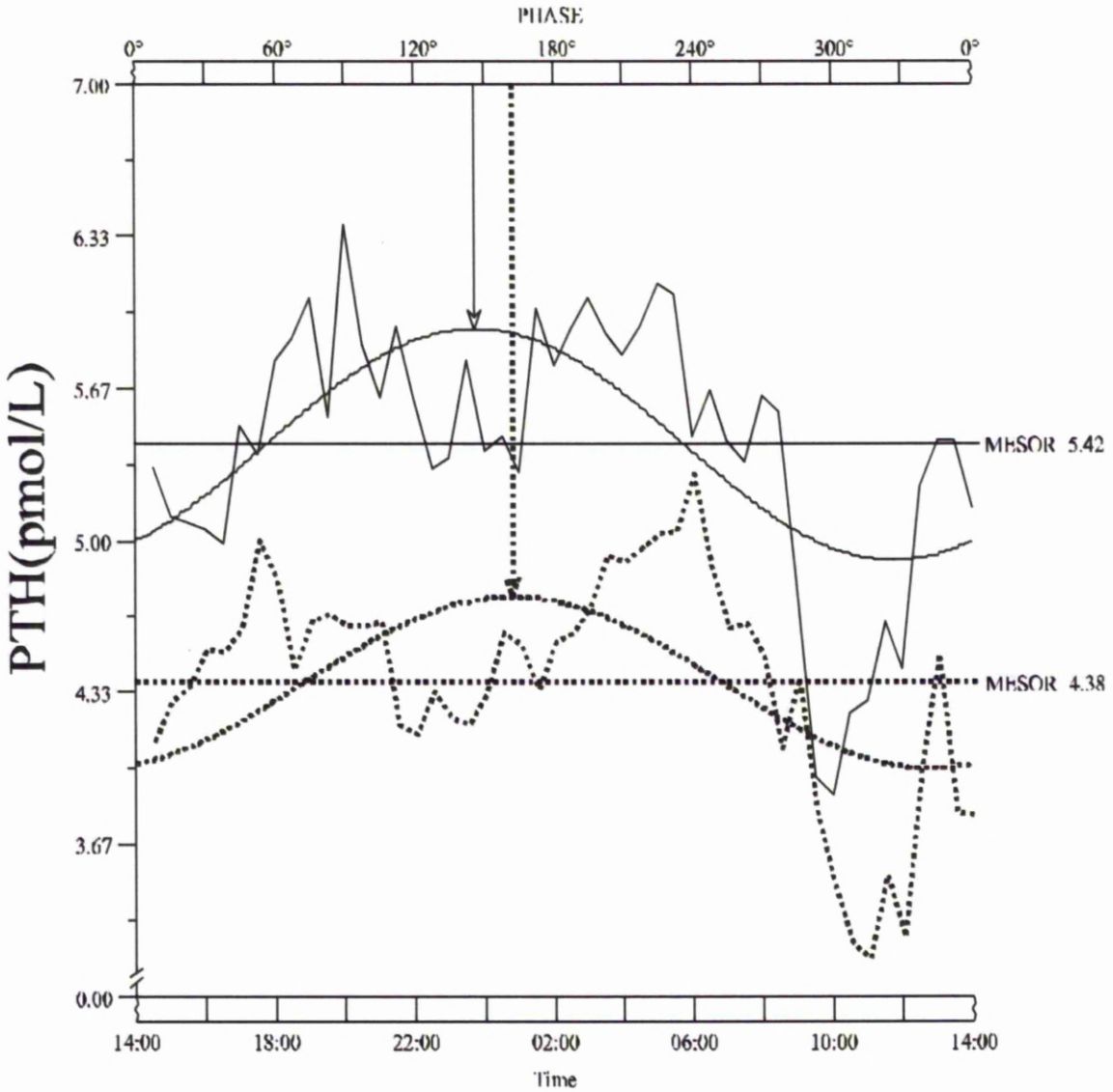
NSψ	no significant difference compared to 6 month
ψ	p<0.05 compared to 6months
ψψ	p<0.01 compared to 6months
ψψψ	p<0.001 compared to 6months

5.3.10. PTH Circadian Rhythmicity

Individual and population cosinor analyses for circulating PTH (Figures 5.3 and 5.4) demonstrated significant circadian rhythms for all healthy controls and all osteoporotic patients at all visits ($p<0.001$) but with differences between patients and controls and changes following GH administration. The mean PTH MESOR was significantly higher in the osteoporotic women than in the controls (5.4±0.3pmol/L versus 4.4±0.3pmol/L, $p=0.03$), but there was no significant difference in the amplitude (0.7±0.1pmol/L versus 0.5±0.1pmol/L for osteoporotic women and controls respectively, $p=0.22$) or acrophase (-156±16 degrees versus -178±16 degrees for osteoporotic women and controls respectively, $p=0.36$). Following GH administration to the osteoporotic women, mean PTH MESOR decreased by 3 months (5.0 ± 0.3 pmol/L, $p<0.05$) with a further decrease at 6 months (4.5 ± 0.2 pmol/L, $p<0.001$) when maximum reduction in mean PTH MESOR was observed. PTH MESOR then rose significantly by 12 months (4.9 ± 0.1 pmol/L, $p<0.05$ compared to 6 months) but remained below baseline (5.3 ± 0.3 pmol/L, $p<0.05$). The amplitude and acrophase of the PTH circadian rhythm did not change significantly following GH administration. A reduction in mean percentage increase (421-423) in PTH concentration between 1400-2300h, without significant change in the maximum percentage increase (424) indicated a narrower afternoon/evening peak, following 3, 6 and 12 months of GH administration. The maximum percentage increase and the mean percentage change in PTH concentration between 2330 and 0800h (424) was significantly lower in the osteoporotic women as compared to the controls representing a less marked nocturnal peak. The maximum and mean percentage change in PTH concentration overnight increased significantly following GH administration indicating restoration of the nocturnal peak.

Figure 5.3. Cosinor-derived PTH circadian rhythms in premenopausal women and older postmenopausal women with low BMD.

Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs for each group and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated for for both groups with differences in MESOR, acrophase and amplitude. The MESOR was highest in the postmenopausal women with alterations to the rhythm that may be contributory to bone loss.



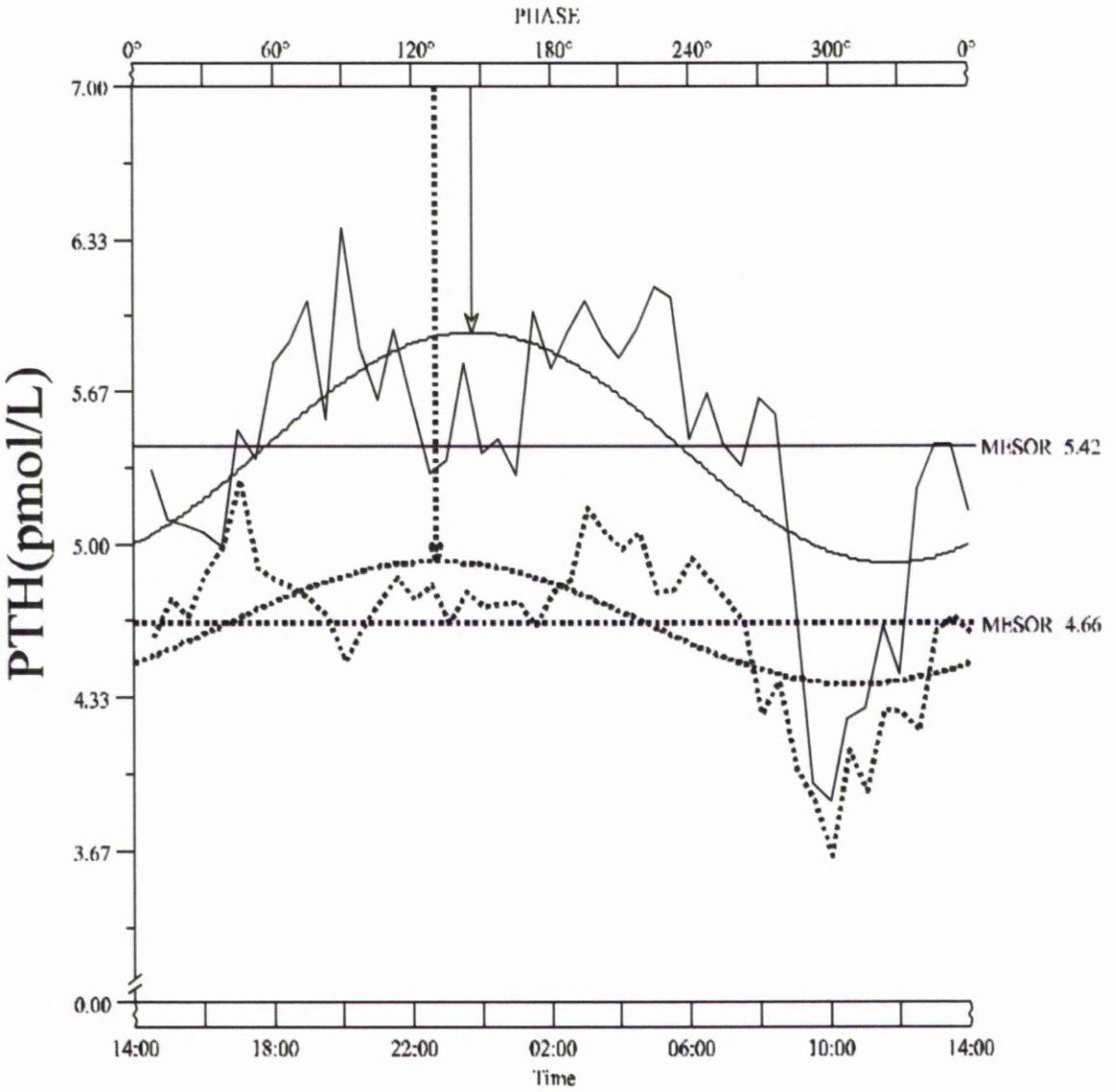
Premenopausal women .....

Postmenopausal women with osteoporosis ——————

Arrows represent the acrophase in degrees.

Figure 5.4. Cosinor-derived PTH circadian rhythms in older postmenopausal women with low BMD, prior to and 6 months after GH administration.

Smooth curved lines respresent best fit cosine curves, straight horizontal lines represent MESORs and the vertical arrows mark the acrophase. Significant circadian rhythms were demonstrated at both time points with a decrease in MESOR following GH administration.



Postmenopausal women with osteoporosis (baseline) ——————

Postmenopausal women with osteoporosis (6 months) ....

Arrows represent the acrophase in degrees.

5.4. Discussion

Postmenopausal women with osteoporosis have lower circulating IGF-1 concentration with higher 24-hour mean PTH and lower NcAMP concentration compared with healthy premenopasual women with normal BMD. GH administration resulted in increased IGF-1 concentration, decreased PTH concentration and increased NcAMP. GH administration also resulted in an increase in $1,25(OH)_2D$, serum ACa, serum PO_4, $TmPO_4/GFR$ and biochemical markers of bone turnover with a greater percentage increase in markers of bone formation than resorption. Our findings indicate a decrease in target organ sensitivity to PTH in postmenopausal women with osteoporosis that increased following GH administration. GH administration restored the circadian rhythm of PTH which was altered in osteoporotic women.

Changes in PTH and bone metabolism in postmenopausal women with osteoporosis (53-55) have previously been mainly attributed to oestrogen deficiency following the menopause, diminished response to vitamin D and to aging itself (316-323). GH effects on PTH in osteoporosis have only been studied in the short term and with the co-administration of other calciotropic agents with variable results including decreased (388), unchanged (136, 388) or increased concentration (250) attributed to increased bone turnover and enhanced mobilisation of skeletal Ca or increased Ca absorption (136). Previous studies have not measured NcAMP, which reflects the activity of PTH in both physiological and pathophysiological states and is a reliable index of PTH function (50). Since NcAMP excretion parallels changes in PTH secretion (252), the reciprocal increase in NcAMP excretion with decreasing PTH concentration, observed in our study indicates increased renal sensitivity to the effects of PTH following GH administration. The observed decrease in NcAMP back to baseline levels at 12 months may be a reflection of

the mean GH dose decrease at 12 months. However, the significantly lower PTH concentration at 12 months compared to baseline in the presence of a NcAMP concentration similar to baseline still suggests an improvement in PTH sensitivity having achieved a new equilibrium. PTH demonstrated a sustained increase between 1400-2300 h with a reduced nocturnal rise in osteoporotic women as previously demonstrated (66). Following GH administration the PTH secretory pattern changed significantly with restoration to a rhythm resembling that similar to healthy control subjects (58, 67, 252) supporting a role for GH in regulating PTH secretory rhythm.

$1,25(OH)_2D$ and Ca absorption decrease with aging and the normal increase in $1,25(OH)_2D$ in response to infusions of PTH is blunted possibly due to decreased renal 25(OH)D 1α-hydroxylase sensitivity to PTH (343). It has also been suggested that women with osteoporosis have a defect in renal Ca conservation (358) and the higher urine Ca excretion in association with the relatively higher PTH concentration in our subjects suggests renal resistance to the effects of PTH may contribute to this. Although the concentrations of 25(OH)D were similar in both groups studied, they were in the low normal range and this may have affected PTH secretion. The initial increase in circulating Ca with no change in urine Ca excretion following GH administration may partly reflect increased renal sensitivity to PTH resulting in renal Ca reabsorption as a direct effect of PTH (425). A possible increase in 1-α hydroxylase activity resulting in increased $1,25(OH)_2D$ production (136, 426, 427) and subsequent Ca absorption may also have contributed (196). The simultaneous increase in urine Ca excretion probably reflects renal regulation of the higher filtered Ca load. By 12 months however the circulating Ca and renal Ca excretion achieve equilibrium possibly with increased Ca utilization for bone matrix formation. PO_4 levels have been shown to increase or remain unchanged in aging postmenopausal women with a negative relationship between PO_4 and vitamin D. In our osteoporotic subjects with normal

vitamin D concentrations we found a lower circulating PO_4 concentration associated with a marginally lower $TmPO_4/GFR$ as compared to controls although the difference was not statistically significant. An initial increase in both the $TmPO_4/GFR$ and serum PO_4 concentration was observed following GH administration possibly as a result of a direct antiphosphaturic effect of GH/IGF-1 (199, 200) and increased $1,25(OH)_2D$ activity (131, 157). Urine PO_4 excretion also increased in parallel with increasing serum PO_4 and therefore a higher filtered PO_4 load before a new equilibrium was established at 12 months when circulating PO_4 and $TmPO_4/GFR$ reached a plateau and PO_4 excretion began to decrease. Increased phosphaturia could also be a reflection of the change in renal sensitivity to PTH during GH therapy but clearly the dominant effect of these hormones on PO_4 is varying with time.

GH administration to osteoporotic patients simultaneously increases markers of both bone formation and resorption and thus no increase in BMD is seen in the short term but BMD increases following prolonged GH administration (388). Our data confirms a simultaneous increase in bone resorption and formation with the increase in bone formation markers becoming significantly higher than resorption only by 6 months, possibly explaining the delay in increase in BMD following GH administration. The sequence of changes in bone turnover markers is different from the response to exogenously administered fixed dose PTH which results in an early increase in bone formation markers preceding any increase in resorption by about a month. The apparent difference may be a result of several individual factors or more likely a combination of these factors. This may include the gradual increase in GH dose, the subsequent gradual increase in circulating GH/IGF-1 concentrations, paracrine and autocrine effects of IGF-1 and other growth factors in the bone microenvironment, an increase in bone cell sensitivity to endogenous PTH and changes in bone cell responsiveness to changes in the circadian rhythm of endogenous PTH.

In conclusion, our results show that lower GH and IGF-1 concentration are associated with target organ insensitivity to the effects of PTH and abnormal PTH circadian rhythm in postmenopausal women with osteoporosis. GH administration restores PTH sensitivity and PTH rhythm with subsequent changes in bone turnover, Ca and PO_4 metabolism resulting in positive bone balance contributing to the delayed increase in bone density demonstrated in previous studies.

Chapter 6

The Putative Role of Osteoprotegerin in Mediating the Effects of Parathyroid Hormone Circadian Rhythm in Postmenopausal Women

6.1. Introduction

Osteoclast resorptive activity demonstrates circadian rhythmicity and is controlled by various endocrine hormones and cytokine factors. PTH is one of the most important of these systemic regulators, controlling bone mineral homeostasis. PTH circadian rhythmicity is well established in healthy individuals (58, 70, 72, 81, 84) and there is increasing evidence that fluctuations in PTH secretion may have an important effect in governing normal bone health, bone turnover and bone remodelling. The nocturnal rise in PTH secretion is blunted in osteoporotic women (66) and PTH circadian rhythm is lost in primary hyperparathyroidism and returns following parathyroid surgery (59). PTH rhythm abnormalities have also been demonstrated in adult GH deficient patients who have a high incidence of osteoporosis (51, 91). PTH has a concentration and duration dependent biphasic effect, and can induce opposing responses resulting in both net bone loss and net bone formation (428-432) and the manipulation of the PTH rhythm presents an important therapeutic possibility for the treatment of osteoporosis by affecting bone turnover (428). At a cellular level the effect of PTH on bone turnover is mediated by the production of factors from osteoblasts that target osteoclast activity (433-437). The recent discovery of OPG and RANKL as the fundamental factors controlling bone turnover has significantly advanced the understanding of the processes involved in osteoclastogenesis and bone remodeling (433-437). Bone remodelling requires the synthesis of bone matrix by osteoblasts along with coordinated resorption by osteoclasts. This process is controlled by osteoblasts through the expression of RANKL and OPG (433-439). OPG acts as a decoy receptor for RANKL and prevents osteoclastogenesis and bone resorption by inhibiting the signals induced by RANKL–RANK interaction (434, 435) and has recently been shown to demonstrate a possible circasemidian rhythm in healthy subjects (440).

Subcutaneous administration of human PTH (1-38) peptide has been shown to induce a rapid and transient decrease in OPG mRNA in both metaphyseal and diaphyseal bone of rats (441). In animal osteoblast cell culture, PTH stimulates RANKL and suppresses OPG expression (441-444). A negative association between OPG and PTH concentrations has been demonstrated in men who are over 40 (445) and human PTH (1-34) administered to postmenopausal women with glucocorticoid induced osteoporosis decreases the circulating concentration of OPG and increases RANKL (446). Animal and human studies have also shown the importance of intermittent PTH injections in increasing trabecular bone mass, whereas continuous PTH infusions favor bone resorption (63-65). The catabolic effect of continuous administration of human PTH (1-38) is associated with inhibited OPG production (429).

The circadian rhythm of PTH correlates significantly with the circadian rhythms of bone resorption marker, βCTX (51) but the factors mediating the rhythm related effect are still unexplained. The recent demonstration of the circasemidian rhythm of OPG raises the possibility of this as a putative pathway mediating this effect. We have investigated the dynamic relationship between circulating PTH, OPG and βCTX over a 24 h period in premenopausal women, postmenopausal women and elderly men.

6.2. Subjects and Methods

6.2.1. Patients and Controls

Subjects were recruited from hospital personnel and from a database of volunteers willing to participate in medical research. Eighteen subjects were recruited for the study, six healthy premenopausal women (mean age 30.2 ± 2.2 years), six healthy postmenopausal non-osteoporotic elderly women (mean age 68.2 ± 2.6 years) and six healthy elderly men (mean age 68.2 ± 2.3 years).

6.2.2. Methods

Study visits occurred as documented in section 4.2.3. The local ethics committee approved the study, and written informed consent was obtained from each patient before recruitment.

6.2.3. Bone Mineral Density

All subjects underwent bone densitometric evaluation as described in section 3.4.Mean age, LS (L2 – L4) and FN T-score are summarized in Table 6.1.

Table 6.1 Characteristics of Study Population

	Age (years)	T-Score	
		Lumbar Spine (L2 – L4)	Femoral Neck
Healthy Elderly Men (n=6)	68.17	1.08	-0.02
± SEM	1.91	0.84	0.32
Premenopausal Women (n=6)	30.17	0.77	0.80
± SEM	2.17	0.36	0.46
Postmenopausal Women (n=6)	68.17	1.22	1.27
± SEM	2.12	0.24	0.25

6.2.4. Biochemistry

PTH, OPG and βCTX were measured on hourly samples using methods as described in section 3.2.

6.2.5. Statistical Analysis

PTH, OPG and βCTX circadian rhythms were assessed as described in section 3.3.3. Following the confirmation of concerted circadian rhythms further analysis of the more extensively studied PTH rhythm was performed as the next step as described in section

3.3.3. Changes in OPG and βCTX concentration were then analysed during corresponding time periods in relation to the changes in PTH circadian rhythm.

Cross-correlation analysis was performed to determine the relationships between the 24 h profiles of PTH, OPG and βCTX as described in section 3.3.4.

The differences between groups were determined using ANOVA for repeated measures taking into account the 25 measurements for each of the 6 individuals in each group. Repeated measures ANOVA assumes normally distributed errors, equal variances and sphericity. The Kolmogorov-Smirnov test was used to confirm normal distribution and Levenes's test for equality of variances. Mauchly's test indicated that the sphericity assumption was violated and degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity (ε=0.62). The between group comparisons of circadian parameters however was performed with n=6 values one for each of the individuals in each group, using students t-test for unpaired data. Significant differences are highlighted (*) in Table 6.2. This comparison is subject to type 2 error given the limited number of individuals in the study and the values of circadian parameters presented in Table 6.2 are MESOR, amplitude and acrophase from the population mean cosinor analysis (CHRONOLAB 3) for the analytes in all 3 groups. Values are expressed as the mean ± SEM. $p<0.05$ was considered significant.

6.3. Results

6.3.1. 24-hour Mean Concentrations

6.3.1.1. PTH

The difference in 24-h mean PTH concentrations between premenopausal women (4.7 ± 0.1 pmol/L) and elderly men (5.4 ± 0.1 pmol/L) approached significance with p=0.08. PTH concentration was highest in postmenopausal women (5.8 ± 0.1 pmol/L) and was significantly higher than in the premenopausal women and elderly men (p<0.001).

6.3.1.2. Osteoprotegerin

24-h mean OPG concentration was significantly higher in the elderly men as compared to the premenopausal women (4.1 ± 0.1 vs. 3.7 ± 0.1 pmol/L; p<0.001). Concentrations were highest in the postmenopausal women (4.8 ± 0.1 pmol/L; p<0.001 when compared to premenopausal women and elderly men).

6.3.1.3. βCTX

24-h mean βCTX concentration was significantly higher in the elderly men as compared to premenopausal women (0.21 ± 0.02 vs. 0.14 ± 0.03 μg/L; p<0.001). βCTX concentrations were highest in postmenopausal women (0.33 ± 0.03 μg/L; p<0.001 when compared to premenopausal women and elderly men).

6.3.2. Circadian Rhythm Analysis

Individual and population mean cosinor analyses demonstrated significant circadian rhythms for PTH ($p<0.05$), OPG ($p<0.05$) and βCTX ($p<0.001$) in all patients. Cosinor-derived population mean circadian rhythms of OPG, PTH and βCTX are presented in Figure 6.1 and population mean parameters for OPG are shown in Table 6.2. The individual cosinor parameters are shown in Table 6.3.

6.3.2.1. PTH

Although the circadian rhythm of PTH was maintained in all groups we observed an initial increase in PTH concentration beginning at 1600 h (Individual range: 1400 h to 1700 h) followed by a later increase beginning at 2400 h (Individual range: 2200 h to 2400 h) in premenopausal women, consistent with previous reports in healthy subjects (6). Elderly men also demonstrated a PTH increase beginning at 1700 h (individual range: 1400 h to 1700 h) followed by a second increase beginning at 0100 h (individual range: 2400 h to 0200 h). To further highlight the differences in secretory pattern between groups, we compared the mean percent nocturnal increase from 1600 h [value at each time point-1600 h concentration)/1600 h concentration X 100] between 1600 h and 2400 h (times of onset of the PTH peaks in young, premenopausal, healthy women) and 2400 h and 0800 h (end of overnight fast) (51, 91) and no significant difference between men and premenopausal women was observed (1600 h-2400 h 7 ± 2% vs. 6 ± 2% ; p=0.4) (2400 h-0800 h 16 ± 2% vs. 11 ± 2%; p=0.7).

A single sustained increase in PTH secretion between 1600 h and 0800 h was observed in the postmenopausal women. The percent increase in PTH secretion between 1600 h and

2400 h was 14 ± 2% in postmenopausal women and was significantly higher as compared to premenopausal women (7 ± 2%; $p<0.05$). No significant difference in mean PTH percent increase was observed between 2400 h to 0800 h when postmenopausal women (15 ± 2%) were compared with premenopausal women (16 ± 2%; $p=0.7$).

6.3.2.2. Osteoprotegerin

In all subjects, the circadian rhythm of OPG secretion was characterized by higher day time concentrations and a nocturnal decrease. Time of onset of OPG decrease was defined as the time of first occurrence of at least 3 consecutive samples lower than the mean levels obtained between 0800 h and 1400 h by more than 1 SD. The nocturnal decrease began at 1800 h in premenopausal women (individual range: 1500 h to 1900 h), 1600 h in elderly men (individual range: 1500 h to 1900 h) and 1600 h in postmenopausal women (individual range: 1600 h to 1800 h). We calculated the percent nocturnal decrease in OPG between 1600 h and 2400 h (time period during which significant changes in PTH secretion were observed). A greater percent decrease [(value at each time point-1600 h concentration)/1600 h concentration X 100] in nocturnal OPG secretion was observed in the postmenopausal women (15 ± 2%) compared to the premenopausal women (2 ± 2%; $p<0.01$) and the men (7 ± 2%; $p<0.05$) between 1600 h and 2400 h. The percent decrease was also greater between 2400 h to 0800 h in post menopausal women as (23 ± 2%) compared to the premenopausal women (3 ± 2%; $p<0.01$) and the elderly men (6 ± 2%; $p<0.01$). No significant difference was observed between the men and premenopausal women.

6.3.2.3. βCTX

βCTX concentrations demonstrated a nocturnal increase beginning at 2100 h in premenopausal women and men (individual range: 2000 h to 2200 h) and at 2000 h in postmenopausal women (individual range: 2100 h to 2200 h). The percent increase in βCTX concentration between 1600 h and 2400 h in postmenopausal women (58 ± 8%) was higher than in premenopausal women (16 ± 8%; $p<0.05$) and elderly men (19 ± 8%; $p<0.05$) with no significant difference seen between premenopausal women and elderly men. The percent increase in βCTX concentration between 1600 h and 0800 h was higher in postmenopausal women (103 ± 12%) as compared to premenopausal women (57 ± 12%; $p<0.05$) and elderly men (60±12%; $p<0.05$).

Figure 6.1. Cosinor-derived circadian rhythms of PTH, OPG and βCTX in healthy elderly men, premenopausal women and postmenopausal women. Higher concentrations with a sustained nocturnal rise of PTH in postmenopausal women were associated with a greater nocturnal decline in OPG and a corresponding higher nocturnal peak in βCTX, compared with healthy elderly men and premenopausal women.

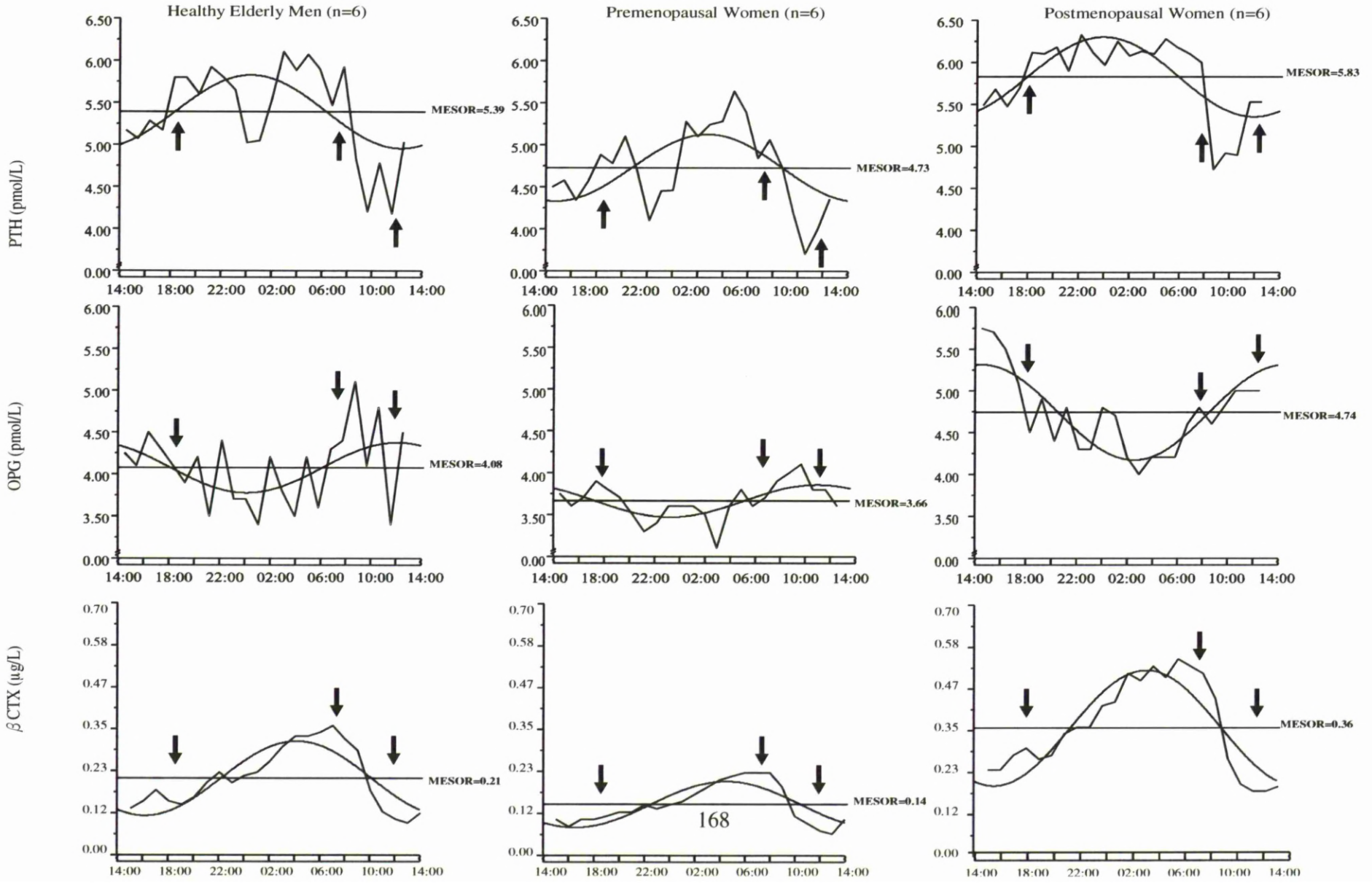


Table 6.2. Mean Circadian Rhythm Parameters of OPG, PTH and βCTX

Parameter	OPG	PTH (1-84)	βCTX
Healthy Elderly Men (n=6)			
Mesor	4.08 ± 0.08 pmol/L	5.39 ± 0.1 pmol/L	0.21± 0.01μg/L*
Amplitude	0.30 ± 0.01 pmol/L	0.44 ± 0.01 pmol/L	0.10± 0.01μg/L*
Time at peak (h)	1205 (1045 - 1325)	0015 (2305 - 0125)	0311 (0243 – 0339)
Statistic significant	$p < 0.05$	$p < 0.01$	$p < 0.001$
Premenopausal Women (n=6)			
Mesor	3.66 ± 0.03 pmol/L	4.73 ± 0.08 pmol/L	0.14 ± 0.01μg/L*
Amplitude	0.19 ± 0.01 pmol/L	0.40 ± 0.01 pmol/L	0.06 ± 0.001μg/L*
Time at peak (h)	1150 (1025 - 1315)	0250 (0130 - 0410)	0330 (0300 – 0400)
Statistic significant	$p < 0.002$	$p < 0.006$	$p < 0.001$
Postmenopausal Women (n=6)			
Mesor	4.74 ± 0.06 pmol/L	5.83 ± 0.06 pmol/L	0.36 ± 0.01μg/L*
Amplitude	0.57 ± 0.01 pmol/L	0.47 ± 0.01 pmol/L	0.16 ± 0.001μg/L*
Time at peak (h)	1430 (1335 - 1525)	2350 (2300 - 0040)	0235 (0213 – 0257)
Statistic significant	$p < 0.001$	$p < 0.001$	$p < 0.001$

Values are expressed as mean ±SEM, and range is presented in parentheses.

* $p<0.05$ compared to other 2 groups

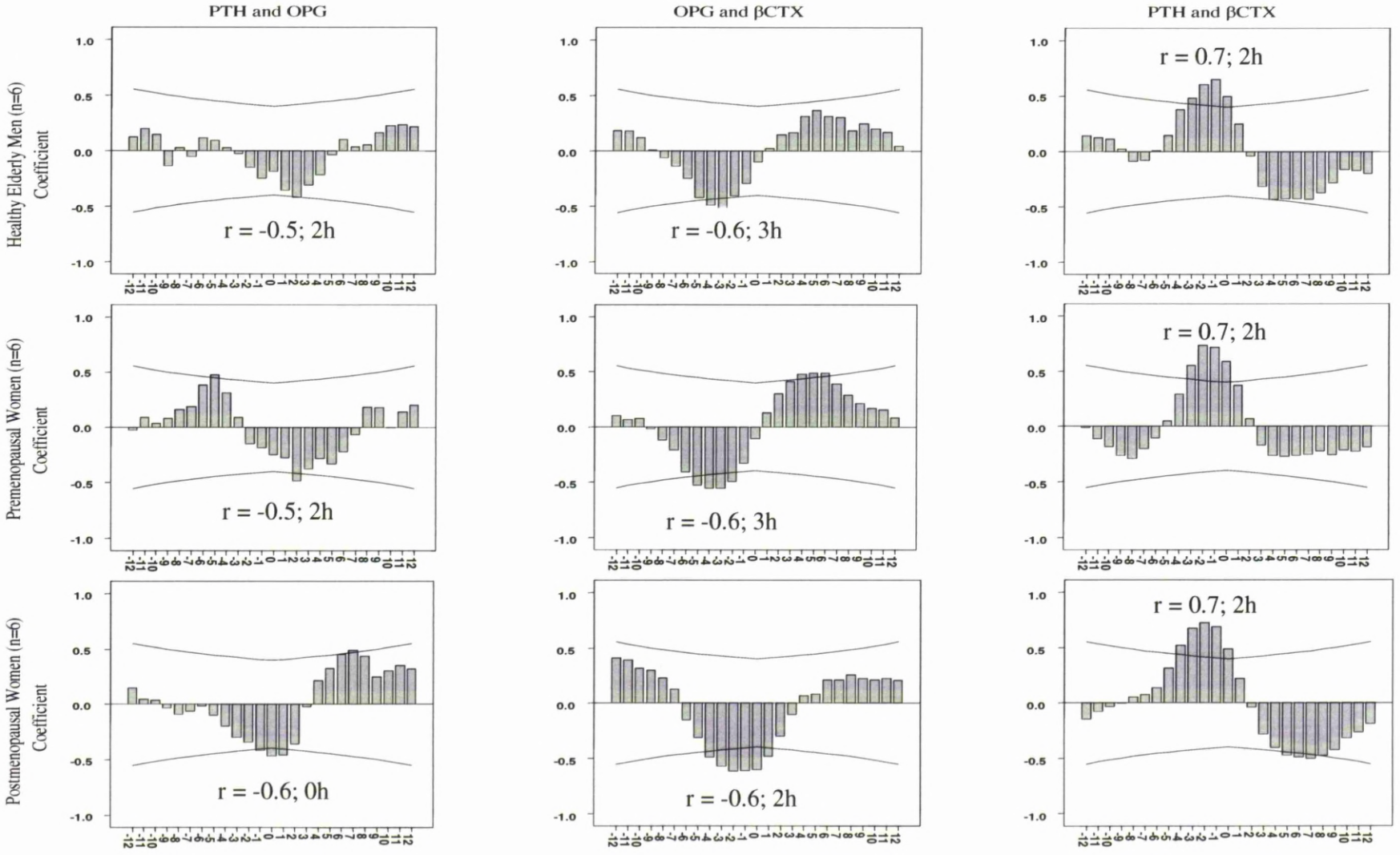
Table 6.3. Circadian Rhythm Parameters of OPG in Individual Subjects

	MESOR (pmol/L)	Amplitude (pmol/L)	Acrophase (h)	p-value
Healthy Elderly Men (n=6)				
1	2.61 ± 0.13	0.27 ± 0.02	1135	<0.05
2	4.71 ± 0.23	0.23 ± 0.02	1045	<0.05
3	5.56 ± 0.14	0.38± 0.02	1325	<0.01
4	2.62 ± 0.14	0.42± 0.02	1222	<0.05
5	4.34 ± 0.09	0.41± 0.02	1204	<0.001
6	4.52 ± 0.41	0.51± 0.03	1240	<0.05
Premenopausal Women (n=6)				
1	3.21 ± 0.05	0.14± 0.01	1236	<0.01
2	3.31 ± 0.04	0.14± 0.01	1025	<0.05
3	6.25 ± 0.16	0.52± 0.03	1124	<0.05
4	3.40 ± 0.05	0.24± 0.02	0946	<0.05
5	3.67 ± 0.08	0.39± 0.02	1254	<0.01
6	2.05 ± 0.05	0.23± 0.01	1315	<0.001
Postmenopausal Women (n=6)				
1	4.74 ± 0.15	0.49± 0.02	1344	<0.05
2	4.90 ± 0.17	0.49± 0.02	1458	<0.05
3	4.05 ± 0.26	0.53± 0.02	1510	<0.01
4	4.23 ± 0.14	0.16± 0.01	1525	<0.05
5	5.80 ± 0.15	0.48± 0.02	1242	<0.001
6	4.29 ± 0.12	0.49± 0.02	1335	<0.01

6.3.3. Cross-correlation Analysis

Secretory patterns of PTH and OPG were inversely related and mirrored each other during a 24 h period (Figure 6.2). Maximal negative correlation between PTH and OPG was observed when PTH changes preceded OPG changes in the opposite direction by 2 hours in premenopausal women and elderly men (r = -0.5). A shift in maximum negative correlation between PTH and OPG rhythms was observed in postmenopausal women to 0 lags (r = -0.6) suggesting that changes in PTH and OPG occurred at the same time in opposite directions. Maximum negative cross correlation between OPG and βCTX was observed when OPG changes preceded βCTX changes by 3 hours in premenopausal women (r = -0.6) and men (r = -0.6) and 2 hours in postmenopausal women (r = -0.7). PTH and βCTX demonstrated a positive correlation (r = 0.7) with a constant lag time of 2 hours in all 3 groups. Cross correlation with log transformed values confirmed these findings.

Figure 6.2. Cross-correlation analysis of PTH, OPG and βCTX in healthy older men, premenopausal women and postmenopausal women. Graphs represent the time lag between changes in one analyte in relation to another. Highest r values representing strength of correlation with time lag in hours is represented in each graph. Relationship between PTH and OPG as well as relationship between OPG and βCTX were altered in postmenopausal women when compared with older men and premenopausal women.



6.4. Discussion

Changes in hormonal concentrations are important for physiological and pathophysiological effects (447, 448). Circadian rhythms are known to exist for bone turnover and bone mineral metabolism and can be altered by changes in PTH circadian rhythm (66, 72, 81, 84, 449, 450). Intermittent PTH injections increase trabecular bone mass, whereas continuous PTH infusion favors bone resorption with inhibited OPG production (63-65, 429). The significant differences in the circadian rhythms, temporal interactions and concentrations of PTH, OPG and βCTX secretion we observed when postmenopausal women were compared with elderly men and premenopausal women suggest that the rhythmicity of osteoclast activity and the increased osteoclast activity in postmenopausal women may be mediated by this pathway.

A circasemidian rather than circadian rhythm for OPG has been reported with a bimodal variation in a mixed group of 9 subjects (mean age of 32.5 ± 7.8 years). The findings were based on 4-hourly samples and a circadian rhythm could not be demonstrated using cosinor analysis due to the limited number of samples, and results were based on data expressed as percentages of the arithmetic mean of each time series (440). Our data confirms a circadian rhythm for OPG with a pattern different to that reported previously. The difference in age and gender of the groups studied, as well as the difference in OPG assay used may have been contributory factors, but more importantly cosinor analysis is dependent on the sampling frequency and bio-kinetic properties of the analyte. Despite its short half-life (451) the circadian characteristics of PTH have been demonstrated with hourly sampling in this study but the characteristics can not however be demonstrated with samples greater than 2 hours apart (452). Thus increased sampling frequency provides a more accurate representation of circadian rhythmicity. The half-life of OPG has been shown to be

approximately 20 minutes in rats (435) but no human data is available. All studies measuring circulating OPG are also limited by the multiplicity of its extra-skeletal sources and the possible discrepancy between circulating concentrations and concentrations in the bone micro-environment. Despite these factors the increased sampling frequency in our study results in a more accurate representation of circulating OPG concentrations over a 24-h period.

The profiles of OPG and PTH in our patients were inversely related and mirrored each other suggesting that the expression of PTH and OPG may be linked to common rhythm generating or common regulatory factors and that fluctuations in PTH concentration over a 24 hour period may directly or indirectly regulate OPG production, consistent with the known suppressive effect of PTH on OPG production *in vitro* and in *vivo*. PTH (1-34) suppresses OPG and stimulates RANKL both *in vitro* and *in vivo* and an inverse correlation between PTH and OPG has been established (429, 441-444, 446, 453). Pharmacological administration of human PTH (1-34) decreased circulating OPG levels in postmenopausal women with glucocorticoid induced osteoporosis (446) and PTH has also been shown to negatively correlate with serum OPG levels in men beyond 40 years after adjustment for age and body weight (445).

Osteoclast activity is regulated via cross-talk between osteoblasts and osteoclasts mediated by OPG and RANKL and blocking RANKL signaling with OPG is able to block the pro-resorptive effects of PTH. Although controversial, PTH may also directly affect osteoclast activity via PTH receptors (116, 454). The maximum correlation between PTH and βCTX remained constant in all 3 groups but in postmenopausal women the temporal relationship between PTH and OPG and OPG and βCTX was altered. It is possible that the constant time lag between PTH and βCTX is a reflection of the direct effect of PTH on osteoclasts

which remains unaltered. The altered temporal relationship between PTH, OPG and βCTX may reflect changes in the indirect effect of PTH on osteoclasts via osteoblast cross-talk. In vitro, PTH (1-34) administration suppresses OPG and stimulates RANKL gene expression within 1 hour (429, 441-444, 446, 453) and in vivo subcutaneous administration of a single injection of PTH (1-38) at 80 μg/kg has been shown to induce a rapid and transient decrease in OPG mRNA in both metaphyseal and diaphyseal bone of rats that is evident by 1 hour (441). Our data indicate that changes in OPG concentration follow changes in PTH by two hours in premenopausal women and in healthy elderly men. Observational data must be interpreted with caution as they do not entirely reflect data following pharmacological administration. In postmenopausal women the temporal relationship between PTH and OPG was altered, with changes in PTH and OPG occurring concurrently. The altered effect of PTH on OPG may be a consequence of the altered hormonal milieu, including the lack of oestrogen following the menopause and/or the declining GH and IGF-1 concentrations with increasing age (277-283) either interfering with the effect of PTH on OPG production by osteoblasts or affecting osteoblast production of OPG directly and thus altering the temporal relationship. The 3 hour delay between changes in OPG and βCTX probably reflects a separate osteoblast mediated component of osteoclast activity which is altered in postmenopausal women via similar mechanisms. These temporal alterations may contribute to the significantly greater nocturnal decrease in OPG concentrations and increased osteoclast activity in postmenopausal women. Interpretation of the temporal relationships reported in our study is limited by the cross-sectional design of the study and dependency of the statistical technique on sampling frequency of the analytes. Further interventional studies designed to induce shifts in the analyte concentrations and more frequent sampling would be required to confirm the exact time taken for PTH to alter circulating OPG and βCTX in these different patient groups.

OPG is a major biological factor that inhibits osteoclast differentiation, formation and function (434, 435, 455, 456) and circulating OPG concentrations are altered in conditions of abnormal bone remodeling (423, 433, 435, 439, 457-461). Results from previous studies using single time point sampling methodology have been inconsistent and while some studies have demonstrated a negative correlation between circulating OPG and bone resorption markers (457, 459, 461) others have not (462-466). The negative correlation of serum OPG concentrations with BMD has also been reported by some (460, 467, 468) but not others (462, 464-466). High serum OPG concentrations have been observed in patients with Paget's disease (457) but not from patients with severe osteolysis (469). These discordant results may reflect the single time point (fasting sample) methodology in sample collection. Our observation that OPG is subject to diurnal variation may help explain some of these inconsistencies. Future studies on circulating OPG must take into consideration this variability.

The majority of data currently supports higher circulating OPG in conditions where bone resorption predominates over bone formation. Higher OPG concentrations have been reported in elderly and postmenopausal women with and without osteoporosis (458-461, 470, 471) and our findings are in keeping with this. Because serum OPG was positively correlated with biochemical markers of bone resorption in these studies this was thought to be a somewhat ineffective counter-regulatory mechanism to prevent further bone loss (439). Alternative explanations are that OPG clearance is decreased in the elderly or there is enhanced release from bone with aging due to microfractures. Because of the contradiction between local OPG levels, which decrease with aging, and the unambiguous findings of increased circulating OPG serum levels with aging, it is unclear whether circulating OPG adequately reflects local OPG production within the bone

microenvironment, especially during aging (445). Several reports have also established an inverse relationship between OPG and bone resorption markers (423, 461, 472). A significantly higher mean nocturnal percent decline in OPG may result in increased nocturnal bone resorptive activity by osteoclasts as observed in our postmenopausal subjects and explain the apparent contradiction and ineffectiveness of higher fasting or 24-h mean OPG concentration in postmenopausal women. The dynamic change in OPG concentration we have observed may be the important regulatory factor for osteoclast function rather than absolute circulating values.

OPG activity goes hand in hand with RANKL activity with the ratio between the two being more relevant to bone cellular activity than absolute values of either taken individually. Most RANKL activity is provided by its expression on the osteoblast cell surface. Current RANKL assays are problematic and not sufficiently sensitive as they measure soluble RANKL that is unstable, is degraded rapidly or binds to OPG to form large stable conglomerates (473) . RANKL profiles were investigated in several individuals from each group using a current available assay (ELISA measurement provided by Biomedica, Oxford Biosystems, UK) but the concentrations of RANKL obtained were all close or below the assay detection limit and no statistically significant variability was observed and this data was not included in the study. Since OPG is a decoy receptor for RANKL and they have an inverse relationship, it is possible that total RANKL may demonstrate diurnal variation opposite to that of OPG but similar to PTH. Future studies with more sensitive RANKL assays would be required to confirm or refute this hypothesis.

In conclusion, we have confirmed a significant circadian rhythm for OPG in different subject groups and propose that altered OPG circadian rhythm may be an additional factor contributing to postmenopausal bone loss. The circadian rhythm of PTH is recognized as a significant regulator of bone turnover and the rhythm mediated effect may be, at least in part, mediated by the rhythm of OPG. Significantly greater nocturnal decline in circulating OPG in postmenopausal women may in part alter the circadian pattern of osteoclast activity on a daily basis resulting in higher nocturnal resorption and net bone loss. The rate of change of OPG concentration could contribute to the rate of bone resorption over and above the absolute circulating OPG concentration. Manipulation of the endogenous rhythm of OPG presents another possible therapeutic target for osteoporosis and our findings warrant future studies aimed at identifying factors that may be utilized to modify the circadian secretion of OPG to prevent bone loss.

Chapter 7

Conclusions

The studies in this thesis have further defined the role of the GH/IGF-1 axis, target-organ sensitivity to PTH and PTH secretory rhythm in the pathogenesis of age related and postmenopausal osteoporosis. The findings also highlight the distinct differences in the patterns of bone loss and underlying mechanisms between men and women.

Various lines of investigation have suggested that GH plays a central role in the development of primary osteoporosis. Advancing age is associated with a reduction in GH secretion and so it has been proposed that a relative GH deficiency in older patients may underlie the development of age-related osteoporosis (280, 290). A relative GH deficiency also occurs following the menopause. The presence of oestrogen is required for GH secretion, thereby providing a mechanism by which oestrogen regulates bone turnover indirectly through its effect on the GH/IGF-1 axis (290, 293, 474, 475). Therefore, a reduction in GH secretion in postmenopausal women may contribute to the development of osteoporosis (293, 474). Despite these facts previous attempts to administer GH therapy in primary osteoporosis have been disappointing. Markers of bone turnover have been shown to increase following the administration of GH to postmenopausal women but with poor tolerability and little benefit in terms of improvement in BMD (378, 384, 385). More recently, however, a 5 year study reported a 15 % increase in BMD following low dose GH therapy, with few side effects reported (388). Despite these findings the underlying mechanisms have not been fully investigated or understood.

The role of insensitivity to PTH and abnormal PTH rhythm has been established in the AGHD model of osteoporosis (51, 91) and the studies in this thesis help define a role for insensitivity to PTH and abnormal PTH rhythm in the development of osteoporosis in postmenopausal women. PTH sensitivity improves and PTH rhythm is restored following GHR in patients with AGHD. These mechanisms contribute to the improvement in bone

turnover and BMD in GH replaced AGHD patients (91). The data presented in this thesis provides evidence that PTH insensitivity and abnormal PTH rhythm contribute to the development of postmenopausal osteoporosis. GH administration to postmenopausal women results in an increase in IGF-1, improved PTH sensitivity and restoration of PTH rhythm in a manner similar to that seen in AGHD patients treated with GH. These changes are associated with significant improvements in bone metabolism and turnover that would result in increased BMD. Furthermore titrated GH doses that demonstrated benefit were well tolerated.

Older men also have lower IGF -1 concentrations with decreased target-organ sensitivity to the effects of PTH. The degree of PTH insensitivity is greater in older men with low BMD. Normal PTH circadian rhythmicity is lost and bone resorption is increased in older men with low BMD but not in older men with normal BMD. Primary osteoporosis in men is less well studied and various etiological mechanisms have been suggested. We propose that the age related decline in GH and IGF-1, modified by the age related decline in bio-available gonadal hormones results in PTH resistance and abnormal PTH rhythm. Both of these mechanisms contribute to the development of bone loss in older men with low BMD.

Although the importance of the secretory rhythm of PTH is well documented (58, 70, 72, 81, 84), the mechanisms by which the altered secretory rhythm increases bone resorption and bone loss is unclear. Our findings suggest that OPG may mediate the rhythm dependent effect of PTH on bone turnover. Loss of normal PTH circadian rhythmicity in postmenopausal is associated with a greater nocturnal decline in circulating OPG. This in turn may account for the nocturnal increase in bone resorption seen in postmenopausal women and hence postmenopausal bone loss.

The development of effective treatments for postmenopausal osteoporosis is particularly important in view of the decline in use of oestrogen therapy following the announcement of detrimental effects of HRT in the Women's Health Initiative study (476). Bisphosphonates currently represent standard treatment of postmenopausal osteoporosis but increases in BMD are limited by the reduction in bone remodelling space occurring as a consequence of reduction in bone resorption (477).

In women with postmenopausal osteoporosis, daily subcutaneous injections of full length parathormone (1-84 PTH) and teriparatide (the 1-34 N-terminal fragment of PTH) showed the largest increases in BMD reported to date for any treatment of osteoporosis, accompanied by an important reduction of the risk of new vertebral fractures (478, 479). Teriparatide was shown to reduce the risk of vertebral and non-vertebral but not hip fractures in postmenopausal and glucocorticoid-induced osteoporosis (479, 480). Although the safety and efficacy of teriparatide have been studied beyond two years of treatment, the generally approved duration of therapy is limited to two years.

While therapeutic alternatives are available for inhibiting bone resorption, options are much more limited with regard to bone anabolic substances. There is a need for new bone active substances aimed at restoring quantitatively and qualitatively normal bone. The safety profile of most bone active substances is at least partially known, but there is considerable potential for improvement (osteonecrosis of the jaw, atypical fractures, interactions with the immune system). Treatment alternatives are needed for patients exhibiting or at risk of side-effects. While a relative fracture risk reduction of up to 20%, 50%, and 70% can be achieved for non-vertebral, hip, and spine fractures, respectively, still 30 to 80% of these fractures cannot be prevented. As osteoporosis is a systemic

disease, there is an obvious need for improving the magnitude of the therapeutic effect at all fracture sites. Therefore limitations currently exist with available effective therapies for the treatment of primary osteoporosis (481).

Although GH has previously been relegated to a forgotten corner of skeletal anabolics, the recent demonstrated benefit in BMD with GH doses that were well tolerated provide some room to reconsider the notion that there may still be a role for GH therapy in the management of primary osteoporosis. Recent studies using adjunctive therapies with GH in AGHD patients have been promising. Similar studies in primary osteoporotic patients may help achieve better improvements in BMD with GH. An understanding of the intricate mechanisms by which GH exerts its anabolic effects is thus essential. It may be possible in the future to harness its full potential as a possible anabolic therapeutic modality for osteoporosis, a disease for which treatment options are still limited.

Chapter 8

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Appendix I: Published Work

1. Joseph F, Ahmad AM, Ul-Haq M, Durham BH, Whittingham P, Fraser WD, Vora JP. Effects of Growth Hormone Administration on Bone Mineral Metabolism, Parathyroid Hormone Sensitivity and Parathyroid Hormone Secretory Rhythm in Postmenopausal Women with Established Osteoporosis. Journal of Bone and Mineral Research. 2008 May;23(5):721-9.

2. Joseph F, Chan BY, Durham BH, Ahmad AM, Vinjamuri S, Gallagher JA, Vora JP and Fraser WD. The Circadian Rhythm of Osteoprotegerin and its Association with Parathyroid Hormone Secretion. Journal of Clinical Endocrinology and Metabolism. 2007 Aug;92(8):3230-8.

Appendix II: Oral Presentations at National and International Meetings

1. F Joseph, BY Chan, JA Gallagher, P Whittingham, S Vinjamuri, WD Fraser and JP Vora. Effect of growth hormone therapy on in vitro cultured osteoclasts in adult growth hormone deficiency. American Endocrine Society Meeting 2006

2. Joseph F, M Ul Haq, S Shimjee, Chan BY, White HD, AM Ahmad, Durham BH, Vinjamuri S, Gallagher JA, JP Vora, WD Fraser. The Circadian Rhythm of Osteoprotegerin and its Association with Parathyroid Hormone Secretion in Elderly Men and Women. Bone and Tooth Society 2005