

The Effects of Stroke on the Skeleton

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By

Kenneth E. S. Poole BM MRCP



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"I was looking forward to my 61st birthday in two days time. I enjoyed life- walks with the wind in my hair, driving to see loved ones, working in my garden for hours. Then my life stopped and I had to learn everything again. How to walk, how to dress and feed myself- everything I took for granted. But having to ask for help was hard- I'd been so independent all my life.

The stroke had wiped out all my left side. I walked with a frame with someone beside me and moving the log of wood that was my leg was exhausting. One doctor said it would be five years before my hand would move. I thought 'never' and worked on it hour after hour until I got one finger to move a fraction. The rest of my fingers came back in three months.

I set myself targets and when I walked with a stick three months before the target month I was well chuffed. But last August, a freak fall broke my left hip. This has hit me harder than the stroke did...."

Letter to Stroke News, Volume 19.2, February 2001.

Declaration

This dissertation is my own work and contains nothing that is the outcome of work done in collaboration with others, except as specified in the text and acknowledgements.

Signed

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This work was inspired by clinical observations recorded by Dr. Jonathan Reeve and presented as a case study at an Addenbrooke's Hospital staff round in 2001 (later published in *Osteoporosis International*, see Appendix 4). I wish to express my deepest gratitude to Dr. Reeve, my supervisor, for his expert guidance and mentorship. I would also like to thank Dr. Liz Warburton and Dr. Nigel Loveridge for their kind support, particularly during my transition from Specialist Registrar in the stroke unit to Clinical Research Training Fellow. I respectfully acknowledge the individual contributions of the patients who volunteered to take part in the clinical trial when they were in the early stages of recovery from a devastating stroke. Collette Rose worked closely with the subjects throughout the study and her commitment and dedication in caring for the stroke patients was a major contribution to the success of the clinical research. Similarly, the excellent staff and facilities of the Addenbrooke's Centre for Clinical Investigation (ACCI) deserve special thanks for providing such a good environment for clinical research.

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Dedication

For Tamsin.

Summary

Stroke is now a well-recognised risk factor for hip fracture. The aim of this study was to elucidate the pathophysiological mechanisms by which hip bone loss occurs in hemiplegia and to test the efficacy of a novel pharmaceutical strategy for preserving bone in stroke patients. Patients who were admitted acutely with a first-ever stroke and who remained unable to walk one week later were studied prospectively for 12 months, with a series of bone mineral density measurements of the hips (dual energy X-ray absorptiometry) in the context of a randomised controlled trial. Untreated patients (n=13) experienced a decline in bone mineral density at the hemiplegic hip that was rapid, with the greatest losses in the trochanteric region of the affected side. This bone loss was prevented by the administration of a single 4 mg dose of the intravenous bisphosphonate, zoledronate (n=14) within 35 days of stroke onset. Computed tomography of the hips in 8 untreated patients more than a year after stroke confirmed that the greatest difference between sides was in the trochanteric region. Serum vitamin D measurements in 44 patients with acute stroke were substantially lower than healthy elderly controls, with 77% of patients in the insufficient range, suggesting that vitamin D insufficiency preceded stroke.

Histomorphometric analysis of iliac bone biopsies from hemiplegic patients 10 weeks following stroke showed normal erosion parameters, but a striking decrease in the surface extent of osteoid when compared with healthy reference values. Unexpectedly, treatment with zoledronate was associated with a significantly higher osteoid surface compared with placebo treated subjects in cancellous, endocortical and cortical bone. Sclerostin, a newly discovered osteocyte-derived protein was studied using immunohistochemical staining of the bone biopsies. Sclerostin is known to be an inhibitor of active osteoblasts, which led to the hypothesis that in stroke, the proportion of osteocytes expressing sclerostin would be inversely associated with the surface extent of bone formation. Histological analysis revealed widespread expression of sclerostin in osteocytes and their canaliculi in all subjects. However, examining individual osteocytes in relation to bone forming surfaces revealed that newly embedded osteocytes did not express sclerostin until after primary mineralisation. It is proposed that this precise pattern and timing of sclerostin expression by osteocytes allows bone formation to continue locally (during remodelling), but prevents excessive new bone formation elsewhere, as seen in the single gene disorder sclerosteosis.

List of Publications

This thesis is based on the following articles:

Poole, K. E. S., Reeve, J., and Warburton, E. A. (2002). Falls, fractures, and osteoporosis after stroke: time to think about protection?. *Stroke*, 33(5), 1432-1436.

Poole, K. E. S., Rose, C., Loveridge, N., Warburton, E. A., and Reeve, J. (2004). A single injection of zoledronic acid may prevent bone loss after stroke: Planned interim analysis of a randomised trial (abstract). *Osteoporos Int*, 15 (Suppl. 2), S1-S64.

Poole, K. E. S., Bezooijen, R. L., Loveridge, N., Hamersma, H., Papapoulos, S., Lowik, C. W., and Reeve, J. (2005). Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. (<http://www.fasebj.org/cgi/reprint/05-4221fjev1>. Published online September 15, 2005. Printed version *in press*). *FASEB Journal*.

Poole, K. E. S., Warburton, E. A., Loveridge, N., Halsall, D. J., and Reeve, J. (2005). A contribution to bone loss in hemiplegic stroke patients may be vitamin D deficiency (abstract). *Stroke*, 36 (Suppl. 2), S37.

Poole, K. E. S., Warburton, E. A., and Reeve, J. (2005). Comment on 'Risedronate therapy for prevention of hip fracture after stroke in elderly women'. (<http://www.neurology.org/cgi/eletters/64/5/811>. Published online May 23, 2005. Printed version *in press*). *Neurology*.

Poole, K. E. S., Warburton, E. A., and Reeve, J. (2005). Rapid long-term bone loss following stroke in a man with osteoporosis and atherosclerosis. *Osteoporosis International*, 16(3), 302-305.

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Thesis Structure

This thesis is structured in the following way. Chapters 1 and 2 are general introduction chapters. The literature review of Chapter 1 introduces the clinical problem of hip fractures after stroke, the assessment of bone loss after stroke and interventions to prevent bone loss in stroke patients. The literature review of Chapter 2 introduces the basic biology of bone, skeletal responses to unloading and how these responses may be assessed histologically. Chapter 2 concludes with a review of the osteocyte and its emerging role in controlling bone formation. Chapters 3, 4, 5 and 6 are original research contributions towards understanding and preventing bone loss after stroke (using a randomised controlled trial, bone densitometry, bone histomorphometry and immunohistochemistry of bone sections). Each of these chapters follows the same format: abstract, introduction, methods, results and discussion. Chapter 7 is a general discussion chapter placing the research findings in context and highlighting potential areas for further work.

Non-Standard Abbreviations

ALP	Alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
BMU	Basic multicellular unit
CI	Confidence interval
CSA	Cross sectional area
CV	Coefficient of variation
DAB	Diaminobenzidine
DXA	Dual-energy x-ray absorptiometry
hQCT	Hip quantitative computed tomography
HSA	Hip Structural Analysis
MMA	Methylmethacrylate
NTX	Amino-terminal telopeptide of type 1 collagen
OC	Osteocalcin
PBS	Phosphate buffered saline
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
QCT	Quantitative computed tomography
RCT	Randomised control trial
ROI	Region of interest
RR	Relative risk
RT	Room temperature
SD	Standard Deviation
SE	Standard Error
TRAP	Tartrate resistant acid phosphatase
um	Micrometers
vBMD	Volumetric BMD
ICTP	1-carboxy C-telopeptide
1,25(OH) ₂ D	1,25 dihydroxy-vitamin D (active)
25OHD	25 hydroxy-vitamin D

Chapter 1. Falls, Fractures and Osteoporosis Following Stroke



Figure 1.1. Computerised tomogram of a 62 year old female with acute stroke. Her left arm and leg hemiplegia were caused by a cerebral infarction shown as an area of hypodense tissue in the right internal capsule (arrowed).



Figure 1.2. Plain x-rays from the same patient less than a year later following a fall from standing height. A sub-capital fractured neck of femur is clearly seen (arrow) on the hemiplegic left side.

1.1 Hip Fractures after Stroke

1.1.1 Epidemiology of Hip Fractures after Stroke

Stroke was defined by the World Health Organisation (1978) as a clinical syndrome of vascular origin, typified by rapidly developing signs of focal or global disturbance of cerebral function lasting more than 24 hours or leading to death. Stroke is a major cause of disability and death (Bonita, 1992). Figures from the UK show that the estimated number of new strokes each year is close to 100,000, with a steep rise with age (Bamford *et al.*, 1988). Additionally, an estimated 70,000 fractures of the hip occur annually in the UK, also rising steeply with age (Dolan and Torgerson, 1998). Patients who survive an acute stroke face numerous early and late complications and of these, hip fracture is perhaps the most serious and disabling. Up to 30% of patients with a fractured neck of femur die within a year of the acute event (Keene *et al.*, 1994), but survivors face pain, disability and loss of independence.

Recognition of hip fracture as a consequence of stroke began in the 1950's (Peszczynski, 1957) and 60's (Hodgkinson and Brain, 1967; Howell, 1965; Moskowitz, 1969), with authors reporting a propensity for fracture on the hemiplegic side. In a retrospective study, Peszczynski (1957) reported that 23 out of 150 hip fracture patients undergoing rehabilitation had a previous stroke, with 14 of the fractures occurring within a year of the stroke. Since then there have been several incidence and prevalence studies (Chiu *et al.*, 1992; Dennis *et al.*, 2002; McClure and Goldsborough, 1986; Mulley and Espley, 1979; Poplingher and Pillar, 1985; Ramnemark *et al.*, 2000; Ramnemark *et al.*, 1998) as well as two large cohort studies based on hospital discharge data (Dennis *et al.*, 2002; J. Kanis *et al.*, 2001). Mulley and Espley (1979) found 57 (3.9%) patients with evidence of previous stroke out of 1456 hip fractures admitted to hospital over 4 years. McClure and Goldsborough (1986) found 10 patients (20%) with post-mortem evidence of stroke from 50 patients who died with a fractured femoral neck. Chiu *et al.* (1992) also performed an analysis of 1430 patients admitted with hip fracture, finding 146 (10.2%) with a history of previous stroke and 82% fracturing the hemiplegic side.

Data from Sweden, the UK and the USA on fracture incidence following stroke has been published. Ramnemark *et al.* (1998) reported 154 fractures in 120 subjects from a cohort

of 1139 patients admitted consecutively with acute stroke, followed for a median 2.9 years (Table 1.1). Eighty-four percent of fractures were caused by falls and hip fracture was the most common. The hip fracture was at the femoral neck in 56% and inter-trochanteric in 44% of stroke patients (which was similar to the ratio observed in the Swedish general population over that time (Lofman *et al.*, 2002)). Hip fracture was 2-4 times more likely than in an age-matched reference population. Median time until onset of first fracture was 24 months.

Table 1.1 Cohort studies of fracture rates following stroke

<i>Cohort Studies</i>	<i>Sweden</i> (Ramnemark <i>et al.</i> , 1998)	<i>UK</i> (Dennis <i>et al.</i> , 2002)	<i>Sweden</i> (J. Kanis <i>et al.</i> , 2001)	<i>UK</i> (Dennis <i>et al.</i> , 2002)
Cohort type	Retrospective	Prospective	Hospital Discharge Data	Hospital Discharge Data
Stroke Patients, n	1139	2696	273288	129935
Hospital treated, %	100	65	100	100
Mean age, yr	73	68	74	73
Fractures occurred in , n	120 (10.5%)	81 (3.0%)	24666 (9.0%)	7999 (6.2%)
Maximum follow up, yr	12	8	10	17
<i>Fracture Site</i>				
Hip, n	70 (6.1%)	26 (1.0%)	14263 (5.2%)	4528 (3.5%)
Upper Limb, n	42	29	-	-
Other, n	42	33	-	-
<i>Rate of Hip Fracture</i>				
Per 1000 person years	17	7	-	12.5

Subsequently, Ramnemark and colleagues (2000) examined outcomes in patients with hip fracture and previous stroke. Results showed that survival and recovery of independent mobility after hip fracture was significantly reduced compared to those who had not had a previous stroke. A longer term follow-up study from the USA showed that stroke patients were more likely to have medical complications and remained in hospital significantly longer than others following hip fracture surgery (Youm *et al.*, 2000). Following surgery for hip fracture in Sweden, the risk of a further stroke increased and post-operative death was more frequent than in those without stroke (Ramnemark *et al.*, 2000). This retrospective study also reported the prevalence of previous stroke amongst inpatients with

hip fracture in one hospital, with data gathered over a 17 year period. From 1980 to 1997, the percentage of hip fracture inpatients that had previously had a stroke more than doubled from 16% to 39% (Ramnemark *et al.*, 2000). The hip fracture rate did not increase in the general population during the study period. While the population incidence of stroke did increase, this alone did not account for the percentage increase in prior stroke seen. Potential explanations include changes in stroke severity and recovery in the population over the study duration. Improvements in the medical management of stroke (particularly acute stroke units) in recent years have led to an increase in survival and functional state following stroke (Indredavik *et al.*, 1999).

More recently, Kanis *et al.* (2001) reported a greater than 4-fold increased risk of sustaining a hip fracture in the immediate post-stroke period compared with the general Swedish population. The analysis was based on a coded database of all Swedish hospital admissions over 10 years and included all strokes, not necessarily hemiplegic individuals. Nine percent of patients had a fracture after hospital admission with stroke and 5.2% had a hip fracture (mean follow-up 2.54 years, table 1.1). There was also a striking increase in the risk of hip fracture during the first year in all ages and in both sexes. Although risk decreased in subsequent years after stroke, it remained higher than the age-matched population risk. In this study, the relative risk for hip fracture after stroke was highest in younger subjects, but the absolute risk for fracture was highest in older stroke patients (since age is the greatest independent risk factor for hip fracture).

Dennis *et al.* (2002) showed an increased rate of hip fracture after stroke in the UK, although lower in magnitude than the rate observed in Sweden (table 1.1). Two approaches were used, a prospective cohort study and an analysis of routine hospital stroke discharge data. In the prospective cohort study (2696 stroke patients) the rate of hip fracture was 1.4 times the rate observed in the general population, whereas the UK hospital discharge data suggested it was 1.7 times higher. Increasing age, female sex, pre-stroke dependency and a mental test score of less than 8/10 were associated with higher hip fracture rates. In keeping with previously published data, substantially increased hip fracture rates were observed in the first year following stroke. The difference in relative risk of hip fracture reported between Sweden and the UK remains largely unexplained, but may in part be due to differences in study methodology. A large proportion (over one third) of patients in the

UK prospective cohort study (Dennis *et al.*, 2002) were recruited from a neurovascular clinic, where the stroke severity is typically much less than that seen in patients admitted to hospital with stroke (as in the Swedish studies). The incidence of hip fracture in the general population is also higher in Sweden (2.02/1000) than in the UK (1.44/1000, Kanis *et al.*, 2002), suggesting an environmental or genetic risk factor exists in Sweden, which is enhanced by suffering a stroke.

One study of long-term fracture risk in 378 patients after ischaemic stroke from the USA showed a relative risk of hip fracture of 1.9 (95%CI, 1.3-2.6), compared to that expected from the age-matched population (Melton *et al.*, 2001). However, in this retrospective case-controlled cohort study there was an unexpectedly high rate of hip fractures in the control group, so that the high hip fracture rate following stroke did not appear significantly different. By analysing contemporary medical records for the stroke patients, fracture rates were estimated for three important groups; those with no hemiplegia at stroke diagnosis (Relative Risk 1.0; 95% CI 0.8-1.3), those with functional impairment but who could ambulate independently (RR 1.9; 95%CI 1.2-2.9) and those unable to walk at all or who were bedridden (RR 1.3; 95%CI 0.7-2.4). Fifty-three percent of the stroke patients had hemiplegia in the study. No other studies of hip fracture incidence following stroke have addressed this crucial aspect, i.e. the heterogeneity in stroke type, severity and recovery that occur. Since the term 'stroke' covers a large spectrum of clinical deficits (from mild impairment to profound disability and loss of function), studies relying solely on coded hospital discharge data are unable to determine the fracture risk for the various sub-groups. The study of Melton *et al.* (2001) is important in confirming that the group at most risk of hip fracture following stroke appears to be patients with initial functional disability and hemiplegia who subsequently recover ambulation. Establishing the hip fracture rate for stroke patients based on their baseline deficit, recovery characteristics and stroke type would assist greatly in targeting an intervention to prevent hip fractures. A large study is underway using large epidemiological databases from four countries to address some of these issues (personal communication, Professor S. Cummings, San Francisco, USA).

1.1.2 Mechanisms of Hip Fracture after Stroke

Most hip fractures after stroke are on the paretic side (Chiu *et al.*, 1992), with 84% caused by accidental falls in one series (Ramnemark *et al.*, 1998). Forster and Young (1995) followed 108 patients with mild to moderate disability after stroke and found 73% had fallen in the six months following discharge. Recurrent falls are also common after stroke; in one study of home dwelling stroke survivors more than a year post-stroke, 48% percent fell within a year, with 19% falling just once, but 29% falling repeatedly (Lamb *et al.*, 2003). Lower limb dysfunction and visual impairment are common after stroke, both of which are also important independent risk factors for hip fractures (Grisso *et al.*, 1991).

In the Study of Osteoporotic Fractures (SOF), Nevitt and Cummings (1993) investigated mechanisms of osteoporotic fracture in older women (>65), with 66% of those who sustained a hip fracture falling sideways onto the hip. In a smaller study that compared fallers who fractured a hip with fallers with only soft tissue injury, sideways falling was also significantly associated with hip fracture (Wei *et al.*, 2001). The SOF cohort of 9704 women was recruited from four states in the USA and has been followed for over 17 years for significant medical outcomes including stroke and fracture. Among the 384 patients in the SOF study who suffered a stroke, the rate of subsequent non-vertebral fractures exceeded that of control participants by about 50% within two years of post-stroke follow up (personal communication, Professor S. Cummings, San Francisco, USA). Stroke patients fall to the side of the paresis (Mulley and Espley, 1979) and findings from the SOF study cohort may explain why such ‘hemiplegia-side’ falls commonly result in fracture. The two major factors that determined whether a fracture occurred after a fall were bone density at the hip (which is known to decline rapidly in hemiplegic patients, Chapter 1.2.3) and the ability of the ipsilateral arm to outstretch and cushion the fall (Nevitt and Cummings, 1993). Triceps weakness was an independent risk factor for hip fracture and in hemiplegic patients, this protective response is often absent.

1.2 Bone Densitometry and the Assessment of Bone Loss after Stroke

1.2.1 Dual Energy X-ray Absorptiometry

Osteoporosis is defined as a progressive systemic disease characterised by a low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Consensus Development Conference, 1991). Bone densitometry using dual energy x-ray absorptiometry (DXA) is a non-invasive radiological technique that provides a 2-dimensional or 'areal' view of the 3-dimensional mineralised mass of bone (Seeman, 2002). The x-ray source of the DXA instrument typically produces two photon energies with different attenuation profiles via a collimator beneath the supine patient. The soft tissues and bones of the subject attenuate some of the photon energy before its detection by the scanner arm above the subject. The attenuation of each beam within the scanned region is solved by simultaneous equations to give the absorption by bone alone. This method assumes that soft tissue is uniform and to account for fat interspersed with water density tissue, the region adjacent to bone is taken as a soft tissue standard. The instrument actually measures bone mineral content in grams and area in square centimetres, to give 'areal' bone mineral density in g/cm^2 . Bone mineral density measured in this manner at the hip and lumbar spine is a major determinant of fracture risk (Nevitt and Cummings, 1993). The relationship between BMD and fracture risk is analogous to that between blood pressure and stroke and is equally strong (Marshall *et al.*, 1996). In the SOF study there was a 7 times greater risk of hip fracture after a fall for a 2 standard deviation decrease in femoral neck bone density (Nevitt and Cummings, 1993). In normal adults, peak bone mass is achieved at approximately 30 years (fig. 1.3). Bone density in adults has been estimated to remain constant or to fall at up to 0.5% per year until the menopause (Ensrud *et al.*, 1995; Jones *et al.*, 1994). Following menopause, bone loss rises to 0.5-1.5% per year depending upon years since menopause, site of measurement and measurement technique (fig. 1.3). The following section reviews the literature regarding bone loss caused by stroke as measured by bone densitometry.

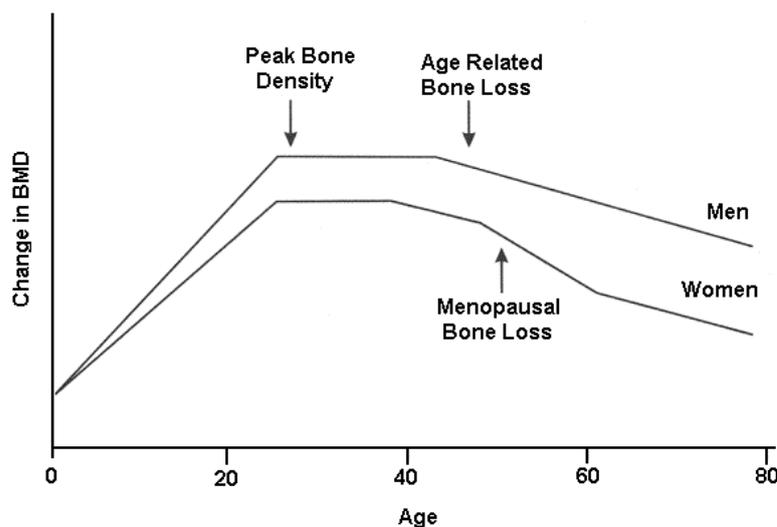


Figure 1.3 Changes in bone mass with aging (Adapted from Jones *et al.*, 1994).

1.2.2 Bone Mineral Density Changes after Stroke

The technique and anatomical site used to measure bone mineral density changes in patients with stroke has varied considerably across studies. Methods employed have included plain x-rays with cortical width assessment, metacarpal computed x-ray densitometry, heel ultrasound and the various modes of DXA (whole body, total hip, femoral neck and hip strength analysis DXA). The earliest investigation reported femoral shaft cortical thickness as a percentage of cortical width from plain x-rays of the hemiplegic and unaffected sides taken at least 6 months post-stroke. There were significant reductions on the hemiplegic side in 10 out of 14 patients (Hodgkinson and Brain, 1967). Changes in femoral cortical bone may be important, since in elderly patients without stroke a reduction in cortical thickness within the femoral neck was associated with hip fracture (Crabtree *et al.*, 2001). Using plain x-rays, Panin *et al.* (1971) compared the cortical thickness of three bones of the upper limb (humerus, radius and metacarpal) between the hemiplegic and unaffected sides of 25 long-term stroke patients. This showed lower cortical thickness on the affected side and that cortical thinning was inversely correlated with muscle function. Examining calibrated forearm x-rays in 74 subjects with hemiplegia (at various time points after stroke, mean 4 years) indicated that cancellous and cortical bone mineral content correlated negatively with forearm function (Prince *et al.*, 1988).

Several studies have reported a reduction in bone mineral density in the limbs of the affected side following hemiplegic stroke (del Puente *et al.*, 1996; Hamdy *et al.*, 1993; . Jorgensen *et al.*, 2000a; Jorgensen *et al.*, 2000b; Pang and Eng, 2005; Prince *et al.*, 1988; Ramnemark *et al.*, 1999a; Sato *et al.*, 1996a; Takamoto *et al.*, 1995). There are relatively few prospective studies using DXA, but these have consistently found the affected proximal femur (total hip or femoral neck) and upper limb to be the sites most sensitive to hemiplegia. Within four months of an acute stroke, Hamdy *et al.* (1995) observed a 9.3% decline in bone mineral content of the hemiplegic upper limb from baseline and a 3.7% decline in the whole femur (n=16). One year after an acute stroke, BMD at the hemiplegic femoral neck had decreased by 10% in 17 initially wheelchair-bound stroke patients, with a 5% decrease in the unaffected femoral neck (Jorgensen *et al.*, 2000a). In another prospective study, 18 patients were followed with total body and total hip BMD measurements 1, 4, 7 and 12 months after acute severe stroke (Ramnemark *et al.*, 1999a). The overall decrease in total body BMD was -2% over the period of investigation, with no loss of BMD from the skull or spine regions. BMD was lost principally from the humerus (-17.4%) and proximal femur (-12.2%) on the hemiplegic side, but there was also a decrease in the proximal femur BMD (-5%) on the unaffected side (fig. 1.4). These changes were independent of changes in body weight (Ramnemark *et al.*, 1999b).

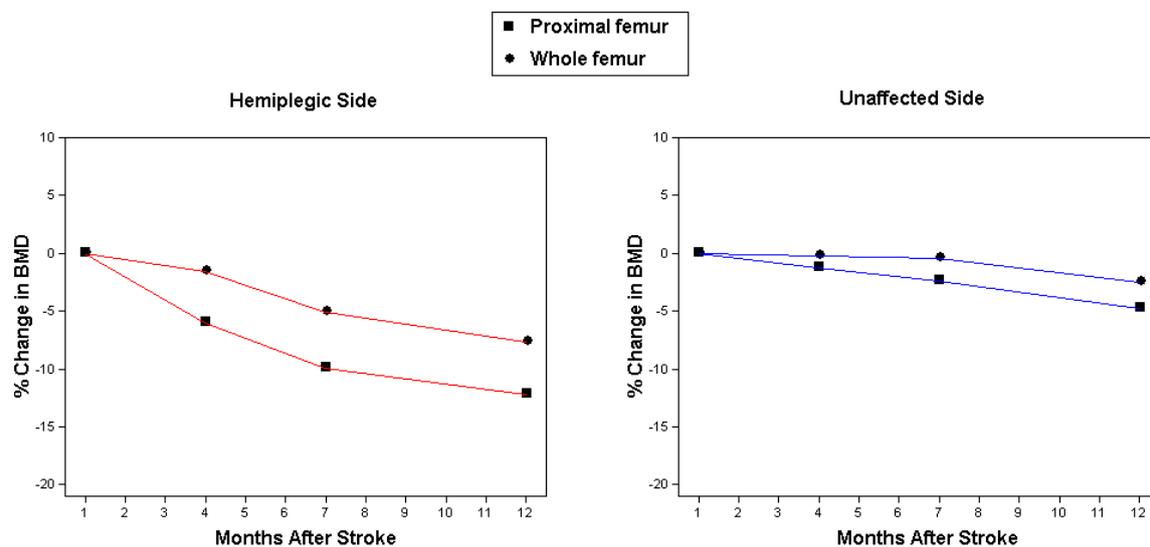


Figure 1.4 Changes in the femoral BMD of 18 stroke patients in the year following stroke. Adapted from (Ramnemark *et al.*, 1999a).

1.2.3 Determinants of Bone Loss after Stroke

Stroke occurs as a sudden neurological deficit, with a wide spectrum of initial clinical symptoms, although patterns of dysfunction are recognised according to the location of the brain lesion and neurological deficits produced (Bamford *et al.*, 1988). Patients with these diverse stroke syndromes have varying degrees of motor recovery, weight bearing and walking function over time. In a cross sectional study, Takamoto *et al.* (1995) found that a low functional ability at assessment was associated with a greater difference between the hemiplegic and the unaffected side BMD. Several studies have investigated the effects of the baseline stroke deficit or functional recovery on prospective changes in bone density at the hip. In the prospective study of Ramnemark *et al.* (1999a, fig 1.4), the presumptive risk factors for bone loss such as motor function, Barthel index and walking ability did not predict an individual's bone loss, possibly because all the patients had severe stroke. Jorgensen and colleagues (2000a) showed that bone mineral density loss at the hip was highest in those who did not bear weight early or relearn to walk within the first 2 months after stroke. They identified a sub-group of stroke patients based on a functional classification (the Functional Ambulatory Category or FAC, reprinted in Appendix 1). Patients who were unable to walk (FAC; 0) at 6 days following stroke had a significantly greater loss of femoral neck BMD at 7 and 12 months than those who could walk, even if they required physical assistance to do so (FAC: 1-5, fig. 1.5). Furthermore, their research suggested that the percentage of body weight borne by the paretic leg was a determinant of bone loss during stroke recovery. Therefore, the FAC may have a role in selecting patients for interventions to prevent bone loss. Although walking recovery and functional ability were predictors in these patients, others argue that specific neurological deficits are responsible for the degree of bone loss, rather than altered loading. Van Ouwenaller *et al.* (1989) suggested that the increased venous pressure found using intraosseus phlebography in the femurs of 6 hemiplegic patients (Chantraine *et al.*, 1979), might be a stimulus to local bone resorption. The increased pressure was believed to be the result of decreased sympathetic nervous system activity. However, the involvement of the autonomic nervous system in stroke is highly variable and in one study of triple phase bone blood flow by scintigraphy, only 5/85 (6%) hemiplegics had altered femoral bone blood flow (Greyson and Tepperman, 1984). Autonomic dysfunction does remain a plausible mechanism for the profound bone loss in the hemiplegic upper limb (Pang and Eng, 2005), particularly since 21/85 (25%) hemiplegic patients in the scintigraphy study had diffuse increased uptake in

the hands and wrists due to reflex sympathetic dystrophy (itself associated with a rapid reduction in bone density, Greyson and Tepperman, 1984). Although a role for autonomic dysfunction in the lower limbs of stroke patients cannot be ruled out, evidence is accumulating that the altered mechanical forces acting on the hip are important determinants of hip bone density following stroke.

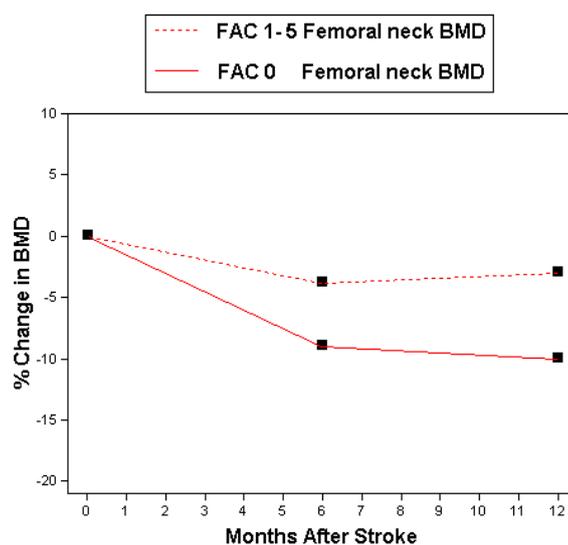


Figure 1.5 Change in BMD in the affected femoral neck in the year following stroke according to functional status at 6 days (FAC score 0, n=23, FAC score 1-5, n=17). Adapted from (Jorgensen *et al.*, 2000a).

Using hip structural analysis (HSA) software, the two dimensional femoral neck image generated by the DXA instrument can be divided into upper and lower femoral neck regions (fig. 1.6). Jorgensen and colleagues (2000b) applied this technique to the DXA images gathered in their study of functional status and BMD (fig 1.5). The reduction in femoral neck BMD observed in stroke patients, appeared to be the result of declining BMD in the lower femoral neck (which is loaded by repetitive compressive forces during normal gait) and not the upper neck. The precision error was reportedly low for the analysis. However, the reproducibility of femoral neck measurements is usually worse than total hip measurements (Patel *et al.*, 2000), owing to the effects of variable lower limb rotation on femoral neck ROI placement (Goh *et al.*, 1995). In stroke, hemiplegic lower limbs may be even harder to position accurately. Therefore, further study is necessary to confirm these findings, preferably with a 3 dimensional imaging technique such as hip quantitative computed tomography (hQCT) which is less prone to positioning errors.

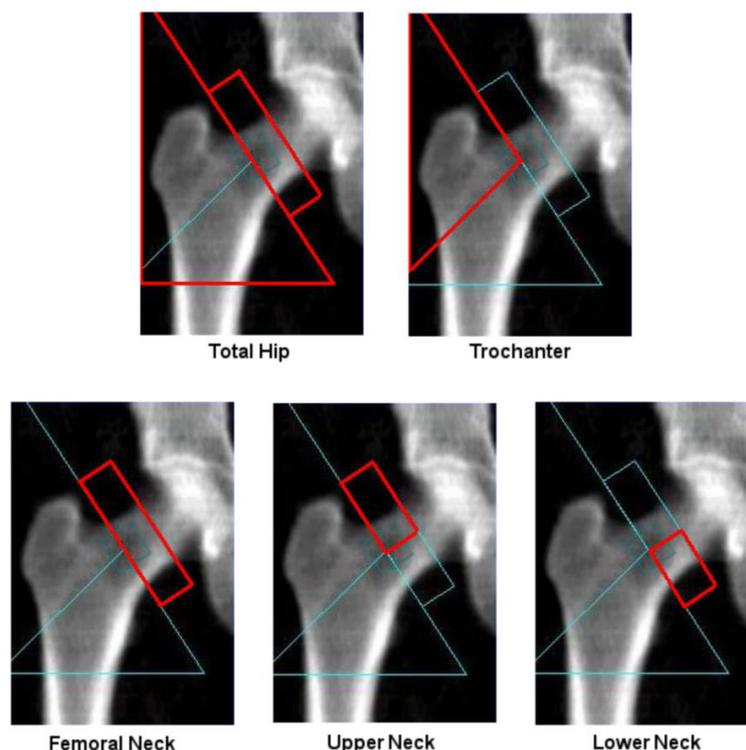


Figure 1.6 Region of Interest (ROI) locations in hip DXA analysis. The positions of the Lunar ROI's (total hip, trochanter, femoral neck) and Advanced Hip Analysis (Upper and Lower neck) are shown.

1.2.4 Computed Tomography and Ultrasound Assessment of Bone Loss

Asymmetrical BMD changes at the hip are not reflected by similar changes at the calcaneus or tibia following stroke. Peripheral quantitative computed tomography (pQCT) studies of both tibiae in a chronic stroke population showed no side-to-side differences in geometric or bone mineral density measurements despite the 6.6% lower femoral neck BMD on the hemiplegic side observed with standard DXA (Fehling *et al.*, 2004). In elderly chronic stroke patients, there were no side-to-side differences in bone mass at the heels using ultrasound when compared to elderly controls (Haddaway *et al.*, 1999), although others have reported that in stroke, the presence of neurogenic oedema in the hemiplegic leg can reduce the accuracy of the technique (Johansen *et al.*, 1997).

The pattern of bone loss in the lower limbs of stroke patients appears similar to that observed during the weightlessness of space flight (where changes were most marked in the hip), but distinct from spinal cord injury which is associated with a more generalised and severe distal bone loss in the lower limbs (Chow *et al.*, 1996). In a spaceflight study,

14 cosmonauts (13 males and 1 female, age 44.6 +/- 4 years) were assessed before and after prolonged periods on the MIR space station (4-6 months). The cosmonauts also lost spine volumetric BMD (vBMD) at a rate of 0.1% per month (Lang *et al.*, 2004). Loss of spinal BMD does not generally occur in stroke patients, although in a patient with pre-existing idiopathic osteoporosis, a rapid decrease of 13% in the lumbar spine was documented in the year following a severe stroke (Poole *et al.*, 2005, Appendix 4). There are no hQCT studies of stroke patients to date.

1.2.5 Bone Mineral Density Prior to Stroke

Stroke can occur at any age, but particularly affects the elderly, with half of all strokes occurring in people over 70 (Bamford *et al.*, 1988). Therefore, the population at most risk of stroke is already at risk of low bone mineral density and fracture. There has been considerable recent interest in common risk factors for osteoporotic fracture and ischaemic stroke. Jorgensen *et al.* (2001) reported hip bone mineral density measurements taken immediately after stroke but before the skeletal changes caused by immobilization and hemiplegia had occurred. Female stroke patients had 8% lower hip BMD than age matched controls without stroke. There is also weak evidence of a correlation between the degree of carotid atheroma (a risk factor for stroke) using ultrasound and whole body bone mineral density in women (Uyama *et al.*, 1997). However, low BMD did not predict stroke in men or women in a large study (Mussolino *et al.*, 2003). Where associations between osteoporosis and stroke do exist, they may be due to shared risk factors. Osteoporotic fractures share certain risk factors with stroke such as reduced physical activity (Gillum *et al.*, 1996), excessive alcohol consumption (Palomaki and Kaste, 1993), smoking (Whisnant *et al.*, 1996) and poor calcium intake (Abbott *et al.*, 1996; Scane *et al.*, 1999). More recently, raised homocysteine levels have been suggested as a unifying risk factor for hip fracture and ischaemic stroke (Sato *et al.*, 2005a). A randomised control trial of vitamin B12 and folate replacement in Japanese patients with ischaemic stroke showed significantly less hip fractures in the treated group, although there was a low absolute risk reduction and relatively low power (Sato *et al.*, 2005b).

1.3 Bisphosphonates and the Prevention of Bone Loss after Stroke

The most appropriate target for preventing bone loss after stroke is the profound increase in bone turnover and osteoclastic bone resorption that occurs soon after the stroke (Kanis

et al., 2001). Bisphosphonates are appropriate drugs for this purpose because they selectively target osteoclast-mediated bone resorption (Flanagan and Chambers, 1991; Sato *et al.*, 1991). There have been three studies using oral bisphosphonates in stroke patients to date (Ikai *et al.*, 2001; Sato *et al.*, 2000a; Sato *et al.*, 2005c). In addition to the expected effect on biochemical markers of bone resorption (a significant reduction), Sato *et al.* also found that inhibiting osteoclastic activity in stroke patients (with etidronate or risedronate) resulted in increased parathyroid hormone, 1,25 dihydroxyvitamin D (1,25(OH)₂D) and biochemical markers of bone formation, relative to placebo treated stroke patients. The authors proposed that the significant increase in 1,25(OH)₂D and osteocalcin (OC, a formation marker, measuring both N and C terminal regions) observed in both the etidronate and risedronate treated stroke patients was the result of reduced inhibition of parathyroid secretion by serum calcium. Bisphosphonate therapy appeared to reduce the elevated ionised serum calcium that occurs commonly in untreated stroke patients (Sato *et al.*, 2000a; Sato *et al.*, 2005c), with a resultant increase in PTH and 1,25(OH)₂D (Chapter 2.2).

Indirect evidence for the application of bisphosphonates after stroke comes from their effectiveness in paraplegia where bone resorption is increased (Minaire *et al.*, 1981; Minaire *et al.*, 1987; Plosker and Goa, 1994). Studies have also indicated that bisphosphonates are an effective counter-measure (even better than exercise) in preventing loss of bone mineral density at the femoral neck during experimental bed rest (Chappard *et al.*, 1989; Grigoriev *et al.*, 1992, Rittweger *et al.*, 2005).

Two studies have been published reporting a beneficial effect of daily etidronate, an oral bisphosphonate on bone loss when administered after acute stroke (Sato *et al.*, 2000a; Ikai *et al.*, 2001). In a double-blind, randomised, placebo control trial involving 98 patients, the BMD loss on the affected side (metacarpal measurements) was reduced to a mean reduction of 2.3% at one year, compared to 4.8% loss in the placebo group (Sato *et al.*, 2000a). In the study of Ikai *et al.* (2001), an interim report at 3 months suggested that patients with a poor functional status had significantly more bone loss on the hemiplegic side (femoral neck) than those with higher functional status. Administration of etidronate was not randomised, nor was there a placebo arm to this study. However, an age-matched control group was followed and at 3 months, the loss of BMD was reduced in the low

functional status group on etidronate, compared to a low functional status control group. Oral bisphosphonates have many practical disadvantages compared to intravenous preparations after stroke. It may be advantageous to intervene with an effective anti-resorptive soon after stroke, as bone resorption has been shown to occur within a week (Sato *et al.*, 2000b). Dysphagia or drowsiness after acute stroke may mean that those at most risk cannot receive therapy. Additionally, etidronate is poorly absorbed from the gastrointestinal tract and patients must avoid food for at least 2 hours before and after swallowing. For the other oral bisphosphonates commonly used in osteoporosis, patients must remain upright, standing or sitting for 30 minutes before and after swallowing. Such drugs may be hazardous to oesophageal mucosa, (even when administered via the NG route) and are therefore not recommended in the presence of swallowing difficulties or an inability to sit or stand up straight (Russell *et al.*, 1999). These factors make it difficult to recommend oral bisphosphonates after stroke. Nevertheless, a recent study by Sato and colleagues (2005c) showed that patients able to stand and swallow after stroke suffered less hip fractures during 12 months of oral risedronate 2.5 mg daily. In this RCT comparing 187 elderly female acute stroke patients (mean age 71.2 +/- 3.8) given risedronate daily for one year with 187 acute stroke controls (mean age 71.6 +/- 4.9), there were significantly fewer hip fractures at one year in the risedronate group (1 hip fracture) than in the placebo group (7 hip fractures). There were several omissions from the paper that were a barrier to assessing the validity and applicability of the results (Poole *et al.*, 2005d). Firstly, the authors admitted that there were no formal power calculations, particularly important in hip fracture intervention trials where the event rate is low (CONSORT guidelines for RCT reporting (Moher *et al.*, 2001)). Secondly, insufficient details were provided of baseline stroke severity, functional status and stroke type, making it difficult to know which stroke patients might benefit from the intervention. Some time after publication, the authors provided additional details that confirmed that the patients had very mild strokes; the mean baseline Barthel indices (+/-SD) for placebo and bisphosphonate groups were 78 (24) and 77 (24) respectively (Sato, 2005d). Therefore, in acute stroke patients with very mild functional impairment and adequate swallowing, risedronate may be effective in preventing hip fractures.

1.3.1 Intravenous Bisphosphonates

Intravenous bisphosphonates are used with great effect as inhibitors of bone resorption, for example in Paget's disease of bone, metastatic and osteolytic bone disease and tumour induced hypercalcaemia (Russell *et al.*, 1999). The hallmark of such diseases is an increase in osteoclast activity. No published work exists for stroke, but intravenous pamidronate was effective in attenuating femoral bone loss after spinal cord injury (Nance *et al.*, 1999) and in healthy volunteers subjected to 90 days of bed-rest (Watanabe *et al.*, 2004). In the study of Nance *et al.* (1999), the method of administration was time consuming (4 hour-long infusions every 4 weeks for 6 months) and 14 patients in total received the drug. The drug was well tolerated and protected against bone density loss, but the dosage frequency was thought to be insufficient and the trial was not randomised.

Third generation bisphosphonates are being evaluated in postmenopausal osteoporosis (Gatti and Adami, 1999). Zoledronate is the most potent intravenous bisphosphonate tested to date (Major *et al.*, 2001). Administration times for the third-generation bisphosphonates are reduced, so that the drugs can now be given over minutes rather than hours. In a phase II study, the use of a single annual injection of zoledronate in postmenopausal osteoporosis was associated with an effect on bone density after 12 months similar to that achieved by 1 year treatment with oral bisphosphonates (Reid *et al.*, 2002). The trial was not designed to show differences in fracture incidence, but was a randomised placebo control trial of bisphosphonate at intervals of 3 months, 6 months or once annually in 351 women. There was an increase in mean hip BMD compared to placebo of +3.3% 1 year after administration. Although third-generation intravenous bisphosphonates such as zoledronate have at least one clear advantage over pamidronate in their ease of administration, there is currently more experience with pamidronate, especially in malignancy and Paget's disease. However, there is favourable efficacy, safety and tolerability data from the use of zoledronate in tumour-induced hypercalcaemia, as well as gradually increasing experience of using the drug in Paget's disease and postmenopausal osteoporosis.

1.3.2 Preventing Bone Loss after Stroke; a Hypothesis

Morbidity and mortality from hip fractures might be reduced by preventing bone loss with a single dose of intravenous bisphosphonate given soon after stroke in the acute stroke

unit. At present, it is not clear which stroke patients would benefit from early bisphosphonate therapy and a clinical trial is necessary to answer important questions regarding safety and efficacy in stroke. The first hypothesis to be tested in this thesis is that a single injection of zoledronate given to newly hemiplegic patients in an acute stroke unit can prevent subsequent bone loss during the first year after stroke. The study design will be a randomised placebo-controlled double-blind trial.

1.3.3 Zoledronate

In a phase II trial of postmenopausal osteoporotic women, the treatment was generally well tolerated, but pyrexia and myalgia occurred more frequently with zoledronate than with placebo (Reid *et al.*, 2002). Other adverse reactions to zoledronate were reported during large trials of ill patients with malignancy induced hypercalcaemia. Acute phase reactions to zoledronate were similar to those reported for other bisphosphonates. Intravenous administration was most commonly associated with a rise in body temperature in approximately 11% of patients. A flu-like syndrome consisting of fever (7%) and bone pain (1%) was also reported. Occasionally cases of arthralgia, fatigue, confusion, thirst, pancytopenia and bradycardia have been reported (1.2%). Impaired renal function (2.3%) was reported in patients in the oncology trials, but other risk factors in this severely ill patient population may have contributed as well (Chang *et al.*, 2003; Wellington and Goa, 2003). There have been case reports of renal toxicity in patients administered zoledronate, most notably for Paget's disease (Markowitz *et al.*, 2003). Case reports of osteonecrosis of the jaw following zoledronate treatment have been substantiated by post-marketing analysis, with formal warnings now issued (Marx, 2003). As with other bisphosphonates, conjunctivitis and uveitis have been reported (Chaplet *et al.*, 2004).

In trials, the reduction in renal calcium excretion associated with zoledronate administration was accompanied by a fall in serum phosphate levels (3.5% of patients). The serum calcium fell to hypocalcaemic levels (6% of patients), with hypomagnesaemia (1%) also reported. Case reports have identified that patients with hypovitaminosis D are at risk of clinically relevant hypocalcaemia (Peter *et al.*, 2004; Rosen and Brown, 2003) with intravenous zoledronate administration. Gastrointestinal reactions, such as nausea (1%) and vomiting (1%) were reported following intravenous infusion of zoledronate. Some cases of pruritus (1%) and chest pain (1%) occurred.

1.3.4 Alternative Strategies to Prevent Fractures

An alternative to bisphosphonate therapy in preventing osteoclast over activity in stroke is calcitonin therapy. Calcitonin (200 iu/day) was tested in a two year prospective randomised double-blind placebo controlled trial of acute stroke patients, with the main outcome being changes in biochemical markers of bone resorption and formation. 18 patients received twice daily nasal calcitonin for two years (with 5 withdrawals including 4 due to the local nasal side effects of therapy) and 16 patients received placebo sprays. Calcitonin administered nasally had no effect on bone markers, although it is possible that the nasal route did not result in sufficient absorption of the drug (Uebelhart *et al.*, 1999).

The decline in hip BMD is one of several factors thought to predispose stroke patients to hip fracture following a fall. Hip fractures rather than soft tissue injuries might occur if recovering hemiplegics were to fall with greater force because of impaired balance (Willig *et al.*, 2003) or because they lack the usual protective reflexes (such as an outstretched hand) to absorb some of the impact (Poole *et al.*, 2002). If the majority of falling stroke patients experienced mechanical forces considerably over the threshold for a fracture, even an effective bisphosphonate might not strengthen the femur sufficiently to prevent such fractures. Based on the risk of falls after stroke there is a case to be made for the use of mechanical hip protectors in at-risk individuals. Mechanical hip protectors have historically been recommended in elderly patients who are at high risk for hip fracture. However the most recent Cochrane meta-analysis and literature review showed no reduction in hip fracture incidence from the provision of hip protectors (RR 1.16, 95% CI 0.85 to 1.59) where randomisation was by individual patient (Parker *et al.*, 2005). Low compliance rates were frequently documented due to problems of comfort and practicality (Hubacher and Wettstein, 2001; Kannus *et al.*, 2000). Therefore, feasibility, efficacy and safety data are required before recommending the use of hip protectors in stroke patients, particularly since stroke survivors have a seven-fold increased risk of falling during dressing (Lamb *et al.*, 2003). Some of these questions were addressed recently by a cohort study of hip protector use in acute stroke patients. They were reportedly difficult to use in an acute setting, compliance was poor and one patient suffered a hip fracture whilst wearing the protectors during a 2 week trial (personal communication, Dr. Kate Hellier, Addenbrooke's Hospital, Cambridge, UK).

1.4 Vitamin D and Bone Loss after Stroke

1.4.1 Vitamin D and Calcium Homeostasis in Acute and Chronic Stroke

Vitamin D insufficiency is common following stroke in Japan (Sato *et al.*, 1999a) and is associated with low BMD in the hemiplegic metacarpal (Sato *et al.*, 1996a) and post-stroke hip fracture (Sato *et al.*, 2001). In a study of 89 patients (39M, 50F mean age 71.2 +/- 11) one week following acute stroke, Sato and colleagues (2000b) found 10% of patients deficient in 25OHD (<10 ug/l), with 63% insufficient (10-20 ug/l) and only 27% with sufficient levels (>20 ug/l). Reduced sun exposure and reduced dietary intake of vitamin D are common in both long-term inpatients and outpatients following stroke (Sato *et al.*, 1996b). One criticism of the work from Japan is the small size of the age-matched control group used for comparison and derivation of 'normal' values. This has ranged from 28 controls (Sato *et al.*, 1996a) to 72 controls (Sato *et al.*, 1999a). The problem of sample size is compounded by the fact that vitamin D and parathyroid hormone levels vary by season (in both Northern and Southern hemispheres (Hegarty *et al.*, 1994; Melin *et al.*, 2001; Pasco *et al.*, 2004; Webb *et al.*, 1988)) and this has not been taken into account by the aforementioned studies. Radioimmunoassay techniques for 25 hydroxyvitamin D necessitate reliable reference ranges and these need to be specific for the time of year and the usual light exposure of the population tested. Detrimental effects on bone turnover have been observed in patients with 25OHD within the 'normal' range (Heaney *et al.*, 2003). The 'normal' range varies depending on the reference laboratory and is usually based on 95% confidence intervals for the general population. Some authorities suggest abandoning the use of absolute values for 25OHD deficiency and insufficiency (O'Shea and Carter, 1998) and instead use a target concentration of 25OHD at which the mean serum PTH concentration starts to increase in population studies thereby eliminating seasonal and geographical differences. These considerations mean that a study of vitamin D in acute stroke with appropriate seasonal adjustment is required (Chapter 4).

Three mechanisms have been proposed whereby inadequate vitamin D could reduce bone quality post-stroke. The first is profound 25OHD substrate deficiency in chronic institutionalised stroke survivors and a failure to adequately mineralise osteoid i.e. osteomalacia (although histological confirmation of this is absent) (Sato *et al.*, 1999c). Secondly, deficiency of the active form of vitamin D (1,25(OH)₂D) might also play a role

in stroke-induced bone loss. $1,25(\text{OH})_2\text{D}$ is produced by hydroxylation of its precursor 25OHD in the kidney (fig. 1.7)

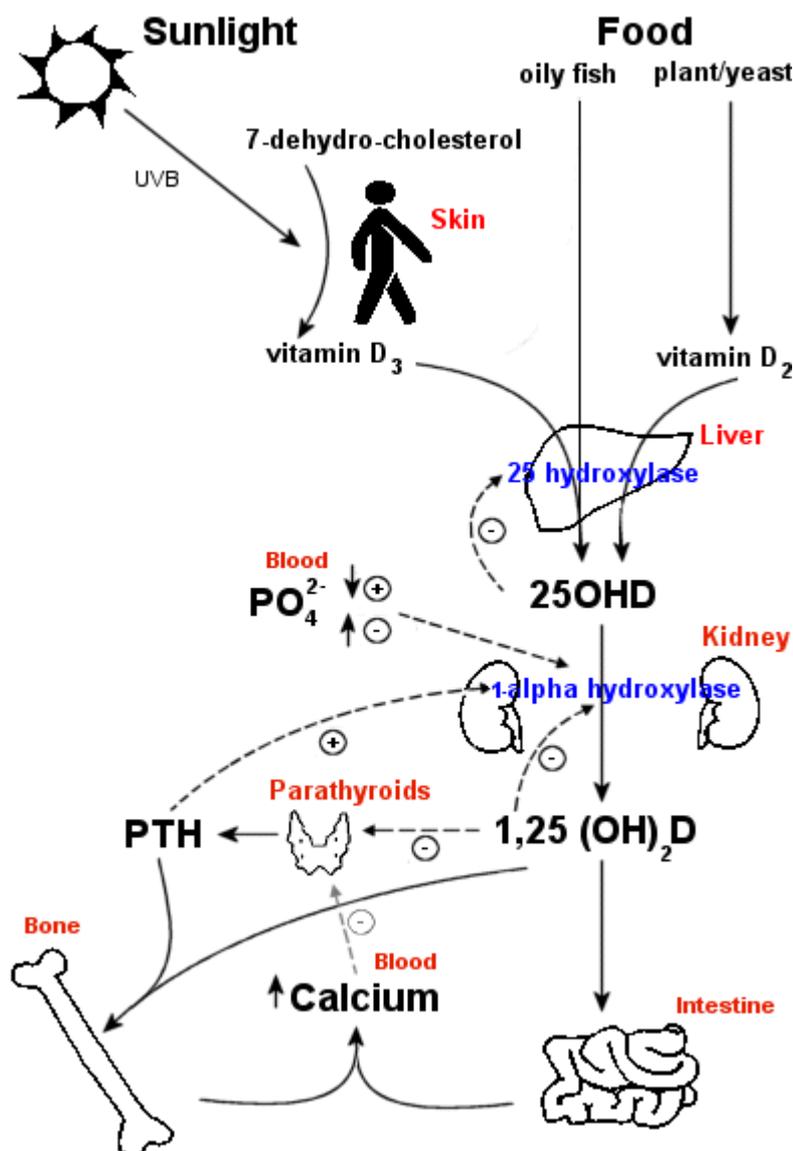


Figure 1.7 The metabolic pathway for vitamin D synthesis and its actions in calcium homeostasis.

Deficiency of $1,25(\text{OH})_2\text{D}$ in immobilized stroke patients may be caused by substrate (cholecalciferol) deficiency or by sub-clinical hypercalcaemia. Hypercalcaemia (from increased osteoclast activity during immobilisation) inhibits parathyroid hormone secretion and therefore production of $1,25(\text{OH})_2\text{D}$ (Sato *et al.*, 1999b). A negative linear correlation between the Barthel index (BI) and ionised calcium in 170 patients with established stroke provided evidence that the degree of immobility determined the calciotropic hormone response (Sato *et al.*, 1999b). A negative correlation was also found between the Barthel

index and the bone resorption marker, ICTP. Stroke in-patients with the greatest degree of immobility had higher ionised calcium (correlated with degree of immobilisation), lower parathyroid hormone levels and reduced $1,25(\text{OH})_2\text{D}$. The $1,25(\text{OH})_2\text{D}$ levels also correlated with metacarpal BMD. A small trial by the same group tested the effect of $1,25(\text{OH})_2\text{D}$ treatment versus placebo after stroke (64 patients, mean 4.8 years post stroke). Bone mineral density loss was of smaller magnitude in the active vitamin D3 treated group and less hip fractures occurred, although the study was not adequately powered for fracture outcomes (Sato *et al.*, 1997). Finally, Sato and colleagues (2004) describe a subgroup of 25OHD deficient stroke patients who are more mobile, have lower calcium, but maintain adequate $1,25(\text{OH})_2\text{D}$ levels. This may be because they appear able to mount the appropriate PTH response to vitamin D deficiency. These patients have relatively higher PTH and hence are at risk of bone turnover changes consistent with secondary hyperparathyroidism. In this subgroup of patients, the PTH levels correlated with BMD in the metacarpal (Sato *et al.*, 2004). Vitamin D insufficiency that leads to a moderate degree of secondary hyperparathyroidism in this fashion may contribute to ‘type II’ osteoporosis (Riggs and Melton, 1990) leading to hip fractures in men and women over 70.

1.4.2 Intravenous Bisphosphonates and Vitamin D Insufficiency

A significant safety issue has been highlighted regarding the routine use of intravenous bisphosphonates in normocalcaemic patients with occult vitamin D insufficiency (Rosen and Brown, 2003). In one case, the administration of a single dose of pamidronate to a normocalcaemic 52 year old woman with sub-clinical osteomalacia (due to intestinal malabsorption) resulted in a profound and life-threatening episode of hypocalcaemia associated with tetany and cardiac arrhythmia. When a patient has 25OHD deficiency, or renal failure with $1,25(\text{OH})_2\text{D}$ deficiency, there may be a reduced ability to absorb calcium from the gut. There is often an increase in PTH secretion and consequently osteoclasts resorb bone mineral, thereby normalising serum calcium. This secondary hyperparathyroidism (resulting from either substrate deficiency in osteomalacia, or reduced 1-alpha hydroxylase enzyme activity in renal failure) is a protective homeostatic mechanism ensuring close control of serum calcium. However, a bolus of pamidronate or zoledronate (which slows calcium efflux from the skeleton) given under these

circumstances may be associated with profound hypocalcaemia since 1,25(OH)₂D (stimulated by PTH) is required for gastrointestinal absorption of calcium (fig. 1.7). Vitamin D insufficiency was also implicated in two cases of profound post-zoledronate hypocalcaemia reported by Fraser and colleagues (Peter *et al.*, 2004).

1.4.3 Vitamin D Status in Acute Stroke; a Hypothesis

Although patients from Japan develop vitamin D insufficiency in the years following stroke, very little is known about vitamin D status in acute stroke patients. The second hypothesis to be tested in this thesis is that newly hemiplegic patients have reduced vitamin D when compared to a healthy age-matched population without stroke.

Chapter 2. Bone Remodelling and Bone Cell Responses to Hemiplegia

2.1. Bone Biology

In order to determine how stroke may affect the skeleton at a cellular level, it is first necessary to understand how bone integrity is maintained. This process is a complex and highly co-ordinated activity carried out by specialised cells (namely osteoclasts, osteoblasts, osteocytes and bone lining cells) in response to a variety of local and systemic stimuli. In this section, the main cell types involved in the maintenance of the adult human skeleton are described, with special reference to remodelling and the analysis of this process using histomorphometry. Thereafter the histomorphometric findings in human models of unloading are presented. The chapter concludes with a review of the critical role played by the osteocyte in bone biology, with reference to the human high bone mass phenotype sclerosteosis, which results from loss of a single osteocyte-derived protein.

2.1.1 Cell Types in Bone

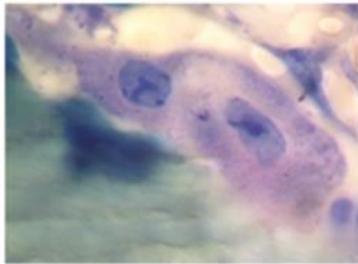
Osteoclasts

The osteoclast is a specialised multinucleated cell formed by fusion of cells of the monocyte/macrophage lineage into a large cell (fig. 2.1 A) with the ability to resorb focal areas of bone (Teitelbaum, 2000). In vitro osteoclast formation from macrophage precursors requires the presence of bone marrow stromal cells (Udagawa *et al.*, 1990), which provide factors essential for osteoclastogenesis; macrophage colony stimulating factor (M-CSF (Udagawa *et al.*, 1990)) and receptor activator of NF kappa beta (RANK ligand, RANKL (Lacey *et al.*, 1998)). It has been suggested that osteoblasts, bone lining cells and stromal cells express these molecules to stimulate osteoclast formation, particularly in response to PTH and 1,25(OH)₂D (Kitazawa *et al.*, 1999; Lee and Lorenzo, 1999). PTH also reduces the expression of the endogenous decoy receptor for RANK, osteoprotegerin (OPG) (Lacey *et al.*, 1998). The differentiated multinucleated osteoclast develops a ruffled membrane border on the bone surface after attachment to the bone matrix (Blair *et al.*, 1989). The extracellular region is acidified by means of a proton pump, maintaining an approximate pH of 4.5 in the sealed space between osteoclast and bone matrix (Silver *et al.*, 1988). This aids in dissolution of the mineral content of bone (Li *et al.*, 1999), before lysosomal enzymes (such as cathepsin K (Gowen *et al.*, 1999)) degrade the collagenous matrix (mostly type 1 collagen), which is endocytosed into the osteoclast

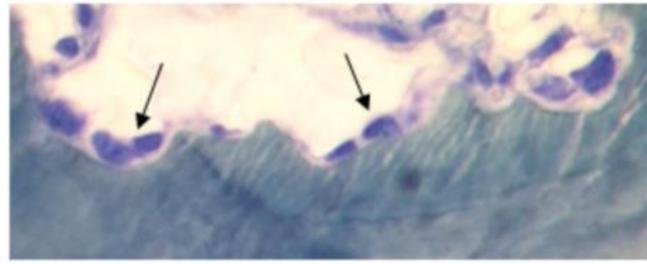
before being transported out of its opposite (non-ruffled) membrane (Nesbitt and Horton, 1997). Events that terminate osteoclast resorption are less well established, although it is known that bisphosphonates, by inhibiting the osteoclastic melavonate pathway can promote osteoclast apoptosis (Reszka *et al.*, 1999).

Osteoblasts

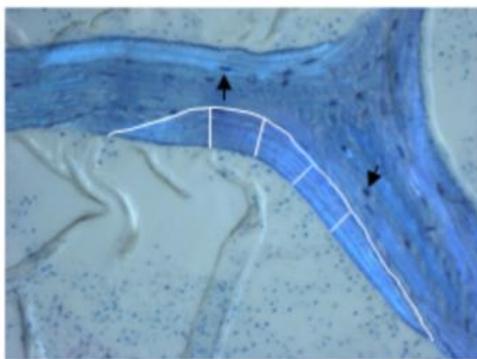
Osteoblasts are found on the surfaces of bone and are responsible for bone formation (fig. 2.1 B). They are derived from mesenchymal stem cells located within the bone marrow or on the periosteal surface. Their main function in maintaining the adult human skeleton is the coordinated secretion of osteoid (the mainly collagenous mixture of bone matrix proteins that is later mineralised to form bone, fig. 2.1 D) in discrete 'packets' during the formation phase of the remodelling cycle (fig. 2.5). A wave of bone formation is brought about by a layer of osteoblasts joined by tight junctions to their neighbours and secreting vesicles of osteoid in the direction of the bone via their specialised plasma membranes (Prele *et al.*, 2003). Each osteoblast is estimated to produce 0.5 μm of matrix per day, with a formation period lasting approximately 100 days (Eriksen *et al.*, 1990). Despite such a specialised function, the osteoblast is not fully differentiated and as an alternative to death by apoptosis (Jilka *et al.*, 1999), osteoblasts can become entombed as osteocytes or change shape to become flat bone lining cells that cease osteoid formation. A number of factors have been found to influence the development and differentiation of osteoblasts including the matrix proteins transforming growth factor beta (TGF-B) and release of bone morphometric proteins (BMP's) that were previously laid down in the bone matrix during an earlier wave of osteoblastic formation (Canalis *et al.*, 2003; Centrella *et al.*, 1991). Other important factors include Cbfa-1 (a transcription factor), secreted proteins such as Wnt and hormones such as parathyroid hormone (Mackie, 2003; Moon *et al.*, 2002).



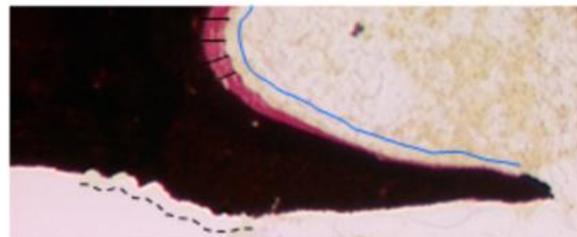
A. Osteoclast, oil immersion x1000. Toluidine blue stain.



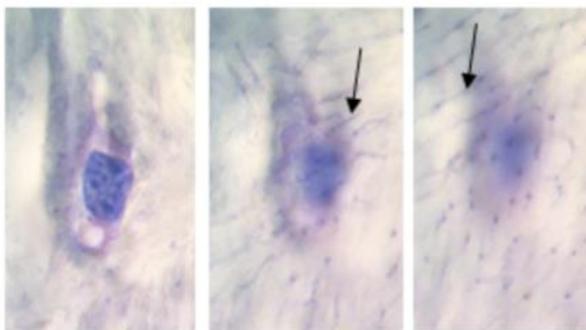
B. Cuboidal osteoblasts (*arrows*) forming bone on a recently eroded surface, oil immersion x252. Toluidine blue.



C. Completed packets of cancellous bone, with the wall thickness marked (*white lines*). Osteocytes are also shown (*arrows*). Polarised light x126. Toluidine blue.



D. Cancellous eroded surface (*dashed line*), Osteoid surface (*solid blue line*) and Osteoid Thickness measurements (*4 solid lines*). x100. von Kossa and van Gieson stain.



E. Osteocyte (*left*) and Osteocytic cytoplasmic projections in canaliculi (*arrows, centre and right*). Oil immersion x1000. Toluidine blue.



F. Bone lining cells on quiescent bone surface (*arrows, flattened nuclei*). Oil immersion x1000. Toluidine blue.

Figure 2.1 Bone cells and selected histomorphometric measurements.

Bone Lining Cells

In the healthy adult skeleton there are relatively few osteoblasts and osteoclasts; the permanent cell populations being osteocytes and bone lining cells (Marotti, 1996). About 5% of the bone surface is covered by osteoblasts, 1% by osteoclasts and the remaining 94% by bone lining cells (Parfitt, 1983), which are flattened surface cells with few intracellular organelles (fig. 2.1 F). The bone lining cell is thought to influence bone formation in either of two ways. Firstly, lining cells may initiate bone formation on an existing bone surface by reverting to an osteoblast phenotype under certain conditions (a process termed *modelling* (Chow *et al.*, 1998; Dobnig and Turner, 1995)). Secondly, bone lining cells may be involved in initiating bone resorption (e.g. through RANK ligand expression (Teitelbaum, 2000)) and a subsequent wave of bone formation (termed *remodelling*). One hypothesis is that bone lining cells can respond to agents such as PTH by a shape change, or retraction that exposes the underlying bone matrix constituents to cells of the osteoclastic lineage (Rodan and Martin, 1981). Perhaps even more than the osteocyte (see below), the precise function of the bone lining cell remains elusive.

Osteocytes

Osteocytes are the most numerous cells in bone (Noble and Reeve, 2000). They are derived from osteoprogenitor cells that have differentiated into active osteoblasts. Only a small proportion of the osteoblasts become osteocytes (fig 2.1 E), by becoming entombed in the fresh matrix layer synthesised by the surface osteoblasts (Palumbo, 1986). Mature osteocytes reside in lacunae (fig. 2.2), having developed direct cellular connections with older osteocytes as they were engulfed and maintained their connections with the overlying bone cells (lining cells, osteoblasts and beyond (Marotti *et al.*, 1992)) via cytoplasmic dendritic processes known as canaliculi (Baud, 1968; Dudley and Spiro, 1961; Palumbo, 1986). The osteoblast undergoes considerable ultrastructural changes as it develops from a plump osteoid-secreting cell into a mature dendritic cell encased in the hard bone matrix. Two distinct stages have been defined in this process. The first is the 'newly embedded' or 'nascent' stage where the osteocyte has a 30% reduction in cell volume and extends thick processes away from the forming surface (Palumbo, 1986). There follows a mature stage where the osteocyte is engulfed in mineralised matrix, cell volume is reduced a further 40% and numerous thin canalicular processes have extended towards the bone surface (Palumbo, 1986).



Figure 2.2 A scanning electron micrograph of a resin impregnated acid-etched piece of human cortical bone. The white resin 'casts' of the osteocyte lacunae and canaliculi stand several microns proud of the bone surface. Image by Dr. Peter Atkinson (Currey, 2002).

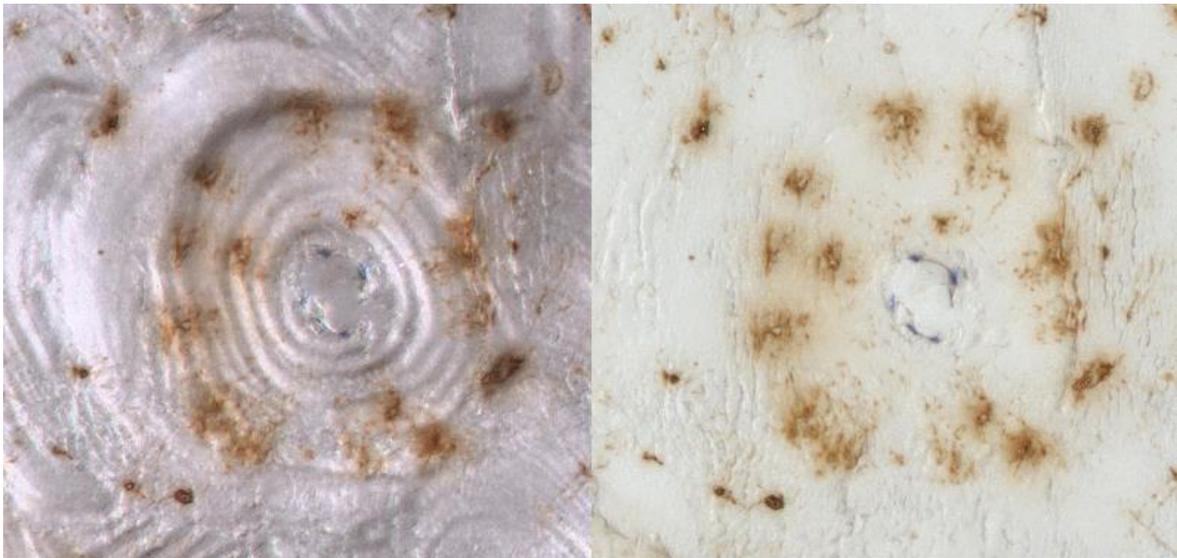


Figure 2.3 Osteocytes and their canaliculi in a newly formed cortical osteon seen using polarised light (left) and bright field microscopy (right). x126 magnification. Osteocytes are stained for the protein sclerostin.

The entire cell and canalicular processes become surrounded by mineralised matrix but within the lacuno-canalicular space is a large surface area for ionic exchange and extracellular fluid (Knothe Tate, 2003). The network of interconnected osteocytes and other bone cells is thought to comprise a functional syncytium (Aarden *et al.*, 1994; Knothe Tate, 2003) for metabolic traffic of substances in the extracellular space in addition to the cell-cell interaction via gap junctions (Doty, 1981). Osteocytes are thought to be the mechano-sensors in bone. They are deformed by fluid flow in their narrow canaliculi

induced by mechanical strain (Burger and Klein-Nulend, 1999). This is thought to enable the osteocytes to translate changes in bone loading into adaptive changes in bone remodelling. An early finding was that loading of bone led to changes in enzymatic (glucose-6-phosphate dehydrogenase) activity in osteocytes (Skerry *et al.*, 1989). Physiological levels of bone strain are thought to produce an anabolic response by enhancing osteocyte signalling via prostaglandins, IGF1, cyclo-oxygenase 2 and nitric oxide (Ehrlich and Lanyon, 2002; Jiang and Cheng, 2001; Turner *et al.*, 2002). Stimulatory signals from osteocytes have generated much theoretical interest. In contrast, Martin used mathematical modelling of bone dynamics to propose a theory based on a single widespread inhibitory signal (Martin, 2000a). The theory states that a loading-induced inhibitory signal from osteocytes carried via the lacuno-canalicular system might be responsible for maintaining bone lining cells in their quiescent state, against their natural tendency to activate modelling or remodelling (Martin, 2000b). A newly discovered osteocyte-derived protein, sclerostin (fig. 2.3), may function as an inhibitory signal in this manner. Experiments using immunohistochemistry to investigate sclerostin expression by osteocytes are reported in Chapter 6 and the literature is reviewed in Chapter 2.3.

2.1.2 Bone Modelling and Remodelling

The skeleton is able to maintain its structure, shape and mechanical properties through two key processes termed modelling and remodelling. Modelling refers to the uncoupled anabolic and catabolic processes that occur during bone growth and shaping, trauma and excessive mechanical loading (Frost, 1990). Remodelling refers to the continuous process of bone turnover throughout life, where removal of bone by osteoclasts (resorption) is balanced by the replacement of bone matrix by osteoblasts (formation) and its subsequent mineralisation (Frost, 1990). Bone remodelling occurs in focal sites named basic multicellular units (BMU's, Frost, 1969), where the processes of bone resorption and formation are thought to be closely coupled. The human skeleton contains millions of BMU's at different stages of activity. About 20% of the cancellous bone surface is undergoing remodelling at any time and remodelling will occur on average every 2 years (known as the activation frequency). Under normal circumstances, resorption of old bone is followed by the formation of an equal quantity of new bone. Remodelling is the process by which maximum strength with minimum mass is actively maintained throughout the skeleton (Wolff's law), while allowing its other critical functions: growth, protection of

internal organs and a reservoir of minerals (Wolff, 1900). The length of the remodelling cycle in human bone ranges from 100 days in cortical bone to 200 days in cancellous bone (Eriksen *et al.*, 1990). The bone remodelling process in cortical bone is distinct, with so-called 'osteonal remodelling' (fig. 2.4) occurring as a 'cutting cone' of osteoclasts moves through the cortex. This is followed by a 'closing cone' of bone formation that results in a completed secondary osteon (fig. 2.3). This 'osteonal remodelling' results in the characteristic appearance of cortical bone sections under the light microscope (fig. 2.3). Schematic diagrams of cortical and cancellous BMU units undergoing remodelling are shown in figures 2.4 and 2.5. Cortical bone is of importance when considering bone loss following stroke since the femoral neck comprises approximately 75% cortical bone and imaging studies suggest that unloading by hemiplegia may preferentially affect this region (Jorgensen *et al.*, 2000b). In femoral neck biopsies from intracapsular hip fracture, regional cortical thinning and porosity (Bell *et al.*, 1999) are found. In such biopsies, extensive coalescence of adjacent resorbing osteons (Jordan *et al.*, 2000) and insufficiently refilled canals (Bell *et al.*, 2000) resemble the changes seen in animal models of disuse (Rubin *et al.*, 1996). Understanding bone remodelling is essential for interpreting the results of bone histomorphometry, which quantifies surface cellular events, architecture and (if timed fluorescent labels are used) dynamics (Chapter 2.2.2).

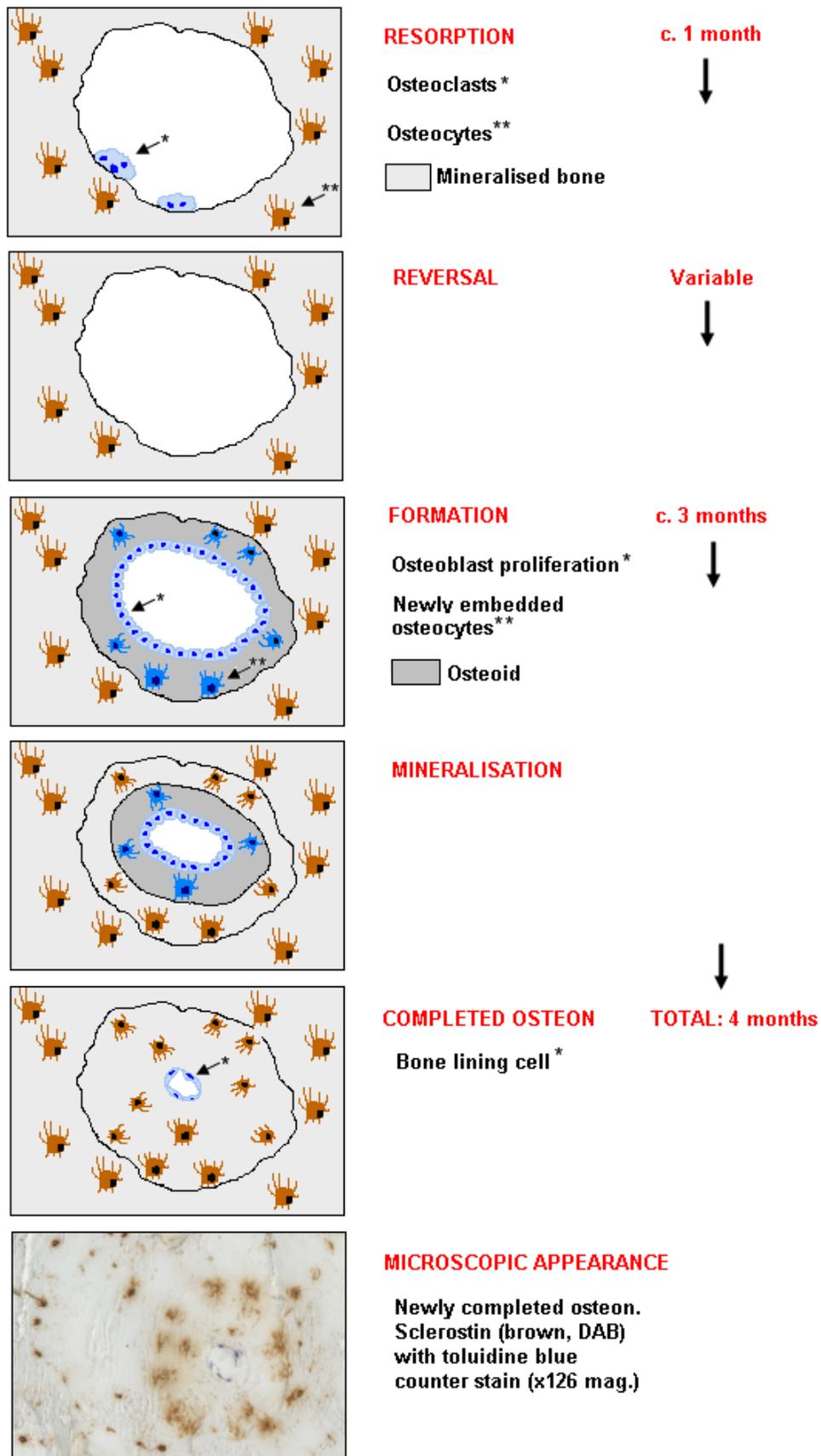


Figure 2.4 Remodelling Cycle in a Single Cortical BMU.

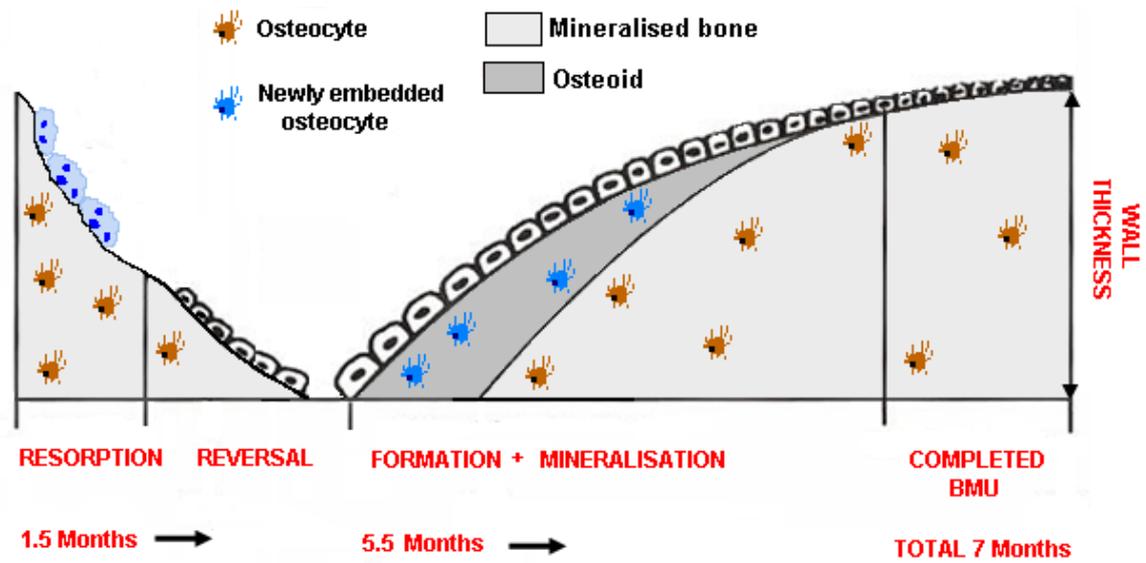


Figure 2.5 Remodelling Cycle in a Single Cancellous BMU.

2.2 The Assessment of Bone Responses to Hemiplegia

2.2.1 Skeletal Responses to Underloading: Human Models

Muscular activity and ambulation are regarded as important factors in the maintenance of the skeletal system in humans. It is generally accepted that a reduction in compression force (i.e. from not bearing weight or weightlessness) and reduced tension forces (i.e. from impaired muscular and ligamentous forces as a result neurological injury) are detrimental to the maintenance of skeletal integrity. In experimental and pathological conditions where humans are subjected to underloading via reduced compressive strains (bed rest studies), or both compressive and tensile strains (i.e. spaceflight and spinal cord injury) bone histomorphometry has been performed. Similarly, biomarker and PTH/vitamin D axis changes have been reported. The major histomorphometric and biomarker findings in studies of underloading in humans are shown in tables 2.1 to 2.3.

Table 2.1 Experiments in human skeletal underloading. Head-down tilt bed rest

Author, Year and Experiment Type	Subjects Sex and Age	Duration (weeks)	Significant Skeletal/Endocrine Significant Changes
Vico <i>et al.</i> , 1987 Paired biopsy study. (Double labelled)	3♂ 32.8+/- 4.5	17	↔ Osteoid parameters ↑ Erosion parameters (ES/BS) ↔ BV/TV Other significant: ↓ MAR
Arnaud <i>et al.</i> , 1992 Leuken <i>et al.</i> , 1990 Biomarker studies.	8♂ 39 +/- 3.5	1	↑ iCa^{2+} ↓ PTH ↓ 1,25(OH) ₂ D ↑ Resorption markers ↔ Formation markers
Palle <i>et al.</i> , 1992 Paired biopsy study. (Not labelled)	5♂ 19-42	17	↓ Osteoid parameters (OS/BS) ↔ Erosion parameters ↔ BV/TV
Zerwekh <i>et al.</i> , 1998 Paired biopsy study. (Double labelled)	9♂ 2♀ 34 +/- 11	12	↑ Ca^{2+} ↓ PTH ↓ 1,25(OH) ₂ D ↑ Resorption markers ↓ Formation markers ↔ Osteoid parameters ↑ Erosion parameters (ES/BS) ↔ BV/TV Other significant: ↓ Osteoblast surface

Table 2.2. Experiments in human skeletal unloading. Space flight

Author, Year and Experiment Type	Subjects Sex and Age	Duration (weeks)	Skeletal/Endocrine pathophysiology Significant changes
Collet <i>et al.</i> , 1997 Biomarker Study.	2 ♂	4 (n=1) 26 (n=1)	↔ Resorption markers ↓ Formation markers
Caillot-Augusseau <i>et al.</i> , 1998 Biomarker Study.	2 ♂ 36-40	26	↔ Ca ²⁺ ↓ PTH ↑ Resorption markers ↓ Formation markers

Table 2.3 Experiments in human skeletal unloading. Spinal cord injury

Author, Year and Experiment Type	Subjects Sex and Age	Duration (weeks)	Skeletal/Endocrine pathophysiology Significant changes
Minaire <i>et al.</i> , 1974 Biopsy study. Cross sectional (Double labelled)	28	25 (mean)	↓ Osteoid parameters (OV/BV)* ↑ Erosion parameters (ES/BS)* ² ↓ BV/TV* ³ Other significant: * up to c.10 weeks then normalised * ² up to c. 16 weeks then gradually normalised * ³ up to c. 20 weeks then stable 50% decrease in cortical thickness by 40 weeks
Stewart <i>et al.</i> , 1982 Biochemistry Study.	14		↔ Ca ²⁺ ↓ PTH ↓ 1,25(OH) ₂ D Other significant: ↑ Urinary Ca ²⁺ excretion
Roberts <i>et al.</i> , 1998 Biomarker Study.	30		↑ iCa ²⁺ ↓ PTH ↑ Resorption markers ↔ Formation markers

Studies of healthy volunteers subjected to experimental head-down bed rest protocols between 1 and 17 weeks duration have reported consistent skeletal and endocrine changes. In young healthy volunteers, a rapid increase in bone resorption occurred in response to head-down bed rest (Leuken *et al.*, 1990; Rittweger *et al.*, 2005; Vico *et al.*, 1987;

Watanabe *et al.*, 2004; Zerwekh *et al.*, 1998). Paired iliac biopsies were obtained from 19 patients in three separate studies (Palle *et al.*, 1992; Vico *et al.*, 1987; Zerwekh *et al.*, 1998). Cancellous eroded surfaces were increased in two studies (Vico *et al.*, 1987; Zerwekh *et al.*, 1998), but were unchanged in another (Palle *et al.*, 1992). Decreased bone formation was observed in some (Palle *et al.*, 1992; Vico *et al.*, 1987; Zerwekh *et al.*, 1998), but not all studies (Leuken *et al.*, 1990). Two studies involving paired biopsies (before and after bed rest) found a significant decrease in histomorphometric indices of bone formation. Osteoid surface declined significantly during 17 weeks bed rest (Palle *et al.*, 1992) in 5 subjects, while in a separate study the osteoblast-covered surface declined significantly during 12 weeks bed-rest in 11 subjects (Zerwekh *et al.*, 1998). The calcium/parathyroid/vitamin D axis appeared to respond in a consistent fashion during human underloading. In the bed rest studies, serum calcium was increased (as early as 7 days, presumably because of osteoclast activity) inhibiting PTH secretion and 1,25(OH)₂D levels also declined (Arnaud *et al.*, 1992; Rittweger *et al.*, 2005; Watanabe *et al.*, 2004; Zerwekh *et al.*, 1998). In subjects with lumbar disc prolapse who were immobilised for 10 days, serum calcium also increased and 1,25(OH)₂D declined (van der Wiel *et al.*, 1991). A decline in PTH was similarly observed during spaceflight (Caillot-Augusseau *et al.*, 1998) and after spinal cord injury (Roberts *et al.*, 1998; Stewart *et al.*, 1982), although the increase in serum calcium achieved significance only when the ionised fraction was measured (similar to stroke (Sato, 2000c)).

In paraplegic patients, an early increase in eroded surface and decrease in osteoid volume resulted in a significant reduction in bone volume that eventually stabilised (Chantraine *et al.*, 1986; Minaire *et al.*, 1987). Thereafter a new steady state developed, with reduced bone turnover and cortical thinning (Minaire *et al.*, 1974).

Altered skeletal loading in animal species has been simulated using a variety of methods including hind limb unloading and tail suspension in rats, plaster cast immobilisation in dogs, restraint in primates, calcaneal fixation in sheep and surgically isolated ulnae in turkeys. A recent review of animal models of unloading and functional isolation has been published (Giangregorio and Blimkie, 2002). While important insights into skeletal responses have been gained from the use of such models, there remains considerable diversity in skeletal and endocrine regulatory processes amongst these different animal

models. Such models, whilst of great importance in physiological understanding of simulated microgravity and functional isolation, may be less applicable to the study of adult humans with neurological injury and hemiplegia who are initially bed-bound. Stroke in humans is a highly heterogeneous condition encompassing a range of deficits and a recovery pattern and clinical course that is often unpredictable. Therefore, when considering bone loss after stroke in humans, factors such as the degree of functional impairment, initial bed rest and recovery (Jorgensen *et al* 2000a) and parathyroid/vitamin D axis derangement (Sato, 2000c) are key factors alongside alterations in skeletal loading. Animal models of underloading do not reflect these conditions well, so they will not be discussed further here.

2.2.1 Biomarker Studies in Stroke

The mechanism of bone loss at a tissue level in stroke is not well elucidated. Ionic hypercalcaemia from an increase in bone resorption has been implicated in early bone loss after stroke by biomarker evaluation and radio-labelled calcium kinetics (Davie *et al.*, 1999; Sato, 2000c; Sato *et al.*, 1998a; Sato *et al.*, 1998b; Van Ouwenaller *et al.*, 1989). Histomorphometry has not been performed in stroke patients to date, but indirect measures such as biomarker evaluation and bone densitometry have provided clues regarding the pathophysiology of bone loss in hemiplegia. A rapid increase in bone resorption following stroke was suggested by a week-long study of serum 1-carboxy terminal peptide (ICTP) levels (Sato *et al.*, 2000b). The ICTP levels in two groups of long standing hemiplegics were also evaluated, with evidence of persisting elevation to 1 year, but normalisation between 1 and 2 years post-stroke (Sato *et al.*, 1998a). Prospective evidence for a persistent rise in resorption markers (urinary deoxypyridinoline to urinary creatinine ratio) was provided by the 180 subjects in the placebo arm of a risedronate study in acute stroke patients, whose mean values were increased relative to baseline (1st day after stroke) at 6 months (+73.4%) and at 12 months (+35.8%) (Sato *et al.*, 2005c).

Evidence also exists for an early (within one week) suppression of bone formation markers, as a reduction in serum osteocalcin occurred within one week of acute stroke (Sato *et al.*, 2000b). In placebo treated patients from the aforementioned oral risedronate trial, this bone formation index remained suppressed below baseline levels at 6 months (-39.8%) and at 1 year (-28.3%) following stroke. Interestingly, oral risedronate resulted in

significantly less suppression of osteocalcin (-9.8% at 1 year) in addition to the expected effect on reducing urinary deoxypyridinoline (Sato *et al.*, 2005c).

After stroke, bed-rest type immobility with generalised skeletal ‘underloading’ occurs initially, but this may be combined with profound local ‘unloading’ of affected limbs due to neurological injury. The biomarker studies described above cannot distinguish the localised skeletal changes from the generalised changes that occur after stroke. For instance, bone densitometry studies of stroke patients have established that the hemiplegic proximal femur, loaded by tensile and compressive forces during normal gait, is particularly vulnerable to bone loss (Chapters 1.2.2 and 1.2.3). Therefore, bone tissue analysis with histomorphometry is required.

2.2.2 Histomorphometry in Stroke

Quantitative analysis of bone histology ('histomorphometry') is a valuable research tool in the assessment of bone turnover, remodelling and structure. In contrast to non-invasive techniques such as bone imaging and bone turnover marker assessment, bone histomorphometry enables the study of bone's cellular activity and structure in a single exercise. The usual site of bone biopsy for histomorphometry is the ilium (fig 2.6). When examining histological sections of bone with the microscope it is possible to distinguish and measure features indicative of past and present bone remodelling. Static bone histomorphometry is the quantitative analysis of these features. Dynamic histomorphometry using timed tetracycline labels permits estimation of current remodelling activity in the biopsy specimen and estimation of kinetic variables (Frost, 1969). To date there have been no histomorphometric studies of bone from stroke patients.

Sites of bone resorption can be identified microscopically (fig. 2.1 A and D). Staining frozen bone sections with tartrate resistant acid phosphatase (TRAP) aids in the identification of cells from the osteoclast lineage. The eroded surface and osteoclast number are among the most difficult to identify reliably, both within and between operators (Compston *et al.*, 1986), although the use of polarised light may aid identification sites of resorption (Vedi *et al.*, 1984). The eroded surface measurement includes sites of previous resorption (in reversal) as well as present resorption. The surface extent and volume of unmineralised osteoid are simple indices to determine using special

bone stains (fig. 2.1 C and D). The surface extent of fluorescent label (laid down at the mineralisation front) is known as the mineralising surface and the percentage mineralising surface is usually calculated as the double-labelled bone surface plus half the single labelled surface. The distance between paired labels can be used to determine the rate at which new bone is mineralising in micrometers per day (known as the mineral apposition rate). The mineral apposition rate is higher in cortical bone (fig. 6.6 J) than cancellous bone. Derived indices based on mathematical reconstruction of the dynamics of the bone remodelling cycle can provide additional estimations of interest such as the likelihood of a surface remodelling cycle on any part of the bone surface (activation frequency) and the bone formation rate, but require sufficient surface remodelling events to be reliable. Measurement of the wall thickness (mean thickness of completed BMU's) can give an indication of how well the resorption space created by the osteoclasts has been filled in by osteoblasts (fig. 2.1 C and 2.5). The method of performing and analysing iliac bone samples using histomorphometry is given in Chapter 5.3 and the relevant formulae are listed in Appendix 2. In Chapter 5, differences in ilial bone histomorphometric parameters between stroke patients are assessed by taking a single trans-iliac biopsy from 14 volunteers at a single time point (approximately 10 weeks post-stroke). In determining the effects of hemiplegia and zoledronate on the skeleton, examination of bone from the proximal femur would be preferable, but it is not a site that can be easily and safely biopsied (a well-recognised limitation). Nevertheless, the ilium may be a reasonable surrogate in stroke since the muscles of hip abduction and flexion used during walking attach to the ilium (fig. 2.6).

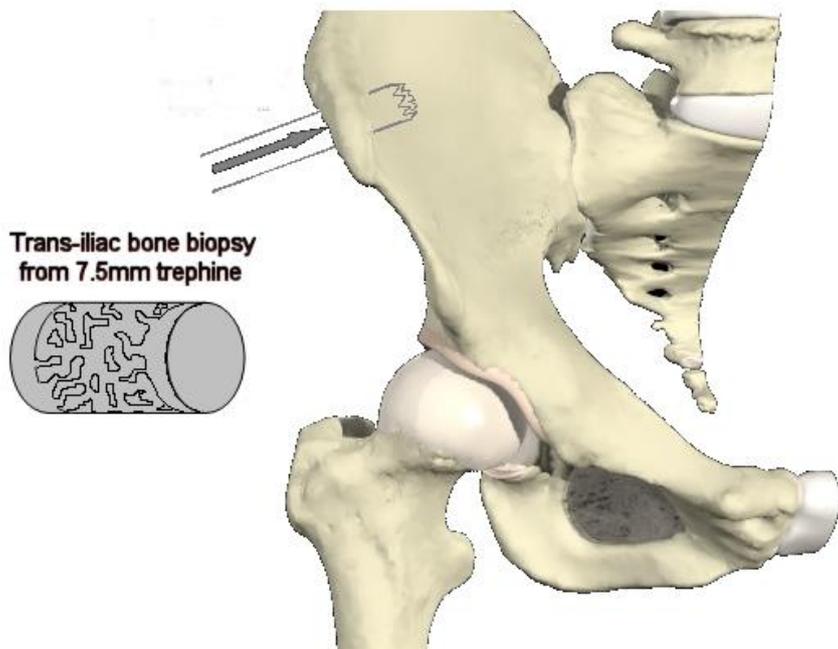
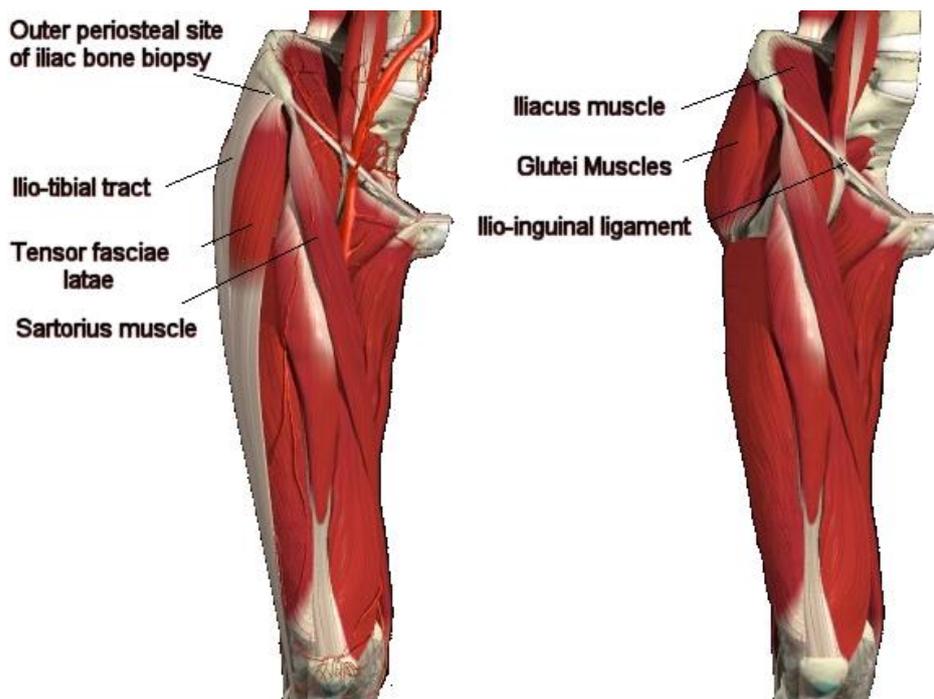


Figure 2.6 Muscular attachments to the right anterior ilium (*above left*). Deeper attachments (*above right*). Use of the modified Bordier's trephine to take a 7.5 mm core biopsy of a site 2 cm inferior and 2 cm posterior to the anterior superior iliac crest (*below*). Modification of image from Primal Pictures 2003.

2.2.4 Skeletal Responses to Hemiplegia; A Hypothesis

Bone histomorphometric and biomarker studies in healthy humans have suggested a cellular response to skeletal unloading involving an early increase in both osteoclastic resorption and serum ionised calcium. This may be followed by impaired bone formation mediated by inhibition of the PTH/vitamin D axis. Biomarker studies have indicated a similar response to hemiplegia. The third hypothesis to be tested in this thesis is that hemiplegic patients treated with zoledronate or placebo have altered bone remodelling parameters as assessed by histomorphometry.

2.3 Osteocytes and the Control of Bone formation

Advances in cell biology, cell culture and genetic disciplines have led to a greater understanding of individual events in the bone remodelling cycle at a cellular level, with osteoclast and osteoblast molecular biology at the forefront. Despite these advances in understanding of bone's cellular physiology, the precise role of the osteocyte (which is ideally located to influence surface modelling and remodelling events via the lacunocanalicular network) remains obscure. This is in part because of difficulties in reproducing the intricacies of the osteocyte's mineralised surroundings, spatial arrangement and cellular connections in an experimental system. Although 'osteocyte-like' cell lines have been studied (for instance the chick MLO-Y4 cell line (Kato *et al.*, 1997)), there are uncertainties concerning how much these cells share in common with osteocytes in human bone.

There are technical challenges in studying a cell that is both dendritic and encased in hard mineral in its native environment. Although osteocytes in adult bone have an approximate half life of 25 years (Knothe Tate *et al.*, 2004), the cells undergo a change in morphology and possibly function over time (Palumbo, 1986). Nevertheless, the unique spatial arrangement of osteocytes permits accurate evaluation of protein expression using immunohistochemical staining, with further information available if tetracycline labelling is used. Accordingly, the role of osteocytic proteins in promoting or inhibiting osteoclastic resorption has been studied by careful immunohistochemical staining of bone specimens from human fracture and control as well as animals subjected to diverse loading conditions. This approach has led to discoveries of osteoclast attraction to areas of osteocyte apoptosis (Noble *et al.*, 2003) and more recently an osteocytic response involving up-regulation of HIF 1- α and osteopontin in conditions of disuse (Gross *et al.*, 2001; Gross *et al.*, 2005).

In contrast to their roles in modifying bone resorption by osteoclasts, the role of osteocytes in influencing bone formation was largely theoretical (Marotti, 1996; Martin, 2000b) until the recent discovery of 'sclerostin', the secreted product of the SOST gene. Sclerostin was found to be an osteocyte-derived inhibitor of active osteoblasts. An understanding of the

critical role of timely inhibition of bone formation by osteocytes in humans necessitates a brief review of the single gene disorder sclerosteosis, where SOST function is lost.

2.3.1 Sclerosteosis and Impaired Osteocyte Control of Bone Formation

Sclerosteosis (OMIM 269500) is a rare autosomal-recessive disease that manifests as a systemic skeletal syndrome with a high bone mass phenotype and markedly increased bone formation (Beighton *et al.*, 1976a). The disease has been identified primarily within the Afrikaner population of South Africa (Beighton, 1988; Beighton *et al.*, 1984; Hamersma *et al.*, 2003; Treswell, 1958). Clinically, sclerosteosis patients display hyperostosis (fig. 2.7 and 2.8) with narrowing of skull foramina and subsequent compression of cranial nerves, leading to pain, facial palsy (paralysis), hearing loss, speech impediment and headaches, often presenting before 4 years of age (Beighton *et al.*, 1977; Beighton *et al.*, 1976a; Beighton and Hamersma, 1979; Epstein *et al.*, 1979; Hamersma *et al.*, 2003). Other clinical features of sclerosteosis include syndactyly, tall stature, radial deviation of the terminal phalanges, nail dysplasia and strong dentition. Although surgical resection of bone can relieve symptoms, patients are at risk of sudden death because of raised intracranial pressure (Beighton, 1988; Beighton *et al.*, 1976b).

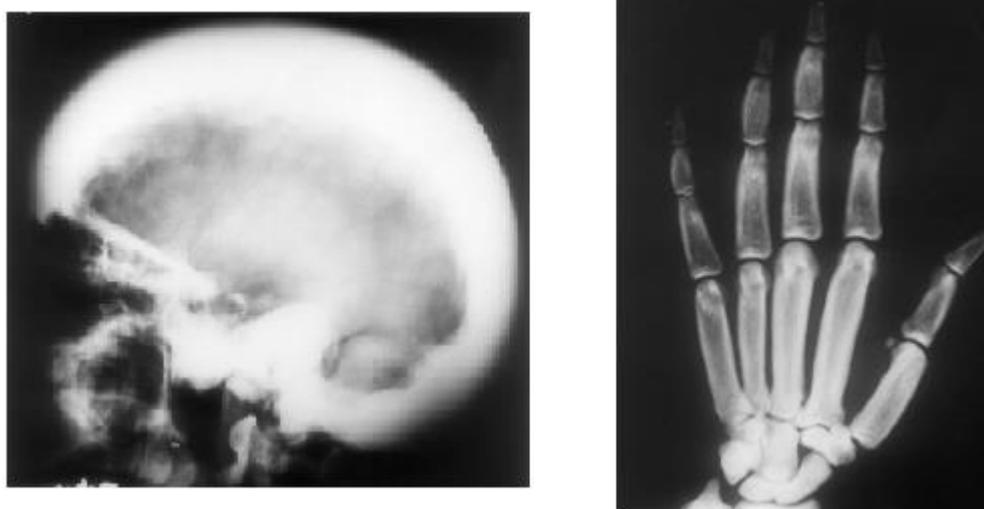


Figure 2.7 Lateral skull radiograph of a patient with sclerosteosis (left). Gross calvarial thickening is evident. AP radiograph of the hands of an adult with sclerosteosis. The shafts of the tubular bones are widened and irregular with marked cortical hyperostosis (Hamersma *et al.*, 2003).



Figure 2.8 30 year old subject with sclerosteosis. Note the unilateral facial nerve palsy (left), syndactyly repair (left 3rd and 4th phalanges), angulation of the distal phalanges and large mandible (Hamersma *et al.*, 2003).

Histomorphometry in such patients reveals a low-normal eroded surface but doubling of the cancellous bone volume and labelled surface. There is also a 5-fold higher osteoid volume, raised mineral apposition rate and bone formation rate (Stein *et al.*, 1983). In addition, craniotomy specimens reveal thickened cortical bone, with an elevated osteoid surface but normal osteoid thickness.

Sclerosteosis patients typically have single nucleotide mutations in the SOST gene located at chromosomal region 17q12-21, the commonest of which is a cytosine to thymine substitution resulting in a premature stop codon (Brunkow *et al.*, 2001). Consequently, a non-functioning transcript is produced. The SOST gene contains two exons and is predominantly transcribed in bone and cartilage, but also found in very low levels in liver, kidney and placenta (Brunkow *et al.*, 2001). The product of the SOST gene is ‘sclerostin’, a deduced 213-amino acid protein and a member of a large family of cysteine-knot containing secretory proteins. Evidence is increasing that sclerostin, an inhibitor of bone formation is solely expressed in osteocytes (Poole *et al.*, 2005b; van Bezooijen *et al.*,

2004). In vitro experiments have shown that sclerostin is an inhibitor of active osteoblasts whose precise cellular target is presently the subject of debate (Li *et al.*, 2005; Sutherland *et al.*, 2004; van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003). It was postulated that sclerostin released from osteocytes could control the proliferation and differentiation of osteoprogenitors/pre-osteoblasts as well as mature osteoblasts (Winkler *et al.*, 2003). In vitro experiments showing that sclerostin could directly inhibit bone morphogenic protein (BMP) actions (Sutherland *et al.*, 2004; Winkler *et al.*, 2003) were not reproducible in independent studies using physiological concentrations of sclerostin (van Bezooijen *et al.*, 2004). Recent evidence from several groups has confirmed that sclerostin binds LRP-5 and 6 and inhibits Wnt canonical signalling (Li *et al.*, 2005) (van Bezooijen, personal communication).

Various groups have attempted to localize SOST mRNA and sclerostin protein in human and animal tissues. In situ hybridisation was used to localise SOST in 15.5 day old embryonic mice. SOST expression was observed in mineralising tissues e.g. palate, rib, mandible, cervical vertebrae and skull bones (Winkler *et al.*, 2003). Immunohistochemical staining of fixed and decalcified human bone tissues (surgical site unspecified) using rabbit monoclonal antibodies raised against human sclerostin was also reported. Osteocytes and cell processes in both cortical and cancellous bone stained strongly for sclerostin, but staining was also reported (weakly) in osteoblasts and chondrocytes. (Winkler *et al.*, 2003). Although osteoblasts in culture have been shown to express SOST mRNA and sclerostin protein at a late stage (>c.18 days), a panel of 30 mouse monoclonal antibodies tested on human bone biopsies failed to show any osteoblast or lining cell immunostaining (van Bezooijen *et al.*, 2004). However, osteocytes, particularly those deep in the cortex were consistently positive in those experiments (van Bezooijen *et al.*, 2004).

Although no human over-expression phenotype has been described, the function of sclerostin was assessed using an in vivo model by over-expressing human sclerostin in transgenic mice (Winkler *et al.*, 2003). These mice displayed thin cortices, disorganised architecture, reduced cancellous bone volume and impaired lamellar bone formation. The osteoid surface and osteoid area were significantly lower than in wild type controls. Bone resorption parameters were not affected.

2.3.2 Osteocytes and the Control of Bone Formation in Stroke; a Hypothesis

Osteoid surface and volume were significantly reduced in iliac crest specimens from hemiplegic patients (Chapter 5). Histomorphometric analysis of bone from animals created to over-express human osteocytic sclerostin showed similar global reductions in osteoid surface and volume. The fourth hypothesis to be tested in this thesis is that in the iliac bone of stroke patients, there is an increase in osteocytes expressing sclerostin, which results in reduced osteoblast activity and osteoid surfaces. To test the hypothesis, the relationship between the percentage of osteocytes expressing sclerostin and the surface extent of osteoid in stroke will be examined in cortical and cancellous bone.

Chapter 3. A Single Injection of Zoledronate Prevents Hip Bone Mineral Density

Loss after Stroke

3.1 Abstract

Hip fractures are a significant complication of stroke. In addition to an increased risk of falling, patients lose bone rapidly from the hemiplegic hip due to increased bone resorption and reduced bone formation. This study evaluated the efficacy of a single dose of zoledronate, an intravenous bisphosphonate, in preventing bone loss after stroke. In a 1-year randomised double-blind placebo controlled clinical trial, 27 newly hemiplegic patients (6F, 21M) with acute stroke were assigned to receive 4 mg of the study drug (n=14) or placebo (n=13) within 35 days. Both groups received calcium and vitamin D from shortly after admission. The primary outcome measure was the bone mineral density (BMD, Lunar Prodigy) at both hips, at baseline, 6 and 12 months. After 1 year, patients in the placebo group had a significantly greater reduction in BMD in both hips than those in the zoledronate group (hemiplegic side $p=0.0003$, unaffected side $p=0.002$; repeated measures ANOVA). The mean total hip BMD was unchanged in the hemiplegic hip of the zoledronate group (mean 0.0% change, 95%CI -1.3,+1.3), while in the placebo group BMD decreased substantially at this site (mean -5.5% 95%CI -8.2,-2.8), with the greatest decrease observed in the trochanteric region (mean -8.1, 95%CI -11.8, -4.5). On the unaffected side the mean change in total hip BMD was +1.0% with zoledronate (95%CI -0.1, +2.5) vs. placebo -2.7% (95%CI -4.8, -0.62, $p=0.002$). Computed tomography (hQCT) of the hemiplegic and unaffected hips in 8 untreated patients one year after stroke confirmed that the greatest difference between sides was in the trochanteric region. The mean cortical thickness of a single slice through the femoral neck was also significantly lower on the hemiplegic side. Reductions in serum calcium and phosphate occurred more commonly following zoledronate than placebo, but otherwise the treatment was well tolerated. In conclusion, a single infusion of zoledronate given within 35 days of acute stroke protected against the deleterious effects of hemiplegia on hip bone mineral density.

3.2 Introduction

Stroke patients are prone to falls, particularly onto the hemiplegic side. Hemiplegia and subsequent immobility predispose these patients to disturbed bone physiology, resulting in reduced bone mineral density (BMD) in the hemiplegic hip. These factors result in a substantial increase in hip fractures in both sexes and across all age ranges after stroke (Kanis *et al.*, 2001). Most hip fractures after stroke are on the paretic side (Chiu *et al.*, 1992), with 84% caused by accidental falls in one series (Ramnemark *et al.*, 1998). Several prospective (Hamdy *et al.*, 1993; Jorgensen *et al.*, 2000a; Ramnemark *et al.*, 1999a) and cross-sectional (del Puente *et al.*, 1996; Takamoto *et al.*, 1995) studies have shown a reduction in BMD in the hip of the affected side following hemiplegic stroke. Bone loss of the greatest magnitude occurred in those unable to walk one week following stroke (Jorgensen *et al.*, 2000a; Ramnemark *et al.*, 1999a). Zoledronate (Novartis, Basle, Switzerland), a new potent intravenous bisphosphonate, has a short administration time (15 minutes), is well tolerated and has long-lasting effects on hip BMD in postmenopausal women (Reid *et al.*, 2002). Zoledronate is therefore a good candidate intervention to prevent bone loss after stroke, particularly since the infusion could be easily administered in an acute stroke unit soon after stroke onset (Poole *et al.*, 2002). The aim of the present study was to investigate whether the early use of a single dose of intravenous zoledronate in patients admitted to an acute stroke unit could prevent the decrease in BMD in the hemiplegic hip of stroke patients. The study consisted of a randomised double-blinded placebo-controlled trial of intravenous zoledronate (4 mg) to prevent bone loss in hemiplegic patients with acute stroke. Bone mineral density in both hemiplegic and unaffected hips of hemiplegic patients was assessed and patients were randomised to receive a single infusion of either 4 mg zoledronate or placebo within 35 days of acute stroke. Serial measurements of hip BMD were made at 6 and 12 months in addition to detailed assessments of stroke recovery and functional status. Secondary outcome measures included within-subject differences in volumetric BMD and femoral neck cortical thickness by hip quantitative computed tomography (hQCT) of the hemiplegic and unaffected hips in selected patients.

3.3 Methods

Study Population

The study population consisted of patients admitted to the Lewin Stroke Unit at Addenbrooke's Hospital between October 2001 and July 2004. For inclusion, males and females aged 40-89 were approached as soon as possible after admission with first ever stroke. Patients were eligible if they were previously independently walking, had clinical and CT evidence of stroke (haemorrhagic or ischaemic), were unable to walk one week following stroke (Functional Ambulatory Category, FAC; 0 or 1) and could give written informed consent to the study. Exclusion criteria are shown in table 3.1. During assessment for eligibility, the first exclusion criterion for each patient was recorded as shown in the far right column (393 patients in total).

Table 3.1 Exclusion criteria for the RCT. The first reason for exclusion was recorded for each subject

<i>Reason for Exclusion</i>	<i>Patients, n</i>
1. Patient not walking independently prior to stroke or previous stroke causing hemiplegia	44
2. Stroke not affecting the lower limb (Functional Ambulatory Category (FAC) >1 at assessment 7 days after stroke or posterior circulation stroke)	149
3. Unconsciousness or terminal illness	13
4. Pre-existing dementia or cognitive impairment	32
5. Aphasia/ significant language impairment	45
6. Renal*/hepatic impairment	12
7. Aged <40 and >89	35
8. Known or baseline osteoporosis or prior treatment with a bisphosphonate, corticosteroids or unilateral bone disease affecting BMD or prior hip fracture or osteosynthetic material at the hip, e.g. hip replacement	26
9. Unable to randomise and give infusion within 35 days of stroke, e.g. tertiary referrals from another hospital	37
10. Current treatment with an aminoglycoside antibiotic	0

*Renal impairment was added as a protocol amendment, see section 3.4

Written informed consent was obtained in accordance with the Second Helsinki Declaration and Royal College of Physician guidelines. The study was approved by the

regional committee for research ethics. The flow of patients through the trial is shown in figure 3.1. Patients underwent dual energy x-ray absorptiometry scanning of both hips as soon as possible after consenting. Patients with baseline osteoporosis (T score <-2.5 at either hip, n=3) were excluded before randomisation and offered alternative therapy. Study patients received calcium (1g) and vitamin D (800 IU) from the day of consent until the end of the study (12 months). Due to the discovery of a high prevalence of vitamin D insufficiency amongst stroke inpatients of all ages immediately after admission (Chapter 4) and emerging evidence of severe hypocalcaemia in patients with vitamin D deficiency given potent intravenous bisphosphonates (Peter *et al.*, 2004; Rosen and Brown, 2003) a protocol amendment was made. The vitamin D level was assessed as soon as possible after admission and if it was <10 ug/l, patients were supplemented with 100,000 IU of ergocalciferol before infusion. Thereafter calcium and vitamin D were given as per protocol. Central randomisation was used, with random numbers generated and the code sealed and kept centrally by a designated trials pharmacist who, after receiving notification of a study participant by the trial team, was responsible for preparing the colourless drug or placebo infusion in 50 ml coded sachets (according to the random number sequence) on the day of infusion. The trial team then collected the coded 50 ml sachet from a receptionist. The infusion was administered over 15 minutes followed by a single bag of 250 ml of 0.9% sodium chloride solution over 2 hours. Both the administering team, receptionist and the patients were blinded to the treatment allocation and preparation. A single planned interim analysis was conducted involving the 16 patients who had completed the study protocol at the end of the second year of the study (Poole *et al.*, 2004). The trial continued beyond the interim assessment because the difference between groups did not achieve the nominal significance level of $p=0.001$ for an interim test (Haybittle, 1971; Peto *et al.*, 1977). The trial was issued with the following International Standard Randomised Controlled Trial Number; ISRCTN57438091.

3.3.1 Assessment of Hip Bone Mineral Density

BMD was measured using a narrow angle fan-beam dual energy x-ray absorptiometer (Lunar Prodigy, enCORE software V6.8 2002, Lunar, Maddison, WI). Scans of both hips were performed in all patients using the automated 'dual femur' program at recruitment, 6 months and 12 months after the first measurement. All scans were obtained by a single operator (KESP) to minimise positioning errors. The "copy region of interest" function

was used to ensure duplicate ROI placement and hip orientation on follow-up scans. The 'total hip' and 'femoral neck' regions were determined automatically as described in the Lunar manual. The short term coefficient of variation (CV) was determined by repeating the BMD measurement on the hemiplegic side of 3 stroke patients 3 times with repositioning in between. CV was 1.03% (total hip) and 1.12% (femoral neck), as shown in Appendix 1. The longitudinal drift assessed by encapsulated aluminium spine phantom measurements was <1%.

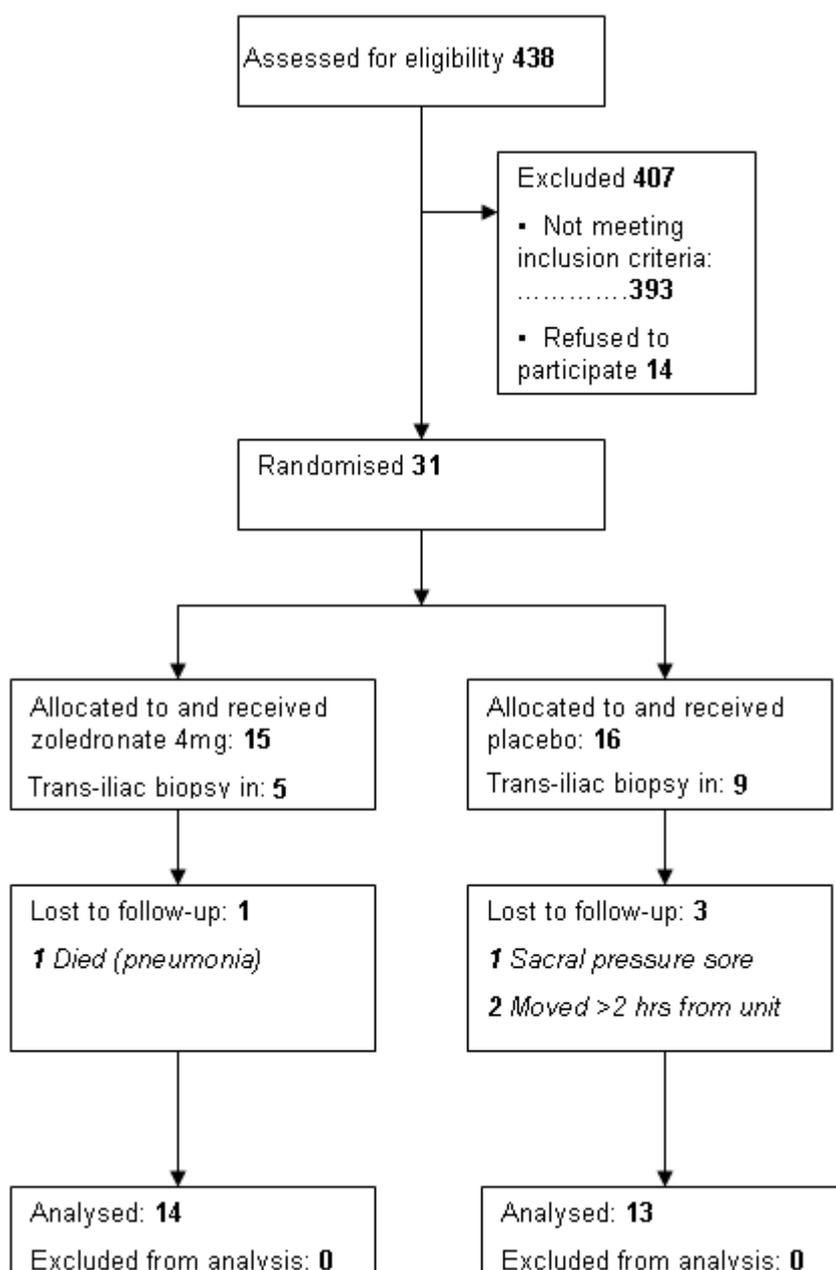


Figure 3.1 Flow of patients through the Randomised Control Trial.

3.3.2 Assessment using hQCT

From the study population, 8 subjects who had undergone a $>3\%$ decrease in total hip BMD over the 12 month study period (as measured by 2D DXA at the hemiplegic hip) additionally consented to a supplementary scan of both hips using hQCT. The scan was taken after completion of the one-year protocol (mean 22 months after stroke ± 10.9), to assess region-specific differences in volumetric BMD and to estimate femoral neck cortical thickness. Patients were positioned on an ergonomic solid-state phantom containing hydroxyapatite equivalent (Mindways, Austin, Texas, USA) in the CT scanner and a scout image was acquired from the iliac crests to the lesser trochanters. A CT image was then taken using the Siemens Somatom 16 helical scanner (1 mm slice thickness, 'B20s' reconstruction kernel). The 16-detector multi-slice scanner permits high quality 3D reconstruction. CT images were transferred to a computer workstation and processed using commercially available software (Mindways, Austin, Texas, USA) that reconstructed the 3 dimensional image and extracted measures of volumetric BMD (vBMD) and bone size. The processing task involved automatic calibration of the CT images from Hounsfield units to equivalent concentrations (g) of mineral content (calcium hydroxyapatite) against the Mindways solid phantom. The software-determined regions of interest (ROI's) from the proximal femur on both sides were the 3-dimensional equivalents of standard 2D axial DXA ROI's (fig. 3.2). For each region, vBMD (g/cm^3), BMC (g) and bone volume (cm^3) were calculated. The entire region analysed at both proximal femora included the greater and lesser trochanters and femoral neck. In addition, a minimal femoral neck cross-section area (CSA) program was used to select a single slice through the femoral neck for analysis of cortical thickness (fig 3.3). The minimum CSA of the femoral neck was selected as this technique gave the most reproducible and distinctive location precision in tests by the software designers (personal communication, Dr. K. Brown, Mindways software). Cortical thickness was defined as the mean distance between the inner and outer edge of the cortical shell. A recognised limitation of hQCT analysis is that the spatial resolution of the CT scans is larger than the smallest cortical thickness (e.g. the superior femoral neck that may be only 2 pixels wide, fig 3.3). CT pixels at cortical edges tend to measure falsely low densities because of the significantly lower density of the surrounding soft tissues. Therefore where cortical bone is thin, a greater proportion of pixels (representing cortical bone) are only partially filled giving rise to a measurement error known as the 'partial volume' effect. The Mindways algorithm appeared to overcompensate for partial volume

errors. While partial volume errors may affect true estimates, they are unlikely to mask within-subject differences. Therefore, regional differences in estimates were reported with the contralateral hip as a control so that measurement error is constant within the same patient. The longitudinal drift assessed by scanning a solid-state QCT phantom was <1%. The short term CV was determined for the software extraction process (5 extractions of a single hemiplegic hip) as rescanning patients with CT was not ethically permissible. CV for vBMD were 0.46% (total hip) 0.26% (femoral neck) and 0.87% (trochanter). The CV for cortical thickness using the automated minimal CSA selection process was 1.87%. The field of view in the case of hQCT scans was 300 mm with a 512 x 512 pixel matrix. The slice thickness was 1.0 mm. This yielded a pixel size of $300/512 = 0.5859$ mm.

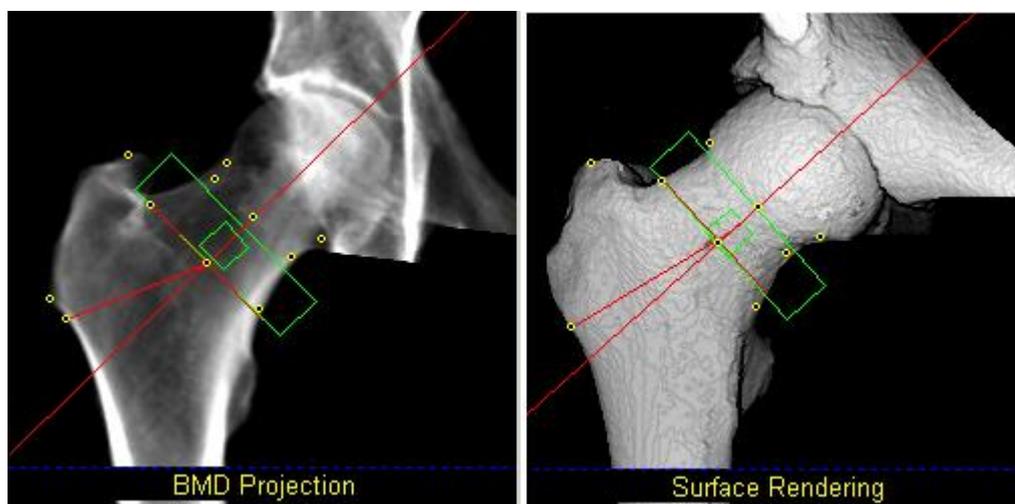


Figure 3.2 hQCT of the right total hip region. Images produced using Mindways© software.



Figure 3.3 Single slice through the hemiplegic femoral neck selected using an automated minimal cross-sectional area technique using Mindways© software. A Anterior P Posterior S Superior I Inferior femoral neck region.

3.3.3 Assessment of Stroke Severity and Functional Status

Three functional scoring systems were employed. Firstly, the physical dependency of the patient was assessed at 0, 6 and 12 months using the modified Barthel score (Mahoney and Barthel, 1965). In this functional dependency test, a score of 100 represents independence whilst a score of 0 denotes total dependence on others. Stroke severity was assessed using the long-term score of the Scandinavian Stroke Scale (SSS), which has a maximum score of 48 (Scandinavian Stroke Study Group, 1985). Assessment was made of the motor power of the hemiplegic side only, with face, arm, hand and leg power scored in addition to walking ability. The Functional Ambulatory Category (FAC) was measured at each of the time points to assess the degree to which each stroke patient was dependent upon assistance for ambulation (Holden *et al.*, 1984). Scales are reproduced in Appendix 1.

3.3.4 Stroke Imaging, Classification and Biochemical Tests

CT brain scan results were recorded at baseline. The clinical stroke syndrome was evaluated using the Oxford Community Health Classification (Bamford *et al.*, 1988). Serum calcium, phosphate, albumin, urea, creatinine and liver function tests were measured at baseline (pre-infusion). Serum intact parathyroid hormone and vitamin D (25OHD) were also measured. Following the infusion, serum calcium, phosphate, magnesium, urea, creatinine and liver function tests were measured daily for 5 days and then at 10 days.

3.3.5 Statistical Analysis

Power calculations based on the data of Jorgensen *et al.* (2000a) indicated that 15 patients per group were required to have a probability of at least 0.8 (at the 5% level of significance) of rejecting the null hypothesis (that the change in BMD would be the same in both groups) if the total hip BMD in the populations differed by 10% at 12 months. Data were analysed using the JMP statistical package (v 4.0, SAS institute, Cary, NC, USA). After consulting a statistician (Dr. S. Kaptoge), the following analyses were performed. A fixed effects repeated measures ANOVA model was fitted with time as the repeated measure. This model was used to compare total hip BMD changes (and changes in sub-regions) between treatment groups. It was also used to assess the effects of the following covariates; age, baseline 25OHD, SSS, Barthel index, FAC as well as the following nominal covariates; sex and stroke type, on BMD data from the hemiplegic and unaffected

sides. The functional scales (SSS, Barthel and FAC) were added to the model independently due to their expected inter-dependence. The Greenhouse-Geisser correction (GG) was applied when the sphericity assumption was not met. For the hQCT data, the principal outcome measure was the difference in vBMD between sides. To test their significance, difference scores (in g/cm^3) were first calculated and then compared against a hypothesised mean of zero using the paired t-test. The significance of any post-infusion symptoms was determined by comparing the proportions of patients from each group with the symptoms and testing the difference in proportions (Appendix 1). The paired t statistic was used to compare change scores in calcium between drug and placebo groups (from pre infusion to day 5 post-infusion).

For

3.4 Results

Study Subjects

The baseline characteristics of the stroke patients in the study are summarised below (table 3.2). There was a high prevalence of baseline vitamin D insufficiency in this acute stroke population (median 25OHD level 34.9 nmol/l, IQR 29.0, 44.4), with no difference between groups. The commonest reasons for exclusion were that patients were ambulant 7 days after stroke or did not have hemiplegia affecting the lower limb (38%, table 3.1).

Table 3.2 Baseline characteristics of stroke patients

<i>Measurement</i>	<i>Zoledronate 4 mg[*]</i>	<i>Placebo[*]</i>	<i>p Value</i>
<i>Demographics</i>			
Age, years	66.9 (11.7)	72.9 (10.4)	0.167 ‡
n (females, males)	14 (4, 10)	13 (2, 11)	
haemorrhage	2	3	
infarction	12	10	
<i>Baseline Total Hip BMD</i>			
Hemiplegic, g/cm ²	1.033 (0.2)	1.014 (0.2)	0.775 ‡
T-score	-0.2 (1.4)	-0.5 (1.2)	
Unaffected, g/cm ²	1.046 (0.2)	0.996 (0.2)	0.452 ‡
T-score	-0.1 (1.6)	-0.6 (1.1)	
<i>Functional Parameters</i>			
Baseline SSS, /48	27.4 (6.6)	24.7 (7.5)	0.320 ‡
Baseline Barthel, /100	43.9 (19.2)	33.9 (22.2)	0.217 ‡

^{*}Mean (standard deviation) ‡ Student's t-test

Bone Mineral Density

BMD was similar at baseline in the two groups. After 1 year, patients in the placebo group had a significantly greater reduction in BMD in both hips than those in the zoledronate group (hemiplegic side $p=0.0003$, unaffected side $p=0.002$; repeated measures ANOVA). The percentage change in BMD for the three main regions of interest (total hip, femoral neck and trochanter) over 1 year is shown in (fig 3.4). The mean total hip BMD was unchanged in the hemiplegic hip of the zoledronate group (mean 0.0% change, 95%CI -1.3,+1.3), while in the placebo group BMD decreased by -5.5% at this site (95%CI -8.2,-2.8). The greatest reduction was in the trochanteric region of the hemiplegic hip where

BMD decreased by -8.1% (95%CI -11.8, -4.5) in the placebo group. On the unaffected side the mean change in total hip BMD was +1.0% with zoledronate (95%CI -0.1, +2.5) vs. placebo group -2.7% (95%CI -4.8, -0.62, $p=0.002$). The greatest reduction on the unaffected side was also in the trochanteric region (-4.5%, 95%CI -7.6, -1.3).

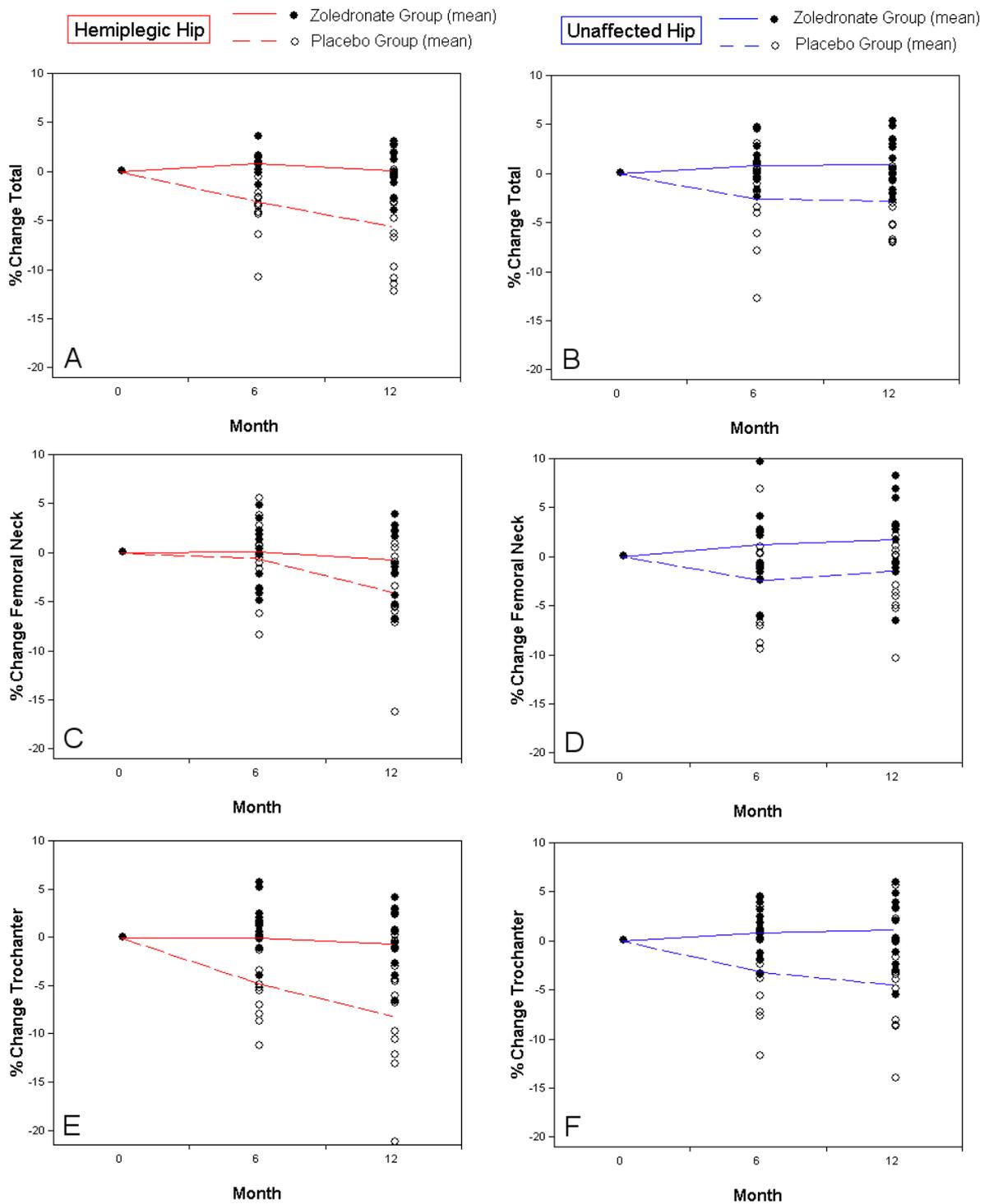


Figure 3.4 Percentage change in main outcome variables with time. Significance of time*treatment interaction by repeated measures ANOVA: total hip BMD A p=0.003** B p=0.002** Femoral neck BMD C p=0.139* D p=0.216* Trochanteric BMD E p=0.003* F p= 0.013** (*unadjusted F test, **adjusted test).

In the repeated measures ANOVA of the hemiplegic side BMD, the effects of age, stroke type, baseline 25OHD, smoking status, SSS, Barthel index and FAC were insignificant. This left one major interaction term; treatment with zoledronate ($p=0.0003$, GG correction). The same was true for the unaffected side BMD, with the significant interaction term also being treatment with zoledronate ($p=0.002$, unadjusted epsilon). Repeated measures ANOVA was performed with SSS, Barthel and FAC respectively as the outcome variables, drug treatment as the between-subjects factor and time as the repeated measure. There were significant mean improvements in the functional and severity scales over time in both groups, but no significant effects of treatment on recovery (SSS; $p=0.693$, Barthel; $p=0.822$, FAC; $p=0.495$ GG correction). There was no significant difference between groups in functional scales at the 12 month time point (SSS $p=0.21$, BI $p=0.40$). The change in BMD in g/cm^2 over 12 months was plotted against the change in SSS or Barthel index (fig 3.5) and in both cases, the changes in functional scales tracked the changes in BMD poorly. This signifies that in these patients, functional recovery did not predict change in bone density well (even in the placebo group), suggesting that changes in SSS or BI were unlikely to have accounted for the changes in BMD observed in these patients.

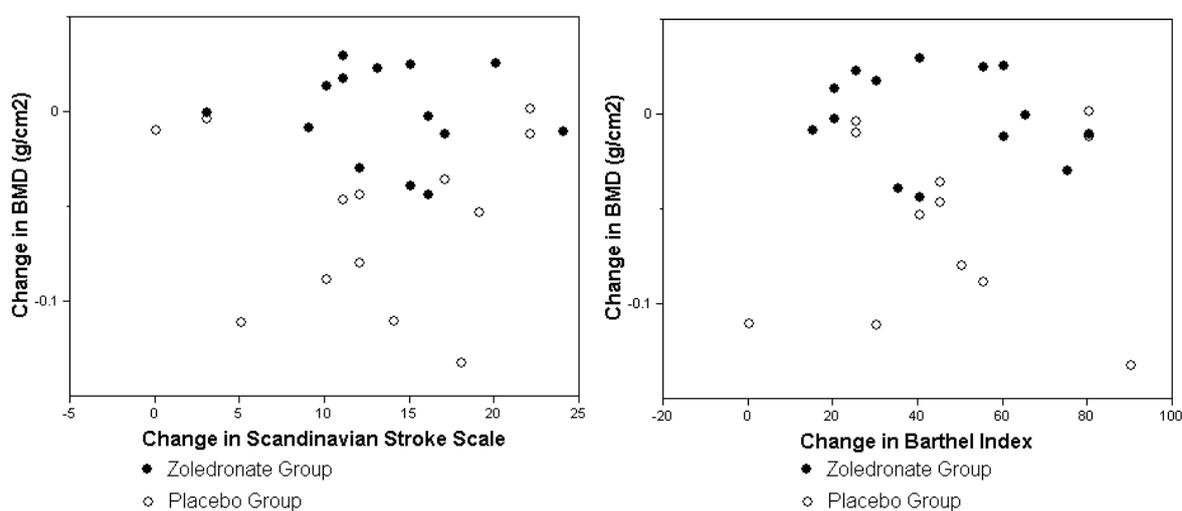


Figure 3.5 The changes in Scandinavian Stroke Scale (*left*) and Barthel Index (*right* $p=0.40$) vs. changes in total hip BMD over 12 months.

Falls and Fractures

There were no fractures in either group during the study. Falls from a standing height or less occurred in 21 stroke patients (72%). The median number of falls per patient was 2 (Range 0-17).

Adverse Events

The treatment was generally well tolerated. Post-infusion symptoms that were significantly more common in the zoledronate treated group were; serum corrected calcium less than 2.1 mmol/l (7 treated vs. 0 placebo patients, $p=0.0003$ for the difference) and post infusion serum phosphate less than 0.8 mmol/l (10 vs. 0 patients, $p<0.0001$). The mean serum calcium concentration in the zoledronate group changed from pre-infusion 2.27 (0.1) mmol/l to 2.13 (0.2) mmol/l 5 days post-infusion ($p=0.03$). In the placebo group mean calcium did not change significantly 2.3 (0.1) mmol/l to 2.32 (0.1) mmol/l ($p=0.21$). Changes in mean serum calcium are shown in (fig 3.6). There were 4 episodes of 'acute phase' symptoms post infusion (malaise and pyrexia) although the difference between groups did not achieve statistical significance (3 with zoledronate and 1 with placebo, $p=0.114$). In addition, there was one adverse event in the zoledronate group. A subject with pre-existing moderate chronic renal impairment had a prolonged severe hypocalcaemic response (nadir 1.55 mmol/l) to an infusion of zoledronate. Urea and creatinine also rose transiently in this patient. The hypocalcaemia was clinically asymptomatic and biochemical parameters returned to baseline levels after the administration of calcitriol. In response, the inclusion criteria were amended to exclude patients with renal impairment or increases in creatinine according to guidelines released by the manufacturer in an amended product schedule.

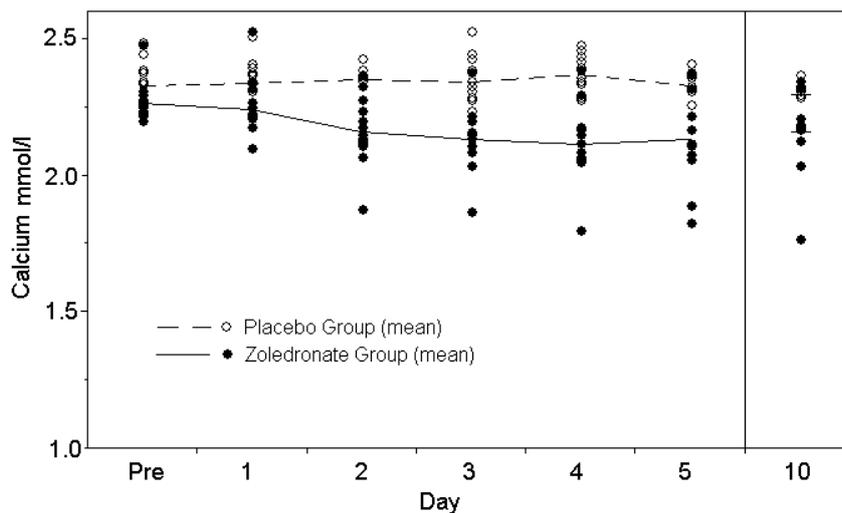


Figure 3.6 Changes in serum calcium from one day pre infusion to day 10 post infusion by group.

hQCT Study Subjects

The hQCT patients were all from the placebo group. The characteristics of the subjects are shown in table 3.3.

Table 3.3 Characteristics of 8 stroke patients selected for hQCT

<i>Measurement</i>	<i>hQCT patients*</i>
<i>Demographics</i>	
Age, years	71.9 (11.3)
n (females, males)	8 (2, 6)
<i>Mean change in Total Hip BMD at 1 year</i>	
Hemiplegic (%)	-5.5 (3.9)
Unaffected (%)	-2.3 (3.6)
<i>Functional Parameters at 1 year</i>	
SSS, /48	34.6 (10.5)
Barthel, /100	77.5 (28.9)
<i>Timing of CT scan</i>	
Months after stroke onset	22 (10.9)

*Mean (standard deviation)

There were statistically significant differences (with the hemiplegic hip significantly lower) in the total hip region vBMD (mean percentage difference -6.8 95%CI -12.3, -1.2), trochanter vBMD (mean % difference -11.2 95%CI -19.0, -3.4) and single femoral neck slice mean cortical thickness (mean % difference -10.1 95%CI -20.8, 0.6) as shown in figure 3.7. The difference in vBMD for the femoral neck ROI did not achieve significance (mean % difference -4.3 95%CI -9.6, 1.0).

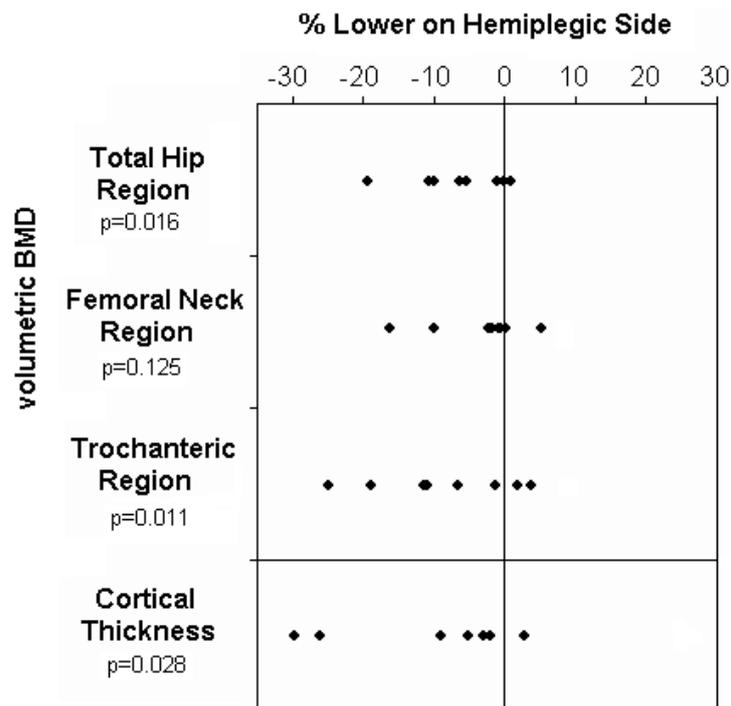


Figure 3.7 Volumetric BMD and Cortical Thickness differences: % lower on the hemiplegic side. p- values relate to unit differences between measurements (g/cm^3 for vBMD, mm for cortical thickness).

3.5 Discussion

In this RCT, a single intravenous dose of zoledronate administered within 35 days of admission effectively prevented bone loss in patients with hemiplegia who were unable to walk unaided one week after stroke. This is the first study to demonstrate the efficacy of bisphosphonates in preventing the substantial loss of bone that can occur in the hemiplegic hip during the first year after stroke. In the total hip region, the mean BMD declined in the placebo group by -5.5% and in the trochanteric region by -8.1%, although there was considerable variation between patients (total hip 95%CI -8.2,-2.8, trochanteric 95%CI, -11.8, -4.5). That zoledronate therapy prevented the loss of BMD in the hemiplegic hip was demonstrated convincingly. This has important implications for the bone health of stroke patients. Stroke patients have a propensity for injurious falls towards the hemiplegic side (Mackintosh *et al.*, 2005). In one large series, 82% of stroke patients admitted with a hip fracture had sustained the fracture on the side of the hemiplegia (Chiu *et al.*, 1992). The present study confirmed the trend for falling, with 72% of patients falling within 12 months, although there were no recorded fractures in either group. Although there are no equivalent studies for comparison in stroke, osteoporotic women in a phase II trial of a single annual injection of zoledronate had a mean increase in femoral neck BMD of +2.5% at 12 months with zoledronate treatment (Reid *et al.*, 2002). In contrast, the mean femoral neck BMD on the hemiplegic side of zoledronate treated stroke patients remained stable at +0.1% change (95% CI -2.5,+2.7) in the present study. This suggests that the zoledronate is acting as an effective countermeasure in stroke, by preventing bone loss but not by increasing BMD.

Patients with stroke have a wide spectrum of initial neurological deficits, coupled to varying patterns of motor recovery, weight bearing and walking function. In this study, subjects were unable to walk independently 7 days after stroke, the aim being to target the intervention at the stroke patients most likely to sustain significant bone loss. Jorgensen *et al.* (2000a) found that this patient group had a significantly greater loss of femoral neck BMD at 7 and 12 months than those who could walk (even if they required assistance) one week after stroke. However, in another prospective study of hip bone loss in more severely affected stroke patients, the presumptive risk factors for bone loss such as motor function, Barthel index and walking ability did not predict an individual's bone loss (Ramnemark *et*

al., 1999a). Similarly, despite a wide range of BMD responses in the present study, the covariates sex, 25OHD, SSS, Barthel, FAC and baseline BMD did not explain the variance in BMD response in either group. There appear to be unmeasured factors that determine the magnitude of bone loss in moderate to severely affected stroke patients. One possible determinant of bone loss is the duration of unloading of the hemiplegic leg (which was the sole predictor of bone loss in a study of unilateral bone loss after lower limb fractures (Van der Wiel *et al.*, 1994)) but this is hard to define accurately in stroke. Another is that autonomic nervous system dysfunction and bone blood flow could affect hip BMD on the affected side (Van Ouwenaller *et al.*, 1989).

The decision to select stroke patients with lower limb hemiplegia for the clinical trial was made partly because of the risk of subsequent bone loss in these subjects. However, this group were also studied because they were considered to be at high risk of subsequent hip fracture. Declining hip BMD is one of several factors thought to predispose stroke patients to hip fracture following a fall. However, the possibility remains that the fall dynamics in recovering hemiplegics result in falls of greater force, without the usual protective reflexes (such as an outstretched hand) to absorb some of the impact (Poole *et al.*, 2002). There is now a need for a carefully designed fracture prevention study evaluating zoledronate or a similar drug in a large acute stroke population. Which patients should be targeted for such an intervention is an immediate challenge to the design of a fracture outcome trial. Epidemiological studies have so far provided evidence of the age-adjusted risk of fractures after stroke (with the highest relative risk in younger stroke patients, but higher absolute risk in the elderly) and their timing (highest fracture risk within one year of stroke) (Dennis *et al.*, 2002; Kanis *et al.*, 2001). However, these studies mainly used hospital coding data and consequently could not determine the fracture risk among groups of patients categorised by their stroke deficits and recovery characteristics. In one small retrospective multivariate analysis it was only stroke patients with initially moderate disability (but able to walk with aids if needed) who had a high risk of subsequent fracture and patients with more or less severe initial disability did not differ significantly from controls. With improved risk estimations, intervention may be better targeted to patients at the highest risk of subsequent fracture.

In acute stroke units and during subsequent stroke recovery, falls and fractures are increasingly recognised among the most costly and devastating complications (Poole *et al.*, 2002). The most common reason that patients were ineligible for this study was that they presented with mild functional stroke deficits, i.e. subjects who were able to walk one week after the event, or had no lower limb involvement (38%). One recent RCT evaluating oral risedronate 2.5 mg daily in acute stroke and continued for a year showed a reduction in hip fractures (1 versus 7) in a group of patients with very mild stroke deficits (Sato *et al.*, 2005c). Patients in the zoledronate study had mean (SD) baseline Barthel indices for placebo and bisphosphonate groups of 33.9 (22.2) and 43.9 (19.2) respectively compared to the 78 (24) and 77 (24) reported by Sato and colleagues. Thus, there may be several approaches available to the stroke unit physician: intravenous zoledronate in those with moderate to severe stroke (FAC 0 or 1) and oral risedronate in those with milder strokes, if swallowing function and an upright posture are achievable early.

Although zoledronate was generally well tolerated, two issues arose from administering the drug in stroke patients. Firstly, there was an unexpectedly high prevalence of baseline vitamin D insufficiency. Recent reports have highlighted the risks of administering potent intravenous bisphosphonates to patients with vitamin D insufficiency (Peter *et al.*, 2004; Rosen and Brown, 2003). The study protocol was amended to allow high dose vitamin D repletion prior to infusion in those with very low vitamin D. Secondly, a prolonged hypocalcaemic episode in a study patient with chronic renal impairment prompted a protocol amendment excluding all patients with renal failure (pre-existing or acquired) from the study. Even in those without established renal failure, regular assessment of renal function (particularly changes in renal function) and hydration status should be undertaken in stroke patients selected for intravenous bisphosphonate treatment.

The greatest loss of bone as assessed by serial DXA was from the trochanteric ROI (fig. 3.4 E and F). This is a similar finding to that reported by Van der Wiel *et al* (1994) in 16 patients followed with DXA for one year after an unstable fracture of the leg. Those patients remained in bed for approximately 10 days and were unable to bear weight for 8 (+/-2) weeks before gradually reintroducing weight bearing through the affected side, a similar pattern to reambulation in the stroke patients studied here. Also in agreement with that study, the changes in femoral neck BMD in these stroke patients (fig. 3.4 C and D)

appeared to occur later than at the trochanter (although changes at this site did not achieve statistical significance). The decline in trochanteric rather than femoral neck BMD may be explained by the higher rate of remodelling in the more cancellous bone of the trochanter compared to the largely cortical bone of the femoral neck (Iversen *et al.*, 1989).

The hQCT studies in 8 of the stroke study patients confirmed vBMD differences of greatest magnitude in the trochanteric region (mean 10% lower on the hemiplegic side, 95% CI -17.3, -1.7). The main muscles of hip abduction and internal rotation that maintain the stability of the trunk during normal locomotion are gluteus minimus and gluteus medius (fig. 3.8). The tendons of these powerful muscles attach to the greater trochanter and exert substantial tensile forces during walking. In stroke where there is initial motor paralysis and an abrupt cessation of walking activity, it is possible that the lack of mechanical force through this region (normally highly loaded) is a cause of the rapid decline in BMD and vBMD at the trochanter. Cancellous bone is also sensitive to the anabolic effects of small amplitude, high frequency applied forces, as demonstrated by Rubin *et al* (2001) in an animal model. The loss of bone from this predominantly cancellous region in stroke patients might also reflect a reduction in the small strain forces that would normally accompany such activities as maintaining posture and standing.

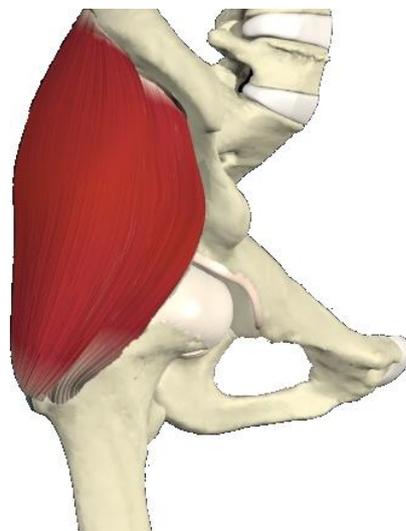


Figure 3.8 The glutei (minimus and medius) and their attachments to the pelvis and greater trochanter.

Unlike the findings of Jorgensen *et al.* (2000b), there were lower magnitude changes in the femoral neck ROI than in the trochanteric and total hip ROI's using DXA in these patients.

Using hQCT with a narrow slice width (1 mm), accurate assessment of cortical thickness at the femoral neck is technically feasible for both difference and absolute values (Prevrhal *et al.*, 2003). There was a significantly lower mean cortical thickness (of a single femoral neck slice) on the hemiplegic side than on the unaffected side. There was no significant difference in mean femoral neck cross sectional area between sides (fig. 3.9). This suggests that the difference in cortical thickness between sides was not due to a differences in periosteal diameter, but differences in the endosteal diameter (fig. 3.10). This is similar to hQCT findings recently reported from pre and post space flight studies of 14 cosmonauts (Lang *et al.*, 2004).

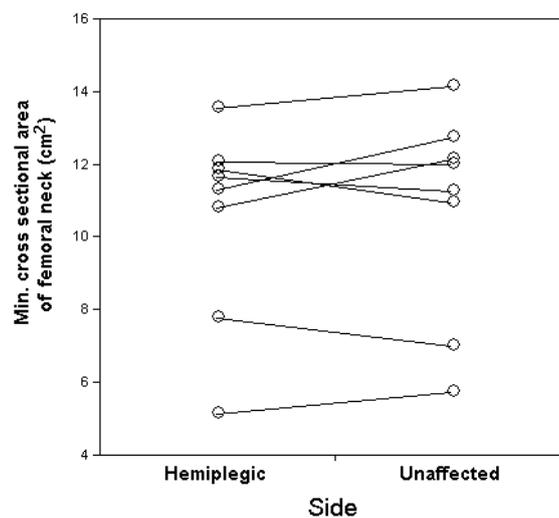


Figure 3.9 Minimal cross sectional area of the femoral neck slice for 8 subjects. Mean hemiplegic side femoral neck slice area was 10.5 cm² (95%CI 8.3, 12.8). Mean unaffected side slice area was 10.8 cm² (95%CI 8.3, 13.2). p=0.97 for the difference.

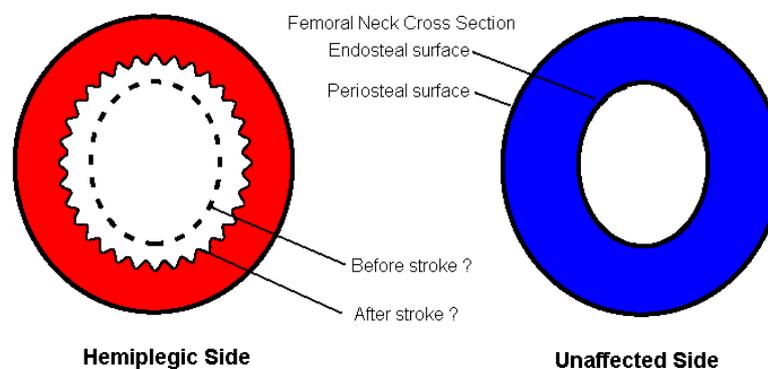


Figure 3.10 Schematic for the effects of hemiplegia on the femoral neck. It should be noted that cortical thickness in the human femoral neck is not uniformly distributed (see fig. 3.3).

In summary, intravenous zoledronate effectively prevented bone loss after stroke in patients with hemiplegia. It is the first agent clearly shown to attenuate bone loss at the hemiplegic hip as assessed by DXA. With this evidence of a beneficial effect on BMD, further studies should be undertaken to assess the effectiveness of zoledronate in preventing hip fractures after stroke. Preventing hip fractures in stroke patients should remain a goal of rehabilitation in the acute stroke unit and onwards into the community.

Chapter 4. Hypovitaminosis D in Acute Stroke

4.1 Abstract

Stroke leads to a reduction in bone mineral density, altered calcium homeostasis and an increase in hip fractures. In long-term stroke survivors, there is an association between reduced vitamin D and post-stroke hip fractures. Although vitamin D deficiency in long-term stroke survivors is well documented, less is known regarding levels in acute stroke. The serum 25-dihydroxyvitamin D levels of 44 patients admitted to an acute stroke unit with first ever stroke were compared with results obtained by measuring 96 healthy ambulant elderly subjects every 2 months for a year. Statistical Z-scores of serum vitamin D were then calculated, using seasonal adjustment for the month of sampling. The mean Z score of vitamin D in acute stroke was -1.4 standard deviation units (95%CI -1.7, -1.1), with 77% of patients falling in the insufficient range. Reduced vitamin D was identified in the majority of patients with acute stroke throughout the year and may have preceded stroke. Vitamin D is a potential risk marker for stroke and the role of vitamin D repletion in enhancing musculoskeletal health after stroke needs to be explored.

4.2 Introduction

Stroke patients are prone to falls, particularly onto the hemiplegic side. Hemiplegia and subsequent immobility predispose these patients to disturbed bone physiology, resulting in reduced bone mineral density (BMD) at the hemiplegic hip. These factors result in a substantial increase in hip fractures in both sexes and across all age ranges after stroke (Kanis *et al.*, 2001). Reduced vitamin D levels may further increase risk for bone loss and fractures. In long-term stroke survivors, there are associations between low vitamin D, low BMD and post-stroke hip fracture (Sato, 2000c). Insufficient vitamin D can impair bone mineralization, increase bone loss through secondary hyperparathyroidism, impair muscular function, increase the likelihood of falling and contribute to the risk of hip fracture (Pasco *et al.*, 2004). It may also predispose to severe hypocalcaemia when bisphosphonates are administered. Seasonal periodicity in vitamin D is recognised in both Northern and Southern hemisphere populations (Hegarty *et al.*, 1994; Ono *et al.*, 2005; Pasco *et al.*, 2004). The aim of this study was to test examine whether serum vitamin D was reduced in acute stroke patients when compared with healthy ambulant elderly subjects, after controlling for seasonal variation. This information has not been available to date. There is limited evidence that reduced vitamin D is itself a risk factor for hypertension and stroke, via indirect mechanisms such as compensatory hyperparathyroidism (Sato *et al.*, 2003) or loss of renin-angiotensin system inhibition (Li *et al.* 2002).

As awareness of hip fractures following stroke grows (Poole *et al.*, 2002), understanding baseline characteristics such as the vitamin D status of stroke patients is of increasing importance. A large trial of oral bisphosphonates to prevent bone loss in stroke was published recently (Sato *et al.*, 2005c). One aim of the present study was to establish baseline vitamin D levels in hemiplegic stroke patients before entry into a randomised controlled trial of preventing bone loss after stroke using an intravenous bisphosphonate (Poole *et al.*, 2004). Recent case reports have highlighted the risk of developing hypocalcaemia after administering potent intravenous bisphosphonates to patients with vitamin D deficiency (Peter *et al.*, 2004; Rosen and Brown, 2003). Vitamin D assessment and repletion might therefore be necessary before such intravenous bisphosphonates can be administered routinely in stroke.

4.3 Methods

Forty-four patients with acute stroke were sequentially consented into the study, using inclusion and exclusion criteria from the protocol of a randomised clinical trial of intravenous zoledronate to prevent bone loss after stroke (Chapter 3). Serum samples were taken from the patients (28M, 16F, median age 73 IQR 60-80.5) within 30 days of a first ever stroke. Prior to their stroke, patients were healthy and independently mobile. Other inclusion criteria were hemiplegia involving the lower limb, stroke confirmed by CT scan, an inability to walk independently 1 week after stroke and sufficient cognition and language function to give informed consent. Exclusions were cognitive impairment, aphasia, previous hip fracture, pre-existing bone disease (including primary hyperparathyroidism), steroid treatment, vitamin D or calcium supplementation and renal or liver impairment. Serum 25-hydroxyvitamin D (25OHD) levels were measured by radioimmunoassay (IDS Ltd, Boldon, UK) in a UK Chemical Pathology Accredited laboratory. Coefficients of intra-assay and inter-assay variation were less than 8% and 10% across the range of 25OHD concentrations measured. Previously, 96 healthy free-living elderly volunteers (47M, 49F median age 69, range 65-74) had serum samples taken every 2 months for a year to establish a normal range adjusted for season. Details of these patients have been published (Hegarty *et al.*, 1994). After consulting a statistician (Dr. S. Kaptoge), the following analyses were performed. A seasonally adjusted Z-score of 25OHD was calculated for each stroke patient by comparison with the monthly mean and standard deviation of 25OHD (after log-transformation to achieve normalisation) from the healthy elderly reference range. Baseline DXA scans of both hips (Lunar Prodigy, Madison, WI) and parathyroid hormone (PTH) levels were assessed in the subjects. The Z scores were tested against a hypothesised mean Z score of 0 using Student's t-test. The experimental protocol was approved by the Cambridge Regional Ethics Committee.

4.4 Results

The median time from admission to sampling was 14 days (IQR 10-22). No linear relationship was observed between 25OHD levels and the time from admission to sampling (*adj. r*²=0.02, *p*=0.77). A comparison between the vitamin D levels in the stroke patients and the mean (+/- 2SD) for the healthy elderly controls is shown in figure 4.1. The mean Z score of 25OHD for acute stroke patients was -1.4 (95%CI -1.7, -1.1). This was highly statistically significant (fig. 4.2. *p*<0.0001). Thirty-four patients (77%) had a serum 25OHD level below 50 nmol/l, regarded by many as the lower limit for good health. To produce the mean and +/- 2SD curves of figure 4.1, the log 25OHD levels of healthy subjects were regressed against the sine day of year and the three curve equations (mean, +2SD, -2SD) were produced from the linear regression and back transformed (Barker *et al.* Proceedings UK NEQAS meeting, Cardiff, 1996). Neither PTH nor 25OHD levels correlated with total hip BMD. Linear regression showed no significant association between log 25OHD and log PTH (*adj. r*²=0.04, *p*=0.15). Computed tomography indicated that infarction was the cause of 36 strokes, with haemorrhage the cause in the remaining 8. Median baseline Barthel score (maximum 100) was 37.5 (IQR 20-55) and the median long term score (maximum 48) of the Scandinavian Stroke Scale at baseline was 27.5 (IQR 20-33).

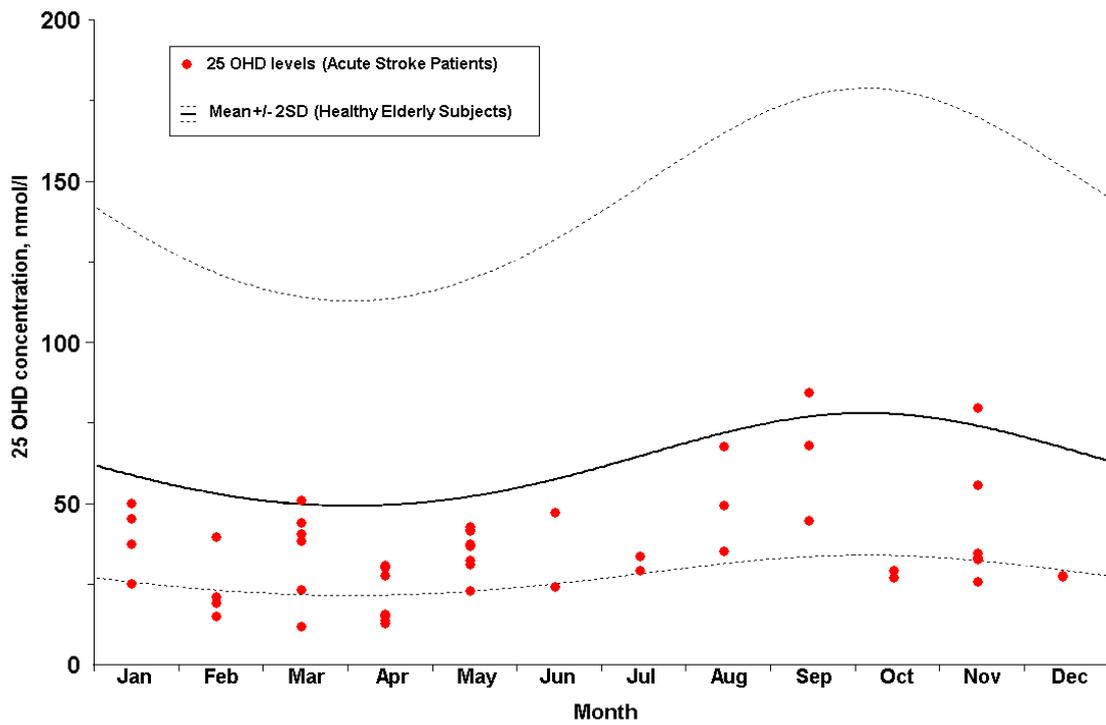


Figure 4.1 Distribution of 25 hydroxyvitamin D (nmol/l) by month in stroke patients. Mean (solid line) and +/- 2SD curves (dashed lines) of healthy subjects measured repeatedly throughout the year are superimposed.

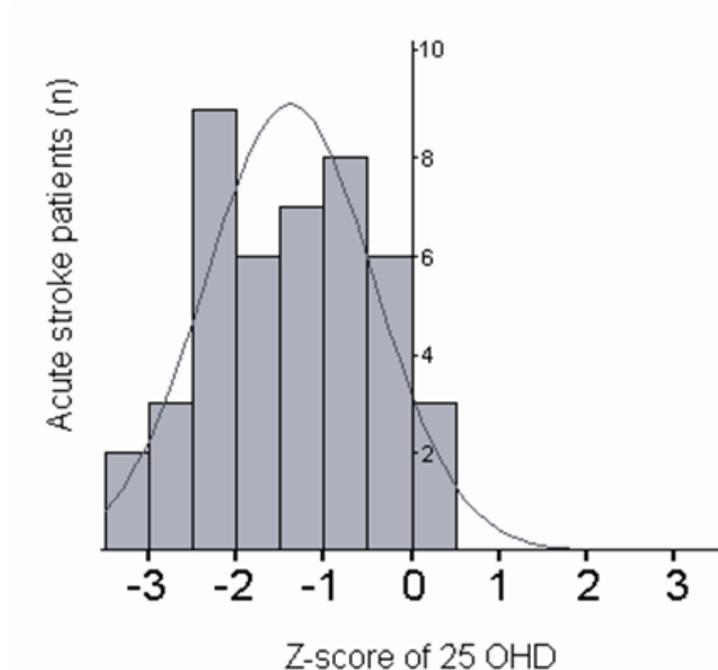


Figure 4.2 Seasonally adjusted Z-scores of 25 hydroxyvitamin D in acute stroke patients.

4.5 Discussion

These results show for the first time that, independently of season, hemiplegic patients from an acute stroke unit have 25OHD levels substantially lower than healthy elderly subjects. In fact only 3/44 patients had values that exceeded their mean control values. The prevalence of vitamin D insufficiency among these patients was greater than that observed in general medical inpatients in the USA without stroke (Thomas *et al.*, 1998) and of similar magnitude to that observed in inpatients with stroke from Japan (Sato *et al.*, 2000b). However, the study of Sato and colleagues (2000b) was not controlled for seasonal changes in 25OHD (Ono *et al.*, 2005).

In contrast to other medical inpatients, the bone health of stroke patients is additionally threatened by generalised and localised skeletal unloading due to hemiplegia, poor nutrition and a greatly increased risk of falls. The 25OHD insufficiency observed was unlikely to be due to a decline in existing stores (which are largely found in body fat) because there was no interaction between serum 25OHD level and the number of days from stroke to sampling. Normal stores (in vitamin D replete subjects) are sufficient to maintain serum vitamin D levels for at least 3 weeks. This makes it probable that the observed reductions in vitamin D preceded stroke. The usual relationship between log 25OHD and log PTH was not observed in this group of stroke patients. This is in keeping with reports suggesting that because of increased bone resorption secondary to stroke, an increase in ionised calcium may partly suppress PTH secretion (Sato, 2000c). Reduced synthesis of 25OHD or vitamin D binding globulin can be associated with certain acute illnesses (such as hepatic failure, nephrotic syndrome or severe malnutrition) but acute stroke is not known to halt 25OHD synthesis or its binding globulin (Cooke and Haddad, 1989). Nevertheless, serial measurements in stroke are needed before confidently excluding this possibility. Although vitamin D-binding protein was not measured, no association between serum vitamin D-binding protein and serum 25OHD was observed in a study of 290 acute medical inpatients with a variety of illnesses (Thomas *et al.*, 1998).

In conclusion, vitamin D insufficiency is common in acute stroke patients, may precede admission and is highly prevalent in the years following stroke as sun exposure and dietary vitamin D decline (Sato *et al.*, 1996b). Vitamin D as a potential risk marker for stroke

warrants investigation, since the serum values reported here are likely to have preceded stroke. It has been suggested that the relevant risk factor for stroke associated with 25OHD insufficiency may be hypertension associated with compensatory secondary hyperparathyroidism (Sato *et al.*, 2003). Others have shown that active vitamin D (1,25(OH)₂D) insufficiency leads to raised renin and angiotensin II activity and hypertension in mice (Li *et al.*, 2002).

After stroke, vitamin D repletion might provide health benefits to the musculoskeletal system in acute stroke patients by conserving bone, restoring muscle strength and reducing falls (Bischoff *et al.*, 2003), although no trials have been conducted in stroke to date. In one study of 122 women (mean age 85.3 years) in long stay geriatric care, a single intervention with vitamin D and calcium supplementation over a 3 month period reduced the risk of falling by 49% and increased muscular function compared with calcium alone (Bischoff *et al.*, 2003). In addition, stroke patients should be screened for vitamin D deficiency before intravenous bisphosphonate therapy is considered. A larger scale survey of vitamin D status in acute stroke patients should be undertaken and meanwhile consideration should be given to vitamin D replenishment in patients with hemiplegic stroke.

Chapter 5. Stroke and Bone Health; A Histomorphometric Analysis

5.1 Abstract

The effects of zoledronate treatment and hemiplegia on iliac bone histology were studied in stroke patients randomised into a double-blind placebo-controlled trial. In addition, histomorphometric parameters from both groups were compared to four published reference ranges from healthy patients of a similar age. Patients received a single dose of zoledronate 4 mg or placebo within 35 days of stroke in addition to daily calcium and vitamin D3 therapy. Single trans-iliac biopsies were obtained 10 weeks following stroke from 14 patients; 5 from the zoledronate group and 9 from the placebo group (mean age \pm SD 70.7 \pm 11.0). Half of the biopsies were from the hemiplegic side, the remainder from the unaffected side. Histomorphometry was performed on undecalcified sections stained with Von Kossa and toluidine blue, using a microscope with a drawing arm and a digitiser. Basic dynamic indices were measured on unstained sections from 8 patients. Additional frozen sections were stained for tartrate resistant acid phosphatase (TRAP).

The mean eroded surfaces were similar in the zoledronate and placebo treated stroke groups and similar to reference subjects without stroke. However, there were significantly less TRAP positive cells in the zoledronate treated biopsies than in the placebo treated stroke group. Unexpectedly, osteoid surface was significantly higher in the zoledronate group than the placebo group, at cancellous and endocortical sites ($p=0.008$ and $p=0.002$ respectively). However, both the zoledronate and placebo groups had significantly lower osteoid surfaces than reference values. Zoledronate treatment and baseline 25 hydroxyvitamin D together accounted for 72% of the variance in cancellous osteoid surface by multiple regression. Osteoid width did not differ between groups, but osteoid volume was also higher in the zoledronate group ($p=0.012$). The mineralising surface and mineral apposition rate were not significantly different between the stroke groups, but the mineralising surface in the placebo group was significantly lower than values from reference subjects without stroke. Wall thickness, was slightly higher in the zoledronate group ($p=0.033$) than placebo. There were no differences between hemiplegic and unaffected side biopsies for any parameter.

Untreated stroke patients therefore had lower osteoid surface, osteoid volume and mineralising surface than reference subjects without stroke. Zoledronate treatment of stroke patients was associated with significantly higher osteoid surface and volume. It seems likely that the mechanisms underlying the differences in osteoid surface with zoledronate treatment in stroke are indirect and may be due to altered calciotropic hormone responses in hemiplegia. These results imply a generalised skeletal imbalance between resorption and formation in stroke patients, with a reduced surface extent of new bone formation that may be improved by early treatment with zoledronate.

5.2 Introduction

Prior work has demonstrated that a single infusion of zoledronate given within 35 days of acute stroke protected against the deleterious effects of hemiplegia on hip bone mineral density. After a stroke, there is a reduction in bone mineral density in the hemiplegic hip as assessed by dual-energy x-ray absorptiometry (Chapter 1.2.2). Despite the magnitude and rapidity of these effects, very little is known regarding the mechanisms that occur at the bone tissue level in stroke patients. Biochemical bone marker studies have suggested a generalised increase in bone resorption and reduced bone formation in the first week after stroke compared to controls (Sato *et al.*, 2000c) (Chapter 2.2.1), with persistence of the raised resorption markers as well as suppressed formation markers throughout the first year (Sato *et al.*, 1998a; Sato *et al.*, 2005c). Although bone density reduction following stroke is site specific (with the greatest losses at sites such as the affected hip (Jorgensen *et al.*, 2000b)), more generalised bone changes resulting from bed-rest and alterations in calciotropic hormone regulation may also be important in these patients. Year-long treatment with oral bisphosphonates after acute stroke has been associated with suppression of bone resorption markers, but also a significant increase in bone formation markers (Sato *et al.*, 2005c). Sato and colleagues (2000c) proposed that bone formation might be suppressed after stroke due to a combination of reduced PTH and active vitamin D, the result of raised ionised calcium inhibiting the parathyroid gland. Bone histological studies in carefully selected patients are essential to understand pathogenetic mechanisms and direct appropriate therapy because to date only indirect markers of bone formation and resorption have been studied. The aim of this study was to assess indices of cancellous, endocortical and cortical bone turnover in the iliac crest 10 weeks following stroke and to evaluate the effects of bisphosphonate treatment on histomorphometric parameters by comparing patients given a single dose of zoledronate after stroke with those given placebo. Secondary aims were to investigate the relative contributions of the side of biopsy (hemiplegic or contralateral), baseline 25 hydroxyvitamin D (25OHD), stroke severity and functional status on bone histology in these patients.

5.3 Methods

Approval for the study was obtained from the Cambridge local research ethics committee. All the study participants were admitted to an acute stroke unit with first ever stroke and were taking part in the randomised placebo controlled trial of 4 mg zoledronate versus placebo to prevent bone loss in hemiplegia. Patients received calcium (1g) and vitamin D (800 IU) daily during the trial. Full inclusion and exclusion criteria for this trial are documented in Chapter 3.3. Additional exclusion criteria for this study were disorders of blood coagulation, warfarin therapy, respiratory disease and obesity. Patients had been independent and ambulant before admission with a stroke and all had hemiplegia affecting the lower limb. They were unable to walk one week following stroke. Of the 29 trial subjects, 14 patients were randomly allocated to a single trans-iliac bone biopsy approximately 10 weeks after the stroke occurred (mean 10.4 +/-1.9 SD). A further 9 patients declined the procedure, 5 were taking oral warfarin therapy (and were therefore excluded) and in one subject the biopsy attempt was unsuccessful. The trans-iliac bone biopsies were taken between January 2002 and October 2003. There were 3 women and 11 men, aged between 56 and 89 (77.1 +/- 11) years. Five patients received zoledronate and 9 received placebo infusions. In order to explore the side-specific effects of hemiplegia on bone turnover, 7 patients had a biopsy from the hemiplegic side and 7 trans-iliac biopsies were taken from the contralateral side (table 5.1).

Table 5.1 Details of patients undergoing bone biopsy

<i>Side of Biopsy</i>	<i>Zoledronate 4 mg</i>	<i>Placebo</i>
Hemiplegic side	2	5
Unaffected side	3	4

Although stroke patients typically lose bone mineral density from the hemiplegic proximal femur (Jorgensen *et al.*, 2000a), this site is not accessible for biopsy. However, the iliac bone (and particularly the iliac crest) has numerous attachments from large muscles involved in normal ambulation and therefore may be subject to abnormal loading in the hemiplegic and contralateral sides following stroke (fig. 2.6). All patients underwent DXA examination of both hips at study entry, repeated at 6 and 12 months. Two scales were used to evaluate patients' activity and motor power at study entry and immediately prior to

biopsy. These were the Barthel Index (/100) (Mahoney and Barthel, 1965) and the long term score (/48) and lower limb power score (/6) of the Scandinavian Stroke Scale (Scandinavian Stroke Study Group, 1985). Mean histomorphometric parameters from the stroke patients were also compared with mean values from four published reference ranges of healthy adults (Clarke *et al.*, 1996; Freemont, 1995; Recker *et al.*, 1988; Vedi *et al.*, 1982). The reference values were derived from the four published ranges as follows. Each of the reference ranges were published as means and SD's for particular age subgroups (e.g. 50-59, 60-69, 70-79 etc.). Data was only used from subjects in the total age range 50-90 (i.e. the age range of the stroke patients). For each of the published reference ranges, a single overall mean and standard deviation for values from 50-90 year olds was calculated by weighting the overall mean and SD by the number of patients in each subgroup.

Trans-iliac bone biopsy

Trans-iliac crest biopsies were obtained using a 7.5 mm internal diameter modified Bordier's trephine using local anaesthetic infiltration and intravenous sedation by the same operator (KESP). Ten patients received double demeclocycline labelling before the biopsy (300 mg demeclocycline twice daily for 2 days, then a ten day gap, followed by 300 mg twice daily for 2 days, followed by a biopsy 4 days after the last dose, Frost, 1969). Biopsies were coded and the histomorphometric analysis was performed "blind" by the same observer (KESP). Dr. S. Vedi performed the eroded surface measurements in both stroke and control groups. Administration of two doses of demeclocycline separated by a known interval enables assessment of the dynamic indices of bone turnover. Stroke patients were prescribed two courses of 300 mg demeclocycline twice daily, with the two-day courses separated by a 10-day gap. The biopsy was performed four days after the last dose. The bone biopsy procedure used was a modified version of the Mayo Clinic percutaneous iliac bone biopsy method under intravenous midazolam sedation (Hodgson *et al.*, 1986). For reasons of accessibility and safety, a site 2 cm inferior and 2 cm posterior to the anterior superior iliac crest was the preferred site of biopsy (fig. 2.6). Briefly, after sterile preparation of the region of the iliac crest, up to 10 ml of 1% lignocaine was infiltrated to the skin, subcutaneous tissue, muscle and proximal periosteal surface in a wide field. A 2 cm incision was made in the skin and subcutaneous tissue before blunt dissection to the iliac bone surface. In a modification to the published method (Professor J E Compston, personal communication), a steel needle with an obturator was advanced just

under the outer periosteal surface with gentle strikes with a small steel weight. After removal of the obturator, up to 5 ml of lignocaine was infiltrated under resistance into the periosteum. By replacing the obturator and advancing the needle apparatus all the way through the iliac cancellous bone and the inner cortex with further gentle strikes, the periosteum of the inner cortex was anaesthetised in a similar fashion. The outer periosteum was located with a toothed guide sleeve and obturator that was then replaced with the modified Bordier's trephine once the teeth had engaged the periosteal surface. Using a gentle rotary motion, the trephine (with a 7.5 mm internal diameter) was advanced all the way through both cortices. After two complete revolutions in each direction, the trephine was withdrawn, the biopsy ejected and taken immediately to the laboratory. Complete biopsies contained two cortices, intervening trabeculae and marrow. The wound was closed with up to 3 vicryl sutures and a pressure bandage applied. Because of the need to remain with the patient throughout this stage, immediate tissue processing was carried out by a laboratory technician (Mr. Alan Lyon), who was also responsible for embedding the biopsy in methylmethacrylate, sectioning the resin block using a microtome and all routine histological staining of the tissue (fig 5.1).

Tissue preparation

The histological sections on which histomorphometry is performed are two dimensional images showing the cut profile of the relevant three dimensional structures on which are measured widths, perimeters and areas. Fresh cores of bone were cut in half longitudinally using a small fretsaw blade. One half of the biopsy was snap frozen in polyvinyl acrylic using a -70 degree hexane chilled bath and immediately transferred to a -80 degree freezer. The other half was embedded in methylmethacrylate (British Drug House Chemicals Ltd, Poole, Dorset). After embedding, 8 um undecalcified sections were cut with a Jung Polycut microtome (Leica, Milton Keynes, UK) and stained with the following techniques: Von Kossa/Van Gieson stain for osteoid measurement, trabecular surface and structural parameters and toluidine blue stain for eroded surface and wall thickness (using polarised light) before mounting on slides with DPX (Fisher Chemicals). Example measurements and stains are shown in figure 2.1 C and D. The staining methods are described in detail in Appendix 2. Additional 15 um sections were examined for fluorescent labels using ultraviolet light microscopy and a filter. Undecalcified sections are necessary for accurate assessment of bone structure and retention of fluorescent (demeclocycline) label.

Demeclocycline (like tetracycline) binds bone matrix at sites of osteoid mineralisation, but is otherwise cleared rapidly from the circulation into the urine. The correct administration of time-separated demeclocycline tablets results in well-defined lines of an orange-yellow fluorescence when an unstained section is observed using fluorescent light and a 365-nm filter (for an example in cortical bone, see fig. 6.6 J). For all cancellous indices at least two sections from levels separated by more than 150 μm (a reduced thickness due to the limitations of using a half core biopsy) were analysed from each biopsy to avoid replicate sampling of a single surface event. Twelve samples contained two complete cortices with intervening cancellous bone and two samples had only one complete cortex. Since small samples can provide misleading data due to limited sampling, statistical analysis was performed with and without values from the two incomplete biopsies.

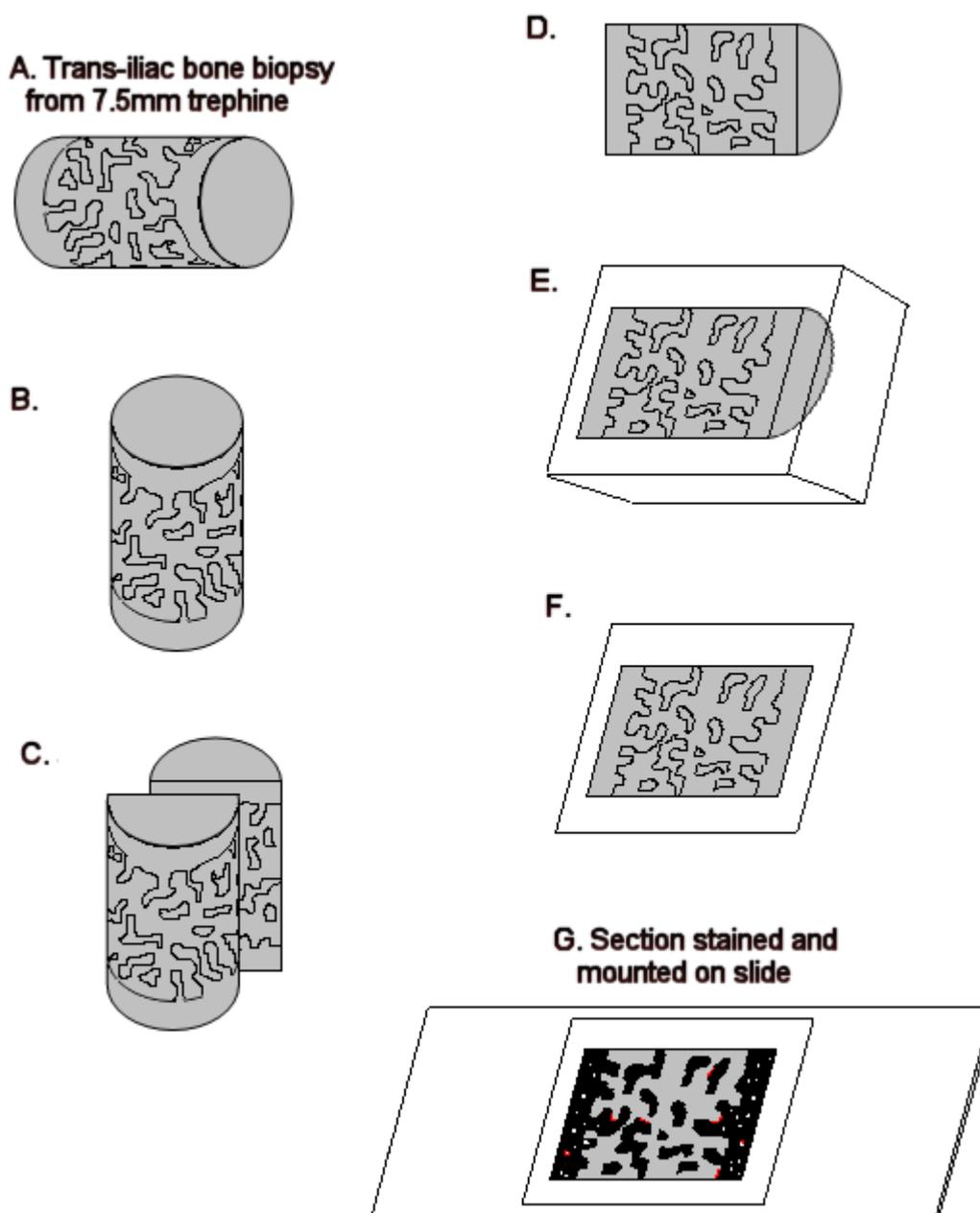


Figure 5.1 Schematic showing how bone biopsy cores were processed. The fresh core (A) was placed vertically in a 7.5 mm diameter chuck (B) and sawed in half using a fretsaw (C). The half biopsy was fixed (D) before it was embedded face down in methacrylate (E). 8-micrometer slices were cut from the block face (E to F) and stained as free-floating sections before mounting on glass slides (G).

5.3.1 Histomorphometric Analysis

Histomorphometric assessment was made using a Summasketch II (Summagraphics, Fairfield, CT, USA) digitizing tablet and modified cursor with an LED point light source. Sections were visualised with a Reichert-Jung Polyvar (Reichert Optische Werke, Vienna, Austria) binocular transmitted light microscope with a macro-dual drawing attachment.

Cursor tracings were automatically transmitted to a computer and analysed by a program designed to calculate the desired perimeter and area values (Garrahan *et al.*, 1986). Cancellous bone was defined as the area bounded by, parallel to and separated from the endocortical surface by 250 μm for the purposes of this study. The remaining surface including the entire endosteum was analysed separately. Osteoid thickness (O.Th) was measured at x200 magnification, bone surface (BS) at x80, wall thickness (W.Th) using polarised light at x125 and eroded surface (ES) at x200. Based on the geometric probability density function, measures of mean apparent widths were transformed into 3D mean apparent thicknesses by multiplying by $\pi/4$ (Kragstrup *et al.*, 1982). All histomorphometric measurements are described using nomenclature approved by the American Society for Bone and Mineral Research (ASBMR, Parfitt *et al.*, 1987), with the exception of 'cortical osteoid area', Ct.OAr/BAr (see Cortical Histomorphometry). 3D quantities (thickness, surface and volume) are reported for cancellous bone.

Static Parameters

Definitions: 1) Cancellous bone volume (BV/TV %); volume of mineralized bone and osteoid as a percentage of total bone tissue volume. 2) Osteoid surface (OS/BS %); surface extent of osteoid as a percentage of cancellous bone surface. 3) Osteoid thickness (O.Th μm): mean osteoid thickness. 4) Osteoid volume (OV/BV %); volume of osteoid as a percentage of bone volume. 5) Eroded surface (ES/BS %); eroded surface as a percentage of cancellous bone surface. For O.Th, a minimum number of 20 osteoid seams were analysed from each biopsy. All osteoid widths greater than 3 μm were included in the analysis (Freemont, 1995). The exact formulae used to calculate histomorphometric parameters are listed in Appendix 2.

Basic Dynamic Parameters

Single labels only were found in two biopsies (with no double labels in cancellous or cortical bone, table 5.2). Eight biopsies were found to contain sufficient demeclocycline double-label for analysis. Definitions: 1) Wall thickness (W.Th μm); mean thickness of completed bone remodelling units. For W.Th a minimum of 25 bone remodelling units were measured for each biopsy. 2) Mineralising surface (MS/BS %); the surface extent of labelled (double-labelled + half single-labelled surface, $dL + \frac{1}{2} sL$) surface to cancellous bone surface. 3) Label thickness (L.Th μm); mean distance between double labels. 4)

Mineral apposition rate (MAR $\mu\text{m}/\text{d}$); label thickness divided by labelling interval. 5) Bone formation rate (BFR/BS $\mu\text{m}^3/\mu\text{m}^2/\text{d}$); amount of new bone mineralised per day per unit of cancellous bone surface.

Table 5.2 Identification of fluorescent labels in bone biopsies

<i>Label status</i>	<i>Drug (patient code)</i>	<i>Placebo (patient code)</i>
Double label seen	3 (kp5,7,10)	5 (kp1,2,12,13,14)
Single label only	0	2 (kp 4,8)
Not labelled	2 (kp 6,9)	2 (kp 3,11)

Endocortical Histomorphometry

The endocortical surfaces were defined for the purposes of this study as both inner bone surfaces and cancellous surfaces up to 250 μm into the medullary cavity parallel to the plane of the inner surfaces. Osteoid surface (Ec. OS/BS) was measured in the same manner as the cancellous OS/BS.

Cortical Histomorphometry

Mean cortical thickness (Ct. Th) was measured automatically after drawing periosteal and endocortical surfaces from three sections and using correction for section obliquity. Cortical osteoid area (Ct. OAr/BAr) and cortical porosity (%) were measured using a novel image analysis technique. The entire Von Kossa stained cortex was captured at 100x magnification (2 cortices per biopsy) using automated montaging software. Canals were defined on the cortex using a digital drawing pen and osteoid was filled in. The osteoid area was measured directly using ImageJ software (ImageJ 1.32e, NIH, USA available to download free at <http://rsb.info.nih.gov/ij/>) and osteoid area as a percentage of cortical bone area was calculated. Assumptions of isotropy used to convert 2D to 3D values in cancellous bone are not met in cortical bone (Parfitt, 1983; Parfitt *et al.*, 1987), so mean cortical areas in 2D cannot be extrapolated to mean volumes in 3D. Therefore, Ct. OAr/B.Ar is reported, acknowledging that the correct ASBMR nomenclature is to use either 2D or 3D referents only in a single publication (Parfitt *et al.*, 1987).

5.3.2 Statistical Analysis

This was a single biopsy design since interval biopsy designs are more susceptible to sampling uncertainties and are less ethically acceptable. Hauge *et al.* (2001) found that the single biopsy approach overcame the sampling uncertainties of the interval approach for selected measurements where there was a large magnitude of difference between groups. Data was analysed using the JMP statistical package JMP (v 4.0, SAS institute, Cary, NC, USA). After consulting a statistician (Dr. S. Kaptoge), the following analyses were performed. Significance was determined by Student's *t*-test and Dunnett's *t*-test when comparing stroke patients with reference data. For non-normally distributed data, significance was determined by use of the Wilcoxon/Kruskals-Wallis 2 sample test. A factorial model was used to explore the differences in OS/BS in stroke patients. The model had the following covariates; treatment (drug/placebo), side biopsied (hemiplegic/contralateral), age, interval from stroke to biopsy, SSS, BI and baseline 25OHD. Standard least squares regression was used to assess significance in the model. Intra-observation (precision) was as follows: BV/TV 1.9%, OS/BS 4.2%, O.Th 2.4%, and W.Th 3.1%. Since one of the four reference ranges was produced by a local histomorphometry expert (Dr. S Vedi), it was also possible to calculate coefficients of inter-observer variation for selected parameters, by analysing the same sections independently. The inter-observer coefficients of variation were BV/TV 9.1%, OS/BS 11.3%, O.Th 8.8%, and W.Th 8.2% (see Appendix 2).

5.4 Results

There were no differences between the hemiplegic side and unaffected side biopsies for any parameter. For example, the hemiplegic side values of BV/TV (mean 21.2, 95%CI 17.5, 24.8), ES/BS (5.0, 95%CI 3.7, 6.2) and OS/BS (3.9, 95%CI 1.0, 6.8) were not significantly different from the unaffected side values of BV/TV (18.0, 95%CI 14.3, 22.0, $p=0.21$), ES/BS (6.5, 95%CI 5.3,7.8, $p=0.09$) and OS/BS (5.4, 95%CI 2.5,8.3 $p=0.44$). For subsequent analyses, the stroke patients were grouped instead by drug or placebo treatment irrespective of the side biopsied (as discussed in section 5.5). Table 5.3 shows static and basic dynamic parameters in stroke patients grouped by treatment with either zoledronate or placebo. The groups were comparable in terms of age and severity of stroke (as assessed by the SSS and BI) at the time of biopsy.

Table 5.3 Histomorphometry in Stroke Patients; Zoledronate and Placebo Groups

Measurement	Zoledronate 4 mg*	Placebo*	<i>p</i> Value [‡]
<i>Demographics</i>			
Age	67.2 (52.8,81.6)	72.7 (64.3,81.0)	0.40
n (males, females)	5 (1, 4)	9 (2, 7)	
Interval** (weeks)	9.3 (7.6,10.8) [‡]	10.7 (9.8,11.8) [‡]	0.20 [§]
SSS/48	29 (21,37)	28 (22,39)	0.81
Barthel Index/100	58 (40,76)	57 (34,80)	0.96
25OHD (mmol/l)	41.4 (20.5, 62.7)	42.9 (29.5, 56.7)	0.88
<i>Static parameters</i>			
Number	5	9	
BV/TV (%)	20.1 (12.6,27.7)	19.3 (16.2,22.3)	0.75
OS/BS (%)	7.7 (2.8,12.6)	3.0 (1.6,4.3)	0.008
OV/BV (%)	0.64 (0.16,1.75)	0.31 (0.18,0.45)	0.013
O.Th (um)	6.8 (5.6,7.9)	6.3 (5.4,7.3)	0.53
ES/BS (%)	5.5 (3.0,8.0)	5.9 (4.7,7.1)	0.64
W.Th (um)	32.0 (27.1,37.0)	28.3 (24.4,29.1)	0.033
<i>Frozen Sections</i>			
TRAP ⁺ cells (/mm ²)	0.05 (0.01,0.17) [‡]	0.35 (0.11,0.52) [‡]	0.023[§]
<i>Bone Mineral Density</i>			
Δ Total Hip BMD ^a (%)	+1.4 (-0.2,+3.0)	-3.3 (-6.4, -0.2)	0.013
Δ Total Hip BMD ^b (%)	+1.9 (-2.0, +5.7)	-0.5 (-2.7, +1.7)	0.16

*Mean (95%CI) unless otherwise stated, [‡]Median (interquartile range). [‡]Student's t-test unless otherwise stated, [§]Wilcoxon test. **Interval from stroke to biopsy. ^aHemiplegic & ^bUnaffected change over 6 months.

The ES/BS was not significantly different between groups, but the zoledronate treated group had less TRAP⁺ cells per mm² than placebo. The OS/BS was significantly greater in zoledronate treated patients than placebo treated patients. A standard least squares regression model to predict OS/BS confirmed two main effects with a significant interaction term; treatment with zoledronate and baseline 25OHD. Treatment with zoledronate explained 46% of the variance in OS/BS (p=0.0076). A lower baseline 25OHD in those treated with zoledronate predicted a higher OS/BS at the time of biopsy in this model. Together, the two factors explained 72% of the variance in OS/BS (table 5.4 gives the relevant linear equations). The interaction profiles indicated that 25OHD made no significant difference to placebo group OS/BS but a lower 25OHD was associated with higher OS/BS in the drug treatment group. The other covariates were not significant determinates of the OS/BS. The results remained significant after removing values generated from the two incomplete biopsies.

Table 5.4 Multiple Linear Regression Equations using a Least Squares Model to predict OS/BS (%)

<i>Drug</i>	OS/BS % =	11.5 - 0.23(25OHD) - 0.24 (25OHD-17)
<i>Placebo</i>	OS/BS % =	6.9 - 0.23(25OHD) + 0.24(25OHD-17)

There was no significant difference in MS/BS (double-label plus half single-label) between zoledronate and placebo treated stroke patients (table 5.5). However, values were substantially lower than would be expected in both groups (see table 5.7). MAR and BFR/BS were similar in the two groups.

Table 5.5 Basic Dynamic histomorphometry in Stroke Patients: Zoledronate and Placebo Treated

<i>Measurement</i>	<i>Zoledronate 4 mg*</i>	<i>Placebo*</i>	<i>p Value ‡</i>
Number	3	5	
<i>Dynamic parameters</i>			
MS/BS (%)	2.1 (0.8)	2.2 (1.2)	0.94
MAR (um/d)	0.7 (0.3)	0.7 (0.2)	0.96
BFR/BS (um ³ /um ² /d)	0.016 (0.01)	0.015 (0.01)	0.89

*Mean (SD), ‡ Student's t-test

The zoledronate treated stroke patients had significantly higher endocortical OS/BS and cortical OAr/BAr than placebo treated patients (table 5.6), with no difference in cortical porosity or thickness.

Table 5.6 Endocortical and Cortical Histomorphometry in Stroke Patients

<i>Measurement</i>	<i>Zoledronate 4 mg*</i>	<i>Placebo*</i>	<i>p Value ‡</i>
<i>Parameters</i>			
Number	5	9	
Ec. OS/BS (%)	14.0 (10.1,17.8)	5.1 (2.2,8.0)	<u>0.0018</u>
Ct. OAr/BAr (%)	0.36 (0.19,0.52)	0.20 (0.03,0.27)	<u>0.049</u>
Ct. Porosity (%)	5.6 (3.0,8.1)	5.5 (3.6,7.4)	0.97
Ct. Thickness (um)	701 (527, 875)	866 (643, 1088)	0.30

*Mean (95% CI), ‡ Student's t-test

Histomorphometry 2: Stroke vs. Published Reference Ranges

Key parameters measured in stroke patients were compared with four published age matched reference ranges (table 5.7). For the purposes of this study, the mean values from stroke patients were considered to be significantly different from the mean reference values if the stroke value differed from all four reference values (fig. 5.2, $p < 0.05$).

Table 5.7 Histomorphometric Parameters in Stroke Patients vs. Healthy Reference Ranges

<i>Value</i>	<i>Stroke</i> [*]	<i>Stroke</i> [*]	<i>Freemont</i> [§]	<i>Compston</i> [§]	<i>Clarke</i> [§]	<i>Recker</i> [§]
<i>Treatment</i>	Zoledronate	Placebo	-	-	-	-
<i>Demographics</i>						
Age range	56-84	56-89	51-90	51-80	50-89	55-74
n (m,f)	5 (1,4)	9 (2,7)	77 (49,28)	28 (16,12)	21 (21,0)	23 (0,23)
Country	UK	UK	UK	UK	USA	USA
<i>Parameters</i>						
BV/TV (%)	20.1 (6.1)	19.3 (3.9)	16.7 (4.9)	22.3 (4.3)	19.1 (2.4)	20.2 (5.0)
OS/BS (%)	<u>7.7</u> [‡] (3.9)	<u>3.0</u> [‡] (1.7)	12.5 (3.8)	26.8 (11.8)	11.8 (2.0)	15.8 (6.8)
OV/BV (%)	1.0 (0.6)	<u>0.3</u> [‡] (0.2)	2.1 (0.9)	4.0 (2.2)	1.4 (0.4)	1.7 (1.0)
O.Th (um)	6.8 (0.9)	6.3 (1.3)	8.3 (3.3)	6.1 (2.1)	9.2 (4.2)	8.8 (2.0)
ES/BS (%)	5.5 (2.0)	5.9 (1.6)	4.3 (1.7)	1.6 (0.7)	7.2 (1.5)	3.9 (1.9)
W.Th (um)	32.0 (4.0)	27.1 (3.4)	37.0 (4.3)	34.9 (3.0)	33.1 (2.5)	29.4 (3.6)
<i>Basic Dynamic</i>						
n	3	5	53	28	21	23
MS/BS (%)	2.1 (0.8)	<u>2.2</u> [‡] (1.2)	7.2 (3.4)	11.1 (5.5)	n/a	6.8 (4.3)
MAR (um/d)	0.7 (0.3)	0.7 (0.2)	0.6 (0.2)	0.6 (0.1)	0.5 (0.1)	0.5 (0.1)

* Mean (SD), §Grand mean (SD). **Underscore**[‡]: mean significantly different from all four reference means using Dunnett's t-test $p < 0.05$. (Clarke *et al.*, 1996; Freemont, 1995; Recker *et al.*, 1988; Vedi *et al.*, 1982)

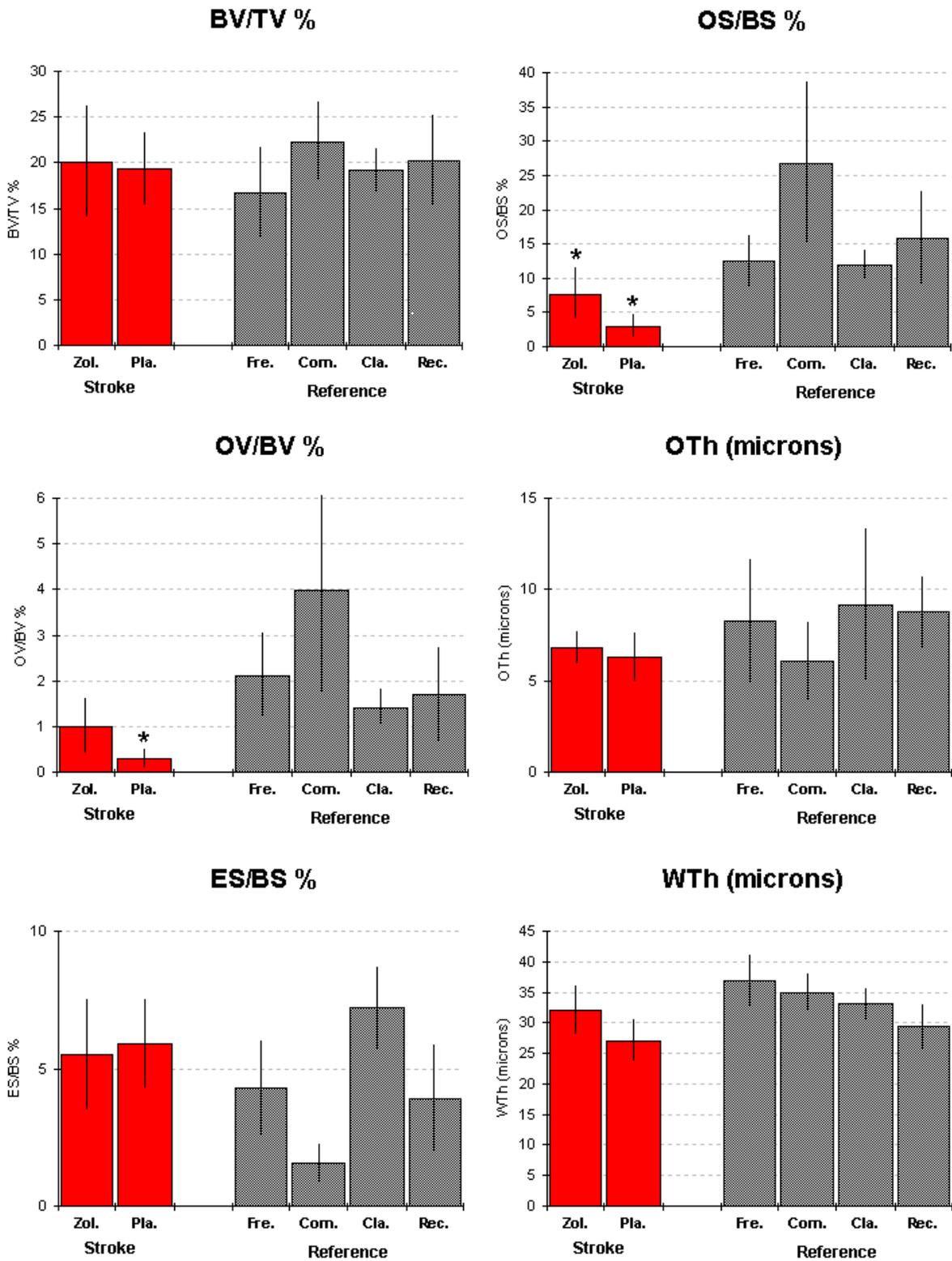


Figure 5.2 Comparing mean and SD of parameters in stroke patients (Zoledronate:Zol and Placebo:Pla) with the grand mean and SD of reference data (Freemont:Fre, Compston: Com, Clarke:Cla and Recker:Rec). (Clarke *et al.*, 1996; Freemont, 1995; Recker *et al.*, 1988; Vedi *et al.*, 1982). * Significantly different from all four reference values p<0.05.

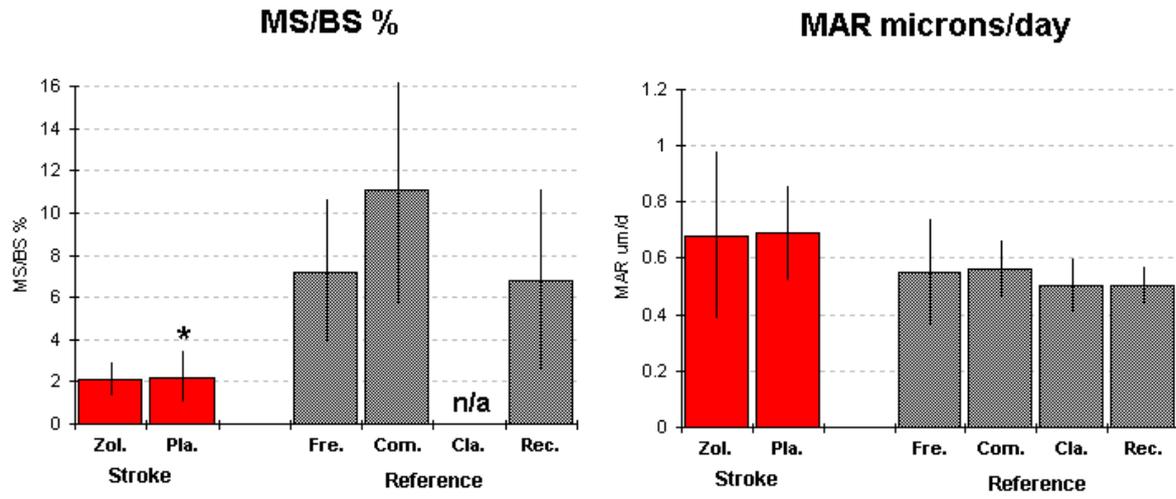


Figure 5.2 (continued from previous page).

5.5 Discussion

This study is the first to evaluate bone histology in hemiplegic patients. The main finding was that zoledronate treatment within 35 days of acute stroke was associated with a significantly higher osteoid surface and osteoid volume (in cancellous and endocortical regions) when compared with placebo treated stroke patients. However, the mean osteoid surface measurements in both treated and untreated stroke patients were significantly lower than values from four published reference ranges of healthy subjects without stroke. Although the surface extent of osteoid was significantly different between zoledronate and placebo treated stroke groups, the osteoid thickness, mineralising surface and mineral apposition rates were not significantly different. In addition to a lower mean osteoid surface, placebo treated stroke patients also had significantly lower mean osteoid volume and mean mineralising surface than the reference ranges. The mean mineral apposition rates and bone formation rates in both stroke groups were similar to the reference values (fig. 5.2). This is in contrast to findings using zoledronate at various doses in postmenopausal osteoporosis where the only changes in bone formation indices reported following zoledronate treatment were 70-80% reductions in the bone formation rate and mineral apposition rate (Reid *et al.*, 2002).

The eroded surfaces in the stroke patients were not significantly different from the reference values of the healthy subjects. Neither were there any differences in the mean eroded surface measurements between hemiplegic patients given zoledronate or those given placebo within 35 days of stroke. However, there were significantly less TRAP positive cells (thought to represent osteoclasts and their precursors) in the zoledronate treated group. Zoledronate has previously been reported to inhibit osteoclastic resorption. A reduction in mean eroded surface of 39% was reported in the iliac bone of women administered zoledronate at various dose intervals in a phase II trial in postmenopausal osteoporosis (Reid *et al.*, 2002). The mean time from the acute stroke to infusion of zoledronate was 25.4 days (SD +/-4.7) in the present study subjects. Therefore, not only would existing osteoclasts have had a period of time to continue resorption activity, but new bone multicellular units (BMU's) may have been initiated after (or as a result of) stroke but before the zoledronate infusion occurred. Biomarker studies in stroke (and other

studies of human immobilisation) have suggested that increased osteoclastic activity can occur within a week of the event (Arnaud *et al.*, 1992; Sato *et al.*, 2000b).

The new finding in stroke of a normal mean eroded surface and reduced mean surface extent of formation raises the possibility that there is a failure of coupled bone formation at eroded surfaces. With an overall mean eroded surface (both groups combined) of 5.8% (95%CI 5.0, 6.4), the mean osteoid surface appears inappropriately low at 4.7% (median, IQR 1.7,7.8). The osteoid surface is generally substantially higher than the eroded surface in healthy subjects (Clarke *et al.*, 1996; Freemont, 1995; Recker *et al.*, 1988; Vedi *et al.*, 1982). The present results suggest that one possible explanation for bone loss in hemiplegia might be an increase in the remodelling space through failure of the coupled formation wave that would normally accompany bone resorption. There may be local or systemic factors that are responsible for this altered dynamic; for instance, reversal may be prolonged or osteoblast recruitment may be impaired. The eroded surface simply represents where bone resorption is occurring and has occurred previously. To understand the dynamics of events at the BMU level requires the calculation of derived dynamic indices based on assumptions about normal bone remodelling. This was not feasible using the present data due to errors associated with extrapolation of the very few surface bone formation events. The generation of more dynamic data on remodelling events in stroke is a priority for future studies.

The findings of low osteoid and mineralising surfaces in the ilium of stroke patients are of considerable interest when considered alongside studies using circulating bone formation biomarkers in stroke. In one such study, Sato *et al.* (1988a) reported a decrease in circulating osteocalcin (OC) in subjects with acute hemiplegia, with values suppressed throughout the first year after stroke compared to subjects without stroke. That the osteoid surface was considerably higher in bisphosphonate treated patients from the present study was unexpected and there are no similar studies of intravenous bisphosphonates in stroke available for comparison. However, in a recently published trial of an oral bisphosphonate (risedronate) in acute stroke, bone formation markers were significantly increased at 6 and 12 months post-stroke by once daily risedronate therapy, in addition to the expected reduction in bone resorption markers observed with this treatment (Sato *et al.*, 2005c). A similar pattern of increased bone formation markers was reported recently in healthy

volunteers during 90 days of bed rest who were treated with a single dose of intravenous pamidronate at commencement (Rittweger *et al.*, 2005). Although a stimulatory effect of zoledronate on osteoblastic differentiation and activity has been observed *in vitro* (Chaplet *et al.*, 2004; Viereck *et al.*, 2002), clinical studies of humans and animals (without stroke) treated with the drug have consistently shown reductions in bone remodelling activity, of both formation and resorption (Pataki *et al.*, 1997; Reid *et al.*, 2002). The higher osteoid surface observed in stroke patients treated with zoledronate might be the result of an indirect mechanism rather than a direct effect of the drug on osteoblasts. One possibility is that a reduction in serum calcium after bisphosphonate treatment in stroke results in a sustained increase in parathyroid hormone and 1,25(OH)₂D levels that enhance osteoblast recruitment to eroded surfaces. Zoledronate treatment in acute stroke would reverse steps 3 to 8 in the schematic of stroke-induced bone loss shown in figure 5.3, leading to conditions more favourable to osteoblast recruitment. In the clinical trial, the mean serum calcium concentration in the zoledronate group changed from 2.27 (0.1) mmol/l (pre-infusion) to 2.13 (0.2) mmol/l (5 days post-infusion, $p=0.03$ for the change). In the placebo group mean calcium did not change significantly from 2.30 (0.1) mmol/l to 2.32 (0.1) mmol/l ($p=0.21$). The reduction in serum calcium after zoledronate might also explain the contribution of a low baseline 25OHD in predicting a higher OS/BS in the multiple regression model, since low 25OHD is a known risk factor for a prolonged hypocalcaemic response after intravenous bisphosphonates (Peter *et al.*, 2004; Rosen and Brown, 2003). A prolonged reduction in serum calcium (as a result of zoledronate treatment in patients with insufficient vitamin D) might result in a sustained increase in PTH secretion. Such an effect was shown previously in stroke patients with insufficient 25OHD treated with etidronate (Sato *et al.*, 2000a) and risedronate (Sato *et al.*, 2005c) and 25OHD replete bed-rested volunteers treated with pamidronate (figure 7.2, Rittweger *et al.*, 2005; Watanabe *et al.*, 2004), but prospective data on calciotropic hormones were not collected in the present study. Assessment of biomarkers and calciotropic hormone levels during treatment of stroke patients with zoledronate should now be undertaken.

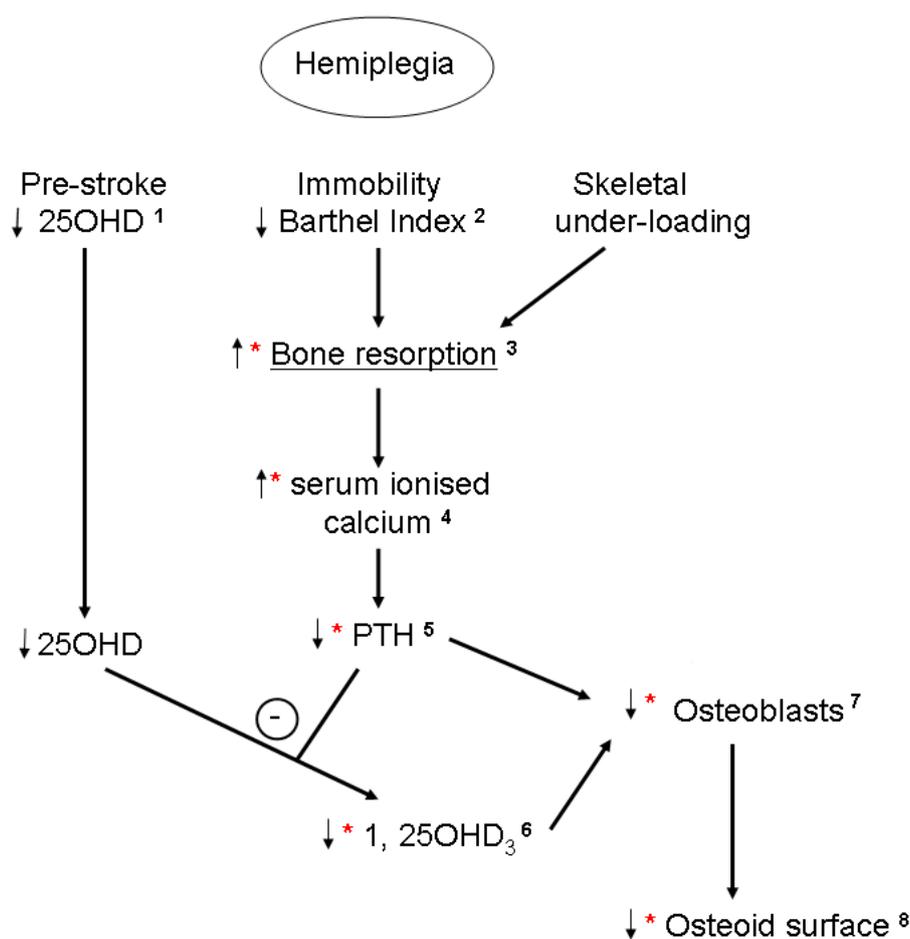


Figure 5.3 Schematic of the effects of hemiplegia on bone turnover. Evidence for each link is as follows. 1. Chapter 4, Correlations between 2, 3, 4, 6 and 7 based on biomarker studies. (Sato *et al.*, 2005c; Sato *et al.*, 2000b; Sato *et al.*, 1998a), 8: present work.

In the present study, there were no differences in iliac bone parameters between the hemiplegic and unaffected sides, which is in contrast to the marked BMD changes at the hemiplegic and unaffected hips observed concomitantly in the patients (table 5.3). Using DXA, hemiplegia has been shown to have both generalised and localised effects on the skeleton (Poole *et al.*, 2002; Ramnemark *et al.*, 1999a). Iliac bone differences therefore seem representative of the generalised (or indirect) skeletal effects of stroke, rather than those directly due to altered mechanical loading or muscle forces. It has been suggested that the generalised bone loss after stroke occurs because of changes in calciotropic hormone secretion such as raised ionised calcium inhibiting parathyroid hormone (fig. 5.3). Raised ionised calcium is considered to be a consequence of reduced mechanical loading or muscle forces leading to increased osteoclastic bone resorption at specific sites (for example the hemiplegic hip and arm).

Histomorphometric and biochemical studies in paraplegic patients and healthy volunteers subjected to experimental bed-rest protocols provide a useful comparison for the results observed in stroke. In agreement with the present findings, a decrease in cancellous osteoid surface was observed in one study of prolonged head-down bed-rest (Palle *et al.*, 1992) and the osteoblast-covered surface was significantly reduced in another (Zerwekh *et al.*, 1998). Oral and intravenous bisphosphonates have also been evaluated histologically in human models of unloading. Treating bed-rested healthy volunteers with etidronate for 120 days did not prevent an increase in the cancellous eroded surface, but did decrease the osteoclast number (Chappard *et al.*, 1989). In contrast to the findings using zoledronate in stroke patients, Vico *et al.* (1987) showed that daily oral etidronate therapy in bed-rested volunteers led to significant reductions in osteoid surface over 120 days. However, a combination of supine exercise and etidronate led to less suppression of the osteoid surface in that study. More recently, Watanabe *et al.* (2005) and Rittweger *et al.* (2005) reported results from the same study of healthy young volunteers observed during 90 days of head-down bed rest. Seven subjects were pre-treated with a single dose of pamidronate 90 mg, nine undertook a resistance exercise regime throughout the bed-rest period and nine subjects received no countermeasures. Serum PTH was increased relative to baseline in the pamidronate group (but not the other groups) until at least 44 days post-infusion of the drug (Rittweger *et al.*, 2005). The peak mean PTH concentration was over twofold higher than in the other groups. Serum bone specific alkaline phosphatase increased significantly from baseline in the pamidronate group over the same period before declining below baseline thereafter (Watanabe *et al.*, 2004). Serum osteocalcin on the other hand rose briefly then gradually declined throughout the bed rest period in the pamidronate group (Watanabe *et al.*, 2004). Biochemical markers of bone resorption were significantly reduced in the pamidronate group up to 44 days post infusion. There were no histomorphometric data.

The main limitations of the present study are the sample size and the lack of a contemporary control group of healthy subjects for comparison. There are large inter-individual variations in histomorphometric parameters and even larger regional sampling variations within the ilium itself (Hauge *et al.*, 2001). Consequently, in a single biopsy design such as this, differences must be of large magnitude in sufficient patients in order to achieve statistical significance. Where significance is achieved with small numbers (such

as the osteoid surface) this should be interpreted with caution given the predicted sample size needed to detect large magnitude between-group differences. The multiple regression model for predicting OS/BS was similarly based on a small sample size. With regard to the lack of a control group (which was not within the study protocol), comparison of stroke values with reference values from published data was the only alternative available. Such an approach is far from ideal, but does put the results from individuals with stroke into the broader perspective by comparing across several reference ranges. Finally, compliance with the demeclocycline dosing regimen could not be guaranteed in the present study, leading to loss of data relating to dynamic measurements. Examining a morning urine sample from patients after each two-day demeclocycline course (under fluorescent light) is a recommended approach for future studies.

In summary, histomorphometry differences observed in iliac biopsies appear to be indicative of the more generalised bone loss that occurs after stroke. The principal findings were that stroke patients had lower forming and mineralising surfaces than healthy reference values. Zoledronate treatment was associated with a higher osteoid surface than placebo treated patients. These results help to explain the deleterious effects of stroke on the skeleton and demonstrate the beneficial effects of zoledronate therapy in patients with hemiplegia.

Chapter 6. Sclerostin and the Osteocytic Control of Bone Formation in Stroke

6.1 Abstract

Osteocytes are the most abundant cells in bone and are ideally located to influence bone turnover through their syncytial relationship with surface bone cells. Examining the dynamics of bone remodelling led several investigators to propose a widespread osteocyte-derived inhibitory signal that would prevent excessive surface bone formation by osteoblasts. Such signals remained enigmatic until the recent discovery that human osteocytes secrete sclerostin, an inhibitor of osteoblasts. Absent sclerostin results in the high bone mass clinical disorder sclerosteosis, a condition characterised by excessive surface bone formation.

Using histomorphometry, hemiplegic stroke patients were previously shown to have reduced forming and mineralising surfaces compared to reference values from healthy subjects. Likewise, transgenic mice over-expressing human sclerostin display greatly reduced osteoid and mineralising surfaces. Here, a novel hypothesis was tested: that the percentage of osteocytes expressing sclerostin would be inversely associated with the surface extent of osteoid in the bones of stroke patients. Sclerostin was detected using immunohistochemistry of frozen sections of bone from the iliac crest of hemiplegic patients who underwent trans-iliac biopsy 10 weeks after an acute stroke. The results were compared with osteoid parameters measured previously in resin-embedded sections from the same biopsy.

The majority of mature osteocytes in mineralised cortical and cancellous bone were positive for sclerostin with diffuse staining along dendrites in the osteocyte canaliculi. No linear association was observed between the percentage of osteocytes expressing sclerostin and osteoid surface measurements in either cortical or cancellous bone. However, by employing a novel section mapping technique with automated microscopy on serial sections, new insights into the precise control of bone formation by osteocytes were elucidated. Within cortical osteons in the formation phase, newly embedded osteocytes were negative for sclerostin staining but became positive at or after primary mineralisation. These findings provide for the first time *in vivo* evidence to support the concept that osteocytes secrete this inhibitory signal after they become embedded in a mineralised

matrix to limit further bone formation by osteoblasts. Therefore, sclerostin production by osteocytes may regulate the linear extent of formation and the induction or maintenance of a lining cell phenotype on bone surfaces. In doing so, sclerostin may act as a key inhibitory signal governing skeletal microarchitecture.

6.2 Introduction

Osteocytes, the most abundant cells in adult human bone are generally acknowledged to sense loading stimuli and regulate remodelling and bone turnover processes (Knothe Tate *et al.*, 2004). Despite recent advances in biological understanding of other bone cell types, relatively little is known regarding the mechanisms by which osteocytes influence bone renewal, partly because the cells exist as a syncytial network within a mineralised matrix that makes them inaccessible and partly because the mineralised matrix itself may influence their behaviour. From the available evidence, interesting concepts have been advanced suggesting that osteocytes produce a factor that inhibits osteoblasts and lining cells (the retired, end-form of the osteoblast that covers 94% of the bone surface (Parfitt, 1983))

Marotti (1992, 1996) hypothesized that mature osteocytes send an inhibitory signal to osteoblasts at a bone-forming surface via their newly formed canalicular processes, causing the adjacent osteoblast to slow osteoid formation. Martin (2000a, 2000b) then proposed that the same signal maintained bone lining cells in a quiescent state, against their natural tendency to reactivate the remodelling process. In this way, the exponential decline in bone formation rate at forming sites could be explained as new osteocytes were formed (Martin, 2000a), with the density of osteocytes determining the completed osteonal wall thickness and Haversian canal size for optimal osteocyte nutrient exchange (Metz *et al.*, 2003). In addition, the correct balance between bone formation and its inhibition could be maintained if production of the signal was sensitive to mechanical loading (Reeve, 1986). Until recently no candidate factors had been identified that were osteocyte-derived osteoblast inhibitors.

Recent *in vitro* experiments have shown that the secreted protein product of the SOST gene, sclerostin, is a negative regulator of osteoblasts and might be exclusively osteocyte derived (van Bezooijen *et al.*, 2004). As such, it might be a good candidate to fulfil the functions suggested by Marotti and Martin. How sclerostin inhibits the function of osteoblasts has been the subject of intensive recent study (Li *et al.*, 2005; Sutherland *et al.*, 2004; van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003).

There were several aims of the present study. The first was to determine at what stage normal osteocytes express sclerostin in human bone samples from stroke patients, with a detailed analysis of spatial and temporal expression patterns. The second aim was to investigate the relationship between the percentage of osteocytes expressing sclerostin and the surface extent of osteoid in the stroke patients. Patients with stroke were previously shown to have reduced forming and mineralising surfaces compared to reference values from healthy subjects (Chapter 5). The working hypothesis is that widespread expression of sclerostin by osteocytes could reduce osteoblast recruitment to eroded surfaces or enhance osteoblast differentiation into quiescent bone lining cells. Under such conditions, an inverse association would be expected between the percentage of osteocytes expressing sclerostin and the surface extent of osteoid.

An additional aim was to define the relationship between sclerostin expression and markers of bone formation within the same osteon using precise serial sections enabled by a new sectioning technique. Finally, evidence was sought whether sclerostin might influence the recruitment of osteoblasts to the osteocyte phenotype, by comparing a surrogate marker of osteocyte recruitment (the lacunar density) within osteons of patients with absent sclerostin (sclerosteosis) and controls. If sclerostin acts as a dominant signal responsible for local osteocyte recruitment (Marotti, 1996; Marotti *et al.*, 1992), a reduction in the number of osteocyte lacunae per square millimetre of bone (osteonal lacunar density/mm²) would be expected in the bones of subjects with sclerosteosis.

6.3 Methods

Fourteen patients underwent trans-iliac biopsy 10 weeks after an acute stroke. Details of the 14 subjects and the biopsy protocol are provided in Chapter 5.3. The fresh biopsy specimens were cut in half longitudinally. One hemi-biopsy was embedded in methylmethacrylate for histomorphometry (Chapter 5.3). The other fresh hemi-biopsy was briefly dipped in a 5% solution (w/v) of polyvinyl alcohol (PVA) and then chilled in N-hexane and stored at -80°C prior to sectioning with a cryostat (Brights, Huntingdon, UK). Sections (10 μm thick) were captured onto 50 mm by 10 mm strips of Ultraclear tape (TAAB Laboratories Equipment, Berkshire, UK). The experimental protocol was approved by the Cambridge Regional Ethics Committee.

6.3.1 Immunohistochemistry

Immunohistochemistry is based on incubating high-affinity antibodies on tissue sections to detect patterns of expression for specific protein antigens. An indirect immunoperoxidase method first described by Hsu *et al* (Hsu *et al.*, 1981) and known as the avidin-biotinylated enzyme complex (ABC) method was used. Primary antibody binds to specific epitopes of the antigen within the section. A secondary antibody labelled with multiple biotin molecules is added. An avidin-biotin complex is then added which binds to the biotin of the secondary antibody, amplifying the signal. This is visualised by the addition of a substrate and chromagen to the enzyme, which produces a colour reaction at sites of protein expression that can be assessed using bright field microscopy. A panel of 30 mouse monoclonal antibodies were generated against full length human sclerostin and tested on human bone biopsies (van Bezooijen *et al.*, 2004). The antibody used for this study was hybridoma supernatant H7 of the IgG-1k isotype. The specificity of the antibody was tested by immunostaining with clone H7 in surgical bone biopsies from six sclerosteosis patients. Such patients have a premature C69T stop codon at the first exon of the SOST gene and are unable to translate SOST mRNA into sclerostin protein. Hence, their bones are the ideal negative control tissue. No immunostaining was seen after staining these tissues. After air drying, frozen sections were incubated in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Sections were washed with phosphate buffer solution (PBS) and then blocked in normal serum (Vectorstain Elite Universal ABC kit; code PK-6200; Vector Laboratories, CA, USA) for 1 hour. Thereafter sections were

incubated overnight in a humid chamber at 4°C in mouse monoclonal antibody IgG clone H7 (2.21 mg/ml) at a dilution of 1:1200 or the same concentration of non-immune mouse IgG (Vector Laboratories, code 1-2000). After washing twice with PBS, sections were incubated with biotinylated anti-mouse/rabbit secondary antiserum (Vector; PK-6200) for 30 minutes. After washing, the sections were incubated in a solution of avidin/biotin enzyme complex for 1 hour (Vector; PK-6200). Immunoperoxidase activity was visualised by reaction with a diaminobenzidine (DAB) substrate (Vector; SK-4100). Sections were rinsed in distilled water before counterstaining with toluidine blue (Sigma-Aldrich Toluidine Blue 0.2 mg/ 100 ml pH 4.2, code T-3260) for 2 minutes. Sections were passed through graded alcohols and cleared in xylene before mounting in DPX (Fisher Chemicals). The staining protocol is given in full in Appendix 3.

Alkaline Phosphatase staining of frozen bone sections

This method was described by Bell *et al.* (1997). Ten μm thick cryosections were reacted for 10 minutes at 25°C in 2 mmol/l alpha-naphthyl acid phosphate (monosodium salt) (Sigma-Aldrich, Dorset, UK), magnesium chloride (2 mmol/l) and Fast Red TR (1 mg/ml, Sigma) on 0.1 M barbitone buffer at pH 9.2. They were rinsed in 1% acetic acid and washed before mounting in Farrant's Medium (Nustain, Nottingham, UK).

6.3.2 Image Analysis

Using the following image analysis procedure, the proportions of sclerostin-positive and sclerostin-negative osteocytes were calculated for each of the 14 subjects. The distance of each osteocyte (sclerostin-positive or negative) from its closest surface was also calculated. Cortical bone was captured at x10 magnification using bright field illumination and automated montaging software with a resolution of one pixel per μm . These were large fields up to 7 mm². Full resolution bitmap images were analysed using a PC with a digitising tablet and drawing pen (Summasketch II plus, Summagraphics, USA). Osteocytes positive for sclerostin were marked on the image using PaintShop Pro v7.00 (JASC software, Banbury, UK) as well as negative osteocytes, the Haversian canal surfaces and endosteal/periosteal bone surfaces (fig. 6.1, right panel). In the case of irregular or elliptical Haversian canals (e.g. canal 1, fig 6.1) the canal cross-section was filled with several touching circular masks of varying radii. The images were analysed automatically using Scion Image (version beta 4.0.2) to give a number and location (X-Y

co-ordinates) of the approximate centre of each osteocyte (X_o, Y_o fig. 6.1) and the centre and area of each Haversian canal or canal circular mask (X_{c1}, Y_{c1} fig. 6.1). A software macro was used to convert the bone surfaces into a series of X-Y co-ordinates (X_p, Y_p fig. 6.1). The distance between osteocytes and their nearest surface (Haversian canal or endo/periosteal surface) was calculated automatically using an Excel spreadsheet (Loveridge *et al.*, 2002). The spreadsheet calculated the distance from every osteocyte X_o, Y_o to every other XY coordinate (field edge, Haversian canal/mask centre, bone surface) in the field using Pythagoras' theorem (equations, fig. 6.1). The canal or mask radius was automatically subtracted in the case of distance from Haversian canals. The output from the spreadsheet was the distance of the osteocyte from its closest surface/edge and a numerical designation for the relevant surface/edge (canal/mask number, periosteal/endosteal surface number, image edge). This information was exported to JMP (v 4.0, SAS institute, Cary, NC, USA). Osteocytes that were nearest to the cut edge of the biopsy or the field edge were excluded. In fig 6.1, the osteocyte (X_o, Y_o) is nearest to canal 1.

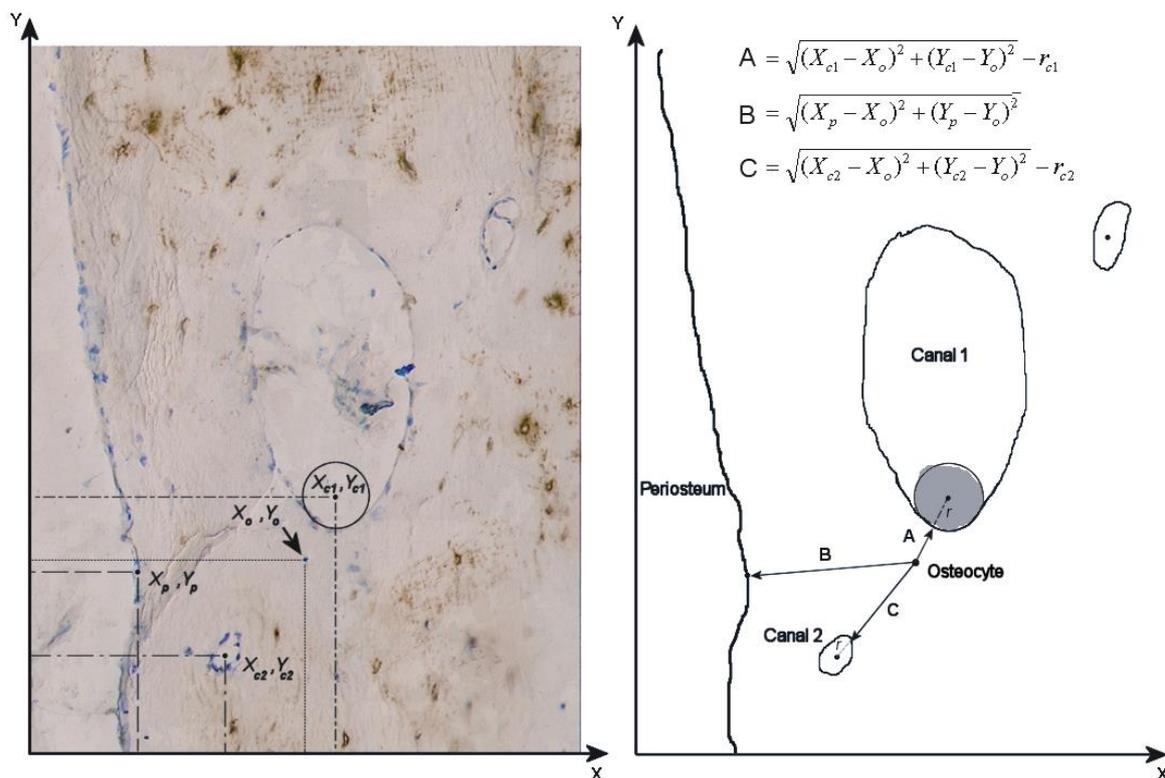


Figure 6.1 Distance from surface measurements (illustration). In this example, a single sclerostin negative osteocyte (coordinates X_o, Y_o) has been marked on the image. The distances of the osteocyte from canal mask 1 (grey hand-drawn area, converted to a circle with radius r_{c1}), the periosteum and canal 2 were calculated using Pythagoras' theorem (equations A, B and C respectively). Distance A is the distance of the osteocyte from its closest surface/edge in this example.

The sclerostin status of osteocytes within 150 μm of the intact periosteal surface were evaluated separately. For cancellous bone analysis, 5 fields (482x364 μm) per section were captured from each of 13 biopsies (one biopsy had no cancellous bone due to sawing artefact at the embedding stage).

Osteoid Surface Measurements

Cancellous osteoid surface and volume were calculated, using sections cut from the half-biopsy specimen that was embedded in methylmethacrylate (Chapter 5.3). Cortical osteoid perimeter and area were also measured using methods described previously (Chapter 5.3).

Serial sections (4 x 10 μm sections)

Serial sections were taken from the biopsies of 5 patients known to have good demeclocycline double-labelling. Section 1 was a control section stained as above, with non-immune mouse IgG at the primary stage and toluidine blue counter stain (fig. 6.6 H). Section 2 was stained for sclerostin as above (fig. 6.6 G). Section 3 was dehydrated, cleared and mounted without staining for fluorescence microscopy (fig. 6.6 J). Section 4 was stained for alkaline phosphatase (fig. 6.6 I). It appeared that the majority of osteons and cortical BMU's contained sclerostin positive osteocytes. There were fewer osteons containing mixed (positive and negative) osteocytes and very few consisting of exclusively sclerostin negative osteocytes. To define this relationship within osteons, sections were first captured using bright field illumination as before. The montage was repeated without moving the section under polarised light. Montages of unstained sections were taken using fluorescence illumination. Cement lines, Haversian canals and bone surfaces were drawn on the polarised image to create a mask. The mask was then transferred onto an image of the same cortex captured under normal light and modified by marking the sclerostin status of each osteocyte. Finally, the modified mask was transferred onto the image of the serial section stained for ALP. Using this method only those osteocytes clearly within an intact osteon were assessed (282 osteons containing 2812 osteocytes in total).

Sclerostin status of newly embedded osteocytes

Using high power objectives (from x20 to x100 Oil immersion), 7 osteons in total were identified that displayed both alkaline phosphatase staining and at least one fluorescent label on the serial section. Images were processed as before. Osteocytes that were located

between the first chronological fluorescent label and the bone surface, (n=28) were identified as ‘newly embedded’ (fig 6.2). The sclerostin status of the osteocytes was recorded.

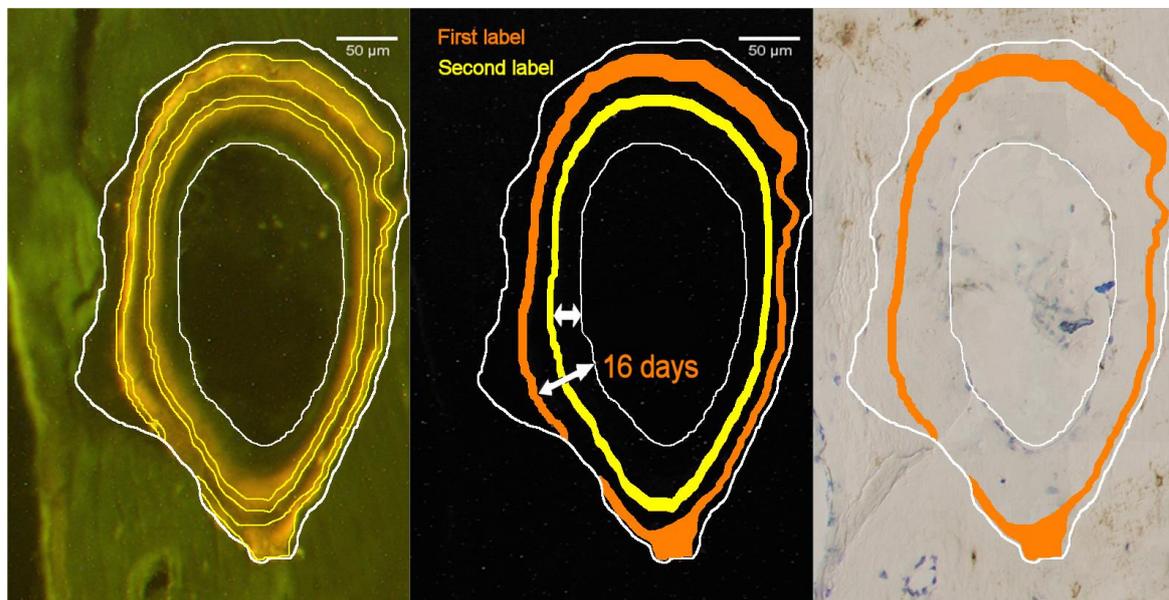


Figure 6.2 Two masks (yellow lines) were drawn on the fluorescent image (left) to mark the location of the demeclocycline labels. The middle image shows that the number of days from the first (orange) label to the osteon surface was 16 days. The orange mask was transferred to the sclerostin-stained section (right) and the sclerostin status of any osteocytes (newly embedded) between the mask and the osteon surface was recorded.

Sclerostin status of osteocytes beneath eroded surfaces

20 ALP negative sites with an eroded surface were identified in the cortex/endocortex using polarised light and toluidine blue stained serial sections. 188 osteocytes located within 150 µm of the eroded surface were analysed for sclerostin status and distance from the eroded surface on the adjacent sections.

Lacunar density in Osteons from patients with Sclerosteosis and Controls

Undecalcified embedded mastoid bone specimens removed at operation were stained using Goldner's trichrome in 3 cases of sclerosteosis (1F, 2M; ages 8,11 and 13) and 3 controls (3M; ages 7,7 and 8). Osteons were identified as before. The mean lacunar density (lacunae/mm²) was calculated by image analysis for 14 control osteons and 47 sclerosteosis osteons.

6.3.3 Statistical Analysis

After consulting a statistician (Dr. S. Kaptoge), the following analyses were performed. Where data was normally distributed Student's t-test was used. Where two measurements were made within the same biopsies, matching pairs t-test was used. The Wilcoxon/Kruskal-Wallis test was used to test non-normally distributed data. The relationship between the sclerostin status of osteocytes within an osteon and ALP status of the osteon was examined by categorising osteons into 3 groups depending on the sclerostin status of the osteocytes within them (1. All sclerostin positive, 2. Mixed and 3. All sclerostin negative) with a subsequent contingency analysis. The relationship between surface osteoid parameters and the percentage sclerostin positive osteocytes was explored using linear regression.

6.4 Results

In undecalcified, unfixed human bone, osteocytes were the sole cell type expressing sclerostin (fig. 6.3 A). In cortical osteocytes from 14 subjects, the median percentage positive for sclerostin was 84.5% (IQR 76.8, 89.4). In cancellous bone the median percentage positive was 72.4% (IQR 67.7, 83.3, $p=0.08$ for the difference, Wilcoxon test). Osteoblasts and lining cells were consistently negative, whether located in Haversian canals (fig 6.3 C), BMU's or at the periosteum (fig 6.3 D). Osteoclasts were found to be negative for sclerostin staining. There was no specific staining seen in serial sections when non-immune mouse IgG was used instead of primary anti-sclerostin antibody (fig. 6.3 B). Images captured at higher power (fig. 6.3 E) confirmed the location of sclerostin in canaliculi and lacunar walls. A montaged image of a pair of osteocytes (fig 6.3 F) taken under oil immersion shows extensive staining along the canaliculi. There was no significant difference in percentage sclerostin expression (cortical or cancellous) in the 5 patients administered zoledronate compared to those given placebo ($p=0.69$ cortical, $p=0.11$ cancellous).

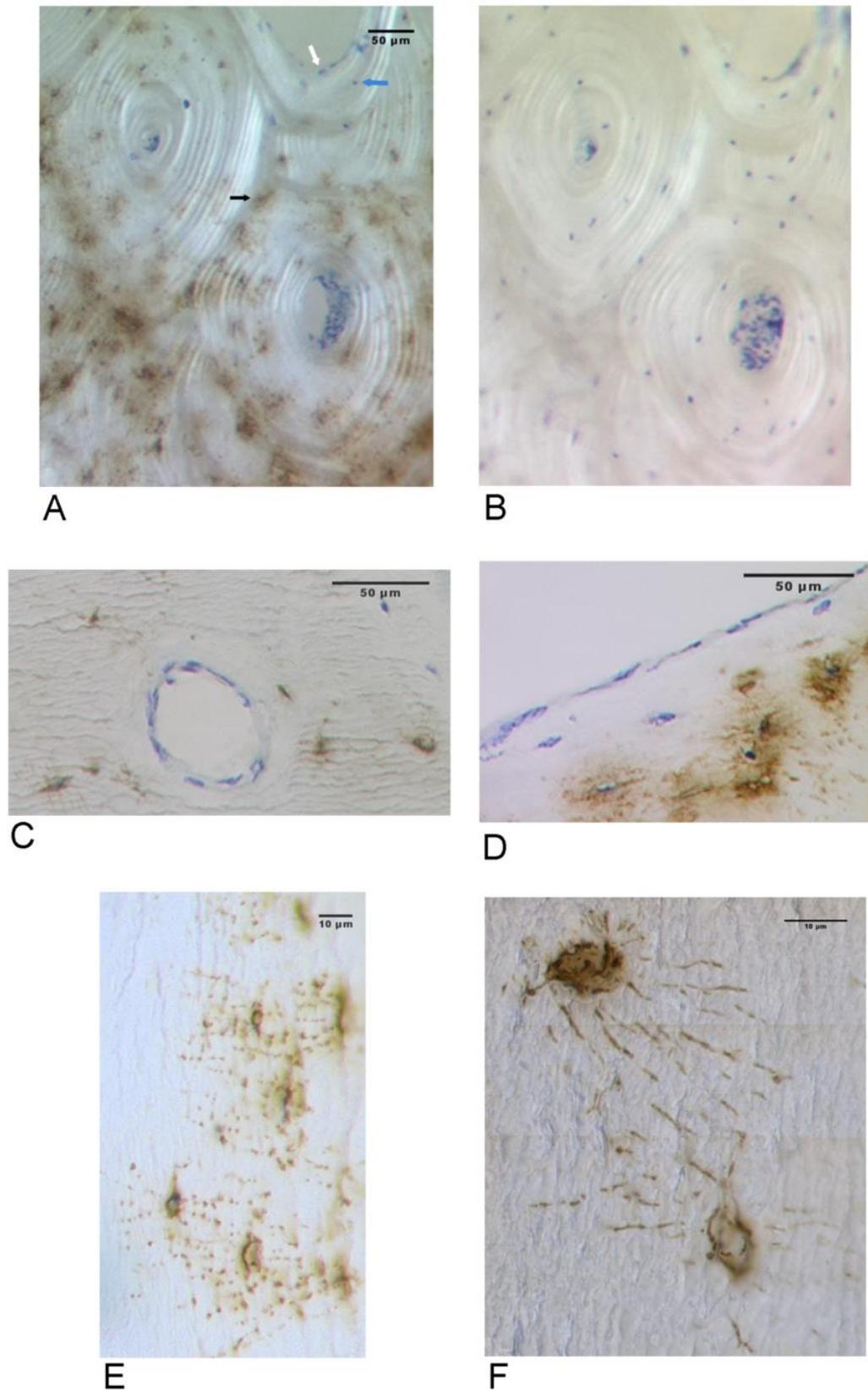


Figure 6.3 A. Sclerostin staining in osteocytes, toluidine blue counter stain, cortical osteons, polarised light, x63 B. Absence of staining with non-immune mouse IgG, x63. C and D. Osteoblasts negative for sclerostin staining in an osteon (C) and at the periosteal surface (D), x126. E. High power bright field image showing canalicular staining, x252. F. Montage stack image of osteocytes, oil immersion, x1000.

Sclerostin Expression in Stroke; Relationship with Osteoid Surface

Sclerostin protein expression was examined in the bone biopsies to establish whether a relationship existed between the percentage osteoid surface observed in stroke and the percentage of osteocytes expressing sclerostin. Figure 6.4 is a plot of the osteoid measurements against the percentage sclerostin-positive osteocytes for cortical bone and cancellous bone respectively. There was no linear relationship for either cortical (r^2 0.01 $p=0.76$) or cancellous bone (r^2 0.02 $p=0.63$). Similarly, when the percentage of sclerostin positive osteocytes was added to the multiple least squares model of Chapter 5 (table 5.3), prediction of the cancellous osteoid surface (OS/BS) was not improved. There were no significant differences in sclerostin expression between patients who received zoledronate or placebo injections (fig. 6.4).

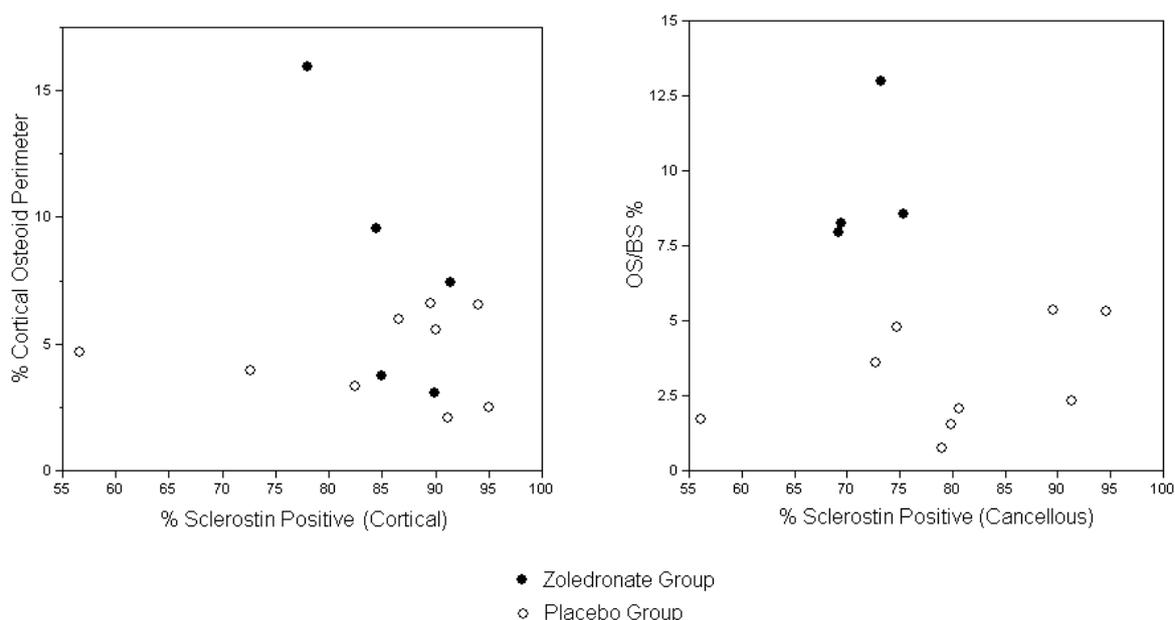


Figure 6.4 Relationship between osteoid indices and % sclerostin positive osteocytes.

Distances of Sclerostin positive osteocytes from surfaces in cortical bone

The distance of each cortical osteocyte to its closest surface (i.e. Haversian canal or endo/periosteal surface) was calculated for sclerostin positive and sclerostin negative osteocytes in 14 biopsies. Because the distributions of distance from closest surface were approximately log normally distributed, the data was described by the median and inter-quartile range. For the entire population of osteocytes, the median distance from the closest surface (MDS) was calculated (fig. 6.5). Sclerostin negative cells were located closer to

surfaces (MDS 57 um, IQR 33, 96, n=1098) than sclerostin positive cells (MDS 102 um, IQR 68, 144; n=5133). The difference between medians was highly statistically significant, $p < 0.0001$. The range of MDS among individual patients was 81 um to 133 um (sclerostin positive) and 39 um to 89 um (sclerostin negative).

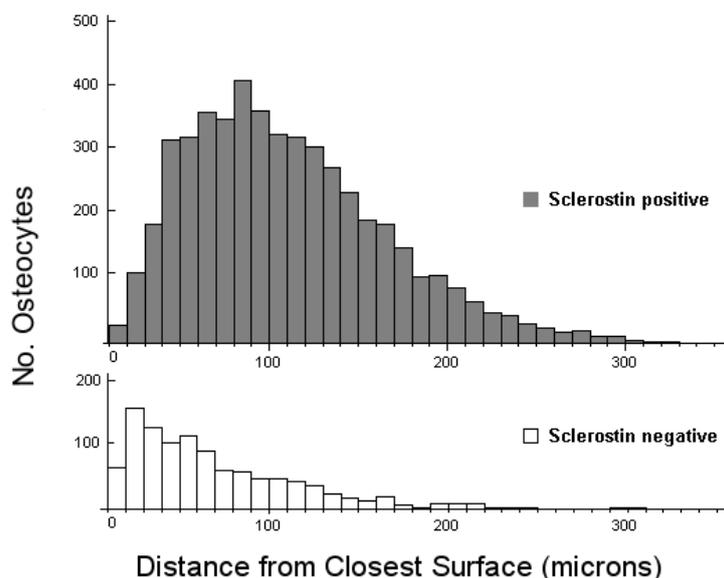


Figure 6.5 Distance of cortical osteocytes from their closest surface, by sclerostin status (in 14 samples).

Analysis of osteons, forming surfaces, eroded surfaces and periosteal surfaces

In total 282 osteons were analysed (table 6.1). By categorising osteons based on their sclerostin status, contingency analysis confirmed that quiescent (ALP negative) osteons were more likely to contain all sclerostin positive osteocytes whereas forming (ALP positive) osteons were more likely to contain sclerostin negative or mixed osteocytes, $\chi^2 = 33.5$, $p < 0.0001$ (table 1). Analysis of high power images confirmed that 27 of 28 (96.4%) recently embedded osteocytes (between the first fluorescent label and the bone surface) were negative for sclerostin (fig. 6.2 and fig. 6.6).

Table 6.1 . Osteocytes within osteons; by sclerostin status (positive, negative, mixed)

	Osteocytes all Positive (%)	Osteocytes mixed (%)	Osteocytes all Negative (%)
Quiescent osteons ALP -ve (n=235)	66	32.3	1.7
Forming osteons ALP +ve (n=47)	23.4	66	10.6

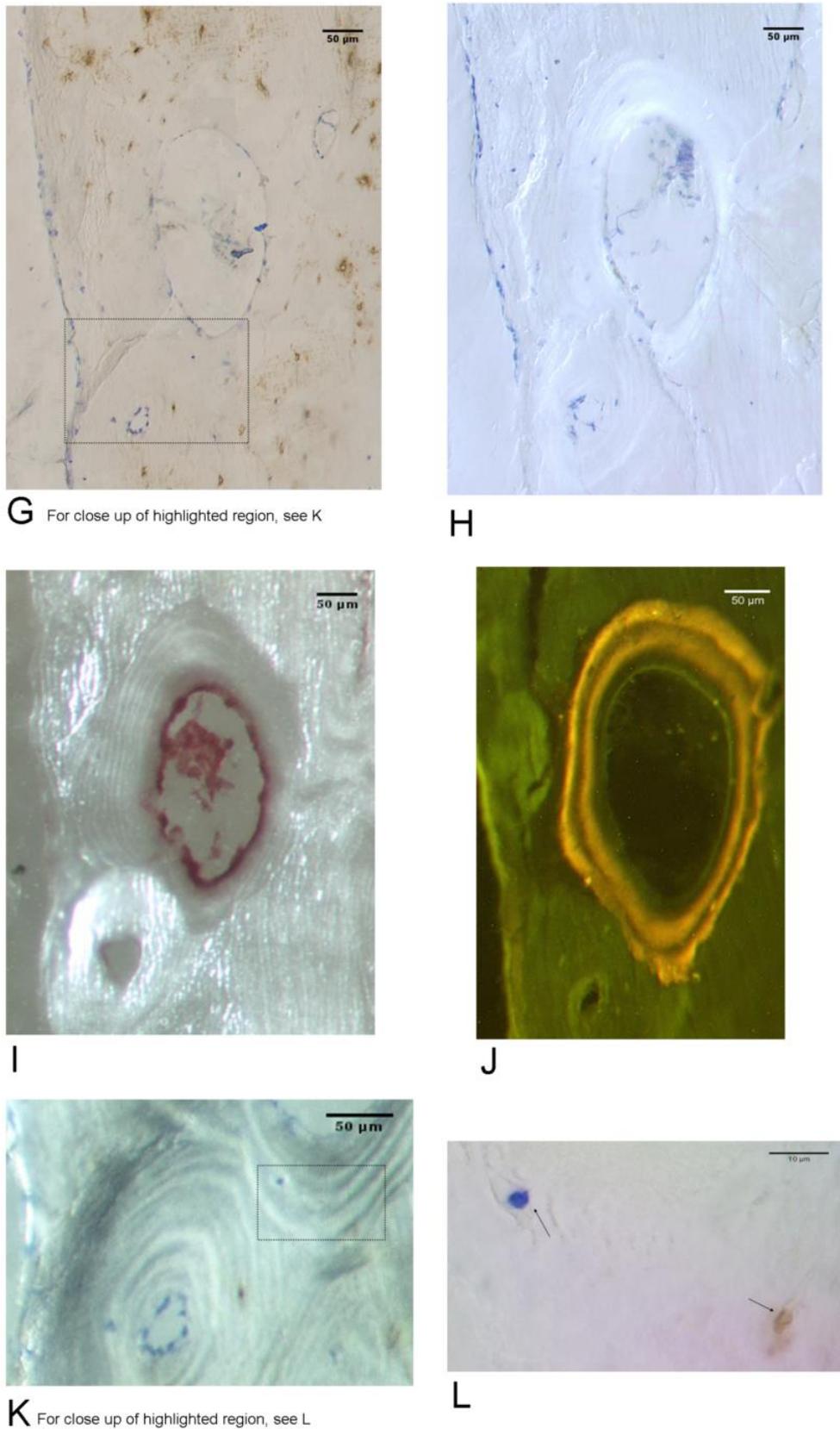


Figure 6.6 H, I, J and K. Serial sections of cortical bone stained for sclerostin (G, toluidine blue counter stain, x126), with non-immune mouse IgG (H, x126), alkaline phosphatase (I, x63) and under a fluorescent filter (J, x63). K and L. High power image of positive and negative osteocytes within osteon shown in G (K, x200 and L, x630).

Within 150 μm of an eroded surface, 90.4% (170/188) of osteocytes were sclerostin positive, which was not significantly higher than the overall % sclerostin positive osteocytes for the relevant biopsies ($p=0.19$). Within 150 μm of the periosteum, 74.8% (437/584) of osteocytes were sclerostin positive which was not significantly different from the overall % sclerostin positive osteocytes from the relevant biopsies ($p=0.94$). However, plotting the percentage of negative osteocytes in 10 μm bands parallel to the periosteal surface revealed a distribution with the highest percentage sclerostin negative osteocytes close to the surface (fig. 6.7).

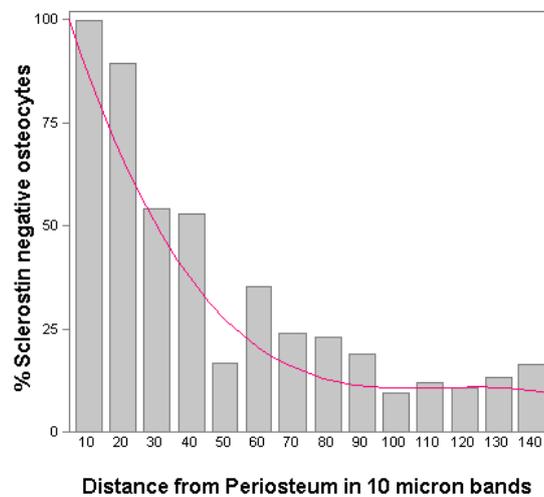


Figure 6.7 Distribution of percentage negative osteons in 10 μm bands parallel to the periosteal surface.

Osteonal lacunar density in mastoid specimens from Sclerosteosis vs. Controls

There was no significant difference in osteonal lacunar density between patients with sclerosteosis (mean lacunar density $292/\text{mm}^2$, SE 28.5) and controls ($258/\text{mm}^2$ SE 15.6, $p=0.30$).

6.5 Discussion

The results of this study confirm earlier work suggesting that osteocytes are the principal class of bone cells expressing sclerostin. The results demonstrate that sclerostin secretion by new osteocytes is a delayed event so that they must in some way mature or receive a signal that triggers sclerostin expression. These findings are consistent with the concept that newly embedded osteocytes secrete sclerostin after the onset of mineralisation to inhibit cortical bone formation and osteon infilling by cells of the osteoblast lineage (fig. 6.8). The majority of completed cortical osteons (66%) contained only sclerostin positive osteocytes, indicating that production of this inhibitory signal is prevalent in osteocytes after their surrounding matrix is mineralised. These findings are in keeping with in vitro experiments using cultures of differentiating osteoblast-like cells since SOST mRNA expression was not detected in undifferentiated cells but occurred after the onset of mineralisation (van Bezooijen *et al.*, 2004). The murine cell populations in such in vitro systems may include osteocyte-like cells embedded in mineralising nodules, so that it is conceivable that the low level SOST mRNA seen was derived from osteocytes (van Bezooijen *et al.*, 2004).

It was shown that the majority of osteocytes stain for sclerostin with a trend towards a higher percentage of cells sclerostin-positive in the cortex (median 85.9%) than in cancellous bone (median 74.2%, $p=0.08$). Osteoblasts, lining cells and periosteal osteoblasts did not exhibit sclerostin staining in iliac bone (fig. 6.3). Exploring the spatial relationships of sclerostin-positive osteocytes within osteons indicated that sclerostin-negative osteocytes were significantly closer to bone surfaces (Haversian canals, periosteal and endosteal surfaces) than the more numerous sclerostin-positive osteocytes. By examining serial sections for sclerostin expression, alkaline phosphatase activity and double-tetracycline labelling, the close spatial relationships between sclerostin-negative osteocytes and forming/mineralising surfaces were demonstrated. Recently embedded osteocytes including those within unmineralised osteoid were almost all negative for sclerostin. The more sclerostin negative osteocytes were found within an individual osteon, the more likely the osteon was to be in the process of bone formation, during which retiring osteoblasts become newly embedded as osteocytes. Since the first demeclocycline treatment finished 16 days before the bone biopsy was performed (2-10-2-4 day regimen),

it is estimated that most new osteocytes are negative for sclerostin staining for at least 16 days. Sclerostin protein expression has been found in osteocytes and hypertrophic chondrocytes in human bone biopsies (van Bezooijen *et al.*, 2004; Winkler *et al.*, 2003). Winkler *et al.* (2003) also reported weak staining in osteoblastic cells, but there was no sclerostin protein expression in osteoblasts or bone lining cells in the iliac bone specimens of the present study, although immunohistochemistry may not be able to detect very low protein concentrations. This supports the previously reported absence of staining in osteoblasts (van Bezooijen *et al.*, 2004). Furthermore, there was no apparent sclerostin expression in osteoclasts which is in keeping with reports by Winkler *et al.* (2003) and van Bezooijen *et al.* (2004) but is in contrast to the report by Kusu *et al.* (2003). In the present study, however, 90.4% of osteocytes adjacent to cortical eroded surfaces were positive for sclerostin

In light of this precise temporal and spatial expression of sclerostin by osteocytes, it is perhaps not surprising that there was no linear relationship between the percentage of osteocytes staining positive for sclerostin and osteoid surface measurements for either cortical (r^2 0.01 $p=0.76$) or cancellous bone (r^2 0.02 $p=0.63$, fig. 6.4). Osteoid measurements were made on the methacrylate-embedded half biopsy whereas the sclerostin immunostaining experiments were on fresh cryosections of bone from the remaining half biopsy (fig. 5.1). One reason for the lack of a relationship may have been that the sections on which IHC and histomorphometry were performed were separated by well over 250 μm and therefore different surface remodelling events would be represented in each section. Additionally, with the pattern of sclerostin expression observed (fig. 6.8), it seems more likely that the percentage of sclerostin negative osteocytes in a single section would be determined principally by the number of osteocytes located within unmineralised osteoid in that section. However, as shown in table 6.1, there are certain situations where an osteon has finished forming (no alkaline phosphatase activity at the surface) but not all the osteocytes within that osteon are sclerostin positive (33.7% of osteons in this series were filled with a mixed population of sclerostin positive and negative osteocytes). Thus some of the osteocytes within a completed osteon do not appear to express sclerostin. More work is required to elucidate the mechanisms controlling sclerostin expression by osteocytes.

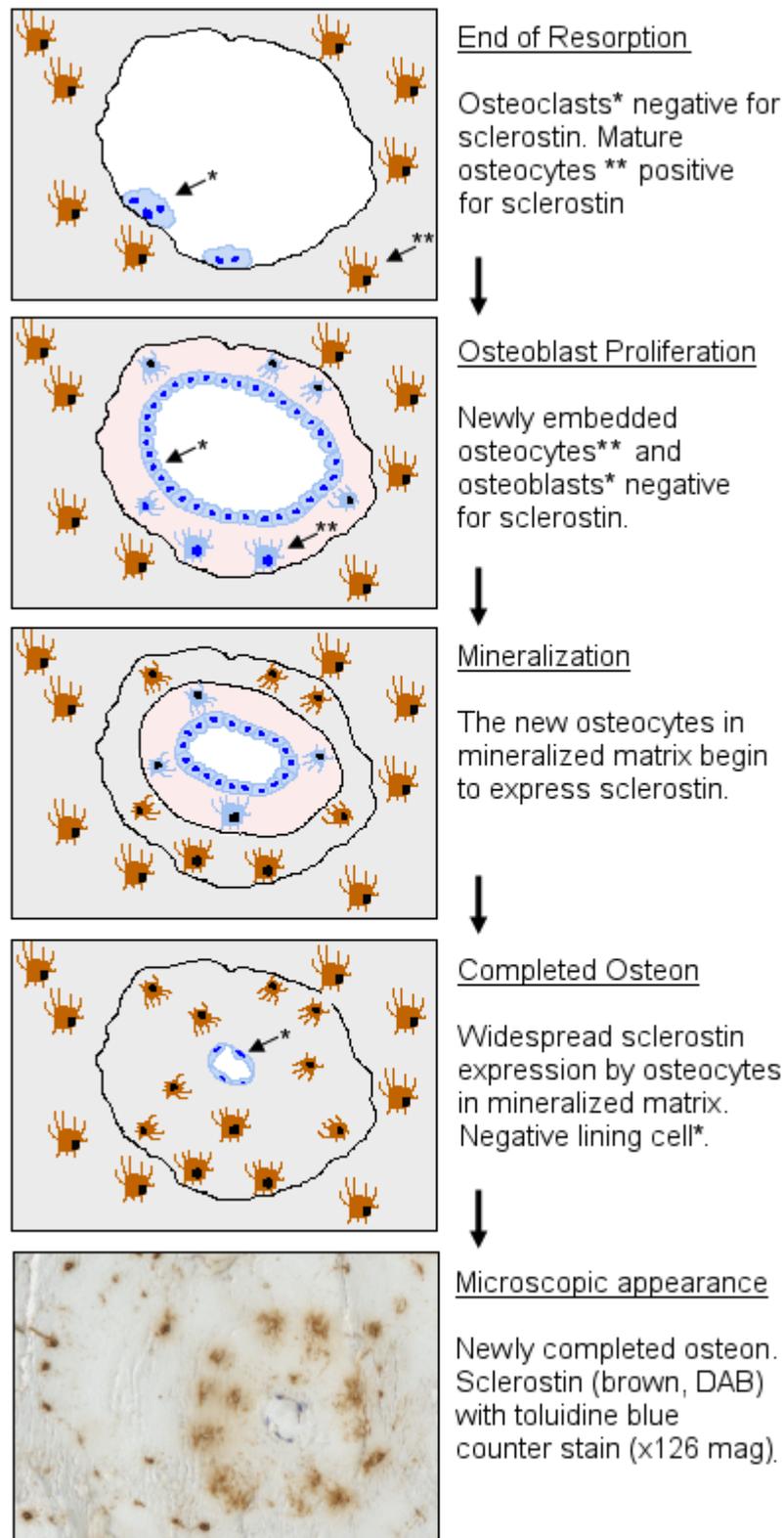


Figure 6.8 A new theory to explain osteocytic control of bone formation by sclerostin signalling.

The high proportion of sclerostin positive osteocytes seen in the study prompted examination of bone samples from patients with sclerosteosis, the disease that occurs due to a single nucleotide mutation in the *SOST* gene and results in absent sclerostin protein (Balemans *et al.*, 2001; Brunkow *et al.*, 2001). There was no suggestion that the recruitment of osteocytes (as inferred from osteonal lacunar density) was altered in the osteons of mastoid bone specimens removed at operation from 3 cases of sclerosteosis. It seems that in sclerosteosis, the balance between the formation of bone matrix and the incorporation of new osteocytes is not grossly disturbed. If sclerostin were to act by globally inhibiting bone lining cells (retired osteoblasts) from activating remodelling (Martin, 2000a), one might expect to see increased activation frequency and eroded surface in sclerosteosis. Published data, although limited, is contrary to sclerostin acting this way, since indices of resorption were not elevated in bone biopsies from sclerosteosis patients, despite a doubling of the cancellous bone volume and labelled surfaces and a 5-fold higher osteoid volume (Stein *et al.*, 1983). The bone lining cell is thought to influence bone formation in either of two ways. First, lining cells may initiate bone formation on an existing bone surface by reverting to an osteoblast phenotype (modelling) under certain conditions (Chow *et al.*, 1998; Dobnig and Turner, 1995). Secondly, bone lining cells may be involved in initiating bone resorption (e.g. through RANK ligand expression (Teitelbaum, 2000)) and a subsequent wave of bone formation (remodelling). Therefore in sclerosteosis it seems more likely either that bone lining cells revert to the active osteoblast phenotype, or less radically that existing osteoblasts are able to maintain their working state for longer, making more bone at each site, enabled in part by a longer osteoblast lifespan.

There is evidence that periosteal bone expansion is a feature of advancing age, even in late adulthood (Allen *et al.*, 2004; Kaptoge *et al.*, 2003; Power *et al.*, 2003). The pattern of decreasing osteocytic sclerostin expression that was observed on approaching the periosteal surface (fig. 6.3 D and fig. 6.7) is noteworthy, as the osteocytes are unlikely to be newly embedded at this site. This arrangement of osteocytic sclerostin expression might have a role in regulating normal periosteal expansion, whereas complete disinhibition of periosteal osteoblasts (due to absent sclerostin) could lead to the cranial canal compression and cortical bone expansion seen in sclerosteosis.

How sclerostin inhibits the function of osteoblasts and bone lining cells has been the subject of intensive recent study. Sclerostin might function as a BMP inhibitor, reducing the differentiation of osteoprogenitor cells (Winkler *et al.*, 2003) and promoting osteoblast apoptosis (Sutherland *et al.*, 2004). However, since sclerostin was subsequently shown not to inhibit early BMP-induced responses *in vitro*, it was suggested that it might act by modulating Wnt signalling (van Bezooijen *et al.*, 2004). Recently Li *et al.* (2005) have found that sclerostin functions as an inhibitor of mature osteoblast activity, by binding to LRP5 and 6 and inhibiting canonical Wnt signalling in osteoblast-like cultures. The therapeutic potential of sclerostin protein antagonism is being explored with the development and testing of monoclonal antibodies to sclerostin in mice and rats resulting in significant increases in BMD at several skeletal sites (Warmington *et al.*, 2004).

These experiments strengthen the supposition that sclerostin is a key inhibitor that plays a central role in determining the normal extent of bone formation and consequently protects against the deleterious effects of uncontrolled bone growth (sclerosteosis). Whether sclerostin expression by osteocytes in a new osteon is associated with the gradual decline in matrix apposition rate seen in normal osteonal infilling (Martin, 2000a), or in modulating the mineralization of that osteon require further study. However, one qualification of the present study is that bone remodelling events in the iliac bones of stroke subjects are not entirely representative of remodelling in healthy human subjects (as demonstrated in Chapter 5). Another possibility is that altered calcitropic hormone levels in stroke patients could influence sclerostin expression by osteocytes. Confirming these findings in bone samples from healthy subjects should be a priority for further work. Similarly, studying sclerostin expression in a variety of skeletal locations is desirable.

This study adds to the growing body of knowledge concerning the osteocytes' regulatory role in maintaining bone structure and strength. These findings are the first demonstration of how live osteocytes might fine-tune bone formation by the timely secretion of an inhibitory signal. The regulation of bone construction is an important function of osteocytes, adding to their recently established role in targeting bone destruction through apoptosis (Noble *et al.*, 2003; Tomkinson *et al.*, 1997; Verborgt *et al.*, 2000) and signalling through biochemical mediators (Gross *et al.*, 2001; Gross *et al.*, 2005). They form a syncytium of cells through their connections with each other and with bone lining

cells. Until now, it has not been possible to attribute an active role for the osteocyte in suppressing the activities of either osteoclasts or osteoblasts. Together with previous work, this study suggests that the osteocyte may influence the local growth and renewal of bone in several ways. By regulating its own survival (Noble *et al.*, 2003) or signalling through molecules such as osteopontin (Gross *et al.*, 2005), it may control the local activity of osteoclasts and by secreting sclerostin it might determine how much new bone is laid down to replace what has previously been removed by osteoclasts. Thus a picture is emerging of the osteocyte as architect of bone's evolving microscopic structure. By showing how restricted sclerostin expression is in bone, the results make the SOST gene product a highly promising target for developing new pharmaceutical approaches to managing osteoporosis.

Chapter 7. General Discussion; The Effects of Stroke on the Skeleton

The aim of this study was to elucidate the pathophysiological mechanisms by which hip bone loss occurs in hemiplegia and to test the efficacy of an intravenous bisphosphonate in preserving bone in stroke patients. In this final chapter, the progress made towards these objectives will be discussed alongside the practical implications of the research findings. In particular, further avenues of investigation will be highlighted and unanswered questions addressed.

Preventing Bone Loss After Stroke Using Intravenous Zoledronate

In Chapter 3, the results of an RCT indicated that the loss of bone mineral density from the hemiplegic hips of stroke patients could be prevented with a single dose of intravenous zoledronate administered within 35 days. This study is the first intervention trial in stroke patients that has proved that bone loss at the hip can be attenuated with a bisphosphonate. The patients were a carefully selected group, chosen because they were at high risk of bone loss, being unable to walk 7 days after stroke. Up to 10% of patients die from an acute stroke (Terent, 2003) but in patients that survive, the majority of patients cannot walk independently one week later (63% in one series, fig. 7.1, Jorgensen *et al.*, 1995). Such patients have a higher rate of hip bone loss during the first year after stroke than those that can walk within a week (Jorgensen *et al.*, 2000a).

While it has been shown that patients initially unable to walk following their stroke suffer the most bone loss, it is not yet clear whether this translates into a higher risk of subsequent hip fracture. A well conducted study aimed at establishing which stroke patients have the highest risk of fracture based on their functional characteristics (Barthel index) and motor impairment (Scandinavian Stroke Scale) on admission and discharge is now a priority, to provide these answers and help direct appropriate preventative therapy. For instance, in the study summarised in figure 7.1, 18% of the acute stroke patients remained unable to walk 28 days later. The results from two small studies indicate that an intervention to prevent hip fracture might not be successful in these patients, since their outlook for a walking recovery (and subsequent falls) appears very poor (Jorgensen *et al.*, 1995) and their risk of subsequent hip fracture may not be elevated (Melton *et al.*, 2001).

At the other end of the spectrum of stroke severity, 149 of 393 patients (38%) assessed for the zoledronate trial (Chapter 3) were walking unaided 7 days after stroke, the single largest reason for trial ineligibility. This figure is similar to the 37% of stroke patients already fully ambulant 7 days after stroke shown below (fig. 7.1). A prospective study (fig. 1.2) showed that these patients will lose less bone mineral density from the hemiplegic hip than more severely affected stroke patients (Jorgensen *et al.*, 2000a). Nevertheless, such patients might still benefit from the potential fracture-reducing capability of intravenous bisphosphonates, since a picture is emerging of an increased hip fracture rate even amongst those stroke patients whose functional deficits are initially mild (Sato *et al.*, 2005c).

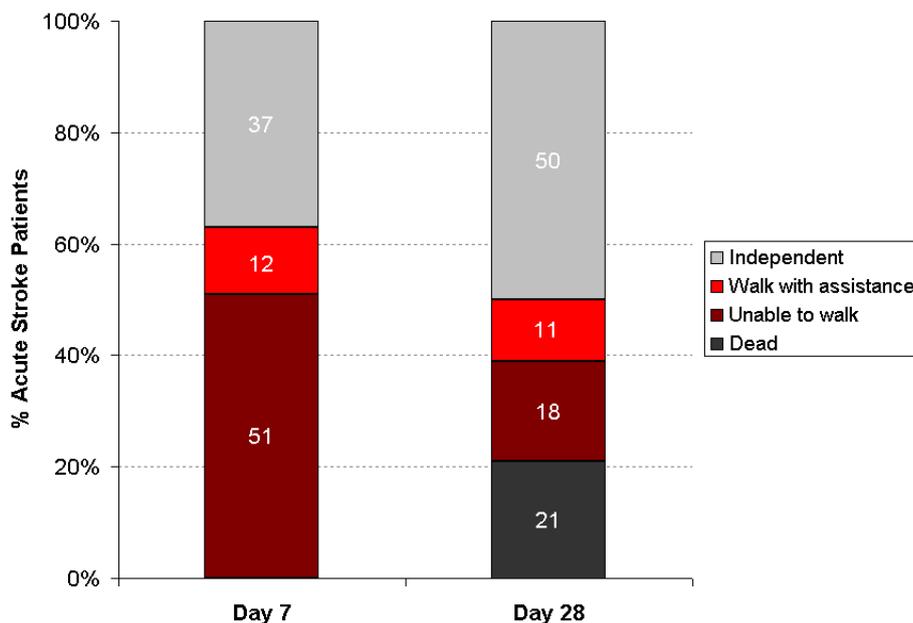


Figure 7.1 Early (7 day) and later (28 day) stroke recovery characteristics in 804 consecutive admissions to an acute stroke unit (Jorgensen *et al.*, 1995).

An RCT aimed at preventing hip fractures after stroke now has much to recommend it. The trial results reported here prove the concept that intravenous zoledronate is effective in preventing the loss of hip BMD in those with moderate to severe motor deficits from stroke. While this work was being conducted, results from an RCT were published showing that an alternative strategy of a daily oral bisphosphonate (risedronate) was effective in preventing hip fractures (within 1 year) in those with very mild motor deficits (Sato *et al.*, 2005c). For future studies, the recommended study design is a multi-centre

RCT, conducted using intravenous bisphosphonates in stroke patients who are unable to walk initially, but who are likely to regain walking ability. An additional possibility is including stroke patients with milder early functional deficits. The new United Kingdom National Research Network for Stroke may have an appropriate infrastructure to conduct such a study. The results of ongoing trials using zoledronate in postmenopausal osteoporotic women will help elucidate the potential fracture risk reduction in a population at increased risk of hip fracture. The advantages of the intravenous single dose approach in stroke (compared with oral bisphosphonates) are that compliance with therapy is guaranteed, swallowing does not need to be preserved and all stroke patients should (under current national guidelines) be admitted to an acute stroke unit. Therefore most patients can be assessed for suitability for the intervention. Importantly, the present study highlighted certain precautionary measures that are needed before intravenous bisphosphonates can be safely administered to stroke patients, including ensuring adequate renal function and vitamin D levels.

Finally, the pathophysiological mechanisms of bone loss in stroke were explored using BMD and hQCT technologies. Interestingly, the trochanteric region was most affected by hemiplegia, a site not considered in published work to date. Conversely, the femoral neck BMD changes were of considerably lower magnitude and developed later. The new technique of hQCT demonstrated that within subjects, the trochanteric and total hip regional vBMD were significantly lower on the hemiplegic than the unaffected side. However, femoral neck cortical thickness (from a single slice of the mid-femoral neck) also differed significantly between hemiplegic and unaffected hips. The algorithm to extract the femoral neck cross-section chose the site with the lowest cross sectional area along the femoral neck axis. That the mean cross sectional area was not significantly lower on the hemiplegic side compared with the unaffected side was noteworthy. The conclusion is that the lower cortical thickness on the hemiplegic side was most likely the result of an increased endosteal diameter (fig. 5.10). Prospective studies using hQCT in stroke should now be undertaken, as well as more detailed studies of the present images, to establish if there are regional differences in cortical thickness that might contribute to femoral neck fragility in such patients. Improved algorithms are currently being developed using the results from the hQCT scanner that will lead to more accurate estimates of cortical and cancellous regional bone changes as well as better cortical thickness estimates.

Vitamin D Insufficiency in Patients with Acute Stroke

In Chapter 4, it was demonstrated that patients admitted acutely with hemiplegic stroke had vitamin D levels that were significantly lower than healthy elderly controls. This study is of great importance to the pre-stroke health and post-stroke musculoskeletal health of hemiplegic patients and must be considered alongside the clinical trial results from Chapter 3. In any trial of intravenous bisphosphonates in stroke, careful consideration must now be given to vitamin D replacement. One approach is the administration of a large dose of intramuscular 25OHD in acute stroke patients at the time of consent into a hip fracture prevention trial. Vitamin D replacement therapy should be considered for all stroke patients, partly because vitamin D is likely to decline further in the years following the stroke. After stroke, vitamin D repletion might provide health benefits to the musculoskeletal system and even reduce falls (Bischoff *et al.*, 2003).

The vitamin D insufficiency observed is striking in comparison to the healthy control subjects and therefore one important avenue for further research is whether vitamin D insufficiency is a risk marker for stroke. It would be possible to test this using a large epidemiological cohort if baseline serum samples were stored. Then, the serum 25OHD of the patients subsequently suffering a stroke in the cohort could be to an appropriate control group drawn from within the cohort. In addition, a larger survey of vitamin D in acute stroke patients should now be undertaken.

Histomorphometry in Stroke Patients

In Chapter 5, the histomorphometric analysis of iliac bone samples resulted in several important observations regarding zoledronate therapy in stroke. Iliac bone samples were not considered to reflect underloading conditions and no side-to-side differences were observed in any parameter. Therefore, these results do not improve understanding of the site specific (localised) bone loss that occurs at sites such as the hemiplegic trochanteric region or mid femoral neck cortex. However, the very low osteoid surfaces found in cancellous, cortical and endocortical bone in stroke suggest that the ilium reflects the more generalised bone loss that occurs in stroke. As an example of this more generalised bone loss, during the RCT, the mean reduction in the unaffected side trochanteric region over 12 months in the placebo group was -4.5%. That the surface extent of bone formation was low

(OS/BS and MS/BS) was in agreement with studies performed in other models of human bed-rest. Of particular relevance, the concept that there is a failure of infilling of eroded areas in the bone of stroke patients was supported. An unexpected finding was that the patients given zoledronate had significantly higher osteoid indices. Study limitations such as the low sample size and that mineralising surfaces did not differ between the intervention groups are acknowledged. However, the linear relationship suggested that a low 25OHD and zoledronate together predict the OS/BS. This generated interest in the role of the PTH/calcium axis in the surface extent of bone formation in stroke. Recent studies using pamidronate before bed-rest in healthy volunteers add to a growing body of literature indicating that the rise in PTH (with even a single dose of intravenous bisphosphonate) is lasting, and may be beneficial to bone formation under conditions of unloading (Rittweger *et al.*, 2005). Increasing PTH might improve bone formation indices in conditions such as bed-rest, stroke, spinal cord injury and space flight, where raised calcium would normally inhibit PTH secretion. In figure 7.2, the results of a longitudinal study of biomarkers and PTH in the bed-rested volunteers given pamidronate, an exercise regime or neither are shown (Rittweger *et al.*, 2005). A similar study in stroke is now needed, to assess the dynamics of the PTH, 1,25(OH)₂D, calcium and bone formation markers.

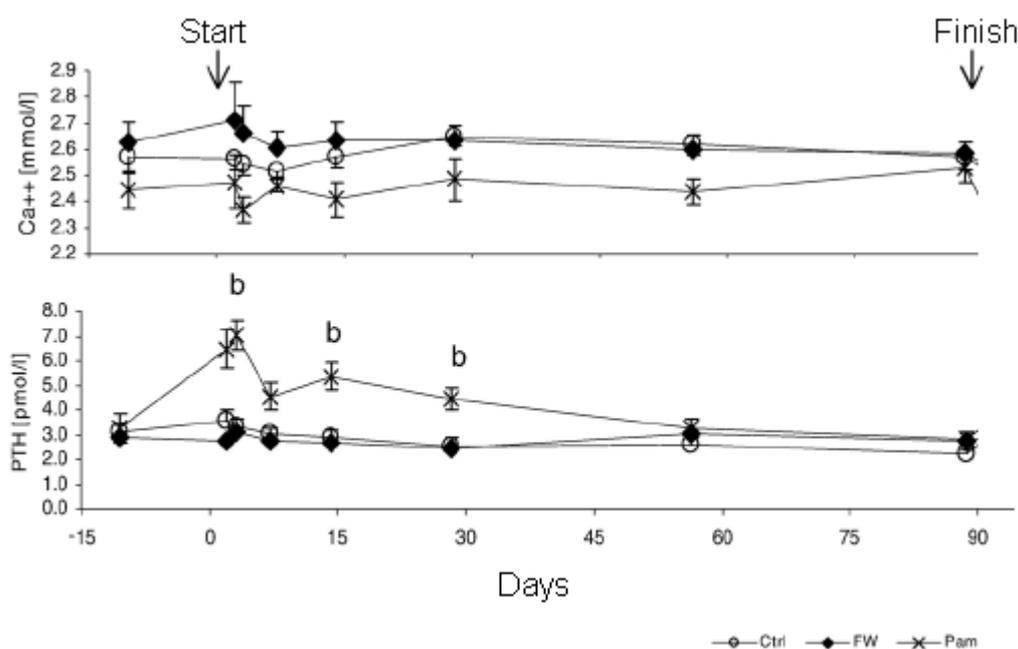


Figure 7.2 Interventions in a bed-rest study: Pamidronate 14 days before bed-rest (Pam) Flywheel exercise throughout (FW) or neither interventions (Ctrl). Note the significant increase in PTH with pamidronate (second graph) which was associated with an increase in bone specific alkaline phosphatase (not shown). Reproduced from (Rittweger *et al.*, 2005).

Sclerostin and the Osteocytic Control of Bone Formation in Stroke

Sclerostin was investigated as a factor that might explain the reduction in osteoid surface in stroke. This was because a ubiquitous inhibitory signal produced by osteocytes and carried by the lacunocanalicular network was an attractive concept to explain widespread inhibition of osteoblast recruitment. In transgenic mice over-expressing human sclerostin, there were reductions in bone surfaces undergoing formation and osteoid surface (Winkler *et al.*, 2003). The theory (that a higher percentage of sclerostin positive osteocytes would be associated with lower surface extent of bone formation) was partly based on an assumption that skeletal underloading would increase inhibitory signalling by osteocytes (fig. 7.3). It became apparent that iliac biopsies from stroke patients were not suitable for investigating underloading effects, since iliac bone histomorphometry failed to show any side-to-side differences in indices of bone remodelling (Chapter 5). Hence, iliac bone from hemiplegic patients is not suitable for examining the effects of skeletal underloading on sclerostin expression by osteocytes. This will need to be tested using a better model of underloading than stroke in humans. However, very recent investigations into the regulation of the SOST gene by parathyroid hormone (Keller and Kneissel, 2005) suggest that it might be premature to abandon the theory that stroke leads to an increase in osteocytic sclerostin expression (fig. 7.3). These investigators recently found that SOST is a target gene for parathyroid hormone, as PTH directly reduced SOST transcription in a PTH-induced calvarial bone formation experiment in mice. After 5 days of human PTH (1-34) injections, SOST mRNA levels were substantially reduced in the calvarial bone. Findings from Chapter 6 would suggest that some reduction in SOST expression would be expected in their model, due to increased newly embedded osteocytes within newly formed (but not mineralised) calvarial bone. However, the magnitude of the reduction in expression (together with several accompanying *in vitro* studies) suggest a direct effect of PTH on downregulating SOST transcription (Keller and Kneissel, 2005). If SOST transcription is directly downregulated by PTH, a chronic inhibition of PTH secretion in stroke (Chapter 1.4.1) might be associated with an increased SOST transcription in osteocytes. More work is needed to substantiate these putative mechanisms. The future avenues for research involving sclerostin concern not only its role in PTH-mediated bone formation but also (because of the present study), the precise osteocytic regulation of osteoblasts and bone lining cells. That patients with sclerosteosis overfill their internal skull foramina with bone highlights a catastrophic failure to regulate periosteal expansion,

mediated by absent sclerostin. The osteocytic regulation of periosteal expansion is an area that should receive increased attention in light of the present results.

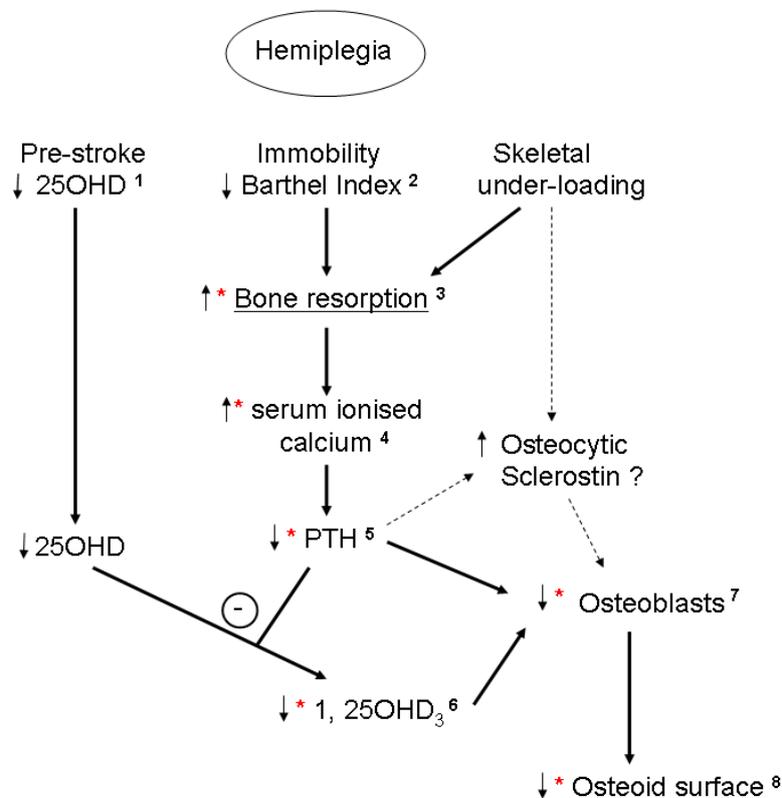


Figure 7.3 Schematic demonstrating the potential role of sclerostin production by osteocytes in reducing bone formation in hemiplegia. PTH has recently been shown to strongly downregulate sclerostin production by osteocytes (Keller and Kneissel, 2005).

In conclusion, the experiments of Chapter 6 have illuminated bone biology by demonstrating the role of osteocytes in determining bone's micro-architectural development through the very precise geographical and temporal expression of sclerostin as a local modulator of mature osteoblast function (Poole *et al.*, 2005b). Consequently, sclerostin antagonism has become one of the most eagerly awaited therapeutic strategies for managing osteoporosis and may yet have a role in preventing bone loss after stroke.

Appendix 1: Zoledronate Trial

Coefficient of Variation Lunar Prodigy Hip DXA

Three scans of the hemiplegic total hip BMD were performed in three individuals with repositioning between each measurement. For repeated measurements BMD1, BMD2 and BMD3, the mean and variance were calculated. The overall mean of the mean measurements was calculated (mean overall). The overall variance was calculated from the mean of the variances.

The coefficient of variation (CV) was calculated by:

$$\frac{\sqrt{\text{variance overall}} * 100}{\text{grand mean}}$$

$$= \frac{\text{global estimate of the SD of the repeat measurements}}{\text{mean}} * 100$$

Table. AT1.1 CV for Lunar Prodigy: Total Hip measurements=1.03%

Patient ID	BMD 1	BMD2	BMD3	mean within	SD within	CV within	Variance overall	Mean overall	CV overall
MG	0.798	0.801	0.798	0.799	0.001732	0.216777	0.000082	0.878667	1.029884
JJ	0.886	0.884	0.886	0.885333	0.001155	0.130426	0.000082	0.878667	1.029884
LW	0.939	0.947	0.969	0.951667	0.015535	1.63239	0.000082	0.878667	1.029884

Table. AT1.2 CV for Lunar Prodigy: Femoral Neck= 1.12%

patient ID	BMD1	BMD2	BMD3	Mean within	SD within	CV within	Variance overall	Mean overall	CV overall
MG	0.728	0.731	0.74	0.733	0.006245	0.851978	0.00008	0.808667	1.105283
JJ	0.826	0.837	0.832	0.831667	0.005508	0.662233	0.00008	0.808667	1.105283
LW	0.86	0.849	0.875	0.861333	0.013051	1.51523	0.00008	0.808667	1.105283

Coefficient of Variation for Mindways hQCT

The precision of Mindways extraction and manual hip rotation in 3 dimensions was calculated by

$$CV(\%) = \frac{SD}{Mean} * 100$$

Table AT1.3 CV for Mindways Total Hip vBMD= 0.40%

patient ID	vBMD	mean	SD	Coefficient of Variation %
PP	1.092	1.093	0.0043589	0.39880137
PP	1.09	.	.	.
PP	1.094	.	.	.
PP	1.089	.	.	.
PP	1.1	.	.	.

Table AT1.4 CV for Mindways Femoral Neck vBMD= 0.26%

patient ID	vBMD Neck	mean	SD	Coefficient of Variation %
PP	0.921	0.9218	0.00238747	0.25900057
PP	0.921	.	.	.
PP	0.92	.	.	.
PP	0.921	.	.	.
PP	0.926	.	.	.

Table AT1.5 CV for Mindways Trochanteric vBMD= 0.87%

patient ID	vBMD Troch	mean	SD	Coefficient of Variation %
PP	0.765	0.7742	0.00676018	0.87318232
PP	0.769	.	.	.
PP	0.78	.	.	.
PP	0.779	.	.	.
PP	0.778	.	.	.

Table AT1.6 CV for Mindways Cortical Thickness (cm)= 1.87%

patient ID	Cortical Thickness, cm	mean	SD	Coefficient of Variation %
PP	0.274	0.27	0.00504975	1.87027869
PP	0.274	.	.	.
PP	0.265	.	.	.
PP	0.273	.	.	.
PP	0.264	.	.	.

Functional Scales

Scandinavian Stroke Scale (Scandinavian Stroke Study Group, 1985)

1. Arm motor power affected side only	6-	Raises arm with normal strength
	5-	Raises arm with reduced strength
	4-	Raises arm with flexion in elbow
	2-	Can move, but not against gravity
	0-	Paralysis
2. Hand motor power affected side only	6-	Normal strength
	4-	Reduced strength in full range
	2-	Some movement, fingertips do not reach palm
	0-	Paralysis
3. Leg motor power affected side only	6-	Normal strength
	5-	Raises straight leg with reduced strength
	4-	Raises leg with flexion of knee
	2-	Can move, but not against gravity
	0-	Paralysis
4. Orientation	6-	Correct for time, place and person
	4-	Two of these
	2-	One of these
	0-	Completely disorientated
5. Speech	10-	No Aphasia
	6-	Limited vocabulary or incoherent speech
	3-	More than yes/no, but no longer sentences
	0-	Only yes/no or less
6. Facial palsy	2-	None/dubious
	0-	Palsy present
7. Gait	12-	Walks 5 metres without aids
	9-	Walks with aids
	6-	Walks with the help of another person
	3-	Sits without support
	0-	Bedridden/wheelchair

Long Term Score /48

The Barthel Index (Mahoney and Barthel, 1965)

- | | | |
|----------------------|-----|--|
| 1. Feeding | 0- | unable |
| | 5- | needs help cutting, spreading butter, etc, or requires modified diet |
| | 10- | independent |
| 2. Bathing | 0- | dependent |
| | 5- | independent (or in shower) |
| 3. Grooming | 0- | needs help with personal care |
| | 5- | independent face, teeth, shaving (implements provided) |
| 4. Dressing | 0- | dependent |
| | 5- | needs help but can do half unaided |
| | 10- | independent (including buttons, zips, laces etc) |
| 5. Bowels | 0- | incontinent (or needs to be given enemas) |
| | 5- | occasional accident |
| | 10- | continent |
| 6. Bladder | 0- | incontinent, or catheterised and unable to manage alone |
| | 5- | occasional accident |
| | 10- | continent |
| 7. Toilet Use | 0- | dependent |
| | 5- | needs some help but can do something alone |
| | 10- | independent (on and off, dressing, wiping) |
| 8. Transfers | 0- | unable, no sitting balance |
| | 5- | major help (one or two people, physical) can sit |
| | 10- | minor help (verbal or physical) |
| | 15- | independent |
| 9. Mobility | 0- | immobile or <50 yards |
| | 5- | wheelchair independent, including corners >50 yards |
| | 10- | walks with the help of one person (verbal or physical) >50 yards |
| | 15- | independent (but may use any aid; for example, stick) >50 yards |
| 10. Stairs | 0- | unable |
| | 5- | needs help (verbal, physical, carrying aid) |
| | 10- | independent |

Total (0-100)

Functional Ambulatory Category (Holden *et al.*, 1984)

0= Nonfunctional Ambulation	Patient cannot ambulate, ambulates in parallel bars only, or requires supervision or physical assistance from more than one person to ambulate safely outside of parallel bars.
1= Ambulator-Dependent for Physical Assistance-Level II	Patient requires manual contacts of no more than one person during ambulation on level surfaces to prevent falling. Manual contacts are continuous and necessary to support body weight as well as maintain balance and/or assist co-ordination.
2= Ambulator-Dependent for Physical Assistance-Level I	Patient requires manual contact of no more than one person during ambulation on level surfaces to prevent falling. Manual contact consists of continuous or intermittent light touch to assist balance or co-ordination.
3= Ambulator-Dependent for Supervision	Patient can physically ambulate on level surfaces without manual contact of another person but for safety requires standby guarding of no more than one person because of poor judgement, questionable cardiac status, or the need for verbal cueing to complete the task.
4= Ambulator-Independent Level Surfaces Only	Patient can ambulate independently on level surfaces but requires supervision or physical assistance to negotiate any of the following: stairs, inclines, or non-level surfaces.
5= Ambulator-Independent	Patient can ambulate independently on non-level and level surfaces, stairs and inclines.

Zoledronate Side Effects

This was a per protocol analysis for side effects, including patients who did not complete the study. The significance of any side effects were determined as follows. Let the proportion of patients with the side effect in zoledronate group = p_1 . Let the proportion of patients with the side effect in placebo group = p_2 . Zoledronate group $n=15$, placebo group $n=16$ (per protocol). To express the difference in proportions:

$$\text{Difference in proportions} = (p_1 - p_2)$$

To calculate the standard error of the difference in proportions:

$$SE = \sqrt{\frac{(1-p_1)*p_1}{15} + \frac{(1-p_2)*p_2}{16}}$$

$$\text{Upper 95\% CI for the difference} = (p_1 - p_2) + (1.96 * SE)$$

$$\text{Lower 95\% CI for the difference} = (p_1 - p_2) - (1.96 * SE)$$

$$\text{Z statistic} = (p_1 - p_2) / SE$$

The z statistic was used to calculate a p value for the difference (2 sided test)[‡] as shown in table AT 1.7.

Table AT1.7 Side effects with zoledronate and placebo infusions

Side Effect within 10 days	<i>n</i> , Zoledronate 4 mg Total=15	<i>n</i> , Placebo Total=16	<i>p</i> Value [‡]
Calcium <2.1 mmol/l	7	0	0.0003
Phosphate <0.8 mmol/l	10	0	<0.0001
Magnesium <0.7 mmol/l	2	4	0.40
Creatinine ↑ >40 umol/l	1	0	0.30
ALT >50 mmol/l	0	3	0.06
Nausea and Vomiting	2	1	0.51
Pyrexia	4	1	0.11
Headache	1	0	0.30
Myalgia	1	0	0.30
Arthralgia	1	0	0.30
Malaise	3	1	0.25
Fatigue	2	0	0.13
Anorexia	1	0	0.30

Appendix 2: Histomorphometry

Staining of Methylmethacrylate Sections

Embedding and staining of methylmethacrylate (MMA) sections for histomorphometry was performed by Alan Lyon, using established methods.

Toluidine Blue Stain (for Methacrylate Sections)

Demonstrating osteoid as light blue/green and other tissue components blue.

Toluidine Blue powder (Sigma-Aldrich chemicals)

Working solution: 1% toluidine blue in deionised water buffered to pH 7.0 (1 ml to 50 ml buffer)

1. Place MMA sections in the working solution for 18-24 hours.
2. Rinse sections in deionised water very briefly
3. Rinse sections in 95% alcohol
4. Wash sections in 100% alcohol
5. Rinse sections in fresh 100% alcohol
6. Rinse in two changes of xylene and coverslip with DPX.

Von Kossa Stain/ Van Gieson Counter Stain (for Methacrylate Sections)

Demonstrating sites of calcium deposition black and osteoid red.

Reagent A: Silver Nitrate 2.5% aqueous, working solution 1.25%

Reagent B: 1% acid fuchsin

Reagent C: picric acid saturated aqueous solution, working solution 8 ml picric acid and 2 ml acid fuchsin

Reagent D: 2.5% aqueous sodium thiosulphate

1. Place MMA sections in filtered working solution of A for 2 hours in good light.
2. Wash sections in deionised water
3. Treat sections with D for 1 minute
4. Stain sections in filtered working solution of C for 15 minutes.
5. Follow stages 2-6 above

Tartrate Resistant Acid Phosphatase staining (for Frozen Sections)

Staining sites of acid phosphatase activity red. (prepare B and C before A).

Reagent A: 18.75 mg of naphthol AS B1 phosphate and 93.75 mg of sodium tartrate are added to 37 ml of 0.1M tri sodium citrate just before use (buffered to pH 4.5 with 2.1% citric acid)

Reagent B: 0.25g of sodium fluoride in 250 ml distilled water

Reagent C: To 250 ml distilled water add 3.4g sodium acetate and buffer to pH 6.2 with acetic acid. Working solution: 18.75 mg of fast garnet to 35 ml of this buffer

1. Cut fresh frozen section on to tape.
2. Place in a bath of pre-warmed reagent A at 37° C
3. Wash rapidly in reagent B
4. Place in reagent C at RT for 2-4 minutes
5. Wash in distilled water twice and cover slip with an aqueous mounting media.

Formulae, Referents and Magnification

The stereological formulae applied in these calculations assume that sampling is unbiased and random and that the structure is evenly dispersed and randomly orientated in space (i.e. isotropic Parfitt, 1983; Parfitt *et al.*, 1987). Measures of mean apparent widths were transformed into 3D mean apparent thicknesses by multiplying by $\pi/4$ (Kragstrup *et al.*, 1982). The following tables give the 2 dimensional measurements taken along with the formulae and referents used to calculate 3 dimensional results (table AT2.1 and AT2.2). In addition, the units of measurement are given.

Light microscopy was performed using a Polyvar light microscope (static parameters) with one set of oculars (x 10), three objectives (x 4, x 10, x 25) and a magnification changer turret (x 0.8, x 1, x 1.25). Fluorescent light microscopy was performed using a Nikon microscope with one set of oculars (x 10), two objectives (x 10, x 20) and a fixed magnification turret (x 1.25). Eroded perimeter was also assessed on the Nikon light microscope. The magnification use to assess the histomorphometric parameters is shown in table AT2.3. The software used for histomorphometry was calibrated for the different magnifications with a stage micrometer and eyepiece micrometer graticule.

Table AT2.1 Primary parameters

Index	<i>Formulae from actual 2D measurements</i>	3D terminology/ referent	3D Units (mag.)
Bone Volume Referent	Medullary Bone Ar- Hole Ar	BV^a (equivalent to B.Ar)	um ³
Bone Surface Referent	Medullary Bone Pm+ Hole Pm	BS^b (equivalent to B.Pm)	um
Tissue Volume Referent	Total number of fields examined *field Ar	TV^c (equivalent to T.Ar)	um ³
Osteoid Surface	$(\Sigma \text{Osteoid Pm} \div (\text{BS}^a) * 100$	OS/BS^d	%
Osteoid Thickness	$(\Sigma \text{Osteoid Wi} \div \text{total number of Osteoid Widths measured}) * \pi/4$	O.Th^e	um
Osteoid Volume (TV referent)	$((\Sigma \text{Osteoid Pm} * \Sigma \text{Osteoid Wi}) \div \text{TV}^b) * 100$	OV/TV^f	%
Mineralised Volume	$(\text{BV}^a \div \text{TV}^c) * 100$	Md.V/TV^g	%
Bone Volume	$(\text{Md.V/TV}^g) + (\text{OV/TV}^f)$	BV/TV^h	%
Osteoid Volume (BV referent)	$((\text{OV/TV}^f) \div (\text{BV}^a)) * 100$	OV/BVⁱ	%
Wall Thickness	$(\Sigma \text{Wall Wi} \div \text{total number of Wall Widths measured}) * \pi/4$	W.Th^j	um

Key: Σ = sum from all fields and all sections examined, Pm=perimeter, Ar=area, Wi=width, Th=Thickness
Hole refers to holes in trabeculae.

Table AT2.3 Dynamic parameters

Index	<i>Formulae</i>	3D terminology/ referent	3D Units
Single labelled surface	$(\Sigma \text{ single labelled perimeter} \div \text{BS}^b) * 100$	sL.S/BS^k	%
Double labelled surface	$(\Sigma \text{ double labelled perimeter} \div \text{BS}^b) * 100$	dL.S/BS^l	%
Mineralising Surface (BS referent)	$\text{dL.S/BS}^l + 0.5 * \text{sL.S/BS}^k$	MS/BS^m	%
Label Thickness	$(\Sigma \text{ label widths} \div \text{total number of label widths measured}) * \pi/4$	L.Th^o	um
Mineral Apposition Rate	L.Th^o \div Labelling period in days	MAR^p	um/d
Bone Formation Rate (BS referent)	$(\text{MAR}^p * (\text{MS/BS}^m)) \div 100$	BFR/BS^r	um ³ /um ² /d

Key: Σ = sum from all fields and all sections examined, d=day, yr= year, Pm=perimeter, Ar=area, Wi=width, Th=Thickness.

Table. AT2.3 Magnification used for histomorphometry

Magnification	<i>Actual 2D Measurements</i>	Stain Used
x 80	Bone Pm, Osteoid Pm (drawing)	Von Kossa
x 125	Wall Wi, Labelled Pm	Toluidine blue
x 200	Osteoid Wi, Osteoid Pm (identification)	Von Kossa
x 250	Eroded Pm, Label Wi	Toluidine blue

Coefficients of Variation for Histomorphometry (Intra and Inter-Observer)

Intra and Inter observer variation

To calculate the individual short term precision of the measurements, five repeated measurements were made on one day. Each measurement was taken from the same section. Short term precision is expressed as a coefficient of variation (CV). There is often inter-observer variation in histomorphometric analysis of bone (Compston *et al.*, 1986). Coefficients of variation were therefore calculated by comparing the results obtained when both observers (KESP and SV) recorded the same parameter from the same sections at least twice (table AT2.4). These values are comparable with published values (Compston *et al.*, 1986).

Table AT2.4 Intra and Inter observer variation (short term precision) expressed as a CV for primary histomorphometric variables

<i>Measurement</i>	<i>Intra observer CV (%)</i>	<i>Inter observer CV (%)</i>
BV/TV	1.94	9.07
OS/BS	4.16	11.31
O.Th	2.39	8.75
W.Th	3.08	8.23
Ct.Th	0.43	-

Intra observer variation

Five repeated measurements were made on one day from the same test section. Coefficient of variation was calculated as:

$$CV(\%) = \frac{SD}{Mean} * 100$$

BV/TV %

Field Cnt	Field Area	Bone area	Bone Pm	Bn OS Le	Cn B Ar/T. Area	Mean	SD	Coefficient of Variation %
25	1572332.13	6604133	113977.2	1957.33	0.16800859	0.168003	0.0032563	1.938243
25	1572332.13	6528881	126170.5	2041.2	0.1660942	.	.	.
25	1572332.13	6500092	113125	1941.38	0.16536181	.	.	.
25	1572332.13	6822012	114121.9	1866.76	0.17355142	.	.	.
25	1572332.13	6564393	121797.7	1896.22	0.16699761	.	.	.

OS/BS %

Field Cnt	Field Area	Bone area	Bone Pm	Bn OS Le	O.Pm	Mean	SD	Coefficient of Variation %
25	1572332.13	6604133	113977.2	1957.33	0.017173	0.016488	0.000686	4.158858
25	1572332.13	6528881	126170.5	2041.2	0.016178	.	.	.
25	1572332.13	6500092	113125	1941.38	0.017161	.	.	.
25	1572332.13	6822012	114121.9	1866.76	0.016358	.	.	.
25	1572332.13	6564393	121797.7	1896.22	0.015569	.	.	.

OWi (um)

N.W. meas	Sum Width	O.Wi	Mean	SD	Coefficient of Variation %
44	242.07	5.50159091	5.406091	0.128997	2.38614185
44	230.7	5.24318182	.	.	.
44	232.86	5.29227273	.	.	.
44	243.07	5.52431818	.	.	.
44	240.64	5.46909091	.	.	.

WWi (um)

N.W meas	Sum	Wall wi	mean	sd	Coefficient of Variation %
144	5417.154	37.619125	38.11806	1.1747	3.08174131
144	5580.272	38.7518889	.	.	.
144	5223.616	36.2751111	.	.	.
144	5623.922	39.0550139	.	.	.
144	5600.041	38.8891736	.	.	.

Cortical Width (um)

N.Widths	Sum	Cortwi	mean	SD	Coefficient of Variation %
12	11389.902	949.1585	948.9132	4.105	0.43260016
12	11411.983	950.998583	.	.	.
12	11442.403	953.533583	.	.	.
12	11380.736	948.394667	.	.	.
12	11309.767	942.480583	.	.	.

Inter-Observer Variation for Primary Parameters

Both observers made three measurements for each parameter after examining 2-3 sections from the same test biopsy. For paired measurements \mathbf{x}_k (KESP) and \mathbf{x}_s (SV), the mean and variance of \mathbf{x}_k and \mathbf{x}_s were calculated. The overall mean for the mean measurements was calculated (grand mean). The overall variance was calculated from the mean of the variances. The coefficient of variation was calculated by:

$$\frac{\sqrt{\text{variance overall}}}{\text{grand mean}} * 100$$

BV/TV %

KP	SV	mean	variance	cv within	variance overall	grand mean	cv overall %
15.3165	13.4647	14.3906	1.71458162	9.0991365	1.96228194	15.4485667	9.06760331
16.55	14.5289	15.53945	2.04242261	9.19680887	1.96228194	15.4485667	9.06760331
17.4476	15.3837	16.41565	2.12984161	8.89028266	1.96228194	15.4485667	9.06760331

OS/BS %

KP	SV	mean	variance	cv within	variance overall	grand mean	cv overall %
9.15	7.19	8.17	1.9208	16.9636388	1.036025	9.0025	11.3063386
10.11	9.56	9.835	0.15125	3.9543338	1.036025	9.0025	11.3063386

OTh (um)

KP	SV	mean	variance	cv within	variance overall	grand mean	cv overall %
4.074	5.015	4.5445	0.4427405	14.6415993	0.19990674	5.1077	8.75363305
6.3692	5.823	6.0961	0.14916722	6.33555427	0.19990674	5.1077	8.75363305
4.745	4.62	4.6825	0.0078125	1.88763156	0.19990674	5.1077	8.75363305

WWi (um)

KP	SV	mean	variance	cv within	variance overall	grand mean	cv overall %
41.15	36.74	38.945	9.72405	8.00703789	10.766925	39.8825	8.22741625
43.25	38.39	40.82	11.8098	8.41876275	10.766925	39.8825	8.22741625

Appendix 3. Immunostaining Methods

The following are new methods developed by the author, with expert technical assistance from Alan Lyon. Frozen sections were taken using a Brights Cryostat, Huntingdon, UK.

New Method for Sectioning and Immunostaining Undecalcified Bone

1. Cutting cryostat sections onto Ultraclear tape. First cut a strip of tape a little wider than the bone sample to be cut. Fold the tape back on itself to a third of its length, creating a non-adhesive end to handle. Cut down the bone block (PVA mounted, snap frozen) until the correct area is being cut at 8-10 μm thickness. Holding the non-adhesive part downward, apply the adhesive part to the face of the block and press firmly with a roller. Hold the non-adhesive part of the tape with forceps and slowly take a section using the manual cryostat lever. The section should be of high quality
2. Let the cut section air-dry at room temperature (RT) for 5 minutes and then place in 100 ml 0.3% hydrogen peroxide in methanol for 30 minutes (to block endogenous peroxide).
3. Wash in phosphate buffered saline (PBS) 2x5 minutes (refresh solution in between)
4. Block in Normal Serum (Vectorstain Elite Universal Kit, code PK-6200; Vector Laboratories, CA, USA) for 30 minutes at RT
5. Incubate in 1:1200 mouse monoclonal anti-human sclerostin antibodies (IgG clone H7, 2.21 mg/ml in PBS) overnight at 4° C in a humid chamber. For control sections use non-immune mouse IgG (Vector Laboratories, code 1-2000) instead of primary antibody. The primary/non-immune serum should be pipetted onto labelled regions of a large glass slide. Then the taped bone section can be placed face down on the solution. Use of a hydrophobic pen (e.g. ImmEdgeTM, Vector) prevents spillage of the solution. This method prevents drying of the section
6. Wash in PBS 3x2 minutes (refresh solution in between)
7. Incubate in biotinylated antibody for 30 minutes at RT
8. Wash in PBS 3x2 minutes (as above)
9. Incubate in Avidin-Biotin Complex (ABC) reagent for 30 minutes at RT
10. Wash in PBS 3x2 minutes (as above)

11. Visualize immunoperoxidase using diaminobenzidine (DAB) substrate (Vector; SK-4100) for 1 minute
12. Wash in deionised water for 5 minutes
13. Counterstain with 0.2 mg/100 ml toluidine blue (see above) 2 minutes
14. Pass through graded alcohols (95%, 100%)
15. Pass through two fresh xylene baths
16. Place a drop of DPX (Fisher Chemicals) on a fresh slide and a drop on a coverslip. With forceps, lay the taped section face up on the DPX on the slide, then apply the coverslip.

New Method for Toluidine Blue Counterstaining

Toluidine Blue powder (Sigma-Aldrich chemicals)

Reagent A: 28.3 g sodium hydrogen orthophosphate in 1 litre distilled water

Reagent B: 21 g citric acid in 1 litre distilled water

Buffer C: Add 25 ml of solution A to 75 ml of solution B pH 4.2 (use sodium hydroxide to buffer to exact pH if necessary)

Working solution is 20 mg of toluidine blue to 100 ml Buffer C, diluted 1:100 with deionised water (0.2 mg/100 ml Toluidine blue).

Method:

1. Stain sections in working solution (filtered before use) for 2 mins and wash sections in distilled water for 5-10 minutes
2. Dehydrate and mount

Appendix 4. Rapid long-term bone loss following stroke in a man with osteoporosis and atherosclerosis.

This abstract is from a case report published in *Osteoporosis International* (Poole, K. E. S., Warburton, E. A., and Reeve, J. (2005). Rapid long-term bone loss following stroke in a man with osteoporosis and atherosclerosis. *Osteoporosis International*, 16(3), 302-305). The entire case report is included as a PDF file after the reference section.

Abstract

Bone loss in humans has been reported where there is reduced mechanical loading such as in spaceflight, spinal cord injury and stroke. Whether osteoporotic patients are susceptible to further bone loss in states of under loading such as hemiparesis is unknown. Here we report the case of a 64 year old man with established idiopathic osteoporosis and atherosclerosis who presented with a right middle cerebral artery territory stroke. Annual bone mineral density measurements were made at the left hip and spine before and after left hemiparesis. The left total hip T score was -3.2 before the stroke. Following stroke, there was rapid and sustained bone loss with a reduction in bone mineral density (BMD) of 21.6% over 3 years despite oral bisphosphonate therapy. There was also an unexpected decline in vertebral bone mineral density after the stroke. This is the first report of the accelerated effect of hemiplegia on bone loss in an already osteoporotic skeleton.

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