Calcium Requirements for Bone growth and Development in Gambian Children.

Thesis

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Calcium Requirements for Bone Growth and Development in Gambian Children

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Preface

This dissertation is the result of my own work and contains nothing which is the outcome of work done in collaboration. No part of this dissertation has been submitted for a degree or diploma or other qualification at any other University. Some preliminary results of this work have been presented at international conferences, and more recently, part of this work has been submitted for publication in "The American Journal of Clinical Nutrition" and has been accepted.

Bakary Dibba

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To my family and the children of Keneba, The Gambia

Abstract

Previous studies have demonstrated that rural Gambian children have poor growth, delayed puberty and low bone mineral status. Their calcium intake is low (300 - 400 mg/d). This study examined the benefit of an increase in calcium intake on growth and bone mineral development in Gambian children. 160 subjects, aged 8 - 12 years old (80 M, 80 F) were recruited into a randomised, double-blind, placebo-controlled study of calcium supplementation.

Bone mineral status was evaluated using single photon absorptiometry of the radius, and ultrasound measurements at the calcaneus. Anthropometry was performed and pubertal status assessed. Dietary intake was measured, and information on subject characteristics, physical activity and lifestyle factors were obtained. Fasting blood and 24h urine were collected for measurement of biochemical indices of calcium and bone metabolism. Data was collected at baseline, after 12 months of supplementation (714 mg Ca /d) and 12 months after withdrawal of the supplement.

Analysis of baseline data showed there were no differences in subject characteristics and bone variables between boys and girls, except that girls had significantly greater triceps skinfold thickness ($p \leq 0.0001$). There were no significant differences between the supplemented and placebo groups in subject characteristics, anthropometry or bone variables at baseline. The Gambian children were smaller by ≥ 1 SD for their age compared with British reference children and the majority were prepubertal.

At outcome, the supplemented group had significantly higher BMC, BMD and size-adjusted BMC at the mid-shaft and distal radius compared with the placebo group. No differences were observed in response between boys and girls or at different ages or pubertal status. There was no significant effect of the calcium supplement on weight, height or bone width. At follow-up, the supplemented group still had significantly higher BMC, BMD and size-adjusted BMC at the mid-shaft radius and, although not significant, a similar trend was seen at the distal radius. The calcium supplemented group had significantly lower concentrations of plasma osteocalcin, parathyroid hormone, phosphate, lower urinary titratable acid and phosphate outputs, and had a greater urinary calcium output than the placebo group at outcome. At follow-up the only difference remaining was a lower PTH in the calcium supplemented group.

The results suggest that Gambian children on a low calcium diet may benefit from a higher calcium intake by lowering bone turnover rate. Further studies are needed to determine the long-term benefit of calcium supplement on bone mineral acquisition of Gambian children.

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Appendices

Glossary of abbreviations used in the thesis

AI	adequate intake
ALB	albumin
ANCOVA	analysis of covariance
APOE	apolipoprotein E
BA	bone area
BGP	bone-specific γ-carboxyglutamic acid protein (osteocalcin)
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BMM	bone mineral mass
BSAP	bone-specific alkaline phosphatase
BUA	broadband ultrasound attenuation
BW	bone width
CaBP	calcium binding protein
CHO	carbohydrate
COMA	Committee on the Medical Aspect of Food Policy and Nutrition
Creat	creatinine
D-Pyr	deoxypyridinoline
DXA	dual energy x-ray absorptiometry
ECF	extracellular fluid
EDE	effective dose equivalent
FAO	Food and Agricultural Organization
FNB	United States Food and Nutrition Board
HNR	Human Nutrition Research
HP	hydroxylysylpyridinoline
IRMA	immunoradiometric assay
LP	lysylpyridinoline
MRC	Medical Research Council
MUAC	mid-upper arm circumference
nBUA	normalised broadband ultrasound attenuation
NCHS	National Center for Health Statistics
NIH	National Institutes of Health
PBM	peak bone mass
PEM	protein energy malnutrition
PTH	parathyroid hormone
QCT	quantitative computer tomography
QUS	quantitative ultrasound
RDA	recommended daily allowance

RNI	reference nutrient intake
SPA	single photon absorptiometry
TAP	total alkaline phosphatase
TRAP	tartrate resistant acid phosphatase
VDR	vitamin D receptor
VOS	velocity of sound
WHO	World Health Organization

1 An overview

1.1. Situation in The Gambia

1.1.1 Calcium intake

Calcium intakes of both children and adults in the rural areas of The Gambia are extremely low and average 300-500 mg/d (Prentice *et al.*, 1993). This is about a third to a fourth of the British and American recommendations (National Academy of Science, 1989; Department of Health, 1992). The diet in rural Gambia is based on cereals, with dark green leafy vegetables, rice, fish and groundnuts as the major sources of calcium (Prentice & Bates, 1993). The low calcium intake is related in part to limited dairy produce in the Gambian diet, which is a rich source of calcium. Although, at present, it is not known how much of the calcium from the Gambian diet is absorbed, it is thought that a high proportion of the calcium intake may be poorly absorbed due to the high phytate and oxalate content of the diet (Allen, 1982, Hongo *et al.*, 1989, Pun *et al.*, 1991, Department of Health, 1992). The calcium intake is low throughout life since even in infancy the calcium intake from breast-milk is substantially lower than in Western countries (Laskey *et al.*, 1990).

Table.1.1 Reference calcium requirements for 8 - 10 year old children compared with measured calcium intake in rural areas of The Gambia

	Calcium intake	
	(mg/d)	(mmol/d)
World Health Organization (FAO/WHO, 1962; 1991)	700	17.5
USA, RDA (National Academy Science, 1989)	1200	30
USA, AI (National Academy Science,1997)	1300	32.5
UK, RNI (Department of Health COMA, 1992)	1000	25
Rural Gambia (Prentice *et al.*, 1990)	400	10

Conversion factor mg/d to mmol/d: calcium ÷ 40

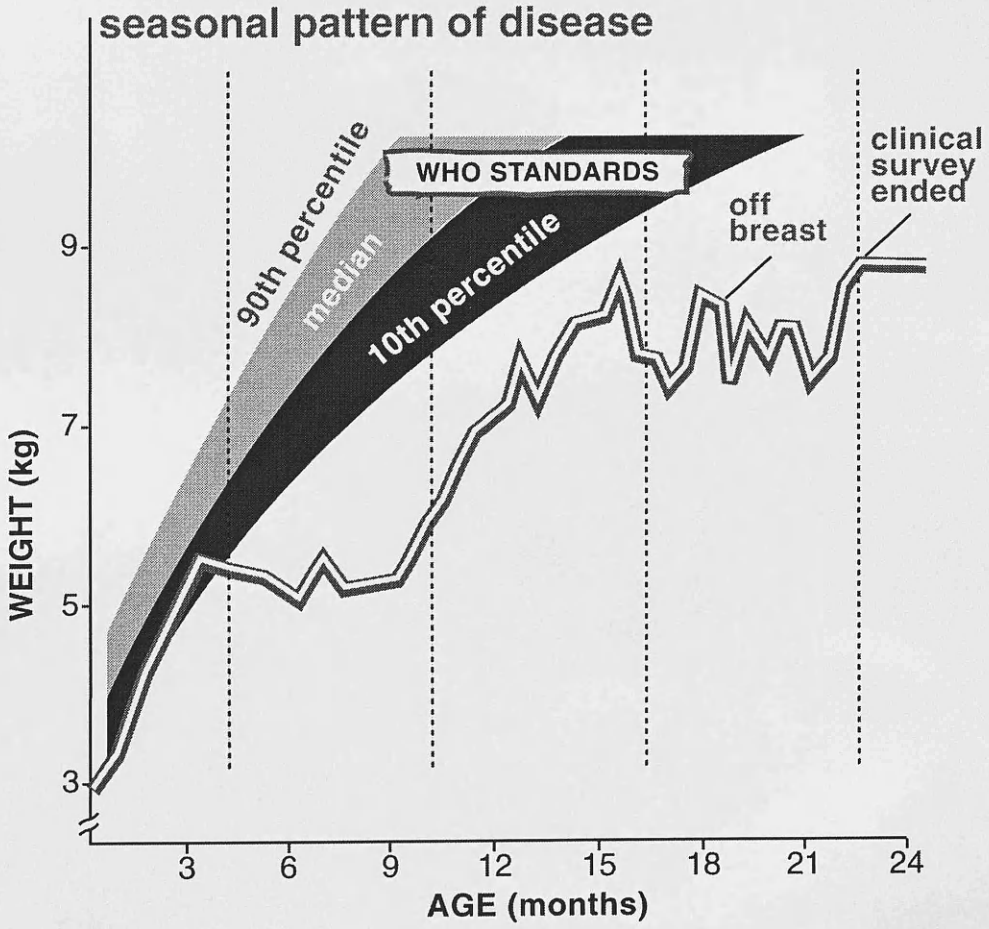
1.1.2 Poor growth and delayed puberty

The growth rates of Gambian children are poor, and the start and completion of puberty are delayed compared with British and American children (Lo *et al.*, 1990). The pattern resembles the growth of children in other developing countries (Waterlow, 1988; Beaton, 1993; Martorell *et al.*, 1994). Gambian children, although born somewhat smaller, grow, on average, at a similar rate to those in the West for the first 3 months of life at a time when they are exclusively breast-fed (Rowland *et al.*, 1977, Whitehead, 1979; Prentice *et al.*, 1990). Thereafter, marked growth faltering occurs so that before one year of age many children have low weight and height for their age. There is little evidence of subsequent accelerated catch-up in weight and height once a deficit has been created, so that by the time they reach later childhood, Gambian children are lighter and shorter than British children (Rowland *et al.*, 1977; Whitehead, 1979; Prentice *et al.*, 1990; Bates *et al.*, 1993, Lo *et al*, 1990). Poor nutrition and

infections are believed to be the main causes of growth retardation in Gambian children (Rowland *et al.*, 1977; Whitehead, 1979).

There is a seasonal pattern of growth and disease in rural Gambia. Growth is particularly poor in the rainy (wet) season and diseases like malaria and diarrhoea are also common in the wet season (Figure 1.1).

Fig. 1.1 Weight velocity chart of a typical rural Gambian child compared with National Center for Health Statistics reference for US children (NCHS).

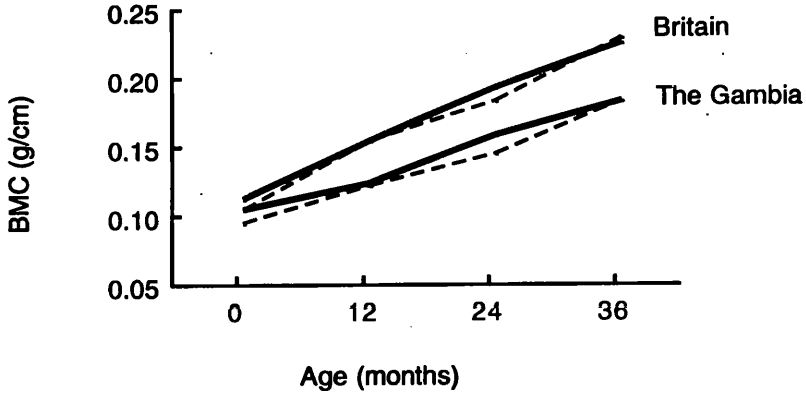


The data is from MRC Dunn Nutrition Unit's clinical surveys in rural Gambia from 1978 - 1980. Data set at each age group is the mean of all the measurements taken during that period. The child has been seen in a weekly infant clinic once a month for a period of two years. The pattern of the graph is typical of how the weight of children in rural Gambia behave throughout childhood depending on the season of the year. The fluctuation of the graph shows the period between weight faltering in the wet season and catch up in the dry season. Diseases such as diarrhoea, pneumonia and other infections are also prevalent in the wet season which also affect growth in addition to poor nutrition. The dotted lines represent seasonal boundaries.

1.1.3 Bone mineral accretion

Previous studies in Keneba, a rural village in The Gambia, have indicated that the bone mineral development of children is low compared with British and American children of the same age, both before and after size adjustment (Prentice *et al.*, 1990; Lo *et al.*, 1990). Differences in bone mineral content (BMC) at the mid-shaft radius between the British and Gambian children averaged 11% at birth and increase to 31% at 3 years (Prentice *et al.*, 1990) (see graph below). The differential was reduced after allowing for the smaller body size of the Gambian children due to their poor growth performance but remained considerable (12% comparing children of 15 kg body weight) (Prentice *et al.*, 1990). It is possible therefore that the low calcium intake of Gambian children could limit their bone mineral accretion rate.

Fig. 1.2



Bone mineral content (BMC) at mid-shaft radius of Gambian and British infants according to age and sex. Continuous line (–) represents boys and dotted line (....) represents girls. Source: Prentice et al., 1990.

1.2 Possible health implications of a low calcium intake

1.2.1 Calcium intake and clinical rickets

There is accumulated evidence to suggest that dietary calcium deficiency may exist unrecognised in countries where habitual calcium intake is low (Pettifor, 1991). A possible association between a low calcium diet and an increased incidence of rickets in young children was well documented more than two decades ago (Maltz *et al.*, 1970; Kooh *et al.*, 1977; Proesman *et al.*, 1988), and was also reported in a more recent study (Pettifor, 1991). These findings were supported by a study in South Africa in which the calcium intake of children with rickets was estimated at 125 mg/day compared with 337 mg/day in controls (Pettifor *et al.*, 1979; Pettifor, 1991). Bone accretion rate in children with rickets was enhanced following treatment with calcium (Marie *et al.*, 1982), suggesting calcium deficiency was the cause of rickets in these children. This hypothesis was further supported by the rapid return to normal of serum calcium concentrations in a group of South African children in a high risk area whose diet was supplemented with 500 mg calcium/day for 3 months (Pettifor *et al.*, 1981).

1.2.2 Calcium intake and growth

Calcium is a primary bone-forming mineral, and adequate amounts must be provided from the diet for bone mass development during growth. Calcium accretion rate during childhood is estimated to be around 200 mg/d and is higher during periods of rapid growth (Prentice & Bates, 1993). Deficiencies of both macro and micro-nutrients may contribute to poor linear growth in early life

(Tomkins *et al.*, 1986; Keller, 1988). Inadequate intake of bone-forming minerals such as calcium have been associated with poor linear growth in children (Prentice & Bates, 1993), and the diets of stunted children are thought to be deficient in calcium (Keller, 1988).

Linear growth retardation is a major problem in the world's poorest nations. Current estimates of the prevalence of stunting in under 5s in Africa, Americas and Asia by the World Health Organization averages around 40% (de Onis *et al.*, 1993; Rivera *et al.*, 1998) as defined by a height-for-age more than -2 SD with regards to the WHO/NCHS reference value (de Onis *et al.*, 1993). Although the actual causes of stunting remain controversial, insufficient dietary intake, such as an inadequate calcium intake, repeated infections and poor standards of living have all been implicated (Keller, 1988, Schurch & Scrimshaw, 1994).

The impact of calcium supplementation on linear growth has been little studied and there are conflicting results on the effect of calcium supplementation. Some supplementation studies using growth as the main outcome have demonstrated no effect of calcium alone or combined with phosphorus on the rate of growth (Pettifor et al., 1981; Aykroyd & Krishnan, 1938). On the other hand, studies of Indian children who consumed relatively low amounts of calcium had a significantly increased gain in height and weight over 3 - 5 months compared with controls (Aykroyd & Krishnan, 1938; Aykroyd & Krishnan, 1939). A recent study in Switzerland showed an association between calcium supplementation and height gain in a group of 7 year old children with low calcium intake (Bonjour *et al.*, 1997).

1.2.3 Calcium intake and bone mineral accretion

Skeletal growth requires an adequate supply of the building blocks of bone of which calcium is the most important mineral. The calcium accretion rate during peak growth can be as high as 400 mg/day (Pettifor, 1991; Kanis, 1994). Some suggest an adolescent requires an intake of at least 1,500 mg/day in order to retain such an amount of calcium (Matkovic & Heaney, 1992). A number of studies have indicated a beneficial effect of moderate to high calcium intake on bone mineral mass (Matkovic *et al*, 1990). Similarly, results from supplementation studies of children have documented a positive effect of a high calcium intake on bone mineral content (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lee *et al.*, 1994).

1.2.4 Calcium intake and peak bone mass

The importance of dietary calcium in the development of the human skeleton has recently received increased attention (Anderson, 1992). A number of retrospective studies have reported an association between a high calcium intake during childhood and peak bone mass (Fehily *et al.*, 1992; Hu *et al.*, 1993; Orwoll, 1991, Slemenda *et al.*, 1991; Metz *et al.*, 1993; Mazess & Barden, 1991; Paganini-Hill *et al.*, 1991). A dispute remains as to whether retrospective studies can account for all the confounding factors, such as the influence of socio-economic factors, physical activities and other lifestyle variables. There is increasing evidence to suggest that a high calcium intake during growth is needed to optimise peak bone mass and reduce risk of fractures later in life (Sandler *et al.*, 1985; Halioua & Anderson, 1989). Attainment of high peak bone

mass has been associated with reduced incidence of fragility fractures (Nordin, 1996, Adebajo *et al.*, 1991). Therefore prospective studies are needed to determine whether consuming high amounts of calcium during adolescence and early adult life will result in continuous, incremental gains in bone mass, sufficient to increase peak bone mass and reduce fracture incidence in later life.

1.3 Evidence of possible problems in The Gambia

1.3.1 Ethnic differences

There are ethnic variations in the amount of bone mass attained at skeletal maturity. Variation in bone mass between ethnic groups is likely to be due to both genetic and lifestyle differences between various ethnic groups. Early studies in Africa and in other black communities have suggested that osteoporosis and related bone fractures are rare in this ethnic group (Adebajo *et al.*, 1991; Solomon, 1979; Prentice *et al.*, 1991). The low incidence of fragility fracture in blacks compared with Caucasians has largely been attributed to blacks having a higher peak bone mass, and lower rates of bone loss in later life than Caucasians (Cooper., 1993; Matkovic *et al*, 1980; Cohn *et al.*, 1977; Ortiz *et al.*, 1992; Harris *et al.*, 1995). However, recent studies in The Gambia have shown that menopausal bone loss occurs in black African women at a similar rate to their European counterparts and that, unlike American blacks, differences in bone mass either in young adult life or in old age cannot account for the difference in fracture incidence (Prentice *et al*, 1991; Aspray *et al.*, 1996). This finding is in agreement with earlier work in South Africa that found no

evidence of higher bone mineral density in African blacks (Walker *et al.*, 1972; Solomon, 1979).

At present, clinical osteoporosis is rare in The Gambia despite the low habitual calcium intake of the population (Prentice *et al.*, 1991; Aspray *et al.*, 1996). The reason for the low incidence of osteoporotic fracture in African blacks is not fully understood, but it is perceived that this could be due to certain lifestyle factors, such as physical activity and muscle strength, rather than a high skeletal mass. Migration of African blacks from rural to urban areas with a more sedentary lifestyle, in search of better life, could lead to an increased incidence of osteoporosis. It is, therefore, important that determinants of bone mass are investigated with a view to optimising the bone mineral content of the skeleton.

The incidence of fragility fractures is likely to increase markedly throughout the world in the next few decades due to the increasing global population of elderly people. The hip fracture incidence is expected to treble to over six million cases a year by 2050 (Cooper, 1993).

Promoting the development of bone mass by growing children is regarded as a possible strategy in preventing osteoporotic fractures. It is therefore, important that the calcium intake of children is optimised to support skeletal growth during adolescence. An optimal calcium intake refers to the levels of consumption that are necessary for an individual (a) to maximise peak adult bone mass (b) to maintain adult bone mass, and (c) to minimise bone loss in later years. The dietary calcium intake of children in rural areas of The Gambia is similar to countries where low calcium intakes have been linked with bone rickets

and yet so far there is lack of evidence to suggest that rural Gambian children suffer from any abnormalities due to low calcium intake. At present, rickets is not a recognised health problem in The Gambia.

Although it is not clear whether the low bone mineral accretion rate of Gambian children reflects their low calcium intake, it is plausible that inadequate dietary calcium intake could slow the rate of bone development in Gambian children and result in poor height attainment and low peak bone mass. Whether a high calcium intake would improve growth and bone mineral development of Gambian children is a question which warrants further investigation and was the subject of the research investigation presented in this thesis.

1.4. Main aims

The main aims of the research were:

- to determine whether poor growth and low bone mineral status of Gambian children could be explained by their low calcium intake, by determining whether calcium supplementation has any effect on the growth and bone mineral mass development of prepubertal Gambian children,

- if calcium supplementation is associated with an effect on bone mineral accretion, to determine whether the increase in bone mineral status is sustained after supplement withdrawal,

- to measure biochemical markers of bone metabolism in blood and urine and to determine the effect of supplement on these indices in order to investigate mechanisms,

- to quantify the calcium intake of rural Gambian children by a direct weighing method,

- to examine the growth pattern of prepubertal Gambian boys and girls in the age range (8 - 12 years) including their passage into puberty,

- to evaluate the bone mineral mass development in pre- and peri- pubertal Gambian boys and girls,

- to compare bone and anthropometric measures of Gambian children with age-matched Cambridge children.

2.1.1 The skeleton

The skeleton is a complex structure, consisting of a variety of tissues and made up of about 200 separate bones (Dequeker, 1988). Bone tissue consists of two substances, the fibrous protein collagen, which is strong in tension, and the mineral apatite (calcium phosphate), which is strong in compression. Both are essential to skeletal function. The adult skeleton is composed of two types of bone, cortical bone which has a hard, compact structure and makes up to 75 - 80% of the skeleton, and trabecular (cancellous or spongy) bone which has a looser structure and comprises 20 - 25% of the skeleton (Kanis, 1994; Dequeker, 1988). Cortical bone is found predominantly in the shafts of long bones and forms the outer shell of trabecular regions while trabecular bone is found at the ends of long bones and in the axial skeleton, the spine and hip.

Cortical bone is made up of cylindrical structures (osteons) with concentric lamellae, which are oriented in the direction of the long axis of the bone (Dequeker, 1988). Trabecular bone is a three-dimensional lattice composed of plates and columns oriented in the direction of the compressive forces. These plates and columns are interconnected by thinner struts (trabeculae) in the non-load-bearing plane (Melsen & Mosekilde 1988), which are vital in providing a framework of maximum support. The structure of trabecular bone becomes disconnected with age, fails to reform, and often disappears, with marked effects on bone strength. However, the resultant effect on overall bone mass may not be substantial. Although trabecular bone accounts for a minority of the skeletal mass, it is metabolically more active and has a higher rate of turnover because its

surface to volume ratio is much higher than cortical bone (Kanis, 1994). Thus bone mineral is lost faster from trabecular bone during periods of bone loss. Trabecular bone is more prone to fracture and so the sites in the body at which it is found are thought to be more vulnerable, particularly in older people (Kanis, 1994; Melsen & Mosekilde, 1988). Proportions of the two types of bone vary quite considerably between different regions of the skeleton (Riggs *et al.*, 1981, Cummings *et al.*, 1985). The bone mineral contributes to the main function of the skeleton, the provision of a strong framework for the body and basis of all its movements. It also plays a biochemical role in the maintenance of calcium homeostasis (Lian & Gundberg, 1988; Ismail *et al.*, 1986).

2.1.2 Skeletal growth

The calcification of the skeleton of the human fetus begins as early as the fifth month of life and from then on the demand upon the maternal supply of bone-forming substances becomes considerable (Dequeker, 1988; Melsen & Mosekilde, 1988). Calcification starts in the clavicle and membranous bones of the skull, which follows rapidly in long bones and spine (Riggs *et al.*, 1981). In the normal full-term infant, the distal femoral and proximal tibial epiphyses are ossified. Calcification of teeth begins by about the seventh month of gestation or after delivery. The formation of healthy teeth is promoted by a diet adequate in protein, calcium, phosphate and vitamins especially vitamin C and D (Cummings *et al.*, 1985; Dequeker, 1988). Hormones such as thyroid hormone and the

active form of vitamin D (1,25 dihydroxy vitamin D) are also crucial in calcification of teeth.

Growth of bone in children takes place at the epiphyseal growth plate of long bones by a finely balanced cycle of cartilage growth, matrix formation and calcification of cartilage that acts as a platform for bone formation (Price *et al.*, 1994). This sequence of cellular events constitutes endochondral ossification. An increase in bone length is followed by an increase in bone diameter and this is achieved by new bone being laid down by the osteogenic layer of the periosteum (Price *et al.*, 1994).

The onset of the adolescent growth spurt is an important event in skeletal development. The period indicates the start of a prolonged acceleration of bone mineralisation in both sexes. During the adolescent growth spurt, the bone mineral content increases at a rate of ~ 8.5%/y (Mazess & Cameron, 1972a, Christenson *et al.*, 1975, Anderson & Rondano, 1996). Even when puberty ceases, adolescents continue to gain bone mass until peak bone mass is attained: for girls the figures are approximately 5% in the cortex of the metacarpals, 6% in the proximal radius, 15% in the distal radius, 9% in the spine (Bonjour *et at.*, 1991; Matkovic *et al.*, 1990, Anderson & Rondano, 1996; Taylor *et al.*, 1997b). This process is known as consolidation (Parsons *et al.*, 1996; British Nutrition Foundation, 1989; Prentice & Bates, 1993). Sex hormones such as oestrogen and testosterone are important in the initiation of the process of growth and bone mineralisation, and probably in the maintenance over many years of the accelerated bone mineralisation (Mazess, 1971; Anderson & Rondano, 1996). This view is supported by observations of Krabbe and associates, and

others which indicated that, in boys with delayed puberty, the maintenance of low levels of serum testosterone postpones the onset of the growth and mineralisation spurt (Krabbe *et al.*, 1979; Frisancho, 1981; Antoniazzi *et al.*, 1995).

The start of puberty and the acceleration in bone mineral accumulation are later in boys compared to that in girls (Bonjour *et al.*, 1991; Peacock, 1991). Some studies found a strong association between bone mineral density and height in children (Mazess & Cameron, 1972a; Glastre *et al.*, 1990) while others found no direct association (Bonjour *et al.*, 1991).

The process of bone modelling takes place from birth until the cessation of longitudinal bone growth (Matkovic, 1996). This is characterised by changes in the shape of the bones. Thereafter, the existing bone tissue within the skeleton is continuously being formed and resorbed with little change in the volume of bone during the remodelling process (Matkovic, 1996). From early life through young adulthood, up to about the 30 years of age, bone formation predominates, resulting in a continuous accumulation of bone mineral mass (Matkovic, 1996).

Peak bone mass is defined as the maximum amount of bone mineral mass achieved during normal growth (Matkovic, 1996; Weaver *et al.*, 1996). The exact age at which peak bone mass is reached varies between different studies. Some studies have shown that the maximum bone mineral mass is attained in the third decade in both sexes and at all skeletal sites (Johnston & Longcope, 1990; Ott, 1990) but others have shown that the attainment of peak bone mass in human occurs earlier (Theintz *et al.*, 1992; Matkovic *et al.*, 1990; Gilsanz *et al.*, 1988). The amount of peak bone mass attained is a major determinant of the risk of age-

related osteoporotic fracture (Hui *et al.*, 1989; Wasnich *et al.*, 1989; Matkovic, 1996). Thus identifying the underlying causes responsible for inadequate accumulation of bone tissue during modelling and skeletal consolidation is important in minimising the later risk of osteoporotic fracture.

2.2 Biology of bone

Three cells are central to bone biology, osteoclasts, osteoblasts and osteocytes, these cells are involved in bone modelling and remodelling.

2.2.1 Osteoclasts

Osteoclasts originate from cells of the monocyte lineage and are usually multinucleated. Osteoclasts resorb existing bone and are active early in the bone remodelling cycle (Parfitt, 1980; Kanis, 1994; Christenson, 1997; Slemenda *et al.*, 1997). The osteoclast attaches the outside edge of its membrane to the mineral matrix on the surface of bone thus forming a microenvironment termed as a sealing zone (Kanis, 1994; Christenson, 1997; Slemenda *et al*, 1997). It is this microenvironment that provides a bone-resorbing compartment at a low pH (Christenson, 1997; Slemenda *et al*, 1997). The function of this microenvironment is to erode bone (Christenson, 1997).

2.2.2 Osteoblasts

Osteoblasts are involved in bone formation (Kanis, 1994; Christenson, 1997). Following erosion of bone by the osteoclast, these cells lay down replacement bone matrix, called osteoid, at the site of bone metabolism (Kanis, 1994; Christenson, 1997). The osteoid become mineralised to form new bone

(Christenson, 1997; Slemenda *et al.*, 1997; Kanis, 1994). The newly formed matrix is then mineralised with hydroxyapatite giving the bone tensile strength (Christenson, 1997). Osteoblasts have one nucleus with an extensive network of rough endoplasmic reticulum, the organelle that is responsible for synthesis of bone matrix protein.

2.2.3 Osteocytes

Osteocytes are derived from osteoblasts (Christenson, 1997). These bone-lining cells covers and surround much of the surface of bones, particularly of the long bones forming a cellular network on bone surface. The calcified bone matrix communicates with the bone surface via the network of osteocytes. The bone extracellular fluid (ECF) is embedded within the network and is separated from the systemic ECF by the lining cells covering the bone surface. In lamellar bone, osteocytes are arranged parallel to the axis of the collagen fibres, and in woven bone, they are bigger and have a much more haphazard distribution. Although these cells are often regarded as metabolically inactive, they are thought to have a role in initiating the bone cycle (Bronner, 1994; Christenson, 1997).

2.3 Bone modelling, remodelling and resorption

2.3.1 Bone modelling

The growth process in bone that leads to the attainment of the mature skeleton is called modelling (Parfitt, 1980; Kanis, 1994). The growth of the human skeleton is thought to take three decades to reach to full adult size and

density (Trotter & Hixon, 1973; Matkovic *et al.*, 1990; Matkovic, 1991). The rate of bone modelling varies in different stages of development (Matkovic, 1991). The process is most active during childhood and adolescence, and enables long bones to increase in diameter, change shape and develop a marrow cavity (Parfitt, 1980; Kanis, 1994).

2.3.2 Bone remodelling in children

Living bone is constantly broken down and reformed as part of the natural process of repair and renewal, a process known as remodelling (Frost, 1969; Parfitt, 1984). In a normal healthy person, the process of bone formation and bone resorption is tightly coupled (Figure 2.1). The imbalance of skeletal formation, resorption, and remodelling during rapid growth results in an increased cortical porosity which has been associated with fracture of forearm (Parfitt, 1994, Kanis, 1994).

Although morphologically well established, the physiological mechanisms coupling bone remodelling and resorption are not fully understood. Bone remodelling is a dynamic process reflecting the balance between bone formation and resorption (Parfitt, 1980; Kanis, 1994). The remodelling cascade is a highly ordered sequence of events starting resorption of a discrete cavity on the surface of bone followed by recruitment of osteoblasts sometime after resorption, and with mineralisation of the newly-formed bone rapidly at first stage of the process but more slowly thereafter (Parfitt, 1980; Kanis, 1994; Prentice, 1997). The process is important for the maintenance and self-repair of skeletal tissue (Parfitt, 1980; Kanis, 1994).

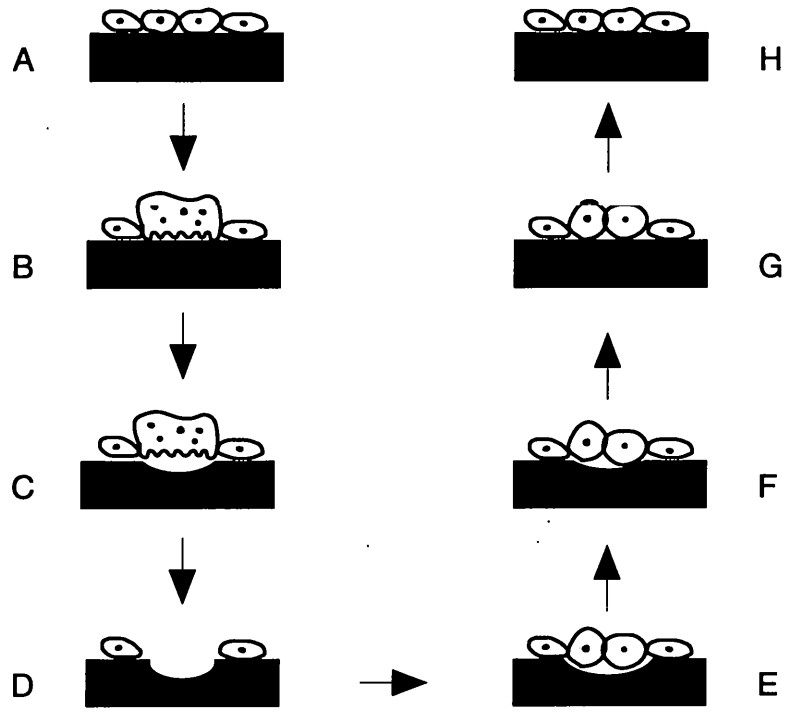


Figure 2.1 Diagram showing the coupling of resorption and formation in bone remodelling starting with a bone surface (A), activation of osteoclasts (B), resorption of old bone by osteoclasts (C), attraction and migration of osteoblasts to bone surface (D and E) and formation of new bone by osteoblasts (F and G). New bone with complete restoration (H). Source: Anderson (1996a).

Two cells are involved in bone remodelling, the osteoblast and the osteoclast. Osteoclasts, the cells responsible for bone resorption are attracted to the newly-formed bone remodelling site where they dissolve away a small, discrete portion of the surface. The resulting resorption cavity is then refilled with pre- mineralised bone tissue by the bone-forming cells, the osteoblasts (Figure 2.1) (Parfitt, 1980; Kanis, 1994; Slemenda *et al.*, 1997; Christenson, 1997). Osteoclasts resorb bone until the resorption cavity is approximately 100μm in diameter and 50μm deep, resorption is then stopped and osteoblasts, the bone-forming cells are attracted and recruited to the remodelling site and refilled the resorption cavity (Kanis, 1994; Slemenda *et al.*, 1997; Christenson, 1997). This results in laying down of bone matrix (osteoid) which become mineralised to form

new bone (Christenson, 1997; Slemenda *et al.*, 1997;. Kanis, 1994). The newly formed matrix is then mineralised with hydroxyapatite (Christenson, 1997). Finally, the remodelled area passes into quiescent phase to complete the bone cycle (Figure 2.1). The whole process is estimated to take 60 - 120 days to complete (Frost, 1969, Kanis, 1991; Kanis, 1994; Christenson, 1997; Slemenda *et al.*, 1997). In children, there is imbalance between bone remodelling and modelling in favour of increase bone mass until 30 -40 years of life, followed by a balanced period where total mass remains constant for a relatively few years before bone loss occurs in middle - and old-age (Christenson, 1997).

2.3.3 Bone remodelling in adults

Bone resorption increases with age, and a negative balance between formation and resorption has been reported in both cortical and trabecular bone after the age of 35- 40 years (Mosekilde *et al.*, 1987; Mosekilde, 1988; Garn *et al.*, 1967). This is thought to be due to a combination of cortical thinning, trabecular perforations and negative calcium balance with advancing age (Parfitt, 1984; Eriksen *et al.*, 1986).

In a normal adult, approximately 10 -15% of skeletal surfaces are involved in the process of remodelling at any one time (Parfitt, 1980; Kanis, 1991; Prentice, 1997). Thereafter, there is a temporal net decrease in bone mineral replacement at the bone remodelling unit and consequently affecting the whole body bone mineral (Parfitt, 1980; Kanis, 1991; Christenson, 1997). In healthy adults, the reversible calcium deficit is estimated at 14000 mg calcium, representing approximately 1.3% of the total calcium in the skeleton (Parfitt, 1980; Christenson, 1997). In men, this is thought to result in failure of osteoblasts

to completely fill the eroded areas to their original dimension, leaving a small deficit at each remodelling unit (Christenson, 1997; Slemenda *et al.*, 1997). In postmenopausal women, the decrease in bone density is thought to be osteoclast-mediated, because the cavity eroded by osteoclasts is deeper than the standard 50μm depth (Christenson, 1997; Slemenda *et al.*, 1997). The oestrogen deficiency that is common in postmenopausal women has been associated with an increase in the frequency of bone remodelling unit activation, leading to accelerate bone loss (Christenson, 1997; Kanis, 1994). In addition, inhibition of bone remodelling has been linked to a spontaneous fracture, which is thought to relate to the inability of the skeleton to repair fatigue damage (Frost, 1960; Parfitt, 1980; Kanis, 1994).

Bone turnover is reduced by high calcium intake due to decreased in osteoclast activity, resulting in lower resorption rate, a reduction in the remodelling space and increase in bone mineral content (Lee *et al.*, 1994; Lee *et al.*, 1995; Slemenda *et al.*, 1993; Prentice, 1997). Conversely, a decrease in calcium intake promotes osteoclast activation, increases bone turnover, which decreases bone mineral (Kanis, 1991; Kanis, 1994; Slemenda *et al.*, 1993).

The amount of calcium normally removed by bone resorption has been estimated to be around 250 mg daily from a total body calcium of 1 kg (Kanis, 1994). In adults in whom longitudinal growth has ceased, it is estimated that up to ninety-five percent or more of the skeletal turnover is accounted for by remodelling of bone, with <5% of turnover attributed to the modelling process (Parfitt, 1980; Kanis, 1994).

2.4 Markers of bone turnover

2.4.1. Biochemical markers of bone formation

2.4.1.1 Osteocalcin

The protein and other substances that are produced, modified, released or degraded by active osteoclasts and osteoblasts during the different phases of cell cycle serves as the biochemical markers for monitoring bone metabolism (Christenson, 1997; Slemenda *et al.*, 1997).

Osteocalcin is a bone matrix γ-carboxyglutamate protein (bone Gla protein, BGP) synthesised by proliferating osteoblasts (Lian & Gundberg, 1988; Karlsson *et al.*, 1992; Christenson, 1997). The osteocalcin produced is released into the circulation and is also incorporated into the bone matrix where it is the most abundant noncollagenous protein. Its synthesis is dependent on vitamin K (Kleerekoper, 1996; Christenson, 1997). Although the exact function of osteocalcin is still not fully understood, it is assumed that most of the newly synthesised protein is incorporated into the bone matrix where it functions by binding to calcium (Christenson, 1997; Slemenda *et al.*, 1997, Fottrell & Power, 1991). The serum concentration of osteocalcin reflects bone mineral metabolism and bone turnover and it is regarded as a specific marker of bone formation (Lian & Gundberg, 1988; Ismail *et al.*, 1986). Serum osteocalcin concentrations in children have been shown to correlate with growth velocity (Cole *et al.*, 1985; Delmas *et al.*, 1986; Johansen *et al.*, 1990; Kanzaki *et al.*, 1992). While most osteocalcin is released at time of bone formation, a small amount of osteocalcin is degraded, released into the circulation and excreted in the urine during bone

resorption (Kanis, 1994; Christenson, 1997). This has raised questions about whether osteocalcin should be considered a marker of bone metabolism or turnover rather than bone formation (Fottrell & Power, 1991; Kleerekoper, 1996), but currently is regarded as the most specific bone formation marker available. Its concentration in blood correlates well with bone mineral metabolism and bone turnover (Lian & Gundberg, 1988; Ismail *et al.*, 1986).

2.4.1.2 Alkaline phosphatase

In bone, alkaline phosphatase is involved in the breakdown of pyrophosphate (Christenson, 1997), a potent inhibitor of calcium phosphate deposition at the extracellular level. A number of isoenzymes of alkaline phosphatase exist, including the bone-specific alkaline phosphatase which is produced by the osteoblast and therefore plays an essential role in bone formation process. It is produced in high amounts during bone formation and its measurement in serum is a measure of bone formation (Canalis *et al.*, 1989, Christenson, 1997).

2.4.2 Biochemical markers of bone resorption

Resorption markers reflect osteoclast activity and/or collagen degradation. Type 1 collagen matrix comprises over 90% of the bone matrix (Christenson, 1997). Type 1 collagen has a triple helix structure formed from three chains, one of which has a high proportion of the amino acids proline and hydroxyproline. It is synthesised as a precursor with large extension peptides on both the carboxyterminal and amino terminal ends (Christenson, 1997). These proteins are cleaved during the secretion and fibril formation process. Crosslinking

occurs between lysine or hydroxylysine residues on nonhelical carboxyterminal or aminoterminal ends known as telopeptides, and the helical portions of an adjacent collagen (Fujimo *et al.*, 1995, Christenson, 1997). This process forms the pyridinoline and deoxypyridinoline crosslinks in the primary structure of type 1 collagen (Fujimo *et al.*, 1995).

2.4.2.1 Deoxypyridinoline (D-pyr)

Deoxypyridinoline (D-pyr) is one of the most specific markers of bone metabolism. D-pyr has only been found in a significant amounts in type I collagen of bone and is released into the circulation following bone resorption (Delmas, 1992; Seibel *et al.*, 1992). Deoxypyridinoline is a cross-linking amino acid of collagen which is excreted in urine both as free amino acid and in peptide-bound form (Black *et al.*, 1989; Uebelhart *et al.*, 1990). A good agreement between total and free form of deoxypyridinoline measured in urine has been reported (Aoshima *et al.*, 1998). However, free D-pyr has the added advantage over total D-pyr in that its concentration in the urine does not vary between day and night, whereas, urinary concentration of total and bound forms of deoxypyridinoline have been shown to have circadian variation (Aoshima *et al.*, 1998). Because of this, free D-pyr is thought to be a better marker than total or bound form D-pyr which are sensitive to time (Aoshima *et al.*, 1998). Measurement of deoxypyridinoline has been used to demonstrate the effect of antiresorptive agents in a number of studies (Seibel *et al.*, 1993; Delmas *et al.*, 1993). The serum concentration of D-pyr has been shown to correlate with collagen breakdown and bone resorption (Eriksen *et al.*, 1993). Several studies indicate that the excretion of D-pyr in urine as free and peptide-bound form, is

more specific and sensitive than resorption markers such as urinary hydroxyproline or calcium excretion (Eyre *et al.*, 1984; Black *et al.*, 1989; Uebelhart *et al.*, 1990; Robins *et al.*, 1991; Garnero *et al.*, 1996a). Deoxypyridinoline is neither absorbed from the diet nor is it metabolised by the liver (Colwell *et al.*, 1993; Fujimoto *et al.*, 1995).

Urinary concentrations of D-pyr in children are higher than those in adults (Fujimoto *et al.*, 1983; Beardsworth *et al.,* 1990). However, little is known about the age-related changes in urinary D-pyr or in bone resorption in children.

Hydroxyproline is the product of post-translational hydroxylation of proline residues of type 1 collagen. Hydroxyproline, pyridinoline, cross-linking amino acids of collagen, hydroxylysylpyridinoline, and lysylpyridinoline, are urinary metabolites that are excreted in urine both as free amino acids and in peptide-bound form and have been widely used in recent years as markers of bone resorption (Eriksen *et al.*, 1988; Christenson, 1997). Urinary hydroxyproline and pyridinoline are not specific for bone and are no longer regarded as markers of choice for bone resorption because these markers are degraded in other tissues as well as bone, and their concentrations in urine are affected by diet. Pyridinoline and deoxypyridinoline are formed via posttranslation of lysine and hydroxylysine respectively (Christenson, 1997).

2.4.2.2 Urinary calcium (Ca)

Urinary calcium concentration either in a 24-hour sample, fasting 2 hour samples, or in a spot or first morning specimen (corrected for creatinine) has been used for assessing skeletal loss in postmenopausal women (Kleerekoper,

1996; Seibel *et al.*, 1992; Seibel *et al.*, 1993). The problem with urinary calcium as a marker of bone loss is that the concentration can be substantially affected by diet, renal function and handling, and excess parathyroid and oestrogen production (Kleerekoper, 1996).

2.4.2.3 Tartrate Resistant Acid Phosphatase (TRAP)

The bone isoenzyme of tartrate resistant acid phosphatase is derived from osteoclasts where it is present in high concentrations. It is secreted by osteoclasts during resorption between bone matrix and membrane sealing zone (Canalis *et al.*, 1989; Christenson, 1997). The enzyme is released into the circulation via leakage after detachment of the sealing zone (Canalis *et al.*, 1989; Christenson, 1997). Though generally classified as a marker of bone resorption, TRAP is not bone specific and changes in serum concentration of TRAP also reflect the contribution of extraskeletal sources (Delmas *et al.*, 1993; Pedrazzoni *et al.*, 1995). Moreover, TRAP is unique among markers of bone resorption since the concentration in serum is thought to depend mainly on the number of osteoclasts, whereas the concentration of other markers depends on the degradation of bone collagen and reflects osteoclastic activity (Delmas, 1993; Pedrazzoni *et al.*, 1995). For this reason and the fact that serum TRAP decreases only slightly and transiently during bone resorption, the serum concentration of TRAP is not frequently used as a marker of bone turnover.

2.4.2.4 Other markers of bone resorption

N-telopeptides are released into the circulation following type 1 collagen degradation by osteoclasts. The fragments readily pass through the kidney into the urine (Goulding & Lim, 1983; Need *et al.*, 1991). N-telopeptides are regarded

by some but not by others as specific for bone tissue breakdown because other collagen-derived tissues are not actively metabolised by osteoclasts (Need *et al.*, 1991). Measurement of urinary concentration of N telopeptide is used for monitoring bone resorption (Need *et al.*, 1991; Ginty *et al.*, 1998).

C-telopeptides fragments are also released into circulation as a result of the osteoclast mediated degradation of type 1 collagen during the bone resorption process (Need *et al.*, 1991; Ginty *et al.*, 1998). For the same reason as N-telopeptide, osteoclasts are not active in the degradation of other type 1 collagen-containing tissues and C-telopeptide fragments are rarely used as resorption markers.

2.5 Growth

2.5.1 Children

Boys are slightly taller than girls during childhood. An exception is in early puberty when girls are very slightly taller than boys, and this sex difference begins in late fetal life (Eveleth & Tanner, 1990; Paynter & Parkin, 1991). There is substantial evidence to suggest that body weight in childhood tracks into adult life with overweight children becoming overweight adults (Kelly *et al.*, 1992; Zack *et al.*, 1979; Rolland-Cachera *et al.*, 1987; Mossberg, 1989; Must, 1992; Guo, 1994). Differences in weight gain and height gain between the sexes vary between different ethnic groups and are partly influenced by socio-economic status (Post & Kemper, 1993). The pubertal growth spurt occurs earlier in girls than boys (Amador *et al.*, 1992; Paynter & Parkin, 1991) but the sex difference in the onset of pubertal growth spurt varies between different ethnic groups and in different environments. By the time they complete adolescence, the height differential between males and females becomes considerable (Eveleth & Tanner, 1990; Amador *et al.*, 1996). The sex difference in longitudinal growth rate is due to the much longer growth spurt in boys compared with that in girls (Eveleth & Tanner, 1990; Paynter & Parkin, 1991). It is not known exactly what factors contribute to variation in growth but genetic factors, nutritional and socio-economic status have all been implicated (Eveleth & Tanner, 1990; Paynter & Parkin, 1991).

2.5.2 Adolescence

Adolescence refers to physical, psychological and behavioural changes occurring around the later stages of childhood. The onset of growth acceleration, the so-called "takeoff" varies significantly between individuals and is also sex-dependent. On average, age at takeoff is 2 years earlier in girls than boys (Abbassi, 1998). The growth spurt during adolescence which varies between sexes, contributes about 15% to adult height, 50% to adult weight, and approximately 40% to the total body bone mineral content (Abbassi, 1998). In boys, adolescent growth spurt is thought to account for a gain in height of 20 cm, with peak velocity of height averaging 10 cm per year (Tanner, 1973). The gain in weight is estimated around 20 kg (Tanner, 1973). In boys, the adolescent growth spurt is thought to account for 17% to 18% of the final adult height (Abbassi, 1998). In girls, the growth spurt is somewhat smaller in magnitude and the peak height velocity averages about 8 cm per year (Tanner, 1973, Abbassi, 1998), and contributes to 17% of the final height (Abbassi, 1998). There is some indication that adult stature may be influenced by age at peak height velocity (PHV) and age at takeoff (Qin *et al.*, 1996). Qin and associates found a negative correlation between adult height and age at peak height velocity, suggesting that the older the age at PHV, the lower the adult height (Qin *et al.*, 1996). A similar association between age at takeoff and adult stature has been reported (Qin *et al.*, 1996). This study suggests that children who spurted earlier had a longer duration of adolescence, and a larger stature increment during adolescence than those who reached the growth spurt later. The observations of Qin and coworkers were based on a Japanese population where average growth potential may be different from that of Africans and those in the West, and it is now known that in

humans, physical growth changes over time are influenced both by genetic and environmental factors. Secondly, in contrast to the study of Qin et al, a recent review on the correlation between adult stature and age at peak height velocity found no significant association between adult stature and age at PHV (Bielicki & Hauspie, 1994). The physiological maturity in adolescence is best determined by radiological examination of the epiphyses, since the closure of the epiphyses is more rapid after the growth spurt (Paynter & Parkin, 1991). Racial differences in the rates of adolescent growth are highly plausible (Eveleth & Tanner, 1990, Paynter & Parkin, 1991).

There are some suggestions that a child's growth velocity during adolescence is pre-determined by his/her growth patterns during early life (Abbassi, 1998; Paynter & Parkin, 1991) but this is highly controversial (Satyanarayana *et al.*, 1981; Hussain *et al.*, 1985; Martorell *et al.*, 1990; Amador *et al.*, 1996). There are conflicting reports from studies examining the relationship between preschool height status to adolescent or adult height (Satyanarayana *et al.*, 1981; Hussain *et al.*, 1985; Martorell *et al.*, 1990, Martorell *et al* ., 1992; Amador *et al.*, 1996). A positive correlation between final height with height at age 5 years was found among Indian adolescents but the height increment from age 5 - 18 years was higher in those girls with a height-for-age less than -4 z-scores of the National Center for Health Statistics (NCHS) reference compared with those with a mean z-score of -2 (Satyanarayana *et al.*, 1981). This indicates that some degree of catch-up growth had occurred. In contrast, a cohort of Nigerian children who were followed from 5 years until 17 years showed that the growth spurt of previously stunted girls was delayed by

about 2 years compared with the less stunted (Hussain *et al.*, 1985). A similar observation was reported in Guatemalan children where length at three years of age was strongly correlated with height at 18 - 26 years (Martorell *et al.*, 1990, Martorell *et al* ., 1992). In a more recent study in West Africa, girls who were stunted at preschool age showed some evidence of catch-up growth between the ages of 5 and 17 years (Simondon *et al.*, 1998). From these studies, it seem clear that the impact of stunting at early age on the final height is only partly known and this is an area that merits further study.

A strong association between skeletal maturation, height, and weight has been found in some (Amador *et al.*, 1996; Amador *et al.*, 1992) but not by others (Johnston & Mack, 1980). Johnston & Mack reported an association between weight and height that was independent of the rate of skeletal maturation (Johnston & Mack, 1980).

Puberty is the time of the greatest sex differentiation after the early intrauterine months. The event is marked by increases in both total fat mass and lean body mass (Goulding *et al.*, 1996). Reproductive maturity is demonstrated by the appearance of secondary sex characteristics: breast development and onset of menstruation in girls; penis and testis development in boys, pubic and axial hair growth and voice change in both sexes (Tanner, 1973). There are changes in the reproductive organs, in body size and shape, in the relative proportions of muscle, fat and bone, and in a variety of physiological functions (Paynter & Parkin, 1991). It refers to the period at which the testes, prostate gland and seminal vesicles, or the uterus and vagina enlarge (Paynter & Parkin, 1991).

Testicular enlargement is usually the first sign of puberty in boys, accompanied by changes in the texture and colour of the skin of the scrotum. A little later the penis starts to enlarge and pubic hair appears (Paynter & Parkin, 1991). The first pubertal event for girls is generally the appearance of breast-stage 2, often called breast bud (Paynter & Parkin, 1991). This consists of an elevation of breast and papilla as a small mound, with slight enlargement of the areolar area. Girls who have seen their first period early had a greater growth velocity with a shorter growth period during adolescence (Paynter & Parkin, 1991; Dhuper *et al.*, 1990; Ito *et al.*, 1995). The pubertal growth spurt lasts approximately two years after menarche in American girls, with a maximum velocity at 12.5 years, and for about two years in American boys, with a peak velocity at almost 14 years (Underwood, 1991; Abbassi, 1998). During this two year-growth spurt, calcium accumulates in the skeleton of girls at a rate of approximately 200 mg/day, and in boys at a rates of roughly 400 mg/day (Abbassi, 1998; Underwood, 1991; Matkovic & Ilich, 1993). In the last thirty years, the mean age of menarche is one year earlier in Western Countries compared with the turn of the century (Underwood, 1991; Abbassi, 1998). The start and duration of pubertal growth differs between children in affluent societies and those in developing countries (Underwood, 1991, Lo *et al.*, 1990).

Evidence from epidemiological studies suggests that the timing of the onset of puberty can be influenced by the level of nutrient intake (Cameron, 1996), with well-nourished children progressing through puberty earlier than undernourished children (Frisch, 1972; Bhalla & Shrivastava, 1976; Cameron, 1996).

Positive correlations between a number of indices of body growth, such as weight, percent body fat and metabolic rate, and the timing of the onset of puberty have been reported in a number of studies (Frisch, 1972; Tanner, 1968; Chowdhury *et al.*, 1977; Frisch & McArthur, 1974; Zacharias *et al.*, 1976). In Western Countries, where reliable written records are available, there is evidence to suggest that the decrease in the age of the onset of puberty over the last 200 years may be related to improvements in socio-economic conditions and nutrition (Roche *et al.*, 1995; Freeman *et al.*, 1995; Rona & Chinn, 1995). The reported negative correlation between socio-economic status and the age of menarche tends to suggest that puberty occurs earlier in better-nourished individuals (Burrell *et al.*, 1961; Laska-Mierzejewska, 1970; Madhavan, 1965; Gopalan *et al.*, 1973). However, it is likely that poor health status and psychological stress, in addition to poor nutrition, contribute to a later onset of puberty in individuals of low socio-economic status. Severe undernutrition has been shown to retard the development of sexual organs and to delay the onset of sexual activity (Biederman *et al.*, 1986).

2.5.3 Difference between boys and girls

There is considerable individual variation in the time of onset and rate of adolescent changes in both boys and girls. On average, boys are of similar weight and height as girls at birth, but boys becomes significantly larger than girls at puberty (Kelly *et al.*, 1992; Paynter & Parkin, 1991). Once longitudinal growth is completed towards the end of adolescent years, men are significantly taller and heavier than women (Hamill *et al*, 1979; Roche & Himes, 1980). There are gender differences in body fat content and these differences begins in

adolescence. Average heights and weight of boys and girls are similar shortly before puberty (Kelly *et al.*, 1992). The sex differences in body composition and the distribution of body fat are less evident in early childhood (Ley *et al.*, 1992) but occur around puberty (Rico *et al.*, 1993; Ogle *et al.*, 1995). Measurements of total body fat, lean and bone mineral content using dual energy x-ray absorptiometry have indicated differences between boys and girls (Ogle *et al.*, 1995; Lazarus *et al.*, 1996; Taylor *et al.*, 1996; Traver *et al.*, 1995). Boys have been shown to have lower body fat than girls of similar age, weight and height (Faulkner *et al.*, 1993; Nelson *et al.*, 1997; Taylor *et al.*, 1997a). Lean tissue mass has also been shown to be higher in girls than boys (Taylor *et al.*, 1997a).

The sex differences in lean and fat mass are largely attributed to differences in sex steroids and the hormonal factors including oestrogen, androgens, cortisol, growth hormone and insulin-like growth factor - 1 (IGF-1). Testosterone concentrations has been shown to correlates with body fat mass in girls during early puberty (de Ridder *et al.*, 1990). Androgen concentration also increases in girls during puberty and this may be responsible for fat distribution (Mueller, 1982; Ogle *et al.*, 1995).

2.5.4 Ethnic differences

Variation in growth performance, body size and composition between different ethnic groups has been recognised for many years (Ulijaszek, 1994). The mean height for 7 year-old boys of 28 European and European-origin populations ranged from 119.1 to 126.5 cm (Ulijaszek, 1994). This is similar to those for African and African descendant populations (119.6 to 126.0 cm) and Indo-Mediterranean populations, but higher than that for Asiatic populations

(118.1 to 122.6 cm) (Ulijaszek, 1994). While in African children, weight-for-height differences reflect to a greater extent environmental influences, the size differences amongst European populations are thought to be predominantly genetically rather than environmentally controlled (Eveleth & Tanner, 1990). American blacks have been shown to have greater muscle mass than age-matched American whites (Cohn *et al.*, 1977).

Ethnic variations in bone mineral mass and hip axis length have been reported by a number of studies (Cohn *et al.*, 1977; Harris *et al.*, 1995; Ortiz *et al.*, 1992; Cummings *et al.*, 1994; Mikhail *et al.*, 1996). Total body bone mineral mass of African-Americans is known to be greater than Caucasians (Cohn *et al.*, 1977; Ortiz *et al*, 1992). Black women have shorter hip axis length than their white counterparts (Cummings *et al.*, 1994; Mikhail *et al.*, 1996).

2.5.5 Factors influencing growth

2.5.5.1 Environmental factors

A child's growth pattern reflects his state of health and nutrition. There are large differences between populations, in height and weight and the age of puberty (Eveleth & Tanner, 1990). Considerable world-wide variation in human growth exists, and within a country, marked differences exist between the well-off and the poor, with the rich growing better than the poor (Eveleth & Tanner, 1990)

2.5.5.2 Genetic factors

The pattern of human growth reflects both environmental and genetic components and it is not always easy to separate genetic from environmental influences (Post & Kemper, 1993; Paynter & Parkin, 1991; Amador *et al.*, 1996). Within a population, there is a wide range of normal growth which is influenced partly by genetic factors (Paynter & Parkin, 1991, Amador *et al.*, 1996). A wide variation in adult height exists between different people and this has been assumed to result from genetic differences. For example, New Guinea Highlander, Japanese, South Indians and the Pygmies of Central Africa are small (Paynter Parkin, 1991). In contrast, American Indians and Polynesians are tall and big (Paynter & parkin, 1991).

2.5.5.3 Diet and poor growth

Growth is at its greatest rate in early infancy and adolescence, and therefore, children need to consume more nutrients at these periods. One obvious reason for poor growth and stunting in poorer countries is the restriction of food. Approximately two-thirds of the population in developing countries do not meet their nutritional needs (Benefice & Malina, 1996). In addition, consumption of animal products is very low in most developing countries (Prentice *et al.*, 1993). Low consumption of calories and animal protein could result in poor growth and delay in puberty (Prentice *et al.*, 1990, Lo *et al.*, 1990). Malnutrition, high morbidity, gastrointestinal dysfunction, poverty and other related environmental factors are known causes of poor growth and delayed maturation of children in developing countries and contribute to the prevention of

complete catch-up growth once malnutrition has occurred (Keller, 1988; Prentice *et al.*, 1990). Inadequate intake and poor bioavailability of the major bone forming minerals, such as calcium, phosphorus, magnesium and zinc could also contribute to the growth retardation of children in developing countries (Bates *et al.*, 1993; Prentice *et al.,* 1990; Burr & Sweetnam, 1980; Elo & Preston, 1992).

Protein energy malnutrition (PEM) which ranges from marasmus to kwashiorkor is common among children of Third World rural communities. Although commonly seen in children aged under three years, PEM predisposes African children to poor growth and is thought to be responsible for delayed maturation of these children (Burr & Sweetnam, 1980; Lo *et al.*, 1990; Elo & Preston, 1992). Protein intake has been positively associated with bone mineral density in children (Chan *et al.*, 1987). Similarly, protein deprivation has also been reported to have an adverse effect on bone (Orwoll *et al.*, 1992). Protein-energy malnutrition in children leads to stunting and has been shown to reduce bone mineral content (Adams & Berridge, 1969; Prentice & Bates, 1993) and delayed skeletal maturation (Adams & Berridge, 1969).

2.5.5.4 Exercise

The underlying mechanism by which exercise enhances linear growth is not clear, but is thought to be mediated by hormonal factors such as growth hormone and somatomedins, that promote growth of long bones. Torun and Viteri (1994) compared 2 - 4 year old Panamanian children recovering from protein-energy malnutrition in a rehabilitation centre, who participated in exercise such as walking on a slope, running, tumbling and climbing stairs, with controls

and found that the active group had a greater linear growth (active, 22± 8 mm, inactive 14± 6 mm, $p<0.0001$). Creatinine-height index was also higher in the exercise group than the control (0.97 ± 0.12, vs 0.89 ± 0.09, $P < 0.05$), suggesting a greater muscle mass and protein repletion. Dietary intake did not differ between groups, thus further supporting the evidence that the difference in height gain between the exercise and control groups could be attributed to physical activity. Bone mass during adolescence and early adulthood is greatly influenced by environmental factors, such as dietary calcium intake and physical activity (Charles *et al.*, 1991; Matkovic, 1996). Physical activity is thought to act as a stimulus for bone growth through the pull of muscle on the ends of the long bones (Goulding, 1994, Rizzoli *et al.*, 1998). Bone turnover and weight gain have been shown to relate to muscle mass even in malnourished children (Goulding, 1994). The strong relationship between growth and muscle mass in children could partly be explained by physical activity. The hypothesis is, as children gain weight and put on muscle, they become more active. The mechanism is thought to stress the bone and stimulate linear growth (Goulding, 1994).

There is evidence to suggest that exercise training can delay the onset of puberty and suppress adult reproductive function (Malina *et al.*, 1978; Warren, 1980; Frisch, 1980; Loucks *et al.*, 1992).

2.5.5.5 Hormones

There is a wide variation in growth and sexual maturation between individuals of the same age (Marshall & Tanner, 1970; Eveleth & Tanner, 1990;

Krabbe *et al.*, 1979). This suggests that the use of chronological age as an indicator of physical development may be inappropriate in puberty. Serum levels of testosterone appear to correlate well with pubertal stage in males (Knorr *et al.*, 1974) and this is thought to be a better measure of physical development than chronological age during adolescence (Krabbe *et al.*, 1979). Although the level of serum testosterone has been used as an indicator of physical development in boys, there have been limited studies on the influence of serum testosterone on bone mineral content (Tanner & O'keefe, 1970; Krabbe *et al.*, 1979). A positive association between growth velocity and a number of hormones have been reported (Lanes *et al.*, 1995; Ross *et al.*, 1986; Kollman *et al.*, 1991; Dhuper *et al.*, 1990). Oestrogens, anabolic steroids and, more recently, growth hormone have been shown to promote bone mineralisation in humans (Krabbe *et al.*, 1979; Riggs & Melton, 1988; Dhuper *et al.*, 1990).

Oestrogen and growth hormone are known to play an important role in bone metabolism and mineralisation (Riggs & Melton, 1988). An association between early menarche and high bone mineral density have been reported by a number of studies and is thought to relate to oestrogen exposure (Fox *et al.*, 1993, Dhuper *et al.*, 1990; Ito *et al.*, 1995). Early exposure to oestrogen has resulted in a high bone mineral density at the spine in adolescent girls (Dhuper *et al.*, 1990). Similarly, low spinal bone mineral density has been linked with a low oestrogen exposure in the same study (Dhuper *et al.*, 1990). Girls in whom menarche had occurred at age 12 years have been shown to have 6% higher bone mineral density than those in whom it occurred at age 16 years (Fox *et al.*, 1993). Age at menarche is thought to be related to the peak bone mass perhaps

because of the positive correlation of early menarche with high bone mineral density (Ito *et al.*, 1995). Oestrogen, anabolic steroids and, more recently, growth hormone have been shown to improve growth velocity of patients with Turner's syndrome (Joss, 1988; Ross *et al.*, 1986; Sadeghi-Nejad *et al.*, 1985, Lanes *et al.*, 1995).

2.5.5.6 Other factors

Large family size has also been associated with poor growth, since malnutrition and infection are common because less food is available to nourish the child (Kuh & Smith, 1993; Billewicz & McGregor, 1981). Short birth intervals mean children receive inadequate child-care and increased exposure to infection. All these are factors that have been implicated with poor growth (Burr & Sweetnam, 1980; Elo & Preston, 1992).

2.6. Calcium in the body

Calcium is a major component of mineralised tissues. There is about 32,500 mmol (1,300,000 mg) of calcium in the body of average healthy adult, of which 99% is within the skeleton and teeth (British Nutrition Foundation, 1989; Willatts, 1987, Hughes *et al.*, 1994). The remainder is present in muscle and throughout the extracellular fluid, where the concentration of ionised calcium is maintained within very narrow limits. Maintenance of ionised calcium is critically important for endocrine and nervous function and muscle activity (British Nutrition Foundation, 1989; Willatts, 1987; Zilva *et al.*, 1991; Charles *et al.*, 1991; Matkovic *et al.*, 1995; Hughes *et al.*, 1994; Ritchie *et al.*, 1998).

2.6.1 Function

Calcium is required for normal growth and development of the skeleton and teeth. Other functions of calcium include control of excitability, release of neurotransmitters, initiation of muscle contractions and as a coenzyme for coagulation factors. Calcium is also an intracellular second messenger (Willatts, 1987, Marshall, 1988, Hughes *et al.*, 1994; Ritchie *et al.*, 1998).

2.7. Calcium homeostasis

Regulation of calcium metabolism is extremely complex and aimed at maintaining plasma calcium at a concentration of about 2.2 - 2.6 mmol/l (88 - 104 mg/l) (Willatts, 1987; Brune & Ullrich, 1992; Cross *et al.*, 1995b; Ritchie *et al.*, 1998). It is this concentration which is critically important for muscle activity, endocrine secretion and nervous system function and in the long term depends on maintenance of extracellular fluid (ECF) calcium by hormonal activity (Cross *et al.*, 1995b).

Bone is metabolically active, and some of its calcium is rapidly exchangeable with the extracellular fluid. The turnover between bone and extracellular fluid is approximately 12.5 mmol/day (500 mg/d). In the kidney, up to 240 mmol/d (9,600 mg/d) of ionised calcium is filtered by the glomeruli. Most of the filtered calcium is reabsorbed in the tubules and normal renal calcium excretion is 2.5 - 7.5 mmol/d (100 - 300 mg/d) (Schaafsma, 1988; Marshall, 1988; Hughes *et al.*, 1994; Cross *et al.*, 1995b; Ritchie *et al.*, 1998). Dietary calcium is ingested into the body, and absorbed, depending on absorbability, from digested food in the intestine.

Plasma calcium is maintained at a constant concentration by a complex homeostatic mechanism that is poorly understood and is closely linked to bone metabolism (Schaafsma, 1988; Cross *et al.*, 1995b). The postulated mechanism for the regulation of plasma calcium is that hypocalcaemia stimulates parathyroid hormone (PTH) production which in turn increases production of 1, 25 dihydroxy vitamin D. 1,25-dihydroxy vitamin D stimulates the absorption of calcium and phosphorus in the intestine and mobilisation from bone (Schaafsma, 1988; Polley *et al.*, 1987; Marshall, 1988; Hughes *et al.*, 1994; Cross *et al.*, 1995b; Ritchie *et al.*, 1998, Peacock, 1998). As the plasma ionised calcium is restored, the concentration of 1,25-dihydroxy vitamin D returns to normal. Raised ionised calcium has the opposite effect, PTH and 1,25-dihydroxy vitamin D production are inhibited, and calcitonin is secreted from the thyroid gland. The main function of calcitonin is inhibition of bone resorption and promotion of calcium and phosphate excretion by the kidney, thus returning extracellular ionised calcium to its normal level (Cross *et al.*, 1995b; Peacock, 1998).

Vitamin D is synthesised primarily in the skin by the action of sunlight (ultraviolet) on the provitamin (Cross *et al.*, 1995b, Hughes *et al.*, 1994; Peacock, 1998). Vitamin D can also be obtained from the diet. There are two forms of vitamin D, cholecalciferol (D_3) is made in the skin by the action of ultraviolet light and is present in animal products, and ergocalciferol (D_2) derived from a provitamin is found in some plants and fungi. Vitamin D itself however, is almost inactive. Both cholecalciferol and ergocalciferol are hydroxylated in the liver and perhaps other organs to produce 25-hydroxy vitamin D, and have similar activity (Willatts, 1987; Cross *et al.*, 1995b). 25-hydroxy vitamin D, however, is still

relatively inactive although it is the major circulating form of the vitamin (Hughes *et al.*, 1994; Cross *et al.*, 1995b; Peacock, 1998). It is transported on a specific globulin binding protein to the kidney, where further hydroxylation occurs to produce several metabolites, the most important of which is 1,25-dihydroxy vitamin D (Cross *et al.*, 1995b; Cross *et al.*, 1995a).

1,25-dihydroxy vitamin D stimulates the absorption of calcium and phosphate from the gut, resorption of calcium and phosphate from bone and of calcium and phosphate from renal tubules. The healing action of vitamin D in rickets seems to be due to its action in raising plasma calcium and phosphate levels (Sower *et al.*, 1990; Hughes *et al.*, 1994; Cross *et al.*, 1995b; Ritchie *et al.*, 1998, Peacock, 1998). 1,25-dihydroxy vitamin D levels are high in conditions of increased demand such as rapid growth (Sower *et al.*, 1990, Cross *et al.*, 1995b; Schaafsma, 1988). The precise action of 1,25-dihydroxy vitamin D in the intestine is not known but it is thought to involve increased production of a calcium binding protein which is raised during periods of active growth and in conditions of low calcium intake (Willatts, 1987; Hughes *et al.*, 1994; Ritchie *et al.*, 1998).

The vitamin D metabolite commonly measured in blood is the 25-hydroxy vitamin D and is regarded as a good marker of vitamin D status (Krall *et al.*, 1989; McKenna, 1992; Dawson-Hughes, 1998). However, its concentration in blood must be interpreted in the light of the marked seasonal variation in circulating levels of this metabolite which may result in several fold differences between winter and summer (Krall *et al.*, 1989; McKenna, 1992; Byrne *et al.*, 1995; Dawson-Hughes, 1998). The concentration of 1,25-dihydroxy vitamin D is

largely independent of the amount of the circulating level of 25-hydroxy vitamin D and is dependent on the plasma calcium concentration (Byrne *et al.*, 1995).

2.7.1 Parathyroid hormone

PTH is secreted by the parathyroid glands and secretion is controlled by two intracellular messengers, calcium and cyclic-AMP (Byrne *et al.*, 1995). PTH increases plasma calcium concentration by activating mechanisms that transfer calcium from bone, glomerular filtrate and the gut to the extracellular fluid. The concentration of ionised calcium in serum is tightly regulated through negative feed back of calcium on the secretion of PTH from the parathyroid glands (Byrne *et al.*, 1995; Peacock, 1998). The reduction of PTH secretion in response to a rise in serum calcium is dependent on the network of a calcium-sensing receptor (Peacock, 1998). Renal regulation of 1,25-dihydroxyvitamin D is partly dependent on PTH secretion (Byrne *et al.*, 1995; Peacock, 1998). PTH acts primarily at kidney by enhancing the tubular reabsorption of calcium and stimulating the formation of calcitriol, but also at the skeletal level by storing calcium (Peacock, 1998; Fassler & Bonjour, 1995). The 1,25-dihydroxyvitamin D produced stimulates intestinal calcium absorption, and down-regulates the synthesis of PTH (Peacock, 1998; Fassler & Bonjour, 1995). Measurement of the blood level of parathyroid hormone which rises in response to low calcium intake is a marker of calcium nutritional status (Fassler & Bonjour, 1995; Prince, 1997). Equally, high calcium consumption leads to decrease secretion of PTH (Fassler & Bonjour, 1995; Prince, 1997).

2.7.2 Calcitonin

The secretion of calcitonin, mostly from the thyroid, is also controlled by intracellular calcium and cyclic AMP. Calcitonin decreases the circulating level of calcium phosphate by direct action on the osteoclasts to inhibit collagen breakdown and to promote phosphate uptake into bone (Aurbach, 1988, Cross *et al.*, 1995b). Calcitonin secretion in young adults is increased by oestrogen and recent studies of the effects of oestrogen in post-menopausal women showed sharp increases in plasma calcitonin (Stevenson *et al.*, 1984; Aurbach, 1988; Cross *et al.*, 1995b). Oestrogen has been shown to prevent menopausal bone loss and this effect could be mediated at least in part by control of calcitonin secretion (Stevenson *et al.*, 1984; Aurbach, 1988). There appears to be a strong association between low circulating level of calcitonin and osteoporosis (Aurbach, 1988, Cross *et al.,* 1995b). Circulating calcitonin levels are lowest in the white women compared to age-matched black women (Stevenson *et al.*, 1984; Hughes *et al.*, 1994; Ritchie *et al.*, 1998). The incidence of fragility fractures due to osteoporosis are lower in Blacks than White Caucasians (Adebajo *et al.*, 1991; Solomon, 1979; Prentice *et al.*, 1991). Low circulating calcitonin concentration has been associated with loss of bone (Hughes *et al.*, 1994; Ritchie *et al.*,1998) and so this may partly account for the low incidence of osteoporosis in blacks compared with Caucasians.

2.8. Calcium requirement for bone growth

During skeletal growth there is a positive input of calcium into the skeleton. This means that dietary intake of calcium must be sufficient to balance obligatory calcium loss and the amount of calcium needed for positive skeletal calcium

balance. In early infancy calcium accretion into bone is estimated to be around 130 - 155 mg/d (3.25 - 3.88 mml/d) (Fomon, 1974; Krebs & Hambidge, 1986). Calcium accretion rates drop rapidly in the first three years of life to reach a nadir of between 70 - 100 mg/d (Pettifor , 1991; Kanis, 1994). Values rise thereafter to peak during the adolescent growth spurt with estimates ranging between 200 and 400 mg/d depending on sex. Boys peak later than girls and have higher peak values (Garn *et al.*, 1972; Prentice & Bates, 1993). The average daily accumulation of calcium for ages 10 to 20 years is 180 to 210 mg in boys and 90 to 110 mg in girls (Committee on Nutrition, 1978).

Calcium needs vary with the stage of skeletal development (Matkovic *et al.*, 1990). The calcium requirements during periods of skeletal modelling and consolidation are high (Nordin *et al.*, 1987; Nordin & Marshall, 1988; Heaney & Recker, 1982; Matkovic, 1991).

Table 2.1 Estimated calcium accretion rates (mg/day) for boys and girls[†]

Age (years)	2	4	6	8	10	12	14	16	18	20
Boys (mg/d)	89	70	70	105	125	173	269	394	166	60
Girls (mg/d)	93	71	83	105	147	217	330	240	101	40

[†]Accretion rates as calculated by Leitch & Aitken, (1959). Source: Pettifor, 1991

Conversion factor mg/d to mmol/d Calcium, ÷ 40

In addition to requirements for growth, part of the calcium consumed is excreted in urine, sweat, faeces, skin and hair. Consequently, growing individuals must be in sufficient positive calcium balance to increase skeletal mass (Kanis, 1994; Charles *et al.*, 1991; Matkovic *et al.*, 1995). Although data on mineral losses in children are scarce, there is evidence that mineral losses are greatly reduced in people accustomed to a very low intake (Widdowson & Dickerson, 1964; Begum & Pereira, 1969, Taylor *et al.*, 1991). It has been argued that assignment of reference values for calcium losses in children is inappropriate, since there appear to be no obligatory calcium losses for children (Department of Health COMA, 1992). However, this hypothesis has been challenged by recent balance studies which reported an obligatory calcium losses in urine, faeces, and sweat for children (Charles *et al.*, 1991; Matkovic *et al.*, 1995).

Calcium balance in the skeleton varies throughout life (Charles *et al.*, 1991). After cessation of rapid growth, bone mineral accretion, the so called skeletal consolidation is thought to continue through late adolescence (Charles *et al.*, 1991; Matkovic *et al.*, 1995).

2.9. Calcium requirements

Calcium needs are greater during adolescence (9 - 17 years) than in either childhood or young adulthood when comparing the maximal calcium retention in the body. This is due to the high velocity of growth during puberty as well as skeletal consolidation towards end of adolescence. All these together exert a major influence on calcium requirements during adolescence. Since up to sixty percent of the weight of mature bone is mineral (Boskey, 1988), mainly in

the form of calcium phosphate, and because bone mineralisation is achieved after the laying down of bone matrix, an important requirement for healthy bone development both during childhood and adolescence is a continuous supply of calcium and phosphate at a level required by the changing needs of growth. Thus calcium requirements during adolescence need to be considered in light of the following features of growth: the intensity and the extent of the pubertal growth spurt, sexual differences in the timing of peak growth (boys develop later), and individual variations in the timing of the pubertal growth spurt (Avioli, 1988). It is likely that all these aspects of growth have a profound effect on calcium and bone metabolism.

The issue of dietary calcium requirements remains unresolved and the question of whether people are eating a diet that is adequate in calcium has been an ongoing debate for over half a century. As early as the beginning of the century, Lusk reported that calcium deficiency was common (Lusk, 1917). However, despite the great attention calcium has received little progress has been made in resolving calcium requirements for bone health. This is reflected in the wide differences in recommendations for calcium from various communities of the world which range from 400 - 1500 mg/day (10 - 37.5 mmo/d) for adults (Department of Health, Committee on Medical Aspects of Food Policy and Nutrition (COMA), 1992; National Academy of Sciences, 1989).

Similar differences are seen in the recommendations for children and adolescents. The World Health Organization (WHO) and the Committee on Medical Aspects of Food Policy and Nutrition (COMA) recommends a daily intake of 700 mg/day and 1000 mg/day respectively for 8 - 10 year olds (FAO and WHO, 1962; Committee on Medical Aspects of Food Policy (COMA), 1992). The World

Health Organization also recommends intakes of 600 - 700 mg/day for 11 - 15 year-old adolescents and 500 - 600 mg/day for those 16 - 19 years old (WHO, 1991). National Research Council recommends 1200 mg/day for these age groups (National Academy of Sciences, 1989) but more recently set AI (adequate intake) of 1,300 mg/day for 9 - 18 year old (National Academy of Sciences, 1997). In a recent review by the Committee on Medical Aspects of Food and Nutrition Policy Subgroup on Bone Health, it was felt that there is insufficient evidence to relate the current UK Reference Nutrient Intake (UKRNI) to bone health, in terms of measures of bone mineral content, bone mineral density, fracture, and biochemical markers and therefore, the factorial method was retained. It was therefore concluded that the available data were insufficient to warrant revising the current Dietary Reference Value for calcium for children and adolescents and other age groups.

Table 2.2 Recommendations for calcium intake[†]

		Calcium (mg/d)		
	Sex	8 - 10 years	11 - 15 years	16 - 19 years
WHO (WHO, 1962; 1980, 1991)	M+F	700	600 - 700	500 - 600
USA, RDA (National Academy Science,1989)	M + F	1200	1200	1000 - 1300
USA, AI (National Academy Science, 1997)	M+F	1300	1300	1300
NIH (National Institute of Health, 1994)	M+F	800 - 1200	1200 - 1500	1200 - 1500
UK, RNI (Department of Health, 1992,				
Bone Health Report, 1998)	M	550	1000	1000
	F	550	800	800

[†] Figures are for both males and females. Conversion factor from mg/d to mmol/d: calcium ÷ 40

Judging by these differences, it is certain that the amount of dietary calcium needed to sustain growth, as well as to provide maintenance, requires much more study, particularly with respect to different populations.

The fact that all these recommendations for calcium intake are based on different criteria further complicates the issue of dietary calcium requirements. For example, the UK Reference Nutrient Intake (RNI) for adolescents is based on a mean retention of 250 mg (6.3 mmol)/d for girls and 300 mg (7.5 mmol)d for boys, and a net absorption of 40 per cent (Department of Health, 1992). In contrast, National Institute of Health Consensus Statements optimal calcium requirements are figures adopted by a panel of experts and not based on any real experimental data (National Institutes of Health, 1994). The Adequate Intake (AI) recently, set by the US Food and Nutrition Board is based on the amount of calcium required to support maximal calcium retention (National Academy of Sciences., 1997). AI is intended to replace the more conventional Recommended Dietary Allowance (RDA) which was based on a calcium intake sufficient to minimise the risk of developing a condition or sign that is associated with calcium deficiency (National Academy Science, 1989).

There are some suggestions that up to twice the WHO recommendation is necessary to maintain the skeletal balance for calcium in young healthy populations (Nordin & Heaney, 1990). However, many researchers have argued that more than half of the world's population are accustomed to low dietary calcium intake (≤ 500 mg/day) and that at present there is little evidence that

such countries are disadvantaged with respect to osteoporosis (Kanis & Passmore, 1989). In early studies from India, children consuming a relatively low calcium intake (~ 300 mg/d) were still able to maintain a sufficiently positive calcium balance to assure skeletal development (Begum & Pereira, 1969; Aykroyd & Krishnan, 1938; Aykroyd & Krishnan, 1939).

It is plausible that populations exposed to more sunlight and with better vitamin D status, like most Africans, may maintain calcium balance more readily on low calcium intake. Therefore, it has been argued by some that current recommendations should be specific to the individual populations and not to be regarded as universal as the FAO/WHO report suggested (WHO, 1991).

There is some evidence however that the dietary calcium intake of a large proportion of the World's children may be insufficient to support optimal linear growth and maximal bone mineralisation (Miller *et al.*, 1988; Lee *et al.*, 1994).

2.9.1 Calcium intakes in different populations

Dietary calcium intakes vary considerably between countries. Much of between country variation in calcium intake can be ascribed to differences in milk consumption. Currently, Finland has the highest mean daily calcium intake in the world at 1300 mg/d (32.5 mmol/d) with approximately ninety percent of the intake from milk and dairy products (Nordin & Marshall, 1988; Nordin 1997). In Britain, the current national average intake of calcium is estimated around 820 mg/day (20.5 mmol/day) and the major dietary sources of calcium are milk and milk products (approx 56%; Department of Health, Committee on Medical Aspects of Food Policy (COMA), Report of the Subgroup on Bone Health, 1998). This is

much lower than the percentage of milk and dairy products in Finland because an extra 14 percent of calcium intake in Britain comes from bread due to fortification of white flour with calcium. In the United States, the current mean calcium intake of the population is calculated around 970 mg/d (24.3 mmol/d) (Nordin & Marshall, 1988; Park *et al.*, 1997), fifty-two-percent of which comes from milk and milk products. In countries where milk consumption is low, such as in China and The Gambia, the calcium intake of the population is extremely low compared with British and America, and averages 300 - 500 mg/d (7.5 - 12.5 mmol/day) (Prentice *et al.*, 1993; Lee *et al.*, 1994).

2.10. Peak Bone Mass (PBM)

Maximising peak bone mass has been associated with reduced incidence of osteoporosis (Cummings *et al.*, 1993; Eisman *et al.*, 1993). The low fracture incidence of osteoporosis in African - American blacks has partly been ascribed to a higher peak bone mass (Cohn *et al.*, 1977; Ortiz *et al.*, 1992; Harris *et al.*, 1995).

2.10.1 Genetic factors influencing bone mass

Genetic factors are estimated to contribute around 80% of the total variance in bone density (Kelly & Eisman, 1993; Eisman, 1995; Peacock, 1995, Rizzoli *et al.*, 1998). However, the relative importance of genetic and environmental factors in determining the variability in bone density is not fully understood. Both genetic and environmental factors influence the amount of peak bone mass attained in early adulthood (Kelly & Eisman, 1993). Physical

fitness, muscle mass and bone mineral density are thought to have genetic determinants (Lortie *et al.*, 1982; Bouchard *et al*, 1986).

Allelic variants have recently been isolated in a number of candidate genes that may be associated with bone health, such as the vitamin D-receptor (VDR) gene, parathyroid-hormone-receptor gene, collagen genes, oestrogen-receptor gene, interleukin-6 gene and apolipoprotein E (ApoE) gene (Sainz *et al.*, 1997, Rizzoli *et al.*, 1998). At present it is not clear to what extent polymorphism in any of these genes influences bone mineral status, bone turnover, or fracture risk (Eisman, 1995; Peacock, 1995). In adolescents, alleles of vitamin D-receptor gene have been shown to be a good predictor of femoral and vertebral bone density in some studies (Sainz *et al.*, 1997) but not others (Peacock,1995). In the Sainz study, girls with the aa and bb genotypes as indicated by the ApaI and BsmI endonucleases had 2 to 3 percent higher femoral bone density and 8 to 10 percent more vertebral bone density than girls with the AA and BB genotypes. The variation in bone density between the variants in the Sainz study could not be accounted for by differences in bone size or in biochemical indices.

Studies of the relationship of vitamin D-receptor alleles and bone density are far from conclusive. Of the 50 or more studies reported to date only half found a significant association between vitamin D-receptor genotypes and bone density and others found no association (Eisman, 1995; Peacock, 1995). There is some evidence to suggest that nutrient-gene interactions may influence the importance of nutrition to bone health (Ferrari *et al.*, 1995). For example the effect of calcium supplements on lumbar spine bone mineral mass and

analogues of vitamin D in older people differs according to VDR genotype (Ferrari *et al*, 1995; Matsuyama *et al.*, 1995) and the transport of vitamin K by lipoproteins is thought to be affected by ApoE genotype (Saupe *et al.*, 1993).

2.10.2 Calcium intake and peak bone mass

Increasing evidence suggests that adequate calcium intake during growth is needed to optimise peak bone mass and reduce risk of fractures later in life (Sandler *et al.*, 1985; Halioua & Anderson, 1989; Matkovic, 1996; Kelly *et al.*, 1994; Theintz *et al.*, 1992; Anderson & Rondano, 1996; Valimaki *et al.*, 1994). Low calcium intake could lead to reduced calcium accretion into bone and this may have a negative impact on skeletal development and ultimately on peak bone mass (Theintz *et al.*, 1992, Anderson & Rondano, 1996; Adami *et al.*, 1994).

2.10.3 Calcium intake, physical activity and peak bone mass

Physical activity in childhood is perceived to be important in maximising peak bone mass (McCloskey *et al.*, 1990; Slemenda *et al.*, 1991; Metz *et al.*, 1993; Ulrich *et al.*, 1996) and is important in the maintenance of bone mass later in life (Halioua & Anderson, 1989; Kriska *et al.*, 1989; Snow-Harter & Marcus, 1991, Ulrich *et al.*, 1996). The association between usual calcium intake and bone mass is thought to be influenced by the level of physical activity (Kanders *et al.*, 1988; Halioua & Anderson, 1989). Both physical activity and adequate calcium intake have been shown to have some effect on the accumulation of radial peak bone mass (Kanders *et al.*, 1988; Halioua & Anderson, 1989). However, the influence of activity on bone mass is a controversial issue. Some studies have indicated that the influence of exercise on bone mass depends not

only on the type and intensity of exercise, but also on the duration of the exercise and the age at which it is practised (Steinberg & Trueta 1981). In addition, some studies have shown that exercise affects both trabecular and cortical bone (Pocock *et al*, 1986), only trabecular bone (Colleti *et al.*, 1989; Krolner *et al.*, 1982), or neither component (Frisancho *et al.*, 1970; Puustjarvi *et al.*, 1991).

Low calcium intake and being inactive during early adolescence are thought to be detrimental to optimising peak bone mass (Tylavsky *et al.*, 1992; Anderson, 1996a). However, the effect of regular physical activity and adequate calcium intake during early life on bone mass in later life is uncertain (Vuori, 1996; Theintz *et al.*, 1992). There are some indications that the effect of physical activity and high calcium intake is not confined to radial bone mass alone, but would be found at other sites in the skeleton (Gleeson *et al.*, 1990; Rockwell *et al.*,1990; Bassey & Ramsdale, 1994).

2.10.4 Sex differences

There are sex differences in the amount of peak bone mass achieved. Peak bone mass is reported to be ~25% higher for males than females, and is believe to be attained around the age of 30- 40 years, and is somewhat earlier in trabecular than cortical bone (Charles *et al.*, 1991; Ito *et al.*, 1995).

2.10.5 Menarche and peak bone mass

Early menarche has been shown to be associated with higher bone mineral density in adulthood (Ito *et al.*, 1995). Using single photon absorptiometry, Fox et al. showed that premenopausal women in whom menarche had occurred at age nine years had six percent high bone density than those in whom menarche

occurred at age sixteen (Fox *et al.*, 1993). Similarly techniques such as dual energy x-ray absorptiometry (DXA) and quantitative computer tomography (QCT) have indicated a positive association between early menarche and higher adult bone mineral density (Ito *et al.*, 1995). Similarly, early exposure to oestrogen has resulted in a high bone mineral density at the spine in adolescent girls (Dhuper *et al.*, 1990). Because of the positive correlation of early menarche with high bone mineral density, age at menarche has been linked to peak bone mass (Ito *et al.*, 1995). Since bone mineral is maintained in part by oestrogen exposure (Ito *et al.*, 1995), it is reasonable to speculate that women with a longer reproductive periods will attain a higher bone mineral content than their counterparts with a shorter reproductive period. For the same reason early menopause is associated with a low bone mineral content in postmenopausal women. Conditions such as anorexia nervosa and certain lifestyle factors, such as early physical training, which induce amenorrhoea or delay menarche can be regarded as risk factors affecting the normal development of peak bone mass in young adolescents.

2.10.6 Physical activity and bone mass in adults

There is some evidence to suggest that women with both adequate calcium intake and high physical activity may be able to maintain their peak bone mass as they progress in adult premenopausal age (Anderson & Metz, 1993). In contrast, Ulrich *et al.* showed physical activity and not calcium intake to be a more powerful enhancer of premenopausal bone mass (Ulrich *et al.*, 1996). This study demonstrated a strong correlation between lifetime weight-bearing exercise and lean body mass, and supported the hypothesis that low muscle

mass, as a result of low levels of physical activity, may be a risk factor for low bone mineral density.

2.10.7 Muscle strength and bone mineral mass

Boys grow more rapidly in muscle strength and co-ordination than girls after the age of thirteen years (Eveleth & Tanner, 1990; Paynter & Parkin, 1991). From the age of 70 and above, it is estimated that muscle strength is approximately 35 - 45% lower than the peak value in younger life (Grimby & Saltin, 1983; Paynter & Parkin, 1991). The decline is sex dependent and varies according to muscle group (Grimby & Saltin, 1983).

One of the tools for testing physical performance is handgrip which correlates with muscle mass in young Caucasian adult males (Bassey, 1990). The hand grip requires the combined action of a number of muscles in both the hand and forearm, and represent a familiar action of functional importance for many activities of daily life. Such as holding onto support, opening containers, using tools, pulling and pumping water out of well. Low handgrip strength in elderly implies considerable functional impairment (Kallman *et al.*, 1990; Bassey & Harries, 1993). Handgrip strength is shown to be more sensitive than body composition measurements in predicting post-operative complications (Klidjian *et al.*, 1980; Webb *et al.*, 1989). To what extent handgrip strength correlates with disease in young adults is not known. It is plausible that grip strength in young adulthood may correlate positively with forearm bone mineral content which may protect fracture of the forearm in later life. A positive correlation between muscle strength and bone mineral mass in children has been reported in a number of studies (Schiessl *et al.*, 1996; Schonau *et al.*, 1996), and this is thought to be due

to the positive effect of muscle strength on bone strength. Increase bone strength has been shown to increase bone mass in children (Schiessl *et al.*, 1998). Similarly, it has been shown that the rate of bone mass formation in girls was reduced following a decline in muscle strength (Schiessl *et al.*, 1998). A high grip strength indicates a high level of activity which could result in high bone mineral mass and lower incidence of fracture (Lau *et al.*, 1993).

2.11 Calcium deficiency

The first evidence that calcium deficiency causes rickets in infants came from two studies in late seventies (Kooh *et al.*, 1977, Maltz *et al.*, 1970). Studies of children with rickets have shown that their calcium intakes were remarkably lower than the recommendations (FAO/WHO, 1962) and this is thought to be responsible for the bone deformities in these children in spite of normal vitamin D status (Pettifor *et al.*, 1981).

Low calcium intake of children in rural South Africa has also been associated with hypocalcaemia, elevated alkaline phosphatase, decreased urinary calcium excretion and a significant decrease in bone mineral content of the radius compared with those children on a high calcium diet (Eyberg *et al.*, 1986; Pettifor & Moodley, 1997).

Children living in rural areas in South Africa where calcium intakes are low have been shown to have low bone mineral density, and significantly higher prevalence of biochemical abnormalities than urban children on high calcium

diet (Pettifor *et al.*, 1981; Eyberg *et al.*, 1986). However, two groups had different biochemical profiles prior to the study, as the criterion for the study. The study children had elevated serum calcium and alkaline phosphatase while these indices were normal in the controls.

2.11.1 Interaction between calcium and fluoride

A high incidence of childhood rickets has been reported in areas of India with high water fluoride concentrations (Teotia *et al.*, 1979). Bone deformities due to endemic fluorosis have also been documented in children (Jackson, 1962; Pettifor, 1991). The biochemical abnormalities of fluoride-related bone deformities are elevated alkaline phosphatase, low urinary calcium excretion and decreased tubular reabsorption of phosphate (Pettifor, 1991). The condition resulted in secondary hyperparathyroidism, elevated 1,25-dihydroxyvitamin D concentrations and osteomalacia (Pettifor, 1991; Pettifor & Moodley, 1997; Eyberg *et al.*, 1986).

In all these studies, the onset of fluoride-related bone disease in children have been associated with low dietary calcium intake. Although the mechanisms that underline the cellular actions of fluoride on bone are poorly understood, it is thought fluoride excess stimulates osteoblast formation and impairs mineral resorption (Pettifor *et al.*, 1981; Pettifor & Moodley, 1997; Eyberg *et al.*, 1986; Dure-Smith *et al.*, 1996). The excess fluoride has also been associated with severe bone deformities which resemble clinical rickets (Pettifor *et al.*, 1981; Pettifor & Moodley, 1997).

2.12. Osteoporosis

Public interest in calcium nutrition and bone disease prevention has greatly increased in recent years because of raised awareness of osteoporosis. Osteoporosis is characterised by low bone mass and microarchitectural deterioration of bone tissue leading to increased incidence of fragility fractures (Food and Agricultural Organization/World Health Organization, 1994). In osteoporosis, both protein and bone mineral are lost but the composition of the remaining bone is normal. The criteria for the diagnosis of osteoporosis rest on bone density measurement. Within 1 standard deviation (SD) of young adult mean is defined by the World Health Organization (WHO) as normal bone mineral mass and bone mineral density while osteoporosis is defined as bone mineral mass and bone mineral density ≥2.5 SD below the young adult mean (Consensus Development Conference, 1993, Food And Agricultural Organization/World Health Organization, 1994; Christenson, 1997).

Osteoporosis constitutes a major burden of morbidity and mortality in many affluent societies. In the United States, for example, fractures due to osteoporosis are estimated to affect well over 20 million Americans over the age of 45 years (Matkovic *et al.*, 1980; Peck *et al.*, 1987; NIH Consensus Conference, 1994) with more than 200,000 hip fractures occurring each year and with 50% of those affected dying within a year of the fracture (Evans *et al.*, 1979; Beals, 1972; Jensen & Tonderold, 1979). Recent estimates of the cost of fracture on the health care system in the United States is around $10 billion per year (NIH Consensus Conference, 1994). It is estimated that one in four European women suffers from osteoporosis by the age of seventy years, and the cost of hip fracture to the

National Health Service in England and Wales is approaching three-quarter billion pounds a year (Compston, 1992). The incidence of osteoporosis is 2 - 3 times higher in women than men in Western Countries (Cooper, 1992), although in countries where overall incidence is lower, the sex ratio is closer to one (Xu *et al.*, 1995; Xu *et al.*, 1996).

Two types of age-related osteoporosis have been identified. Post menopausal (Type 1) osteoporosis occurs within 15 - 20 years of the menopause, affects primarily trabecular bone, and is clinically associated with vertebral crush fractures and wrist fractures (Riggs & Melton, 1986). Senile osteoporosis (Type II), affects both trabecular and cortical bone, and is associated with femoral neck (hip) fractures after the age of 70 (Riggs *et al.*, 1986).

While there is general consensus that high calcium intake will slow bone mineral loss (National Institutes of Health, 1994), there is insufficient evidence to be certain of a direct relationship between dietary calcium intake and bone mineral content. Some investigators (Albanese *et al.*, 1981; Albanese *et al.*, 1983; Matkovic *et al.*, 1990; Matkovic *et al.*, 1979) have shown significant associations while others (Garn *et al.*, 1967; Smith *et al.*, 1989; Sowers *et al.*, 1985) have failed to show any relationship between dietary calcium intake and bone density. Furthermore, calcium intake is known to differ between countries with similar incidence of osteoporotic fractures. For example, the incidence of fragility fractures in Denmark is similar to Great Britain despite the high calcium intake of the Danes (Anonymous, 1984; Jensen & Tonderold, 1979). The lack of an association between dietary calcium intake and bone mineral content may reflect confounding, for example, by body size (Prentice *et al.*, 1994). Studies on

the relationship of dietary factors and bone loss leading to osteoporosis have focused primarily on calcium. However, intakes of other nutrients such as phosphorus, vitamin D and protein have also been studied (Abelow *et al.*, 1992; Harris & Dawson-Hughes, 1994; Hernandez-Avila *et al.*, 1993).

The major cause of postmenopausal bone loss appears to be oestrogen deficiency and the administration of oestrogens can be successful in preventing bone loss (Kanis, 1994). Oestrogen deficiency not only causes an increase in remodelling rate of bone, but also induces an imbalance at remodelling sites (Stepan *et al.*, 1987; Heaney *et al.*, 1978). The role of calcium in the treatment of osteoporosis appears to be via its ability to reduce bone turnover (Nordin & Need 1989), resulting in a decrease in rate of skeletal loss. However, the imbalance between bone resorption and formation caused by oestrogen deficiency in postmenopausal individuals has been shown to be resistant to calcium therapy (Hillyard *et al.*, 1978, Riis *et al.*, 1987; Dawson-Hughes *et al.*, 1990; Elders *et al.*, 1994).

In healthy adults, the deposition of calcium in bone is closely linked with the deposition of bone matrix (Charles *et al.*, 1991; Kanis, 1994; Parfitt, 1980). However, in osteoporosis, the coupling process appears intact but there is an imbalance between the amount of mineral and matrix removed and that incorporated into each resorption cavity which results an increased reduction of skeletal mass (Christenson, 1997; Kanis, 1994, Parfitt, 1980). In postmenopausal osteoporosis, there is evidence to suggest that the imbalance between bone resorption and bone formation at each remodelling site is due to a

decrease in the functional capacity of osteoblasts and their recruitment to previous resorption sites (Charles *et al.*, 1991; Kanis, 1994).

2.13. Calcium absorption

Calcium is absorbed via two routes; a saturable regulated vitamin D-dependent transcellular path, and a nonsaturable path that is paracellular and not acutely regulated (Nellans & Kimberg, 1977; Bringhurst & Potts, 1979; Bronner, 1995; Ganong, 1993). The rate of production of 1,25-dihydroxy vitamin D is increased when the plasma calcium level is decreased and reduced when the plasma calcium level is elevated. The 1,25-dihydroxy vitamin D is concentrated in the nuclear chromatin of the intestinal absorptive cell where it may interact with DNA to code a specific m-RNA which, interacting with the polysomes, leads to the synthesis of calcium binding protein (CaBP) (Nellans & Kimberg, 1977; Bringhurst & Potts, 1979; Bronner, 1995).

The amount of calcium absorbed is thought to depend both on the body requirements and intake until a threshold is reached (Spencer *et al.*, 1984; Matkovic *et al.*, 1995). Absorption increases at low calcium intake and decreases at higher intake (Matkovic *et al.*, 1995; Bronner, 1995). About 40-50% of calcium in the diet is absorbed in the small intestine, (Bronner, 1995; Ganong, 1993) and there is significant absorption in the lower intestine. Some of the endogenous calcium secreted into the gut is reabsorbed, and only a small amount of the filtered calcium is excreted into the kidneys each day (Schaafsma, 1988). At a very high calcium intake, the absorption efficiency may range from 20-80%

(Ganong, 1993). It is estimated that up to about 40% of dietary calcium is absorbed from typical Western diets (Schwartz, 1990; Department of Health COMA, 1992). The extent to which dietary calcium is absorbed and excreted depends on the calcium vehicle and the presence of protein, phosphate, oxalate, etc, in the diet. Calcium absorption is increased during growth and during pregnancy (Matkovic, 1991, Schwartz, 1990). Absorption of calcium is thought to be greater in adolescence than any other stage of life (Matkovic, 1991). Calcium absorption efficiency is high in children and adolescents (Matkovic, 1991, Schwartz, 1990). It has been shown that children on a low dietary calcium intake absorb a great proportion of their calcium intake compared with those on higher intake (Ireland & Fordtran, 1973; Heaney *et al*, 1975; Gallagher *et al.*, 1979; Abrams, 1993; Abrams & Stuff, 1994). This is consistent with the report that fractional calcium varies inversely with calcium intake (Spencer *et al.*, 1969; Ireland & Fordtran, 1973; Heaney *et al.*, 1975; Gallagher *et al.*, 1979).

Absorption of calcium is higher in boys than in girls of all races (Bell *et al.*, 1993). High rates of calcium absorption have been demonstrated in prepubertal black girls compared with prepubertal white girls and are thought to be responsible for the greater increase in bone mineralisation in American black girls during puberty (Abrams, 1993), although, this remains controversial.

Calcium absorption is higher during prepuberty and the early pubertal period and reduced at late puberty (Matkovic *et al.*, 1990; Abrams & Stuff, 1994). Because nearly all the calcium retained from the diet is used for bone mineralisation and remodelling, the current calcium intake of children during the pubertal growth period may be inadequate to ensure maximal bone mineral

retention. Furthermore the diets of most of the world's population vary in the proportions of other nutrients such as protein, sodium and phosphorus, which may affect both mineral absorption and excretion. Fractional absorption in neonates is estimated to be eighty percent from human milk and addition of mineral supplement has been shown to increase the absorption efficiency to eighty-two percent (Liu *et al.*, 1989). However, not all studies have shown such a high absorption (Barltop *et al.*, 1977). In some studies, calcium absorption failed to correlate with age in children (Peacock, 1991).

2.13.1 Dietary factors affecting calcium absorption

2.13.1.1 Chelating agents

Calcium absorption is inhibited by phosphate and oxalate because these anions form insoluble salts with ionised calcium in the intestine (Allen, 1982; Department of Health COMA, 1992). Vegetarian diets are thought to be poorly absorbed due to chelation properties of compounds such as inositol hexaphosphates and oxalate (Allen, 1982; Department of Health COMA, 1992). However, there is controversy over the effect of phytate on calcium absorption and how much calcium is absorbed from a phytate-containing diet is likely to depend on methods of food preparation (Walker, 1985; Murphy *et al.*, 1992). There is some evidence to suggest that baking, fermentation and germination reduce the effect of phytate (Walker, 1985; Golden, 1988; Murphy *et al.*, 1992). The Ca : P ratio is low in the diets of most developing countries and is thought to imply a high phytate intake and a poor calcium absorption (Prentice & Bates, 1993).

2.13.1.2 Protein

There are conflicting reports on the association between protein intakes and bone health. Increased risk of fracture and low bone mineral density have been related to high intake of protein in women (Abelow *et al.*, 1992; Feskanich *et al.*, 1996). This contrasts with studies which either found the reverse effect (Fehily et al., 1992) or found no effect at all (Hernández-Avila *et al.*, 1993). Elderly patients who were treated with protein-rich supplements showed some clinical improvement (Delmi *et al.*, 1990). A high animal protein intake has been associated with a modest increase in urinary calcium excretion (Lennon *et al.*, 1962), this is presumably because these factors increase the urinary titratable acid excretion and because of increased absorption which leads to calciuria.

2.13.1.3 Fibre

There is some evidence to suggest that dietary fibre alone or mixed with constituents such as phytate and oxalate affects calcium absorption (Southgate, 1987; Heaney, 1996a; Heaney, 1996b). However, the results of the effect of fibre on calcium absorption are inconclusive as others reported a long-term adaptation to variation in nutrient availability (Walker, 1985). Studies of Eaton & Nelson have shown that the potential adverse effects of life-long high fibre consumption on calcium bioavailability are negligible provided that other aspects of the diet are adequate (Eaton and Nelson, 1991). Recent studies have shown that many forms of fibre such as those found in dark green vegetables fail to influence calcium absorption (Heaney, 1993; Heaney, 1996a).

2.13.1.4 Vitamin C

A positive association between vitamin C intake and high bone mineral density have been documented in adolescents (Gunnes & Lehmann, 1995), middle-age women (New *et al.*, 1997) and postmenopausal women (Hall & Greedale, 1998). Vitamin C is a cofactor for the hydroxylations of lysine and proline residues required for the formation of collagen (Bunker, 1994; Gunnes & Lehmann, 1995). Pyridinoline is formed from two hydroxylysyl residues, while deoxypyridinoline is synthesised from hydroxylation of lysyl and hydroxylysyl residue. Deficiency of vitamin C has been linked to impairment of bone formation in infants (Franceschi, 1992), and osteoporosis in adults (Lynch *et al.*, 1967; Bunker, 1994).

2.13.1.5 Caffeine

High caffeine intake promotes urinary calcium excretion (Heaney & Recker, 1982; Massey & Wise, 1984; Morgan *et al.*, 1994). Recent longitudinal studies have shown that caffeine promotes bone loss in postmenopausal women with low calcium intake (Harris & Dawson-Hughes, 1994). An association between high caffeine intake and low bone mineral density have been shown in pre- and perimenopausal women (Hernandez-Avila *et al.*, 1993) and in postmenopausal women (Lloyd *et al.*, 1997). However, in a recent review on the effects of dietary factors on calcium absorption, caffeine was thought to have a minimal effect on intestinal absorption of calcium compared with other nutrients (Heaney, 1996b).

2.13.2. Adaptation to low calcium intake

It is possible that individuals accustomed to a low calcium intake absorb more and excrete less calcium to compensate for the low calcium supply.

However, the extent to which such populations can adapt to a low calcium intake is unknown. There are conflicting reports regarding the importance of intestinal absorption of calcium in increasing the risk of osteoporosis, with some literature suggesting decreased absorption in older women with the disease (O'Brien *et al.*, 1998; Dawson-Hughes *et al.*, 1997; Lips *et al.*, 1996; Ott, 1994), while others have shown increased absorption (Hasling *et al.*, 1990; Parsons *et al.*, 1964), and a few studies have demonstrated normal absorption in osteoporotic individuals ((Kung *et al.*, 1998; Bronner *et al.*, 1963).

2.14. Calcium excretion

The excretory routes for calcium are through the intestine (the endogenous faecal calcium), the kidneys (urinary calcium excretion), and the skin (dermal calcium loss) (Peacock 1987; Charles *et al.*, 1991; Matkovic *et al.*, 1995).

The relationship between calcium intake and urinary calcium excretion in adults has been well documented (Nordin & Marshall, 1988; Lemann *et al.*, 1979) with about 5 - 7% of oral load being excreted in urine (Prentice *et al.*, 1995). However, the association between calcium intake and excretion is poor, since the control of calcium balance is via the gut and not by the kidney, and this is supported by the fact that increases in calcium intake is matched by only a moderate excretion of calcium in urine (3% of the dose, Prince *et al.*, 1991).

2.14.1 Ethnic differences in calcium excretion

There are ethnic differences in urinary calcium excretion. The excretion rate is lower in Black than in Caucasian boys and girls and in prepubertal American girls with the same calcium intake (Bell *et al.*, 1993; Abrams, 1993). A

comparative study of Gambian and British adults has also demonstrated a higher urinary calcium excretion in the British compared with the Gambians (Dibba *et al.*, in press). Low calcium and phosphate excretion, higher parathyroid hormone levels and lower 25-(OH)D was reported in a group of young Zairean men living in Belgium compared to Zairean living in Zaire (M'buyamba-Kabanga *et al.*, 1987). A similar low urinary output of calcium has been demonstrated in young Black adult men and women compared with White adults (Bell *et al.*, 1993). It is therefore plausible that ethnic differences in renal excretion of calcium may begin in childhood. This may partly explain the slow rate of bone loss observed in older black Americans (Bell *et al.*, 1993; Abrams, 1993). This observation indicates that differences in renal excretion of calcium may allow for a more positive calcium balance and account for the higher bone mass in American Blacks than in White children (Bell *et al.*, 1991).

2.14.2 Calcium excretion and bone mass

A negative association between urinary calcium excretion and whole body bone mineral content and density have been indicated in a number of studies (Matkovic *et al.*, 1990; Matkovic, 1992; Matkovic *et al.*, 1995). Urinary calcium excretion is one of the important determinants of calcium retention in the body (Weaver *et al.*, 1995). However, the nature of the relationship between dietary calcium and urinary calcium in children is not clear (Matkovic, 1992; Weaver *et al.*, 1995). A number of studies reported a decrease in bone mineral density at range of skeletal sites in adult patients with hypercalciuric syndrome (Barkin *et al.*, 1985; Pacifici *et al.*, 1990; Bataille *et al.*, 1991; Pietschmann *et al.*, 1992; Jaeger *et al.*, 1994). Since calcium intake is an important determinant of bone formation and possibly peak bone mass, it is plausible that high obligatory

calcium loss in the urine may reduce the degree of calcium retention in the body, with a consequent of low bone mineral content and increased risk of osteoporotic fracture.

2.14.3 Dietary factors affecting calcium excretion

2.14.3.1 Sodium

Dietary sodium increases calcium loss in the urine due to a close association between renal tubular mechanisms involved in the reabsorption of these ions (Goulding & Lim, 1983; Sabto *et al.*, 1984; Need *et al.*, 1991; Nordin *et al.*, 1993; Devine *et al.*, 1995; Matkovic *et al.*, 1995; Ginty *et al.*, 1998). This suggests that a high sodium intake could be a risk factor for osteoporosis through its influence on calcium retention and hence on peak bone mass. It has been shown that salt intake influences over night bone loss in postmenopausal women (Goulding & Lim, 1983). Thus it is possible that high salt intake may promote higher calcium excretion leading to loss of bone whereas a low salt intake should help to conserve bone mass by reducing calcium loss in urine. The direct effects of sodium on bone mass of human are equivocal and the magnitude of the changes in urinary calcium excretion attributable to changes in sodium excretion have been disputed (Ginty *et al.*, 1998; Sabto *et al.*, 1984).

2.14.3.2 Potassium

Potassium bicarbonate reduces urinary calcium excretion and enhances calcium retention in healthy adults without altering calcium absorption (Lemann *et al.*, 1989; Lemann *et al.*, 1991). Potassium bicarbonate has also been associated with reduced bone resorption and increased bone formation in postmenopausal women (Sebastian *et al.*, 1994).

A positive association between the dietary intake of potassium and bone mineral density has been found in postmenopausal women (Michaelsson *et al.*, 1995, New *et al.*, 1997) but this association may be confounded by environmental factors and body size considerations.

2.14.4 Calcium balance studies

There is a great controversy surrounding calcium threshold above which the amount of calcium consumed is not related to calcium retention in the body but with faecal and urinary calcium excretion (Matkovic & Heaney, 1992; Weaver *et al.*, 1995) and this is thought to differ between different age groups (Matkovic & Heaney, 1992). In general, higher calcium intake has been associated with a greater calcium retention in adolescents (Matkovic & Heaney, 1992; Weaver *et al.*, 1995; Weaver *et al.*, 1996). In a review of balance studies from 1922 until 1991, Matkovic & Heaney estimated that the threshold intakes of calcium where calcium accretion increase no further (plateaus) were 1480 mg/d (37 mmol/d) for adolescents and 957 mg/d (23.9 mmol/d) for young adults (Matkovic & Heaney, 1992). It was on the basis of these estimates and the results of calcium supplementation studies (Johnston *et al.*, 1992; Lloyd *et al.*, 1993), that a recommendation of 1500 mg/d (37.5 mmol/d) for optimal calcium intake in adolescents was proposed by the National Institutes of Health Consensus Conference on Optimal Calcium intakes in 1994 (National Institute of Health, 1994). Using more sophisticated modelling procedure Jackman et al have more recently estimated the calcium threshold at < 1300 mg/d (32.5 mmol/d) for the same age group (Jackman *et al.*, 1997).

Calcium balance is defined as the difference between intake and output (Matkovic & Heaney, 1992). However, it is difficult to quantify the balance between absorption and excretion, because present methods for measuring intake and output are subjected to large measurement errors (Weaver *et al.*, 1995; Matkovic & Heaney, 1992; Matkovic *et al.*, 1995). It has been argued that most balance studies do not take into account the calcium excreted in the sweat (Dawson-Hughes, 1998). Calcium loss from sweat is estimated to be as high as 65 mg/d (1.63 mmol/d) (Charles *et al.*, 1983). Very little is known about calcium excretion in children. There is some suggestion that adolescents may have lower urinary and faecal calcium excretion and greater net calcium absorption than adults (Matkovic & Heaney, 1992; Weaver *et al.*, 1995).

2.15 Calcium supplementation studies

2.15.1 Calcium supplementation studies in children

A number of retrospective studies have shown an association between high calcium intake in early life and attainment of a greater peak bone mass (Chan, 1991; Fehily *et al.*, 1992; Hu *et al.*, 1993). Hu et al. found a strong association between high milk intake during childhood and bone mineral density in adults and further speculated that a high calcium intake may optimise peak bone mass (Hu *et al.*, 1993). However, the long-term benefits of a high calcium intake for increasing bone mineral status or for reducing fractures have not yet been validated experimentally.

At the start of this study, there were only three published calcium supplementation trials (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lee *et al.*, 1994), and all had shown a significant effect of a calcium supplement on bone mineral acquisition. The timing of the responsiveness to the calcium supplement relative

to the pubertal growth spurt, and the relationship between the dose of calcium supplement and habitual calcium intake are controversial. A supplementation trial of identical twins demonstrated a significant effect of calcium supplement on bone mineral accretion in prepubertal children but not in twins who were postpubertal or went through puberty during the study (Johnston *et al.*, 1992). In contrast, 2 other supplementation studies showed an effect of calcium supplement on bone mineral content and bone density of children who were either postpubertal or went through puberty during the study (Lloyd *et al.*, 1993; Nowson *et al.*, 1997). Since that time several other calcium supplementation studies have shown an effect of a calcium supplement or milk on bone mineral status, mostly in prepubertal children (Lloyd *et al.*, 1996; Lee *et al.*, 1995; Lee *et al.*, 1996; Chan *et al.*, 1995, Cadogan *et al.*, 1997).

2.15.2 Calcium supplementation and follow-up studies

To date there have been three supplementation studies in children in which follow-up data have been collected and all these studies indicated that the gain in bone mineral acquired as a result of calcium supplementation is not sustained after cessation of calcium supplement (Lee *et al.*, 1996; Lee *et al.*, 1997; Slemenda *et al.*, 1993). This raises questions about the mechanisms involved. One possibility is that calcium supplement may exerts its effect by slowing down bone remodelling rate rather than by promoting skeletal growth. The process of skeletal remodelling continues throughout life. Serum concentrations of bone formation and resorption markers are at their highest during rapid growth period, to allow greater bone mass accretion. The mechanism of the effect of calcium supplementation on bone mineral is unknown

but one report suggests that calcium supplementation acts by reducing bone formation as shown by reduction in osteocalcin concentration (Slemenda *et al.*, 1993; Johnston *et al.*, 1992). The reduction in osteocalcin concentration possibly reflected a decrease in osteoblast activity.

The disappearance of the supplement effect in follow-up studies also supports the possibility that the calcium supplement may have acted by altering bone remodelling process. A reduction in remodelling space has been shown to result in apparent increase in bone mass (Heaney, 1994) through a process called bone remodelling transient (see section 2.2). Based on the same concept, the low concentration of biochemical markers of bone remodelling in Blacks compared with Caucasians is associated with a superior bone mass (Kleerekoper *et al.*, 1994, Slemenda *et al.*, 1997). There is also a suggestion that decreased remodelling in calcium supplemented children could be a reflection of calcium-mediated suppression of parathyroid hormone secretion (Slemenda *et al.*, 1997) but this has not been proved.

Whether reduced rates of skeletal remodelling during growth have any long term benefit in children accustomed to a low calcium intake is unknown. Therefore, studies are needed to examine the possible mechanism of action of calcium supplements on bone.

2.15.3 Calcium supplementation studies in adults

The effect of calcium supplementation on bone health in adults is an area of great debate and controversy. A number of calcium supplementation studies have found an association between calcium intake and bone mineral density in

adults (Dawson-Hughes, 1991; Elders *et al.*, 1989a; Elders *et al.*, 1989b, Elders *et al.*, 1991) while others failed to show any association (Dawson-Hughes *et al.*, 1989; Recker *et al.*, 1977). An effect of calcium supplement on the reduction of cortical bone loss has been observed in some studies (Polley *et al.*, 1987; Smith *et al.*, 1989; Elders *et al.*, 1994). Similarly, calcium supplementation in elderly women has been shown to reduce bone loss at the hip (Dawson-Hughes *et al.*, 1990; Nelson *et al.*, 1991; Reid *et al.*, 1993; Chevalley *et al.*, 1994; Prince *et al.*, 1995) and distal forearm (Prince *et al*, 1991). Conversely, calcium supplementation during the early menopause has been shown to be ineffective in reducing trabecular bone loss (Riis *et al.*, 1987; Dawson-Hughes *et al.*, 1990; Elders *et al.*, 1994). In postmenopausal women, the response to calcium supplementation appears to depend on menopausal age (Dawson-Hughes, 1991). An association between calcium intake and fracture incidence has been reported (Matkovic *et al.*, 1979). Supplementation with vitamin D and calcium had resulted in a significant reduction in fracture incidence in a large cohort of elderly women (Chapuy *et al.*, 1992).

2.16. Bone remodelling transient

A bone remodelling transient is a complex series of events occurring within the remodelling apparatus of bone that occurs in response to a change in remodelling rate (Heaney, 1994). These events produce a change in the amount of mineralised bone tissue as well as in calcium balance. The process could be best described as temporary imbalance between the amount of bone being removed and the amount being added. The mechanism behind the bone remodelling transient was first postulated by Frost (Frost, 1973) almost 26 years

ago, and tested by Parfitt in a clinical settings (Parfitt, 1980). It is called a transient because the change in bone mass persists for only one remodelling cycle with the bone mass reverting to its original state when the remodelling change is in the opposite direction (Heaney, 1994).

It is thought that most antiresorptive therapies used for the prevention of osteoporosis work by altering bone remodelling balance. How much of the change in bone reported by calcium supplementation studies represents a remodelling transient and how much represents modelling is not clear. The reason for this is that until recently, the concept of the bone remodelling transient has been poorly understood and hard to apply in a clinical situation (Heaney, 1994).

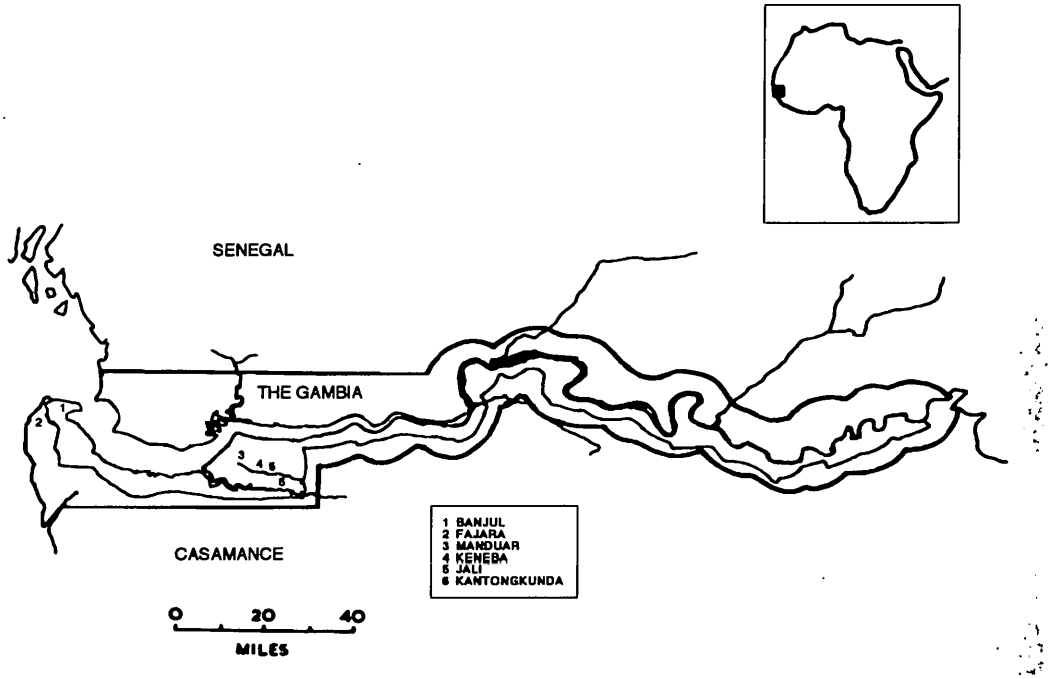
An increased remodelling space results in an apparent loss of bone mass while a reduction in bone remodelling space promotes an apparent increase in bone mass (Heaney, 1994). With the recent advance in technology, it is now possible to monitor the size of the remodelling space of bone by computer simulation which demonstrates that the size of the remodelling transient correlates with the amount of calcium supplement (Heaney, 1994). It is likely that current technologies to measure bone mineral content in vivo also are able to record marked changes in remodelling space given their high precision (Prentice, 1997).

Biochemical markers of skeletal metabolism are thought to reflect both resorption and formation (Kelly *et al.*, 1991; Garnero *et al.*, 1996a; Garnero *et al.*, 1996b; Parfitt, 1994). Because of the tight coupling between bone formation and resorption in a healthy adult, the response of markers to therapies like calcium is

generally regarded as a reduction in the concentration of both formation and resorption markers (Ganero *et al.*, 1994; Ganero *et al.*, 1995). However, in children, these markers are not specific to either modelling or remodelling because of the fact that these processes proceed simultaneously during growth, and the fact that both bone modelling and remodelling involve resorption and formation of bone (Slemenda *et al.*, 1997). Clearly, more studies in children need to include a more detailed investigation of biochemical markers to give insight into mechanisms.

3 Study location and initial design

Figure 3.1 Map of The Gambia showing Keneba and surrounding villages



3.1 Keneba

The study was carried out in the rural subsistence-farming village of Keneba, The Gambia. The Gambia is the smallest country in sub-Saharan Africa, with a population of one million people and occupying an area 30 kilometres wide and 600 kilometres long, either side of the River Gambia.

Keneba is a relatively isolated village situated in the West Kiang district and has a population of 1,800 people. It is 100 miles by road from the capital city

of Banjul, the last 15 miles of which are dirt road. The village is bounded on the south by mangrove swamps, and seven miles to the north by the River Gambia.

Keneba is situated 13^0 20' north of the equator and has two main seasons. The rainy (wet) season begins in July and ends in October and the dry season extends from November to June. The way of life of the people depends on the season of the year. The people are less mobile in the rainy season as this is the time of the year when farmers are busy cultivating their crops and have less money to spend. In contrast, in the dry season, there is sufficient food available and with some money to spend, and men have little or no farming to do. The prevalence of diseases in the West Kiang district is influenced by season. Childhood gastroenteritis and malaria reach their peak prevalence during the later part of the wet season.

3.2 Inhabitants of Keneba

The inhabitants of Keneba are Muslim, predominantly of the Mandinka tribe or descendants of the Jola tribe, with well-defined and distinct roles for men and women according to the Islamic law. The village is divided into compounds which are separated from one and another by a fence, usually made of a variety of local dried plant materials such as millet stalks and occasionally of corrugated iron. People live in compounds defined as a group of families living together where the people in the group are generally related in one way or another. The villagers are mainly farmers. The women are the main work-force in the village and in addition to cooking meals for the family, they are also largely responsible

for the growing and harvesting of the staple food and cash crops. The main cash crop and the major source of dietary protein and fat for the villagers is groundnuts (*Arachis hypogaea*) and is virtually the sole source of income. The staple cereal foods are millet (*Pennisetum typhoideum*), sorghum (*Sorghum margaritiferum*), rice (*Oryza sativa*), suno (*Pennisetum gambiense*) and to a lesser extent, maize (*Zea mays*) and findo (*Digitaria exilis*). Although there are cattle owners, the villagers are predominantly vegetarian. Fish is regularly eaten and is usually purchased from the neighbouring villages of Jali and Tankular.

The diet of children in rural Gambia is based on groundnuts, cereals, and vegetables. The main staple cereals are rice (mano), millet (sanyo), sorghum (kinto), maize (tubanyo) and findo (wild grass). Rice is eaten either as boiled rice or prepared as porridge. All other cereals are eaten as nyelengo, futo or porridge. Boiled rice and cereals eaten as nyelengo and futo are consumed with a sauce, often made with groundnuts (durango). Nyelengo is prepared after washing the grain, pounding and removal of the husk. The flour is steamed with water and dried baobab added during the steaming process. Futo is produced by steaming but the flour is subjected to more numerous steamings than nyelengo. In addition the flour obtained from pounding the grain is sifted to fine powder and a little water is added during stirring to form small balls. The balls are steamed several times to achieve a fine consistency. It is finally removed from heat and pounded again at which time flavourings such as baobab leaf (dried) may be added. Because of the addition of dried baobab leaves during steaming, the calcium contents of nyelengo and futo are higher than other cereal dishes.

Other common methods of preparation include nyankatango, where previously cooked cereal is steamed together with pounded raw groundnuts plus dry fish, locust bean, oil or onion added. Addition of dried fish and locust bean increase the calcium content of nyankatango. Fajiringo refers to a term used for boiling and is usually connected with rice only (manifajiringo). The grains are washed, steamed and then boiled or they may only be boiled without no preliminary steaming. The water is drained off before the rice is fully cooked and it is dried out by steaming. Because of the method of preparation, the water content of cooked rice is high but calcium content low, generally being mostly associated with calcium in the local salt which is added during preparation. Several types of porridge are common in the rural Gambia, depending on method of preparation and consistency. Mono is the thinnest, sato is thickest, while churo refers to a rice porridge of intermediate thickness. All these dishes except mono are eaten with dark green leaves sauces and fish. Dark green leaves, groundnuts and fish are the major source of calcium in the gambian diet (see appendix 7), and special attention needs to be paid to recording the amounts of these in each meal, especially when they are added in small amounts as flavouring, when estimating calcium intakes.

Fruits, groundnuts and vegetables, are common ingredients and the staple diet usually consists primarily of cereals. There is a high prevalence of inadequate intakes of vitamins and minerals, and anaemia and iron deficiencies are common (Bates *et al*, 1993; Bates *et al.*, 1994). The inadequacy of vitamin and mineral intakes may be further complicated by factors which may reduce absorption, which is likely to be poor due to phytate and oxalate content of the Gambian diet (Prentice & Bates, 1993; Bates *et al.*, 1994).

At the time of this study, there was neither electricity nor telephone in the village. Until recently, the main form of communication with the outside world was through the national radio. The first ever television station built in The Gambia was opened two years ago.

All the children in Keneba are breast-fed on demand for 18 - 24 months. Breast-feeding is exclusive for about the first 3 months of life, with traditional weaning foods introduced as a complement after 3 months. All children consume weaning and adult foods by 9 - 12 months. However breast milk remains as a major source of nutrients throughout infancy and early childhood (Prentice *et al.*, 1990).

Previous studies in Keneba have shown that rural Gambia children have a growth pattern that resembles that of Western children in the first 3 - 4 months of life (Hoare, 1994; Weaver, 1994). Growth faltering starts at about the time solid foods are introduced (Weaver, 1994; Prentice *et al.*, 1990; Whitehead & Paul, 1984), caused mainly by repeated illness, particularly, diarrhoea (Rowland *et al.*, 1977; Rowland *et al.*, 1978, Weaver, 1994). Children in Keneba experience severe growth faltering (in terms of both weight and height) during the second six month of life (Prentice *et al.*, 1986; Hoare, 1994). Over half of children in Keneba are below 80% expected weight for age by the time they reach the age of 1 year (Cole, 1993). There is relatively little catch-up once growth faltering has occurred. Introduction of a food supplement aimed at increasing the energy intake of Keneba children failed to improve their growth (Prentice, 1993).

3.3 Effect of season

Food is not abundant at all times of the year and the eating pattern in The Gambia reflects the pattern of food supply. There is a seasonal variation in the pattern of growth of rural Gambian children. Growth is particularly poor during the wet season, when the diet is severely marginal for many nutrients and food stocks from the previous year's harvest are depleted. The problem of food shortages for growth is compounded by increased bacterial contamination of weaning foods (Barrell & Rowland, 1979), reduced standards of mother-care caused by heavy agricultural activities, increased diarrhoeal and other infections, all of which have a detrimental effect on child growth (Rowland *et al.*, 1977; Whitehead, 1979).

In contrast, children grow better in the dry season when there is plenty of food to eat and people are less active due to reduced farming activities (McGregor *et al.*, 1968, Prentice *et al.*, 1981).

The extent to which illness is responsible for growth faltering in Gambian children and its association with inadequate nutrition is still under intensive investigation. In other populations, some studies have shown that up to one third of the total amount of linear growth failure can be attributed to illness (Allen, 1994; Martorell *et al.*, 1975; Martorell & Klein, 1980). Guatemalan children who were free from diarrhoea during the first seven years of their life were 13 cm shorter than well-nourished children in the United States - although 3.5 cm taller than children who had more diarrhoea (Martorell *et al.*, 1975). This study suggests that poor infant growth in the developing countries could be partly related to infection and bacterial contamination of weaning foods (Rowland *et al.*,

1978). Although diarrhoeal infections were thought to be the major causes of growth faltering in rural Gambian children (Barrel & Rowland, 1979; Rowland *et al.*, 1977; Whitehead, 1979), recent reviews on morbidity and mortality have shown that reduction in diarrhoea alone fails to improve growth (Poskitt *et al.*, 1999). This suggests that a combination of undernutrition (Sullivan *et al.*, 1992a; Sullivan *et al.*, 1992b; Lunn *et al.*, 1996) and persistent gastrointestinal disease may be more important (Bunn *et al.*, 1997).

3.4 Low birth weight

Over 50% of children born annually in The Gambia have birth weight less than the World Health Organization cut-off point for low birth weight, of 2500 g (Ceesay *et al.*, 1997). The low birth weight of Gambian babies has been associated with short birth spacing and undernutrition of the mother both before and during pregnancy (Rowland, 1977; Whitehead & Paul, 1984). Provision of a high energy supplement in late pregnancy has been shown to improve the birth weight of Gambian babies (Ceesay *et al.*, 1997).

3.5 The Medical Research Council in Keneba

The study was conducted from the MRC Dunn Nutrition Unit's Laboratory and clinic located in Keneba (recently renamed MRC Keneba). The Medical Research Council (MRC) has had a field station since 1950 in Keneba, which was initiated by Professor Sir I. A. McGregor, the Director of MRC Gambia Laboratory from 1954 - 1973 and 1979 - 1981. Since 1949 all births and deaths in the village of Keneba, and the three neighbouring villages of Kantong Kunda, Manduar and Jali have been recorded by a senior villager, literate in Arabic. In

the early fifties, all the compounds in Keneba were identified and numbered, and this identification was used in the current study.

3.6 The Dunn Nutrition Unit

In 1974 the overseas programme of the Cambridge-based Dunn Nutrition Unit directed by Dr R. G. Whitehead, was transferred to Keneba, The Gambia. Before that Professor Sir Ian McGregor had already highlighted the poor growth performance in the Keneba children (McGregor *et al.*, 1968) and Keneba was an ideal place to conduct scientific research into the aetiology of malnutrition and growth faltering. The infant mortality in the area at that time was ≥ 500 per 1000 live births (McGregor *et al.*, 1968). The initial aim of the programme of the Dunn Nutrition Unit was to identify the nutritional and health related factors that underlay the high mortality, morbidity, and poor nutritional status of the mothers and children in the West Kiang district and to introduce appropriate interventions in a scientifically controlled manner, aimed at improving growth and child health.

Keneba has been the focal point of numerous nutritional and demographic studies by the MRC Dunn Nutrition Unit since 1974. The availability of detailed records of births and deaths of the three study villages which include Keneba over the last 50 years, means that accurate records of birth date are available. Previous studies on growth and calcium requirements of children in Keneba have demonstrated that rural Gambian children have poor growth and low bone mineral status. Their calcium intake is low (300 - 400 mg/d) (7.5 - 10 mmol/d) (Prentice *et al.*, 1990). The availability of this background information on calcium intake and bone growth made Keneba an ideal place to conduct a study to

examine the benefit of an increase in calcium intake on growth and bone mineral development, in children accustomed to a low calcium diet.

3.7. Objectives of the study

3.7.1. Research questions

The main aim of the study was to determine the influence of calcium intake on longitudinal growth and bone mineral development of Gambian children accustomed to low calcium intakes. To do this a randomised, double-blind, placebo-controlled calcium supplementation study was designed and conducted on 160 Gambian children, age 8 - 12 years for 12 months. The study also aimed to provide detailed information about the calcium intake and growth pattern of this age group and to determine the biochemical indices of calcium metabolism before and after calcium supplementation. The age group was chosen to allow recruitment of the target of 160 children required to provide sufficient statistical power (see section 3.8.1 for detail). Because of the delayed puberty of rural Gambian children, most subjects were likely to be prepubertal with some of the older children progressing through to puberty during the study.

3.8 Initial Study Design

This study was designed to be conducted on a group of older Gambian children.

Considerations about an appropriate sample size had to take into account two issues.

1. Sample size needed to be statistically large enough to detect any meaningful change in bone mineral status associated with the calcium supplement over a 12 month period.

2. Recruitment of the sample needed to be achievable within the study period.

In addition, the design of the study needed to take into account the possibility of seasonal effects which had to be minimised. The choice of the calcium supplement also had to be considered in the light of the cost, absorbability and its acceptability.

3.8.1 Sample size and statistical power

Based on statistical considerations, a sample size of 160 children (80 boys, 80 girls) turned out to be an appropriate number. The between-subject coefficient of variation for forearm bone mineral content adjusted for bone width, taken from published data and previous studies in Keneba, is 9% (Johnston *et al.*, 1992; Prentice *et al.*, 1990; Lo *et al.*, 1990; Prentice *et al.*, 1995). A sample size of 160 children provided a treatment group size of 40 in each sex and 80 with sexes combined. This has statistical power to detect a supplement effect on bone mineral status equal to or greater than 5% and 4% respectively (using α = 0.05, 1 - β = 80%). Therefore, a sample size of 160 children (40 boys, 40 girls) in each of the supplemented and placebo groups was expected to give sufficient statistical power to detect meaningful differences in bone mineral status associated with calcium supplementation which are similar to those reported in previous calcium supplementation studies of the same age group in American and Chinese children (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lee *et al.*, 1994). This study was conducted to examine the effect of increase calcium intake in

older children who were pre- or peripubertal. The birth rate of Keneba is such that recruitment of 160 children between the age of 8 - 12 years should be possible over one calendar year.

Calculation of sample size for this study was based on the following equation:

With 40 subjects in each of the supplemented and placebo groups

$$2(1.96 + 0.842)^2 x 9^2/r^2 = 40$$

$$r = 5.64$$

where 1.96 and 0.842 are standard errors at $\alpha = 5\%$ and $\beta = 20\%$ respectively (Florey, 1993) and r = percent change ($\Delta\%$) in bone mineral content. Nine is the calculated between-subject coefficient of variation for forearm BMC adjusted for BW from published data and previous studies in Keneba (see begining of section).

$\therefore$ $r = 5.65\%\Delta$ can be seen at $\alpha = 5\%$ and $\beta = 20\%$.

and for n= 80 in each group,

$$2(1.96 + 0.842)^2 * 9^2/r^2 = 80$$

$$r = 4.00$$

$r = 4\%\Delta$ can been seen at $\alpha = 5\%$ and $\beta = 20\%$.

3.8.2 Effect of season

In order to account for differences due to season, recruitment of subjects was spread over 12 months. To achieve this and to ensure all measurements are completed within a week, the aim was to recruit 4 subjects each week.

3.8. 3 Choice of calcium supplement

Calcium supplementation studies generally use calcium salts especially calcium citrate malate or calcium carbonate (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lee *et al.*, 1994). However, calcium citrate malate is expensive compared with calcium carbonate and this has limited its use in supplementation studies. Calcium carbonate, in the form of palatable orange flavoured chewable tablets, was selected in preference because of its low cost.

Studies comparing calcium carbonate and calcium citrate malate have had conflicting results in adults. Some studies demonstrated no differences in the fraction absorbed between the two calcium salts whether given with meals or under fasting conditions (Smith *et al.*, 1987; Recker, 1985). In contrast, calcium citrate malate have been shown to be better absorbed than calcium carbonate in a number of studies of healthy subjects (Miller *et al.*, 1988; Nicar & Park, 1985; Harvey *et al.*, 1988).

However, results from a calcium supplementation study in children using calcium carbonate (Lee *et al.*, 1994) demonstrated a similar effect of supplement on bone mineral status to studies using calcium citrate malate (Johnston *et al.*, 1992; Lloyd *et al.*, 1993), thus justifying its use in this study. Also calcium carbonate tablets used in a previous calcium supplementation study in Keneba, among lactating women were well accepted by villagers (Prentice *et al.*, 1995).

3.8. 4 Main outcome

The main outcome of the study was forearm bone mineral content and was measured using single photon absorptiometry (SPA). The same technique has been used in two earlier calcium supplementation studies (Johnston *et al.*, 1992; Lee *et al.*, 1994) and is sensitive enough to detect changes in bone mineral status at mid-shaft radius within a duration of 6 - 18 months (Johnston *et al.*, 1992; Lee *et al.*, 1994). A similar duration of supplementation was used in this study. Bone mineral status of the study children was assessed at baseline, at the end of supplementation (outcome) and 12 months after supplementation (follow-up), in order to determine the effect of supplementation on bone mineral accretion rate and whether any effect on bone is sustained after the withdrawal of supplement.

3.8. 5 Biochemical assessment

Blood and urine tests to measure the effect of calcium supplement on biochemical markers of calcium and bone metabolism were also performed in order to give an insight into mechanism. Changes in bone mineral status have been shown to correlate with changes in biochemical markers in the blood and urine, especially osteocalcin, parathyroid hormone, urinary calcium and deoxypyridinoline (Johnston *et al.*, 1992; Sebastian *et al.*, 1994; Matkovic *et al.*, 1990; Matkovic, 1992; Matkovic *et al.*, 1995; Lian & Gundberg, 1988; Ismail *et al.*, 1986; Canalis *et al.*, 1989; Christenson, 1997; Seibel *et al.*, 1993).

3.8.6 Dietary calcium intake

To quantify calcium intake in this age group of Keneba children, a 2 day weighed intake was used. A study comparing group mean values obtained using a 7-day with 2-day weighed intakes in Keneba had demonstrated no difference between the two because of the relative homogeneity of diet in rural Gambia (Prentice *et al.*, 1995; Prentice *et al.*, unpublished data).

3.8.7 Comparative study

A comparative study of age and sex matched Gambian and British children was conducted to examine whether differences in growth and bone mineral development between The Gambian and British infants (Prentice *et al.*, 1990) were also observed in older children.

3.9 Study Design

Table 3.1

	calcium supplementation		
	Baseline	year 1	Year 2
SPA bone measurements	x	x	x
Ultrasound bone measurements	x	x	x
Height, weight, size, grip	x	x	x
Blood sample	x	x	x
24h urine sample	x	x	x
Dietary measurements	x	x	x
Questionnaire	x	x	x

3.10 Scientific and ethical approvals

The investigation was approved by MRC Scientific Coordinating Committee and MRC Gambian Government Ethics Committee. Informed-consent was obtained after the children and their parents, most of whom were illiterate, had been given verbal explanation of the study in their own language (see appendix 1 for detail). The consent form was signed or marked with thumb print by one of the child's parents before the study began.

4 Materials and Methods

4.1 Subjects

One hundred and sixty healthy Gambian children (80 boys and 80 girls) aged 8.3-11.9 years from the village of Keneba, West Kiang district, The Gambia, took part in the study. All the subjects were born and lived in Keneba. Most of the children were Mandinka but four children were from a Fula tribe. The subjects were healthy children with no history of any medical condition known to affect calcium and bone metabolism or of recent bone fractures. None were taking any calcium supplement or antacids at study entry, none smoked or consumed alcohol, and none of the girls was on contraceptive medication.

All subjects were measured three times (at baseline, outcome, and follow-up). Both outcome and follow-up measurements were conducted in each child as far as possible during the same calendar week as baseline measurements. Baseline measurements were conducted in the week prior to the start of supplementation, outcome measurements were made after 12 months (385 ± 41 days), in the week before the supplement was withdrawn, and follow-up measurements were conducted 12 months after supplementation was finished and 24 months after baseline measurements were made (752 ± 27 days).

At each time point, each subject visited the MRC Keneba Laboratory (formerly MRC Dunn Nutrition Group), where bone measurements were made and anthropometry performed. Each subject provided a fasting blood sample collected between 6.30 a.m. and 7 a.m. to avoid introducing errors due to diurnal variation, and each completed a 24 hour urine collection. All subjects completed a questionnaire on lifestyle and calcium intake and a 2-day weighed dietary intake.

4.2. Calcium supplementation

Subjects were stratified by sex and randomised, double-blind, to receive the calcium supplement or a placebo. The randomisation procedure was conducted by a member of staff in Cambridge who was otherwise not involved in the study. The subjects, field and lab staff remained unaware of the allocations throughout the study. Allocations to supplementation group were by randomised permuted block of 4 to ensure that an equal number of subjects were allocated to the supplement and placebo groups each fortnight, to minimise the potential for seasonal confounding. Four children started the study each week to allow recruitment to be spread over one calendar year. All measurements per subject were completed within a 7 day period.

The calcium supplement consisted of two chewable calcium carbonate tablets (Calcichew, Shire Pharmaceuticals Ltd, Andover, UK and Nycomed Pharma Oslo, Norway), containing 500 mg (12.5 mmol) elemental calcium per tablet. The placebo consisted of two tablets of similar shape, taste and texture, produced by the manufacturer of the calcium tablets. Each subject received either the calcium supplement or the placebo, 5 days each week for 12 months, starting the week following baseline measurements. The tablets were dispensed to the subjects at a centrally-located building in the village and were consumed under strict supervision. To achieve regular attendance, a youth club was organised every week-day evening. For children who were unable to attend the youth club for any reason, tablets were dispensed at the child's home by the fieldworkers on the same day. The tablets were administered away from food, in the early evening, between 17.00h - 19.00h. This allowed the supplement to be

consumed between lunch, generally eaten at 14.00h - 15.00h, and dinner, eaten around 20.30h, to minimise possible interference with absorption of other minerals, such as iron and zinc. During the fast month of Ramadan, tablets were consumed later in the evening immediately after the subject had broken fast but before the main meal. Supplements that were missed due to illness or absence from the village were consumed at weekends. The tablets were well accepted, there were no reports of adverse side effects and the overall compliance for each child was 100% in that by the end of the 12 months supplementation period every child had consumed all the tablets that had been assigned to them at the start of the study.

4.3 Anthropometry

Each subject was weighed to the nearest 0.1 kg, in light clothing without shoes. Standing height without shoes was recorded to the nearest 0.1 cm with a stadiometer (CMS Weighing Equipment Ltd, 18 Camden st, London, UK). This requires the subject to stand upright with heels and shoulders against the support, knees and back straight and the head in the Frankfort horizontal plane. Mid-upper arm circumference (MUAC) was measured using a graduated tape and triceps skinfold thickness was assessed using calipers. The mid-upper arm circumference was measured with the arm hanging by the side at the mid point of the arm, measured from the lower border of the acromial process of the scapula to the tip of the olecranon and parallel to the long axis of the arm. The mean of the three measurements was used.

All anthropometry measurements were made on the same day that the subject was scanned.

4.4 Grip Strength

All subjects had their grip strength measured on both hands with a hand grip dynamometer (CMS Weighing Equipment Ltd, London). The subject remained standing during the measurement with the arm straight by the side, resting between each measurement and the next. Three measurements on each hand were carried out and the mean of the 6 measurements was used in the data analysis.

4.5 Lifestyle assessment

A questionnaire was designed to look for possible confounding factors which could confuse the interpretation of any supplement effect or which could explain differences in the growth pattern and calcium intake of children who took part in this study.

Information concerning place of birth, socio-economic grouping, physical activity levels, school enrolment, education attainment, medical and fracture history were obtained using a questionnaire. Additional information on medication was also obtained (see appendix 2).

Families were ranked (A) poor, (B) average or (C) well-off according to whether (A): they experienced food shortages during the wet season, had one or more thatched houses, had no radio or cassette player, both of the parents were farmers with a normal or low yield or the father was a herdsman and there was no wage earner in the family, (B): had no thatched house in the compound but experienced food shortages in the wet season, had parents who were farmers but usually had good yields, came from a family that owned a radio and/or

cassette player, did not have a herdsman father but had no employee in the family or (C) had sufficient food to support the family throughout the year, no thatched houses in the compound and had one or more wage earners in the family.

Nineteen families were considered poor according to these criteria (Category A, 12%), one hundred and twenty-seven children came from families with average earning power (category B, 79%), and 14 were ranked as well-off (category C, 9%). There was no indication of a difference in the distribution of children in the supplemented group or placebo group according to socio-economic group (supplement : placebo, 49% versus 51%). For more details of the outcome of the questionnaire, see appendix 3.

4.6 Activity

The activity patterns of the children were investigated by questionnaire to determine if the supplemented and placebo groups differed in their physical activity (see appendix 4). Although the physical activity questionnaire was based on local customs, all the children were shown to be active and the questionnaire lacked discrimination. There was no indication of a difference in activity patterns between supplemented and placebo groups.

4.7 Urine

A 24-hour urine collection was obtained from each subject at the three timepoints (baseline, outcome, and follow-up) for the determination of daily urinary output of minerals, markers of bone turnover. Urines were collected

during the same week that bone measurements were made. All containers for urine collection and processing were acid-washed prior to use. Three two-litre plastic containers were provided for each collection, and taken to the subject's home. Verbal instructions were given to each subject about the collection with advice as to how to avoid contamination as far as possible. To preserve the urine, the subjects were provided with an insulated "cool bag" in which the urine bottles were surrounded by ice packs. Filled urine bottles were collected during the day and placed in the laboratory refrigerator. Urine samples were processed within a few hours of finishing the collection. Urine from the collection bottles was pooled and the total volume of urine collected from each child was recorded after thorough mixing. Two 20 mls aliquots were acidified with 200µl concentrated hydrochloric acid (HCl spectrosol) and frozen at -20°C. This method had been shown in previous studies to be successful in preventing the deposition of calcium-containing precipitates during storage (Prentice *et al.*, 1995).

4.8 Blood sampling

A venous blood sample was collected from antecubital veins in each subject at the three timepoints (baseline, outcome and follow-up). Samples were taken at the MRC Keneba Laboratory (formerly MRC Dunn Nutrition Laboratory). All samples were collected by venepuncture after an overnight fast (6.30 a.m. and 7 a.m.) with the subject lying down. Approximately 10 ml blood were collected into a lithium heparin monovette and about 2.5 ml into an EDTA monovette, avoiding contamination as much as possible. The blood was kept cold throughout the collection on ice. Samples were spun in a refrigerated

centrifuge and stored at -20°C and within 45 minutes after collection. They were transported to Cambridge and subsequently stored at -80°C.

4.9 Pubertal assessment

Pubertal status of subjects was assessed according to Tanner staging. The assessment of the pubertal status of the girls, based on breast (B) and pubic hair (P) development was carried out by a female paediatric consultant. The pubertal status of the boys was assessed by myself, and was based on genital (G) and pubic hair (P) development. For boys, Tanner stage I suggests pre-puberty, stage II and III are regarded as early puberty, stage IV is advanced puberty, and stage V indicates the end of puberty. For girls, a pubertal growth spurt occurs as breast development reaches Tanner stage II.

However, because of the fact that most of the study children were prepubertal at the end of the study, subjects were grouped as either prepubertal or pubertal. For detail of the method used in this study, see appendix 6.

4.10 Dietary measurements

4.10.1 Weighed intake

Dietary intake at baseline was determined by 2-day direct-weighing in all but one subject (Prentice *et al.*, 1993). Subjects were visited several times in the day during the measurement period starting before breakfast and ending after evening meal. At each visit, all food items consumed and any leftovers were weighed and recipes for all dishes recorded. Consumption of snacks

during and between meals was determined by recall at the next visit. Computation of nutrient intakes was carried out using "Gambian Dido" and 'MW1N4' software programmes (Price & Thompson, 1995) based on McCance and Widdowson's food composition data (Paul and Southgate, 1978), supplemented by information on the composition of Gambian foods (Prentice *et al.*, 1993). The additional information on dietary intake was achieved by analysing raw ingredients and cooked dishes, taking into account foods that are high in calcium but are either eaten in small quantity or infrequently, such as certain vegetables and a small type of fish which is eaten with bones (Prentice *et al.*, 1993). Drinking water in Keneba has only a low concentration of calcium (10 mg/L) (Prentice *et al.*, 1995) and was not quantified. The calcium contents of typical Gambian foods are presented in appendix 7.

4.10.2 Food frequency questionnaire

Dietary intake assessment from weighed-records was supplemented with a calcium frequency questionnaire to give an insight into eating habits over the long-term, and covered the common calcium-containing foods consumed by the subjects. Examples were leaf sauce, baobab fruit and leaf on its own, fruits collected from the bush, milk, fresh and fermented locust bean (appendix 5).

4.11 Bone mineral measurements

4.11.1 Single photon absorptiometry (SPA)

Measurements of bone mineral content (BMC), bone width (BW) and bone mineral density (BMD) at the mid-shaft and distal-radius of the left arm were made using single photon absorptiometry (Lunar SP2 scanner, Lunar Radiation Corporation 313 W. Beltline HWY, Madison, WI 53713, U.S.A). Measurements were made on the left arm unless this had previously been broken, in which case the right was used. The region scanned was encased in a tissue equivalent mould.

The forearm measurement was made at the mid-shaft radius (33% or 1/3 distal site on the ulna). This site was determined by measuring the distance between the mid-point of the ulnar styloid process and the proximal edge of the olecranon and marking the position which is 1/3 of this length from the ulnar styloid process. The instrument made three transverse scans across the forearm at the same position and the mean of the three scans was recorded.

The wrist measurement was made at the 5 mm distal site, defined as the site where the inter osseous (IO) space (distance between the radius and ulna) is 5 mm. This site was identified by performing a rectilinear scan with a step distance of 1.5 mm and using the value where the IO space was closest to 5 mm but between 4 and 6 mm. For both the forearm and wrist scanning speed was 1.0 mm/sec. The area scanned was encased in a tissue equivalent mould. The total set of scans took about 15 minutes to perform for each subject. Bone

mineral content (BMC, g/cm for SPA) was expressed in grams and divided by the bone width (BW, cm) to derive the bone areal density (BMD) in g/cm^2.

4.11.2 Calibration

The instrument was calibrated daily and the long term stability was assessed by measuring phantoms. The overall coefficients of variation over the study period for BMC, BW and BMD of the small phantom (BMC = 0.374 g/cm) were, 1.11%, 0.94%, and 0.82%, and of the large phantom (BMC = 1.196 g/cm) were, 0.52%, 0.41% and 0.53% respectively. These indicate good stability of the measurements over the entire study period.

Table 4.1 Mean and coefficient of variation for BMC, BW, and BMD of small and large phantoms for 1210 individual measurements performed over the 24 months study period.

	Mean	CV(%)
Small		
BMC (g/cm)	0.374	1.11
BW (cm)	0.860	0.94
BMC/BW (g/cm^2)	0.435	0.82
Large		
BMC (g/cm)	1.196	0.52
BW (cm)	1.423	0.41
BMC/BW (g/cm^2)	0.841	0.53

4.12.1 Ultrasound measurements

Broadband ultrasound attenuation and velocity of sound of the left heel were assessed using computer-controlled bone ultrasonography of the calcaneum (CUBA Clinical, McCue, Parsonage Barn, Compton, Hampshire,

SO21 2AS, England). Measurements were performed with the subject's heel positioned between two ultrasound transducers using a gel to ensure coupling between the heel and transducer. A complete series of measurements on each foot took approximately 5 minutes to complete. The results are given in velocity of sound (VOS), a measure of the quality of bone, and broadband ultrasound attenuation (BUA) a measure of the mineralisation of the bone (Brandenburger, 1993; Miller *et al.*, 1993, Moris *et al.*, 1995). Normalised BUA (nBUA), i.e, which takes into account the width of the heel, and velocity of sound (VOS) were used in the data analysis in this study.

4.12.2 Calibration and quality control assessment

The instrument was calibrated daily as in SPA measurements and its stability assessed by measuring range of phantoms provided by the manufacturer.

Table. 4.2 Mean and coefficient of variation for VOS and BUA of all phantoms measured over the study period.

Phantom	Mean	CV (%)	no. of scans
Low BUA1†	39.8	1.26	881
Low BUA2	34.3	0.91	987
Low BUA3	26.7	1.51	438
Medium BUA	60.2	1.42	183
High BUA1	65.5	0.69	1209
High BUA2	58.5	0.61	78
Velocity (VOS)‡	2723	0.40	1098

† Unit of broadband ultrasound attenuation (BUA) is dB/MHZ, ‡velocity of sound (VOS) is in metre/second (m/s).

Calibration of the instrument was performed 2 - 3 times weekly over the study period. Table 4.2 summarises the performance of the instrument over the

36 month period. The results indicated that the machine had attained a reasonable stability during this period.

In vivo reproducibility of the instrument was also determined using a volunteer's foot which was measured weekly. The coefficients of variation (CV) over the study period for VOS and BUA were, 0.65% and 2.10% for VOS and BUA respectively.

4.13. Comparative study

Comparative data were provided from a study conducted by Dr Mary Fewtrell in Cambridge. Subjects were 85 British (45 boys, 40 girls) children aged 8.6 - 11.99 years living in Cambridge, UK. The subjects gave informed written consent to take part in the study. Bone measurements were made with a Lunar SP2 scanner (Lunar Radiation Corporation 313 W. Beltline HWY, Madison, WI 53713, U.S.A) at the same skeletal sites as for the Gambian children. Cross-calibration of the two instruments was made using phantom materials in previous study (Prentice *et al.*, 1995) and gave good agreement. In addition, weight and height measures in the two studies were compared.

4.14 Blood assays

These were conducted by staff of the Bone Indices Laboratory, MRC Human Nutrition Research, Cambridge (formerly MRC Dunn Nutrition Laboratory). Plasma samples for all three timepoints for each subject were analysed together in a randomised order in a single run to avoid bias. A similar method of analysis was used for biochemical markers in urine.

4.14.1 Osteocalcin

Plasma osteocalcin concentration was determined using an immunoradiometric assay (N-TACT Osteocalcin, INCStar Corporation, Stillwater, USA). The method involved the addition of rabbit anti-bovine osteocalcin antibody and ^{125}I bovine osteocalcin to the sample. The mixture was incubated at 2 - 8°C overnight, washed several times with wash buffer, followed by the addition of a complex goat anti-rabbit serum, carrier rabbit serum and polyethylene glycol. The mixture was centrifuged and decanted after a 2 hour incubation at 2 - 8°C. The radioactivity present in the precipitate of each tube was measured using a gamma scintillation counter (1470 Wizard, Wallac Oy, TurKu, Finland). The concentration of osteocalcin present in the sample is inversely proportional to the radioactivity measured.

Between-run precision of the assay was 6% during the study period for a control sample with a concentration of 26 μg/L, measured on each occasion in duplicate.

4.14.2 Plasma Total Alkaline Phosphatase (TAP) and Bone Specific Alkaline Phoshatase (BSAP)

Plasma immunoreactive bone specific alkaline phosphatase was determined using a precipitation method using lectin (Boehringer Mannheim Company). After the total alkaline phosphatase had been determined, bone-specific alkaline phosphatase precipitated using lectin supplied by the commercial kit from Boehringer Mannheim company. The enzyme activity was measured by colorimetry using a Cobas Bio centrifugal analyser (Roche Diagnostica, Switzerland).

4.14.3 Parathyroid hormone (PTH)

Plasma intact parathyroid hormone was determined using an immunoradiometric (IRMA) assay (Incstar Corporation - Stillwater, Minnesota, USA). Only the intact PTH and PTH fragments contain the amino acid sequence (1 - 34) with biological activity. It is this active component of PTH that correlates with calcium nutritional status, and only the intact PTH was measured in this study. The Incstar intact PTH SP IRMA utilises 2 distinct polyclonal antibodies that have been purified using affinity chromatography. The purified antibodies are specific for 2 different regions of the PTH molecules. The first antibody is specific for 39 - 84 amino acid sequence and is bound to a solid phase (Polystyrene beads). The second antibody is specific for the amino acid sequence 1 - 34 and is labelled with ^{125} I. Samples are incubated simultaneously with both antibodies. Intact PTH 1 - 84 contains both the 1-34 and the 39 - 84 amino acid sequences and hence will be bound by both the antibody bound to the solid phase and antibody labelled with iodine-125. Since the antibody coupled to the solid phase is specific for the C - terminal and mid-region fragments in addition to the intact PTH, the capacity of the solid phase is designed to accommodate high concentrations of PTH. This prevents interference with elevated C - terminal and mid-region PTH fragments in measured sample. After incubation, each bead is washed to remove any unbound labelled antibody. The radioactivity present in the remaining bound labelled antibody is measured using a gamma counter (Wallac, Finland). The concentration of intact PTH present in the sample is directly proportional to the radioactivity measured.

4.14.4 Plasma Calcium (Ca), Inorganic Phosphorus (P), Albumin (Alb) and Creatinine

Plasma calcium, creatinine, and inorganic phosphate were measured by colorimetry using a Cobas-Bio centrifugal analyser (Roche Diagnostica, Switzerland). Blood creatinine was measured using a kinetic, buffered Jaffé method without deproteinisation where creatinine reacts with picric acid in alkaline solution to form a yellow-red coloured complex. The rate of colour formation is directly proportional to the creatinine concentration.

Plasma albumin was analysed using a specific immunonephelometric procedure on a centrifugal analyser (Cobas-Bio).

Plasma calcium concentrations were determined using the methyl thymol blue method. The blue-coloured chelate formed by the reaction of calcium ions with methyl thymol blue is directly related to calcium concentration in the plasma. Because up to 80% of plasma calcium is bound to albumin in the blood, serum calcium was corrected for albumin content according to the method described by Willatts (Willatts, 1987).

Albumin-adjusted Ca concentration (mmol/L) = measured Ca concentration (mmol/L) + 0.1 mmol/l for every 4 g/l albumin below 36 g/l, or measured Ca concentration - 0.1 mmol/l for every 4 g/l albumin above 36 g/l.

Inorganic phosphate is measured using phosphomolybdate reaction in which phosphate ions react with ammonium molybdate in acid solution to form unreduced phosphomolybdate complex. The complex thus formed is determined by spectrophotometry.

4.15 Urine assays

These were performed by staff of the Bone Indices Laboratory, MRC Human Nutrition Research, Cambridge (formerly MRC Dunn Nutrition Laboratory).

4.15.1 Urinary Deoxypyridinoline (D-pyr)

Urinary immunoreactive free deoxypyridinoline crosslinks was measured by competitive enzyme linked immunosorbent assay (Pyrilinks-D, Metra Biosystem mountain view, CA, USA) using an ELISA (cups) technique. The method is a competitive enzyme immunoassay, and utilise a monoclonal anti- D-pyr antibody coated on a microtitre stripwell to capture D-pyr. D-pyr in the sample competes with conjugated D-pyr -alkaline phosphatase for the antibody and the reaction detected with a p-nitrophenyl phosphate substrate. The results were expressed as a ratio to urinary creatinine concentration.

Samples, standards and controls were diluted by 1:10 prior to assay. 50 µl diluted standards, controls and samples were then added to strips coated with the anti-D-pyr antibody, 100 µl of the reconstituted enzyme conjugate was added to each well. The strips were incubated for 2 hours at 2 - 8 °C in the dark. The strips were washed three times, 150 µl of the substrate solution was added and incubated for another 60 minutes at room temperature. This was followed by adding 100 µl of stop solution to each well, and the optical density of each well was measured in a microplate reader at a wavelength of 405 nm.

4.15.2 Creatinine (Cr), Calcium (Ca) and Inorganic phosphorus (P)

The concentration of creatinine, calcium, and inorganic phosphate in urine were assayed by colorimetric methods (Cobas-Bio) using centrifugal analyser kits (Roche Products, Welwyn Garden City, Uk.). The methods used for determination of urinary creatinine, calcium, and phosphorus were the same as those used for their blood counterparts.

4.15.3 Sodium (Na) and potassium (K)

Urinary sodium and potassium were measured by flame photometer (IL 943 Flame Photometer, Instrumentation Laboratory, Italy). In a flame photometric measurement, when a substance is exposed to a high temperature, it becomes excited through thermal collision. In the excited state, sodium and potassium atoms emit light with a characteristic set of wavelengths. The intensity of the light emitted is directly proportional to the number of atoms undergoing the transition. The method allows the concentration of Na and K in a sample to be measured directly against a standard solution.

In this study, all urines were diluted prior to analysis using 1% hydrochloric acid. Samples were thoroughly mixed and allow to stand for 5 minutes until all the sediment had settled. 250 µl of the diluted urine was measured by flame photometry. Quality-assurance materials (Lyphochek Normal Urine Control, Bio-Rad, Anaheim, CA, Roche Control Serum N, Roche controls, N & P, Switzerland) were included with all batches of samples to monitor precision and accuracy. All values of the controls used in blood and urine assays were within the expected range.

5 Principles of bone and statistical methods

5.1 Single photon absorptiometry

The principle of absorptiometry is based on the exponential attenuation of penetrating photons as they pass through tissue or similar medium (Cameron & Sorenson, 1963; Mazess, 1971; Wahner *et al.*, 1983; Prentice *et al.*, 1994). When a monochromatic beam passes through a region of the body consisting of two tissues, the intensity of the emerging beam is related to the initial intensity as follow:

$$\ln(I/I_o) = - \mu_a m_a - \mu_b m_b \qquad (1)$$

Where I = final intensity, Io = initial intensity, μ_a =mass attenuation coefficient of tissue a, m_a = mass of tissue a, μ_b = mass attenuation coefficient of tissue b, m_b = mass of tissue b. The mass attenuation coefficients are constant that are characteristic of the tissues at the energy of the photon beam.

The technique of single photon absorptiometry (SPA) was first described by Cameron and Sorenson (Cameron & Sorenson, 1963). With this method, bone mineral content is determined by measuring the transmission of a monoenergetic photon beam from I^{125} (27.5 keV) through bone and soft tissue. The amount of mineral in the path of the photon beam is related to the transmission rates I^*_o and I according to the equation below.

$$m_B = \frac{p_B \ln(I^*_o/I)}{(\mu_B p_B - \mu_s p_s)}$$

In this equation, m_B is the mass of mineral per unit area (g) in the path of the photon beam, μ_B and μ_s are the mass absorption coefficients (cm^2/g) of bone mineral and soft tissue, respectively, and p_B and p_c are their densities. I^*_o is the

beam intensity through tissue outside the bone, and I is the beam intensity after passage through bone and tissue.

5.1.1 Radiation dose

The measurement of bone mineral using single photon absorptiometry involves exposure of the subject to small amounts of ionising radiation (I^{125} gamma-rays) with a maximum strength of 7.4 Gbq (200 millicurie, (mCi)) and a half life of six months. Effective doses for children, which take account of the vulnerability of different tissues to radiation and the penetrating power of the specific radiation energies used for a set of mid-shaft and wrist measurements were calculated to be 0.04 μSv (Dr Philip Dendy, personal communication). The maximum ED for each subject can be compared to typical effect dose of natural background radiation in the Cambridge area (ED = 6 - 7 μSv/day), which is likely to be lower than that in The Gambia, or to a return transatlantic flight in a jet aircraft (ED = 80 μSv), or a standard AP x - ray film of the lung (ED = 50 μSv) (Kalender, 1992).

Table. 5.1.1 Effective dose for shaft and distal radius for both children and adults are presented.

	Adult μSv	Infant μSv
Mid-shaft (3 X1 line scan)	0.09	0.54
Wrist 5 mm site (5 X 1 line scan)	0.18	-
Total	0.27	0.54

5.1.2 Expression of bone mineral measurements

The basic principle of absorptiometric measurements depends on the attenuation of beam of penetrating gamma or x-ray energy photons across the scan region. The attenuation between bone edge is converted into bone mass using calibration materials supplied by the manufacturer.

Bone mineral measurements are commonly expressed as bone mineral content (BMC) and bone mineral density (BMD). However, BMC has more than one unit and has different interpretation depending on the method used. In single photon absorptiometric (SPA) measurement, BMC is defined as bone mineral content (BMC) per unit length of bone and the unit is g/cm. In contrast, in dual energy x-ray absorptiometric method, the results are expressed as bone mineral mass per area scanned and the unit is in gm. This is also called BMC. There have been suggestions that the latter should be called bone mineral mass (BMM) instead of bone mineral content (BMC), to distinguish between the two methods (Jonson, 1993), but the term is yet to be adopted widely.

The other variable commonly used in absorptiometric measurements is an areal bone mineral density (BMD, g/cm^2) expressed as the amount of mineral (grams) divided by bone width (BW, cm) for SPA, and bone area (BA, cm^2) for DXA. These BMD values are not true volumetric density but areal density measurements because absorptiometry cannot measure the depth of bone in the scan path. Secondly, the technique is unable to distinguish between bone and other tissues within the area regarded as bone (Prentice *et al.*, 1994).

Expression of bone mineral measurements as BMD provide information about differences in bone size between individuals, comparison of individuals

with reference populations (Prentice *et al.*, 1994, Jonson, 1993), and is a useful index in the assessment of fracture risk between individuals of different bone and body sizes in the clinical setting.

Expression of bone data as BMD assume that BMC is directly proportional to BMD multiplied by BW, in other words that BMC = k.BMD where k is a constant called BW. However, there is no evidence that BMC is directly proportional to BW, as an increase in BMC is not matched by an equal increase in BW. In many studies, a significant association exists between BMD and BW, showing that the calculation of BMD does not completely correct the variation in BMC due to differences in BW (Prentice *et al.*, 1994).

The relationship between BMC and BW is rather complex and depends on the population and the skeletal site measured. When BMC is not directly proportional to BW, differences in BMD between individuals within a population will be partly due to differences in bone size between individuals. Therefore use of BMD in this case will lead to spurious associations of BMD with other variables such as calcium, grip strength and age, as each of these variables relates with BMC. A procedure to minimise such problems is to adjust BMC for BW, body weight and height using multiple regression models (Prentice *et al.*, 1994). Correct adjustments for bone and body size are important in evaluating the relative BMC for size, otherwise using BMD could result in errors of interpretation, particularly the possibility of reporting artifactual relationships with other size-related variables (Cole & Prentice, 1992; Prentice *et al.*, 1994).

The coefficients and SE's of the relationships between BMC and BW (bone variables were converted to natural logarithms and multiplied by 100) for shaft and distal sites, calculated using baseline data obtained during the study are shown in Tables 5.1.2a and 5.1.2b below.

Table. 5.1.2a Coefficient and SE's for the regression of lnBW on lnBMC for the two sites measured at baseline, for all subjects, boys and girls separately.

	all subjects (n = 160)		Boys (n = 80)		Girls (n = 80)	
	coefficient	SE	coefficient	SE	coefficient	SE
shaft	1.29[a]	0.09	1.24[a]	0.12	1.35[a]	0.13
wrist	1.81[a]	0.13	1.71[a]	0.18	1.90[a]	0.20

Correlations of BMC and BW were; shaft, all subjects, r = 0.76, boys, r = 0.75, girls, r = 0.76; wrist, all subjects, r = 0.74, boys, r = 0.74, girls, r = 0.74. All correlations were significant at p≤0.0001. Significance of difference of power coefficient from 1 (t = [coefficient - 1]/SE) [a] p≤0.0001

The variables BMC and BW were converted to natural logarithms to investigate proportional differences. Analyses were performed using multivariate regression analysis of ln BMC against ln BW (Data Desk 4.1).

There was a highly significant correlation between bone mineral content (BMC) and bone width (P≤0.0001). At both sites, the coefficient of the regression of ln (BMC) on ln (BW) was significantly different from one (Table 5.1.2a) indicating that BMC was not directly proportional to BW but was proportional to BW raised to power 1.3 (mid-shaft) and 1.8 (wrist). Thus in this study full correction for BW was performed in regression models since BMD would not have removed the interference of bone size completely.

Some studies have used body mass index (weight/height^2) to adjust bone measurements by single photon absorptiometry, dual photon absorptiometry,

and dual energy x-ray absorptiometry in an attempt to normalise bone mineral data (Elders *et al.*, 1989b; Pocock *et al.*, 1989; Murphy *et al*, 1994). However, concerns have been raised about the appropriateness of adjusting bone mineral for BMI (Cole & Prentice, 1992), because many studies have shown that measurements of BMC and BMD are positively and independently, correlated with body weight and height (Nilas *et al.*, 1986; Prentice *et al.*, 1991). This suggests that BMC and BMD are predicted by body size and not by adiposity. Therefore use of BMI when adjustment for body size is required could lead to dubious relations appearing or genuine relations being obscured. It has been suggested that both BMC and BMD should be adjusted for both weight and height independently in a multiple regression (Cole & Prentice, 1992).

Table 5.1.2b Coefficient and SE's for regression of weight and height on BMC for mid-shaft and distal radius measured, at baseline for all subjects and boys and girls after adjusting for BW.

	All subjects		Boys		Girls	
	coefficient	SE	coefficient	SE	coefficient	SE
shaft						
weight	2.88[a]	0.32	0.85[a]	0.12	0.71[a]	0.11
height	2.22[a]	0.23	2.25[a]	0.35	2.17[a]	0.32
distal						
weight	1.13[a]	0.10	1.26[a]	0.14	1.05[a]	0.14
height	2.78[a]	0.31	3.03[a]	0.44	2.59[a]	0.43

All data were transformed to natural logarithms prior to analysis. [a] $p \leq 0.0001$ for relationship with BMC.

To overcome problems associated with the use of BMD and to carry out comparisons between groups of different size, a statistical model has been

developed at MRC Human Nutrition Research (formerly MRC Dunn Nutrition Unit) in Cambridge for the analysis of bone mineral data from absorptiometric measurements (Prentice *et al.*, 1994). In this model, adjustment for bone and body size is achieved by entering bone width, body weight and height as independent variables in multiple regression models with bone mineral content (BMC) as the dependent variable, non-significant variables are removed by backwards elimination.

The advantage of this approach is that no assumptions are made about the nature of the relationship between BMC and those variables that are related to size. The method is equally applicable to bone mineral density provided that bone width is use in the model as independent variable.

5.2. Ultrasound

In ultrasound measurement, one transducer, a transmitting transducer is electrically excited to produce a broadband spectra. The ultrasonic wave is transmitted through the heel, which is detected by a receiving transducer - the second transducer and digitised for computer analysis (Gluer *et al.*, 1994; Hans *et al.*, 1994; Jonson, 1993). The two parameters of interest were, BUA and VOS. The VOS (m/s) is the velocity of the ultrasonic wave as it passes through the heel. The BUA (dB/MHZ) is a measurement of the frequency-dependent attenuation of the ultrasonic wave as it passes through the heel (Kann *et al.*, 1995). The measured BUA and VOS are compared with the reference BUA and VOS provided by the manufacturer, expressed as mean value for the young normal and age-matched reference populations.

The measurement of broadband ultrasound attenuation provides information about trabecular bone status. A low broadband ultrasound attenuation (BUA) of the calcaneus has been demonstrated in patients with wrist and spine fracture compared to controls (Agren *et al.*, 1991; Bernecker *et al.*, 1992, McCloskey *et al.*, 1990, Resch *et al.*, 1990). Similar low ultrasound values were reported in individuals with hip fracture when compared with normal population (Baran *et al.*, 1988; Stewart *et al.*, 1994; Sakata *et al.*, 1994; Schott *et al.*, 1995). Most traditional bone densitometer techniques estimate bone mineral density which accounts for about seventy percent of bone strength. However, fragility of bone relates not just to density of bone, but also with bone quality (Brandenburger, 1993; Moris *et al.*, 1995). Qualitative ultrasound provides information on bone architecture and quality (Brandenburger, 1993).

The calcaneum is an easily accessible site and is rich in trabecular bone which develops with age paralleling trabecular bone growth lumbar vertebrae (Miller *et al.*, 1993). Bone ultrasound parameters have been shown to have a negative correlation with age in both males and females (Moris *et al.*, 1995). Studies comparing bone ultrasound and bone densitometer techniques such as DXA in assessing bone status have demonstrated good agreement between the two methods (Moris *et al.*, 1995; Stewart *et al.*, 1994; Mautalen *et al.*, 1995). In the study of Moris et al., the significance of correlation between BUA and dual x - ray absorptiometry (DXA) of the lumbar spine was, $R = 0.67$, $p < 0.01$ for SOS, $R = 0.57$ $p < 0.02$ (Moris *et al.*, 1995). An association was also found between velocity of sound (VOS), broadband attenuation (BUA) and stiffness of the left

and right foot in healthy volunteers (Moris *et al.*, 1995). Quantitative ultrasound (QUS) techniques have recently been introduced as possible alternative methods free of ionising radiation for noninvasive assessment of skeletal status in osteoporosis (Heaney, 1989; Baran *et al.*, 1988; Langton *et al.*, 1984; Antich *et al.*, 1991; Kaufman & Einhorn, 1993; Hans *et al.*, 1993; Smith *et al.*, 1992).

5.3 Statistical analyses

5.3.1 Significance tests

A significance test makes it possible to measure the strength of evidence which the data supply for or against some proposition of interest. For example, in this study, testing the effect of supplement on bone mineral acquisition, one could test the questions, would children have more bone mineral accretion while receiving supplement, and will bone mineral acquisition be different between boys and girls?

In a significance test, we ask the question whether differences in bone mineral mass at outcome between the supplemented and placebo groups; between boys and girls, etc., were small enough to have occurred by chance. If it were the case, then the evidence in favour of there being a difference between the supplemented and placebo groups or between boys and girls would be weak. On the other hand, if the difference between the groups were much larger than one would expect the evidence that there is a real difference between the calcium and placebo group or between boys and girls becomes strong.

To carry out the test of significance we need to assume that there is no difference between the supplemented and placebo groups or between boys and girls. The hypothesis of 'no difference' or 'no effect' is called the **null hypothesis**. This is compared with the alternative **hypothesis** of a difference between supplement and placebo groups or between boys and girls. If the difference between groups is large ($P < 0.05$) the null hypothesis that there is no difference between supplemented and placebo or between boys and girls is rejected. If on the other hand, the difference is small ($p > 0.05$) the data are consistent with the null hypothesis, that there is no difference between the groups. The test of significance of the null hypothesis of no difference is based on the t-distribution.

5.3.2 Student's t-test

The student's t-test is commonly used in statistical analysis to compare two groups or sets of observations, for example, comparing supplemented and placebo groups, boys and girls etc. To use this test, it requires that a probability level is chosen which measures the degrees of freedom of the variance estimate. The Student's t-test is a parametric test, which is based on the assumption that each set of observations is sampled from a population with a normal distribution (Bland, 1987). In this study, 2-tailed t-tests were used to examine differences between supplemented and placebo groups, and between boys and girls.

5.3.3 Pearson product-moment correlation

The product-moment correlation measures linear association between two variables, how one changes with the other. The correlation coefficient r describes the closeness of the relationship between two variables. The correlation coefficient is based on the sum of products about the mean of the two variables. In this method, a positive correlation between two variables exists if the increase in one variable, follows the increases in the other. If one variable decreased as the other increased, the sum of the products is negative, this indicates there is a negative correlation between the variables. When two variables are not related the correlation produces a scatter, there are as many positive as negative products, and the sum is zero. This means there is no correlation between the variables. The value of the sum of products depends on the units in which the two variables are measured. The sum of the products is divided by the square roots of the sums of squares of x and y. This gives the product moment correlation coefficient, or correlation coefficient, denoted by r. The effect of dividing the sum of products by the root sum of squares of deviations of each variables is to make the correlation coefficient lie between -1.0 and +1.0. When all the points lie exactly on a straight line such that y increases as x increases, r = 1. Similarly, when all the points lie exactly on a straight line with negative slope, r = -1.

5.3.4 Regression Analysis

Comparisons of percentage increases and absolute increases in BMC, BW and BMD and body size (weight and height) between the supplemented and placebo groups and between boys and girls were examined using a multiple regression approach. Multiple regression analysis using backward elimination method to remove nonsignificant variables was performed to identify determinants of bone mineral content. Baseline values of the independent variable were included in regression model to adjust for regression toward the mean, except when the influence of BMC itself was examined. To examine the effects of supplement, sex and season, dummy binary variables were used as follows; female = 1, male = 0; supplement = 1, placebo = 0; wet/dry variables were coded as w = 1, d = 0. This test make the assumption that the associations between y-variable and each of the x-variables are linear and that the data are normally distributed (Bland, 1987). All continuous variables except age, sex and pubertal status in this study, were transformed to natural logarithms. Conversion to natural logarithms is a convenient way of expressing proportional differences between groups (Prentice *et al.*, 1990). For example, differences between supplemented and placebo groups or between boys and girls. Transformation of variables to natural logarithms in regression analysis has the added advantage that the regression coefficients provide information in proportional terms about the influence of each factor on the dependent variable. If the coefficient for discrete, binary variables is multiplied by 100, this approximates to the percentage difference between the two groups as defined by

(difference/mean)x100 (Prentice *et al.*, 1994). The level of significance used in this study was ≤0.05.

Examples of regression analysis models set up to investigate the effect of bone width (BW) and sex on bone mineral content (BMC) at the midshaft are given below:

Fig.5.1 data untransformed

Dependent variable is: sbmc
No Selector
R squared = 57.9% R squared (adjusted) = 57.4%
s = 0.0561 with 160 - 3 = 157 degrees of freedom

Source	Sum of Squares	df	Mean Square	F- ratio
Regression	0.681016	2	0.340508	108
Residual	0.494725	157	0.003151	

Variable	Coefficient	s.e. of Coeff	t- ratio	prob
Constant	-0.1090	0.0387	-2.81	0.0055
sbw	0.5694	0.0389	14.6	≤ 0.0001
Sex	0.0021	0.0089	0.237	0.8132

Fig. 5.2 data transformed to natural logarithms

Dependent variable is: Lnsbmc
No Selector
R squared = 57.6% R squared (adjusted) = 57.0%
s = 0.1314 with 160 - 3 = 157 degrees of freedom

Source	Sum of Squares	df	Mean Square	F- ratio
Regression	3.67877	2	1.83939	106
Residual	2.71237	157	0.017276	

Variable	Coefficient	s.e. of Coeff	t- ratio	prob
Constant	-0.7834	0.0149	-52.7	≤ 0.0001
Lnsbw	1.2928	0.0892	14.5	≤ 0.0001
Sex	0.0027	0.0209	0.131	0.8959

Fig. 5.3 data transformed to natural logarithms and multiplied by 100

Dependent variable is: 100*lnsbmc
No Selector
R squared = 57.6% R squared (adjusted) = 57.0%
s = 13.14 with 160 - 3 = 157 degrees of freedom

Source	Sum of Squares	df	Mean Square	F-ratio
Regression	36787.7	2	18393.9	106
Residual	27123.7	157	172.762	

Variable	Coefficient	s.e. of Coeff	t-ratio	prob
Constant	-78.3413	1.487	-52.7	≤ 0.0001
100*lnsbw	1.2928	0.0892	14.5	≤ 0.0001
Sex	0.2743	2.094	0.131	0.8959

In example (i) above,

BMC = 0.57 BW + 0.002 sex + constant

This shows that a 1 cm difference in bone width corresponds to a difference in mid-shaft BMC of 0.57g/cm. The constant is the value of intercept and value for boys is 0.002 g/cm higher. To interpret this finding requires knowledge of typical values of BMC for this age group of children. In this example, there was no evidence of a sex difference in BMC adjusted for BW (p = 0.81).

(ii)

LnBMC = 1.29 LnBW +0.003 sex + constant

This means a 1% change in BW corresponds to a 1.29% change in BMC at the mid-shaft and this result could be interpreted directly without knowledge of units or typical values. The intercept for boys is 0.003 times higher than girls, but no sex difference in size-adjusted BMC was found (p = 0.90),

(iii)

LnBMC = 1.29 LnBW + 0.27 sex + constant

In example (iii) below, the interpretation for BW and constant is the same as (ii), but the difference between boys and girls is expressed as a percentage. Therefore, for the same BW, boys have 0.3% more bone mineral content than girls.

5.3.5 Analyses of variance (ANOVA)

A two-way analysis of variance (ANOVA) test is used to examine whether more than two groups have the same mean or not. ANOVA is a parametric method based on the assumption that samples are from normally distributed populations and have the same underlying variance. In this study, a two-way analysis of variance test was used to test the effects of sex group and treatment effect (calcium or placebo) and their interaction on the variance of the net gains as well as percentage gains in bone measurements and weight and height. The principle of this test is based on the same concept as that described above (significance of test), that is the null hypothesis that there is no difference between the groups (between supplemented and placebo, between boys and girls). The p value for the two-way analysis of variance shows the probability of observing an F value as large as the one observed if the null hypothesis is true. A small p value indicates it is unlikely that the F values observed occur by chance. The test used to compare pairs of groups considered in the study was the Scheffé post-hoc test, which allows the interpretation of significant variation

between the means of two, three or more groups, for example, comparing one group with a number of other groups. Multiple testing is subjected to a high probability of giving false positive results (Type 1 errors) due to chance. Scheffé post-hoc tests have the advantage of controlling and minimising type 1 error. In this study, type 1 error was set at the limit of 5%.

5.3.6 Chi-Squared Distribution

The Chi-squared test is a non-parametric method used to test the degree of association between two variables in which results are tabulated. For example, a cross tabulation of frequencies and is called contingency table or cross-classification. The test can be used even when the observations are not measurements but ranking of the experimental material. For example, by testing the strength of a particular drug in a clinical trial by comparing the treatment and control groups. In a Chi-square test, the null hypothesis is that there is no association between the two variables, the alternative being that there is an association. It requires that the data are from a normal distribution.

5.4 Effect of calcium supplement

The effect of the calcium supplement on bone mineral variables and biochemical markers at outcome were examined after correcting for differences at baseline. The basic model was ln(outcome) = k + ln(baseline) + s/p (1/0). Interaction terms between s/p (s = supplement, p = placebo) and age, sex, and

pubertal status were tested by including s/p multiplied by the variables in the regression model but none were significant.

In this study, differences in BMC, BW, and BMD, and differences in anthropometric variables and biochemical markers were expressed as percent change in calcium supplemented minus percent change placebo from baseline to outcome and follow-up. A similar method was used to express differences between boys and girls.

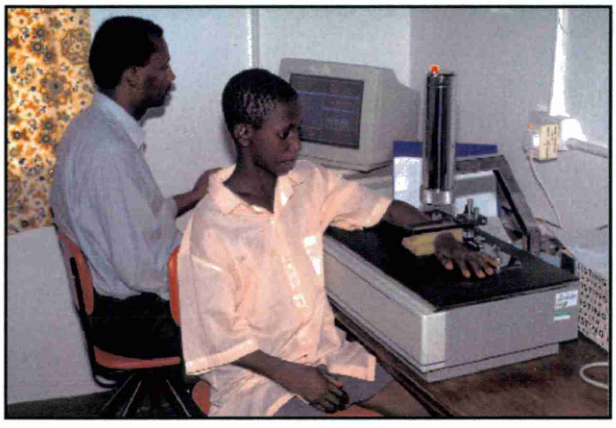
There were no significant differences in subject characteristics or bone variables between boys and girls at baseline, except that girls had significantly greater triceps skinfold thickness (1.6 (SE 0.3), $p \leq 0.0001$) (Table 7.5). The effect of supplement was therefore examined with sexes combined.

5.5 Supplemented and placebo groups by sex

The effect of calcium supplement on bone variables and biochemical markers disappeared when data were grouped by sex, reflecting the decrease in power associated with splitting the data by sex. The effect of the supplement on variables were therefore examined, with sexes combined. A supplement x sex interaction term was included in all regression analyses to examine whether there was any evidence that the effect of the supplement was different between boys and girls.

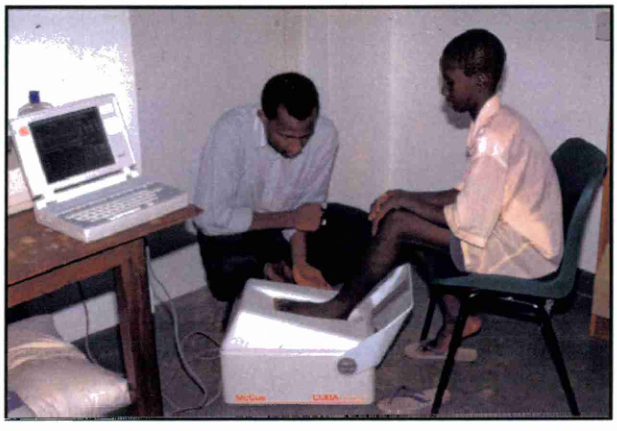
Measurements at MRC Keneba, The Gambia

Subjects arriving at the research room



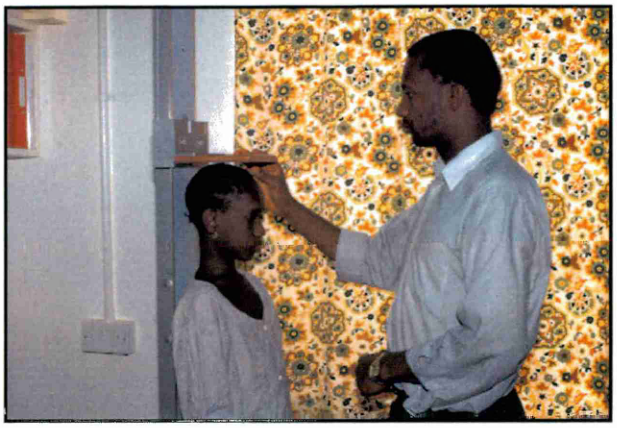
Spa bone scanner

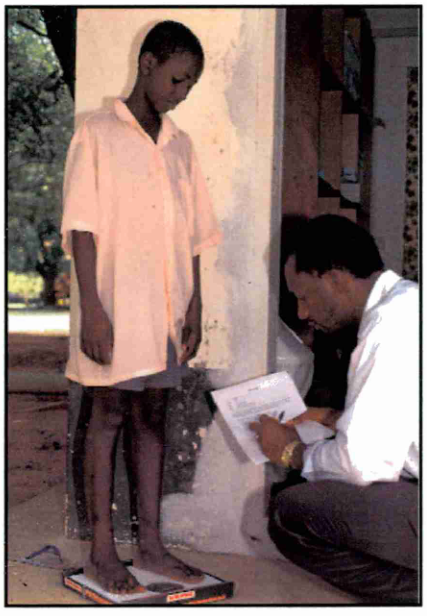
Ultrasound of the heel



Anthropometry measurements and 24 hour urine collections

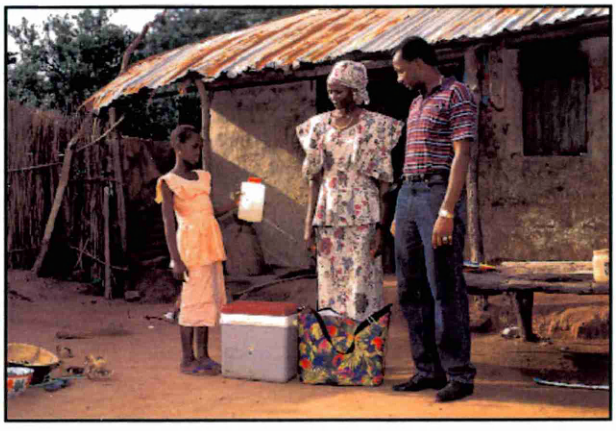
Height measurement





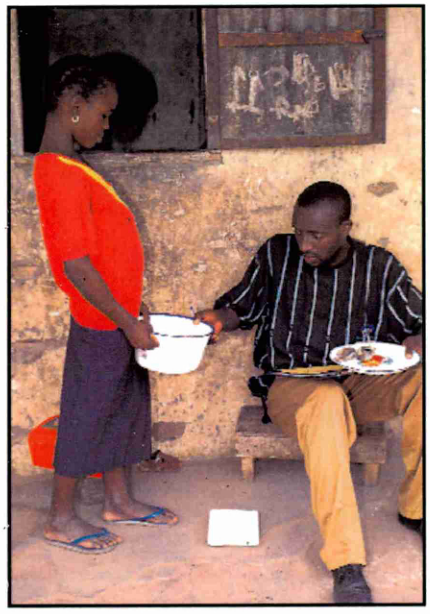
Weight measurement

24 hour urine collections



Food intakes and supplementation

Subjects enjoying their breakfast after overnight fasting



Weighed food intake

A girl having her supplement tablets under supervision at the Youth Club

6 Results: Dietary intake

6.1.1 Calcium and phosphorus intake

The calcium intake of the children was low. The arithmetic mean of calcium intake was 338 SD 141 mg/d (8.45 SD 3.53 mmol/d), phosphorus 834 SD 310 mg/d (26.9 SD 10 mmol/d), and calcium-to-phosphorus ratio, 0.42 SD 0.12 (Table 6.1 and 6.2). The calcium-to-phosphorus ratio of the Gambian diet was substantially lower than that reported in United Kingdom diet (0.7 mg/mg) (Department of Health, 1992).

6.1.2 Sex difference

Table 6.1 gives calcium and phosphorus intakes and calcium-to-phosphorus ratio in boys and girls. There were no significant differences in calcium and phosphorus intake between boys and girls. Calcium-to-phosphorus ratio of the two sexes were identical (boys: = 0.42 SD 0.12 mg/mg, girls = 0.42 SD 0.13 mg/mg).

Table 6.1 Dietary calcium and phosphorus intake and calcium-to-phosphorus ratio for all subjects, and for boys and girls at baseline[†]

	Total (159)	Boys (n = 79)	Girls (n = 80)
Calcium (mg/d)	338 ± 141	355 ± 160	321 ± 118
Phosphorus (mg/d)	834 ± 310	863 ± 307	807 ± 312
Ca:P (mg/mg)	0.42 ± 0.12	0.42 ± 0.11	0.42 ± 0.13

[†] Mean ± SD. No significant difference between the groups in any of the variables. mg/d to mmol/d: calcium ÷ 40; phosphorus ÷ 31.

6.1.3 Effect of age on intake of calcium and phosphorus

Dietary phosphorus intake was significantly negatively related to age ($p = 0.018$) but there was no significant association between calcium intake and age. Differences between sexes and supplement effect on phosphorus intake were therefore examined after adjusting for age.

6.1.4 Differences between the supplemented and placebo groups

There were no significant differences in calcium and phosphorus intake or calcium-to-phosphorus ratio between the supplemented and placebo groups at baseline (Table 6.2).

Table 6.2 Calcium and phosphorus intake and calcium-to-phosphorus ratio for all subjects, and for supplemented and placebo group[†].

	Total (n = 159)	Supplement (n = 79)	Placebo (n = 80)
Calcium(mg/d)	338 ± 141	342 ± 129	334 ± 153
Phosphorus(mg/d)	834 ± 310	825 ± 289	844 ± 330
Ca:P (mg/mg)	0.42 ± 0.12	0.43 ± 0.12	0.41 ± 0.1

[†]Mean ± SD. mg to mmol/d calcium ÷ 40, phosphorus ÷ 31. There was no significant difference between the groups in any of the variables.

6.1.5 Additional calcium intake from the supplement

The additional intake of calcium from the supplement after taking account of compliance and the fact that the tablets were provided on 5 days each week was 714 mg per day (17.85 mmol/d) spread over the entire supplementation period. Calcium supplementation raised the total calcium intake of the supplemented group from 342 to 1056 mg per day (8.55 to 26.4 mmol/d) during

the 12 months as compared with 334 mg per day (8.35 mmol/d) in the placebo group.

6.1.6 Intake of other nutrients including sex differences

The intakes of other nutrients for all subjects, and for boys and girls separately are given in Table 6.3. The results indicate the Keneba children had a low fat but a moderate protein intake. Fat intake was negatively related to age (-7.7 (SE 2.1) p = 0.0004) but none of the other nutrient intakes were influenced by age. Boys had a significantly higher intake of calories, protein, fat and carbohydrate than girls.

Table 6.3 Nutrient intakes for all subjects, divided by sex at baseline[†]

	Total (159)	Boys (n = 79)	Girls (n = 80)
Energy (kcal/d)	1763 ± 447	1867 ± 463[a]	1662 ± 407
Protein (g/d)	55.4 ± 16.6	58.1 ± 17.8[b]	52.8 ± 15.0
Fat (g/d)	38.0 ± 26.0	40.9 ± 29.4[c]	35.3 ± 22.9
CHO (g/d)	326.2 ± 75.9	345.3 ± 71.6[d]	307.3 ± 75.7

[†]Mean ± SD. CHO, carbohydrate. [a.b.c.d] Significantly different between sexes after adjusting for age (regression - data desk 4.1), [a] p = 0.007, [b] p = 0.020, [c] p = 0.034, [d] p = 0.018. kcal to kJ, multiply by 4.18.

6.1.7 Differences between the supplemented and placebo groups in the intake of other nutrients

There was no significant difference between the supplemented and placebo groups in the intake of other nutrients (Table 6.4).

Table 6.4. Nutrient intake for all subjects, and for supplemented and placebo groups at baseline†

	Total (n = 159)	Supplement (n = 79)	Placebo (n = 80)
Energy (kcal/d)	1763 ± 447	1756 ± 418	1771 ± 476
Protein (g/d)	55.4 ± 16.6	55.5 ± 15.5	55.3 ± 17.7
Fat (g/d)	38.0 ± 26.4	37.3 ± 23.6	38.8 ± 29.0
Carbohydrate (g/d)	326.2 ± 75.9	326.9 ± 72.1	325.5 ± 79.9

†Mean ± SD. kcal to kJ, multiply by 4.18

6.1.8 Distribution of calcium intake

The distribution of calcium intake was slightly positively skewed. This was caused by the calcium intake of one subject, which averaged 984 ± 1066 mg/d (24.6 ± 26.7 mmol/d) and varied from 230 mg/d (5.8 mmol/d) on one day to 1737 mg/d (43.4 mmol/d) on the following day. This was due to the consumption on day 2 of an exceptionally calcium-rich dish of millet (sanyo) steamed with dried baobab leaf (naa) and small fish eaten with bones. Otherwise, there was reasonable agreement between measurement of calcium intake for the rest of the children on the two days.

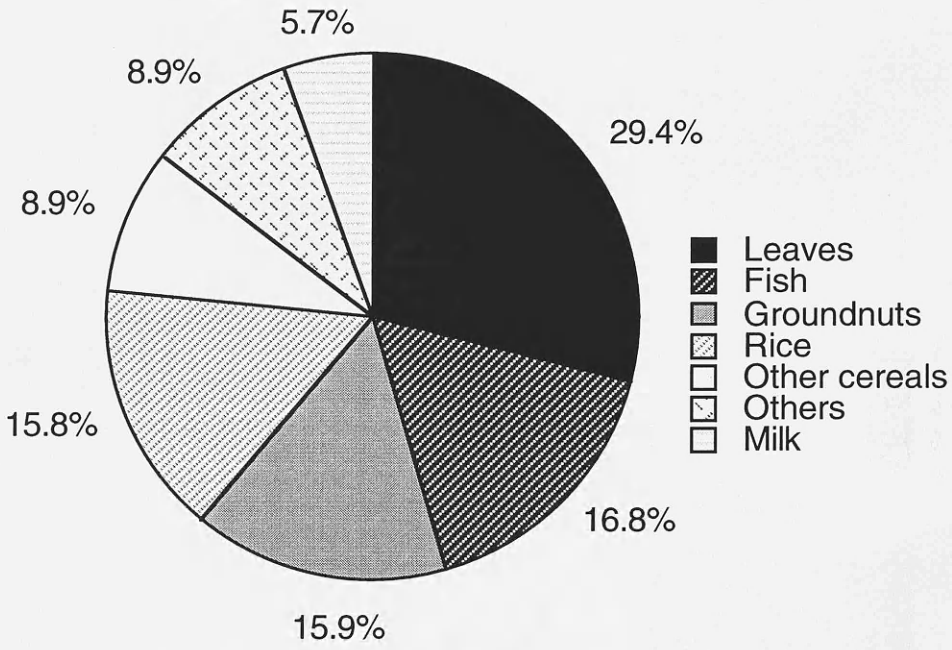
The geometric mean of calcium intake for the group as a whole was 311 mg/d (7.78 mmol/d). The mean ± SD of $\log_e$ was 5.74 ± 0.40.

6.1.9 Major foods sources of calcium

In this study, dark green leaves were the major source of calcium and contributed 29.4% to the mean daily calcium intake (Fig. 6.1). The other major

calcium providers were cereals, fish and groundnuts which contributed 24.7% (of which rice provided 15.8%), 16.8%, and 15.9%, respectively to the daily calcium intake. Milk provided only 5.7% of the mean calcium intake.

Fig. 6.1. Percentage contribution of major foods to the mean daily calcium intake



Although milk and fish were the most calcium rich sources in the diet of the rural Gambia children, milk was very rarely consumed and therefore contributed little to the mean daily calcium intake. Leaf sauces, including those made from dried baobab leaves (naa), contributed more calcium to the diet than any other food items. This is because many of the meals included leaf sauces and traditional leaves sauces are rich in calcium. Cereals together (rice, sorghum, millet, maize, findo) represented the second highest calcium source of the diet. Salt in cooked rice provides most of the calcium in rice dishes. Although, the calcium content of these items is low compared with green leaves, fish and milk,

cereals form a large part of the diet and they are the main staple foods of this community.

6.1.10 Seasonal variation in calcium and phosphorus intake

Calcium and phosphorus intake of subjects in the two seasons (dry and wet season) were compared. The wet (rainy) season begins in July and ends in October and the dry season extends from November to June. The mean calcium and phosphorus intake did not differ between seasons (Dry season : Wet season, calcium: 344 ± 137 vs 331 ± 147 mg/d (8.6 ± 3.43 vs 8.28 ± 3.68 mmol/d); phosphorus: 860 ± 247 vs 803 ± 372 mg/d (27.74 ± 7.97 vs 25.90 ± 12.00 mmol/d).

6.1.11 Differences between supplemented and placebo groups and between boys and girls

There were no significant differences between either the supplemented and placebo groups or between boys and girls in the intake of calcium and phosphorus in the dry or wet season, nor was there an effect of season on calcium and phosphorus intake in either sex or supplement group (Tables 6.5 and 6.6).

Table 6.5 Calcium and phosphorus intake for all subjects, divided by boys and girls and by season.

		Boys		Girls	
	Total	Dry season	Wet season	Dry season	Wet season
	(n = 15)	(n = 44)	(n = 35)	(n = 44)	(n = 36)
Calcium(mg/d)	338 ± 141	368 ± 160	338 ± 161	319 ± 104	325 ± 135
Phosphorus (mg/d)	834 ± 310	886 ± 258	834 ± 361	834 ± 237	773 ± 386

†Mean ± SD. There was no significant difference between the sexes in any of the variables. mg/d to mmol/d: calcium ÷ 40; phosphorus ÷ 31.

Table 6.6 Calcium and phosphorus intake by the supplemented and placebo groups, divided by season.

		Dry season		Wet season	
	Total	Supplement	Placebo	Supplement	Placebo
	(n = 159)	(n = 44)	(n = 44)	(n = 35)	(n = 36)
Calcium(mg/d)	338 ± 141	347 ± 136	340 ± 139	336 ± 122	326 ± 170
Phosphorus (mg/d)	834 ± 310	852 ± 258	869 ± 239	791 ± 326	814 ± 417

Mean ± SD. There were no significant differences between the groups in any of the variables in either seasons. mg/d to mmol/d: calcium ÷ 40; phosphorus ÷ 31.

6.1.12 Seasonal variation in calcium sources

Table 6.7 gives the proportion of calcium contributed by different food sources in the wet and dry season by the subjects. There was a seasonal variation in the types of food contributing to calcium intake. Leaves were abundant in the wet season and were consumed in greater quantity in the wet season but the difference between seasons did not reach significance (leaves, wet vs dry, 33% versus 28%, NS, Chi-squared test). Milk consumption was similar in the two seasons but was slightly raised in wet season. This may have been due to the fact that cows yield more milk in the rainy season when there is plenty of fresh green grass compared with the dry season (milk, wet : dry, 6.8% versus 4.8%, NS). On the contrary, groundnuts were harvested during the early part of the dry season and their contribution to the daily calcium intake in the dry season was two-fold higher than in the wet season (groundnuts, dry:wet, 18% versus 9%, $p \leq 0.0001$, Chi-squared test). A similar amount of fish was eaten in the wet and the dry season (dry : wet, 16% versus 18% NS). Rice was consumed

throughout the year and its intake was also not significantly different between the seasons (dry : wet, 15.5% versus 15.9%)

Table 6.7 Seasonal variation in calcium sources[†]

Foods	Total	Dry season	Wet season
Leaves	30.5	28.0	33.0
Rice	15.7	15.5	15.9
Groundnuts	13.5	18.0	9.0[a]
Other cereals	9.5	8.0	11.0
Fish	17.0	16.0	18.0
Milk	5.8	4.8	6.8
Others	8.5	9.0	8.0

[†] % of total calcium intake. Dry season significantly different from wet [a] $p \leq 0.0001$ (Chi-squared test)

6.1.13 Seasonal variation in other nutrients

6.1.13.1 Differences between supplemented and placebo groups, and between boys and girls

Table 6.8 gives the intake of other nutrients in boys and girls. After adjustment for age, the intakes of calories and fat in girls were significantly greater in the dry season than the wet season. There were no significant differences between dry and wet season in the intake of protein and carbohydrate in girls. The intake of all these nutrients in boys was significantly greater in the dry season compared with the wet season.

After adjustment for age and sex, there were no significant differences between the supplemented and placebo groups in the intake of any nutrient either in the dry or wet season (Table 6.9).

Table 6.8 Daily intake of other nutrients for all subjects, divided by boys and girls and by season[†].

	Total	Boys		Girls	
		Dry season	Wet season	Dry season	Wet season
	(n = 159)	(n = 44)	(n = 35)	(n = 44)	(n = 36)
Energy (kcal/d)	1763 ± 447	2000 ± 401[a]	1698 ± 486	1761 ± 345[e]	1541 ± 448
Protein (g)	55 ± 17	63 ± 17[b]	52 ± 17	55 ± 12	50 ± 18
Fat (g)	38 ± 26	49 ± 25[c]	31 ± 32	43 ± 24[f]	26 ± 18
Carbohydrate (g)	326 ± 76	360 ± 68[d]	327 ± 73	314 ± 67	299 ± 85

[†]Mean ± SD. Significant difference between seasons after adjusting for age were, [a] p = 0.003; [b] p = 0.012 [c] p = 0.006, [d] p = 0.020, [e] p = 0.017, [f] p = 0.0001. kcal to kJ, multiply by 4.18.

Table 6.9 Daily intake of nutrients by supplemented and placebo group, and by season[†].

	Total	Dry season		Wet season	
		Supplement	Placebo	Supplement	Placebo
	(n = 159)	(n = 44)	(n = 44)	(n = 35)	(n = 36)
Energy (kcal/d)	1763 ± 447	1862 ± 397	1899 ± 389	1623 ± 411	1614 ± 528
Protein (g/d)	55 ± 17	58 ± 15	59 ± 16	52 ± 16	50 ± 19
Fat (g)	38 ± 26	46 ± 25	46 ± 24	27 ± 16	30 ± 32
Carbohydrate (g)	326 ± 76	334 ± 64	340 ± 78	318 ± 82	308 ± 79

[†]Mean ± SD. After adjustment for age and sex, there were no significant differences between the groups in either seasons. kcal to kJ, multiply by 4.18.

7 Results: Anthropometry and pubertal status

7.1 Anthropometry results

7.1.1 Baseline anthropometry

Table 7.1. shows the characteristics of the 160 children who took part in the study.

Table 7.1 Baseline characteristics for all subjects, supplemented and placebo groups and for boys and girls separately.

	Total (n = 160)	Supplemented (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
Age (y)	10.3 ± 1.0	10.3 ± 1.0	10.3 ± 1.0	10.5 ± 0.8	10.1 ± 1.1
Weight (kg)	25.2 ± 4.0	25.5 ± 4.0	24.9 ± 4.1	25.5 ± 3.6	25.0 ± 4.4
Height (cm)	132.1 ± 7.2	132.5 ± 6.9	131.6 ± 7.6	132.5 ± 6.6	131.6 ± 7.8

Mean ± SD. There were no significant differences either between sexes or between the groups in any of the variables.

There were no significant differences in subject characteristics between either the supplemented and placebo groups or between boys and girls at baseline (Table 7.1).

Table 7.2 Age, weight and height of the supplemented and placebo group at baseline, by sex.

	Boys		Girls	
	Supplemented (n = 40)	Placebo (n = 40)	Supplemented (n = 40)	Placebo (n = 40)
Age (y)	10.4 ± 0.8	10.5 ± 0.8	10.1 ± 1.1	10.0 ± 1.1
Weight (kg)	25.7 ± 3.3	25.4 ± 3.9	25.4 ± 4.6	24.5 ± 4.2
Height (cm)	132.5 ± 5.7	132.5 ± 7.5	132.6 ± 8.0	130.7 ± 7.6

Mean ± SD. When subjects were divided into sexes, no significant difference was found either between the sexes or between the groups in any of the variables.

When subjects were divided into sexes, there was no significant difference between the supplemented and placebo group in age, weight and height at baseline (Table 7.2).

7.1.2 Z score

Weight, height and body mass index (BMI) in this study were adjusted for age and sex by Z score to compare weight, height and BMI of Gambian children with the age and sex matched British reference (Table 7.3).

There was no significant difference either between the sexes or between the supplemented and placebo groups in any of the variables at baseline. The Z scores for the Gambian children were considerably lower than for the British children with the mean differences more than 1 standard deviation below the British mean for each of the growth variables. This represents 78, 94 and 87 percent weight, height and body mass index for age of the British reference. The results indicate that the growth of rural Gambian children was poor compared with British reference population and confirm the reports from previous studies in Keneba.

Table 7.3 Mean ± SD Z-score for weight, height, and body mass index by group and sex at baseline

	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
weight	-1.67 ± 0.77	-1.86 ± 0.93	-1.82 ± 0.92	-1.70 ± 0.78
height	-1.10 ± 0.77	-1.24 ± 0.94	-1.26 ± 0.85	-1.09 ± 0.87
BMI	-1.53 ± 0.80	-1.62 ± 0.81	-1.53 ± 0.84	-1.62 ± 0.77

BMI, body mass index. No significant differences between the sexes and between groups in any of the variables. Z scores compared with British reference data (Cole, 1990; Cole, 1997).

7.1.3 MUAC, triceps skinfold thickness and grip strength

Both the mid-upper arm circumference (MUAC) and grip strength were positively related to age ($p \leq 0.0001$). After adjusting for age, a positive association between MUAC and pubertal status was also evident indicating greater MUAC in these children who were pubertal (stage II - IV) compared with those who were prepubertal (MUAC, +2.5 (SE 0.8)%, p = 0.003). There was no correlation between either triceps skinfold thickness with either age or pubertal status.

After adjusting for age, there were no significant differences in MUAC, triceps skinfold thickness and grip strength between either the supplemented and placebo groups or between boys and girls at baseline. An exception was for triceps skinfold thickness which was significantly greater in girls than boys (+20 (SE 3.4)%, $p \leq 0.0001$, Table 7.4).

Table 7.4 Baseline anthropometry variables for all subjects, and by group and sex

	Total (160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n =80)
MUAC (cm)	18.1 ± 1.6	18.2 ± 1.6	18.0 ± 1.6	17.9 ± 1.4	18.3 ± 1.8
Triceps (mm)	7.9 ± 1.9	8.0 ± 1.7	7.9 ± 2.1	7.1 ± 1.5	8.8 ± 1.9[a]
Grip (kg)	10.9 ± 2.9	11.1 ± 3.1	10.7 ± 2.7	11.2 ± 2.6	10.7 ± 3.2

[†] Mean ± SD. MUAC, mid-upper arm circumference, Triceps = triceps skinfold thickness, Grip = grip strength . Girls had significantly greater triceps skinfold thickness than boys [a] P = 0.0001. otherwise, there was no significant difference between sexes or between groups in any of the variables.

7.2 Anthropometry measures at outcome

Mean weight and height and other anthropometric variables at outcome are presented in Table 7.5. After allowing for age and pubertal stage, there was no significant difference either between the supplemented and placebo groups or between boys and girls. An exception was in triceps skinfold thickness which was significantly greater in girls than boys as it had been at baseline (P ≤0.0001).

Table 7.5 Anthropometry measures of the supplemented and placebo groups, boys and girls at out come

	Total (n = 160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
Weight (kg)	27.3 ± 4.7	27.7 ± 4.8	26.8 ± 4.6	27.1 ± 3.9	27.4 ± 5.5
Height (cm)	137.2 ± 7.4	137.7 ± 7.1	136.8 ± 7.7	137.2 ± 6.5	137.2 ± 8.2
MUAC (cm)	19.1 ± 1.6	19.3 ± 1.7	18.9 ± 1.6	18.8 ± 1.3	19.3 ± 1.9
Triceps (mm)	8.6 ± 2.6	8.6 ± 2.3	8.6 ± 2.8	7.5 ± 1.6	9.7 ± 2.9[a]
Grip (kg)	14.9 ± 3.3	15.1 ± 3.2	14.7 ± 3.3	15.1 ± 3.2	14.8 ± 3.4

Mean ± SD. MUAC, mid-upper arm circumference; Triceps, triceps skinfold thickness; grip, grip strength. Triceps skinfold thickness significantly greater in girls than boys at outcome, [a] p≤0.0001. There were no significant differences in any of the other variables between sexes or between groups.

7.3. Change in anthropometric variables over 12 months

7.3.1 Differences between boys and girls

After adjusting for baseline value, girls showed a greater percentage gain in weight, height and triceps skinfold thickness than boys (Table 7.6). There were no significant differences between boys and girls in changes in mid-upper arm circumference and grip strength, after correcting for baseline value (Table 7.6). After correcting for baseline value and sex, gain in triceps skinfold thickness, was significantly greater in older children (+0.8 (SE 0.1)mm, $p \leq 0.0001$, regression).

Table 7.6 Change in anthropometric variables in boys and girls over the 12 month period

	Outcome - Baseline		Δ%difference at outcome Boys vs Girls		
	Boys	Girls	Mean	SE	significance
Weight (kg)	1.6 ± 1.4	2.4 ± 1.7	-2.7	0.8	p=0.0009
Height (cm)	4.7 ± 1.8	5.6 ± 2.1	-0.6	0.2	p=0.009
MUAC (cm)	0.9 ± 0.9	1.0 ± 0.8	-0.5	0.7	p=0.43 (ns)
Triceps (mm)	0.4 ± 1.6	0.9 ± 2.1	-10.0	3.5	p=0.005
Grip (kg)	3.9 ± 1.5	4.1 ± 1.9	-2.0	1.8	p=0.27 (ns)

Mean ± SD. Triceps, triceps skinfold thickness, Grip, grip strength, MUAC, mid-upper arm circumference. Data transformed to natural logarithms before analysis (regression). Δ% = percentage difference at outcome after correcting for baseline value and age. After correcting for baseline value, girls had a significantly greater gain in weight, height and triceps skinfold thickness at outcome than boys. After adjusting for baseline value, there were no significant differences in MUAC and grip strength between the sexes. Ns, not significant.

7.3.2 Differences between supplemented and placebo groups

After adjustment for baseline value, age and sex, there was no significant difference between the supplemented and placebo groups in any of the anthropometric measures at outcome (Table 7.7).

After adjusting for baseline value and age, the anthropometric variables in either sex considered separately also did not differ significantly between the supplemented and placebo groups at outcome.

7.3.3 Effect of supplement on weight and height and other anthropometric variables at outcome

After correcting for the initial value, differences in weight and height gain were not significantly different between the supplemented and placebo groups (height, -0.0 SE 0.0%, $p = 0.88$; weight, +0.8 SE 0.8%, $p = 0.32$, Table 7.7). This indicates that there was no effect of supplement on longitudinal growth. There were also no differences in gain in MUAC, triceps skinfold thickness or grip strength.

Table 7.7 Change in anthropometric variables in the supplemented and placebo groups over the 12 month period

	Outcome - Baseline		Δ%difference at outcome Supplement vs Placebo		
	Supplement (n = 80)	Placebo (n = 80)			
			Mean	SE	significance
Weight (kg)	2.2 ± 1.7	1.9 ± 1.4	+0.8	0.8	p=0.32 (ns)
Height (cm)	5.1 ± 2.1	5.2 ± 1.9	-0.0	0.0	p=0.88 (ns)
MUAC (cm)	1.0 ± 0.9	0.9 ± 0.8	+1.0	0.7	p=0.13 (ns)
Triceps (mm)	0.6 ± 1.8	0.7 ± 1.9	-0.4	3.2	p=0.91 (ns)
Grip (kg)	4.0 ± 1.8	4.0 ± 1.5	+1.2	1.8	p=0.51 (ns)

Mean ± SD. Triceps, triceps skinfold thickness, Grip, grip strength, MUAC, mid-upper arm circumference. Δ% = percentage difference at outcome after correcting for baseline value, age and sex. Data transformed to natural logarithms prior to analysis (regression data desk 4.1). Changes in anthropometry variables over 12 month were not significantly different between the supplemented and placebo group. Ns, not significant.

7.3.4 Z score at outcome

There were no significant difference in Z scores for weight, height and BMI relative to the British reference either between the supplemented and placebo groups or between boys and girls at outcome (Table 7.8). There was no increase in Z scores in either the supplemented and placebo group or in boys and girls over the 12 month period. In fact, weight and BMI Z scores for boys and girls and for the supplemented and placebo groups at outcome were significantly lower than baseline (Table 7.9). Height Z scores did not differ significantly between outcome and baseline in either of the sexes or in the supplemented and placebo groups. This further indicates that there was no influence of the calcium supplement on growth. The results also suggest that there was no improvement

in growth of these children over the 1 year period as assessed by Z scores and they continued to fall behind their British counterparts.

There were no sex x supplement interactions seen in percent change from baseline to 12 months in any of the anthropometric variables.

Table 7.8 Z-score for weight, height, and body mass index by groups and by sex at outcome[†]

	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
weight	-1.84 ± 0.79	-2.08 ± 0.99	-2.07 ± 0.94	-1.85 ± 0.86
height	-1.13 ± 0.74	-1.26 ± 0.94	-1.28 ± 0.81	-1.10 ± 0.88
BMI	-1.78 ± 0.83	-2.03 ± 0.89	-1.94 ± 0.86	-1.86 ± 0.88

[†]Mean ± SD. BMI, body mass index. There was no significant difference between either the supplemented and placebo groups or between boys and girls in any of the variables.

Table 7.9 Change in Z scores over 12 months by group, and by boys and girls[†]

	Outcome - Baseline		Outcome - Baseline	
	Boys (n = 80)	Girls (n = 80)	Supplement (n = 80)	Placebo (n = 80)
weight	-0.25 ± 0.40[a]	-0.14 ± 0.33[b]	-0.17 ± 0.36[a]	-0.22 ± 0.38[a]
height	-0.03 ± 0.31	-0.02 ± 0.34	-0.03 ± 0.33	-0.01 ± 0.32
BMI	-0.41 ± 0.77[a]	-0.25 ± 0.54[a]	-0.25 ± 0.61[b]	-0.41 ± 0.72[a]

[†]Mean ± SD. BMI, body mass index. In both sexes, weight and body mass index Z scores were significantly lower at outcome compared with baseline. [a]$p \leq 0.0001$, [b]$p = 0.0002$. In both the supplemented and placebo groups, weight and body mass index Z scores were significantly lower at outcome compared with baseline. [a]$p \leq 0.0001$, [b]$p = 0.0005$. There was no significant difference in height Z scores between baseline and outcome in either boys or girls or in the supplemented and placebo groups. Change in Z scores over the 12 month period was not significantly different between either boys and girls or between the supplemented and placebo groups in any of the growth variables. However, there was a trend towards a greater decrease in weight Z score over the 12 months in boys than girls but the difference between the two was not statistically significant ($p = 0.059$).

7.4 Anthropometry measures at follow-up

7.4.1 Differences between boys and girls

There were no significant difference in weight and height between boys and girls at follow up. Girls were slightly heavier and taller than boys, but the differences between the two did not reach statistical significance. Mid-upper arm circumference and triceps skinfold thickness were significantly greater in girls than boys at follow-up (Table 7.10), but there was no significant difference between the sexes in grip strength.

7.4.2 Differences between supplemented and placebo groups

There were no significant differences between the supplemented and placebo groups in body weight and statural height at follow-up. After adjusting for sex, there were no significant differences between the supplemented and placebo group in MUAC, triceps skinfold thickness and grip strength at follow-up (Table 7.10).

Table 7.10 Mean ± SD of characteristics and anthropometry variables for all subjects, supplemented and placebo and by sex at follow up[†].

	All subjects (n = 160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
Weight (kg)	30.4 ± 5.9	30.9 ± 6.3	29.9 ± 5.6	29.9 ± 4.6	30.9 ± 7.0
Height (cm)	141.9 ± 8.4	142.4 ± 8.1	141.4 ± 8.7	141.5 ± 7.5	142.3 ± 9.2
MUAC (cm)	19.9 ± 2.0	20.1 ± 2.1	19.7 ± 1.9	19.6 ± 1.5	20.2 ± 2.4[a]
Triceps (mm)	8.3 ± 2.8	8.4 ± 2.7	8.3 ± 2.9	7.2 ± 1.5	9.5 ± 3.3[b]
Grip (kg)	16.7 ± 3.6	17.1 ± 4.0	16.3 ± 3.1	16.5 ± 2.7	16.9 ± 4.3

[†] mean ± SD. Girls had significantly greater mid-upper arm circumference and triceps skinfold thickness than boys at follow-up, [a] $p = 0.049$, [b] $p = 0.0001$.

Table 7.11 Change in anthropometric variables over the 2 year period in boys and girls[†]

	Baseline		Follow-up	
	Boys (n = 80)	Girls (n = 80)	Boys (n = 80)	Girls (n = 80)
Weight (kg)	25.5 ± 3.6	25.0 ± 4.4	29.9 ± 4.6[a]	30.9± 7.0[a]
Height (cm)	132.5 ± 6.6	131.6 ± 7.8	141.5 ± 7.5[a]	142.3 ± 9.2[a]
MUAC (cm)	17.9 ± 1.4	18.3 ± 1.8	19.6 ± 1.5[a]	20.2 ± 2.4[a]
Triceps (mm)	7.1 ± 1.5	8.8 ± 1.9	7.2 ± 1.5	9.5 ± 3.3[b]
Grip (kg)	11.1 ± 2.6	10.7 ± 3.2	16.5 ± 2.6[a]	16.9 ± 4.3[a]

[†]Mean ± SD. MUAC, mid-upper arm circumference; Triceps, triceps skinfold thickness; grip, grip strength. Follow-up values were significantly greater than at baseline, [a] p≤0.0001, [b] p = 0.011. An exception was triceps skinfold thickness in boys at follow-up which was not significantly different from 0.

Table 7.12 Change in weight, height, mid-upper arm circumference, triceps and grip strength after 24 month in boys and girls[†]

	Follow-up- Baseline		[#]Δ%difference at follow-up Boys vs Girls		
	Boys (n = 80)	Girls (n = 80)			
			Mean	SE	p value
Height (cm)	9.0 ± 2.8	10.7 ± 3.3[a]	-1.3	0.4	0.0003
Weight (kg)	4.4 ± 1.7	5.9 ± 3.2[b]	-5.3	0.9	≤0.0001
MUAC (cm)	1.7 ± 1.0	1.9 ± 1.2	-1.7	0.8	0.037
Triceps (mm)	0.1 ± 1.5	0.7 ± 2.6[c]	-14.0	3.5	≤0.0001
Grip strength	5.4 ± 1.6	6.2 ± 2.7[d]	-3.5	2.1	0.11 (ns)

[†] Mean ± SD. [#]Δ% = percentage difference at follow-up, after correcting for baseline value. Data transformed to natural logarithms before analysis (regression). Changes in weight, height, triceps and grip strength over 24 months were significantly greater in girls than boys. [a] p=0.0006, [b] p=0.0002, [c] p=0.041, [d] p=0.018. After correcting for baseline value, girls had a significantly greater increases in all cases except grip strength than boys at follow-up. Ns, not significant.

Table 7.13 Differences in weight, height, mid-upper arm circumference, triceps and grip strength between the supplemented and placebo groups after 24 months[†]

	Follow-up- Baseline		*Δ%difference at follow-up		
	Supplement (n = 80)	Placebo (n = 80)	Supplement vs placebo		
			Mean	SE	p value
Height (cm)	9.9 ± 3.1	9.8 ± 3.2	+0.2	0.9	0.85 (ns)
Weight (kg)	5.3 ± 3.1	5.0 ± 2.2	+0.1	0.4	0.89 (ns)
MUAC (cm)	1.9 ± 1.2	1.8 ± 1.1	+0.3	0.8	0.76 (ns)
Triceps (mm)	0.4 ± 2.2	0.4 ± 2.1	-0.4	3.1	0.89 (ns)
Grip strength (kg)	6.0 ± 2.5	5.6 ± 1.9	+0.1	3.3	0.98 (ns)

[†] Mean ± SD. MUAC, mid-upper arm circumference. Triceps, triceps skinfold thickness, MUAC, mid-upper arm circumference. *Δ% = percentage difference at follow-up, after correcting for baseline value. Data transformed to natural logarithms before analysis (regression). There was no significant difference in the incremental gain between the supplemented and placebo group in any of the anthropometric variables. Ns, not significant.

7.4.3 Change in anthropometry over the 24 month period

7.4.3.1 Differences between boys and girls

Both boys and girls had a significant gain in anthropometric variables over the 2 year period (Table 7.11). An exception was in triceps skinfold thickness in boys which was not significantly different from baseline.

Girls had significantly greater gains in triceps skinfold thickness and grip strength than boys (Table 7.12). The incremental gain in mid-upper arm circumference over 24 months was not significantly different between boys and girls (Table 7.12).

After correcting for baseline value, gain in weight and height over the 2 year period was significantly greater in girls than boys ($p \leq 0.0001$ and $p = 0.0003$

respectively, Table 7.12). After adjusting for differences at baseline, girls had significantly greater gain in mid-upper arm circumference and triceps than boys at follow-up (Table 7.12). Gain in adjusted grip strength was not significantly different between boys and girls at follow-up.

7.4.3.2 Differences between supplemented and placebo groups

After adjusting for the baseline value and sex, there were no significant differences between the supplemented and placebo groups, in height and weight increment at follow-up (difference in increment between groups: height, + 0.2 (SE 0.9)%, p = 0.85; weight, + 0.1 (SE 0.4)%, p = 0.89, Table 7.13).

After correcting for baseline value, there were no significant differences between the supplemented and placebo groups in gain in MUAC, triceps and grip strength over 24 months (Table 7.13).

7.5 Z scores at follow-up

Table 7.14 Mean ± SD Z-score for weight, height, and body mass index for all subjects, supplement and placebo groups, and boys and girls at follow up[†]

	All subjects (n = 160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n=80)
weight	-1.92 ± 0.92	-1.82 ± 0.85	-2.02 ± 0.97	-2.04 ± 0.89	1.79±0.93
height	-1.26 ± 0.90	-1.19 ± 0.81	-1.33 ± 0.99	-1.39 ± 0.86	1.13±0.93
BMI	-1.84 ± 0.89	-1.77 ± 0.89	-1.91 ± 0.88	-1.90 ± 0.78	1.78±0.98

[†]Mean ± SD. BMI, body mass index. No significant differences either between boys and girls or between the groups.

There was no significant difference between either the supplemented and placebo group or between boys and girls in Z scores relative to the British reference at follow up (Table 7.14). Z scores for all growth variables measured were still more than 1 SD below the age and sex matched British reference.

There was a significant decrease over the 2 year period in weight, height and BMI Z scores relative to the British reference in boys (Table 7.15). In girls, there was a trend towards lower Z scores in weight and height and BMI relative to British reference at follow-up compared with baseline but only the BMI Z scores were significantly lower than baseline.

In both the supplemented and placebo groups, weight and BMI Z scores relative to the British reference at follow-up were significantly lower than at baseline (Table 7.15). Height Z scores at follow-up did not differ significantly from baseline in either group. There was no difference between the supplemented and placebo groups in change over 2 years in any of the Z scores. The results of the follow-up confirm the observations at baseline and outcome that the Z scores for the Gambian children were remarkably lower than for the British children, and remained so during the peripubertal period.

Sex differences in change from baseline to 24 months in anthropometric variables were independent of supplement effect. There were no sex x supplement interactions in changes from baseline to 24 months in any of the anthropometric variables.

Table 7.15 Change in Z scores over 2 years by supplemented and placebo groups, and by boys and girls[†]

	Follow-up - Baseline		Follow-up - Baseline	
	Boys (n = 80)	Girls (n = 80)	Supplement (n = 80)	Placebo (n = 80)
weight	-0.22 ± 0.36[a]	-0.09 ± 0.46	-0.15 ± 0.44[d]	-0.16 ± 0.40[f]
height	-0.14 ± 0.44[b]	-0.04 ± 0.49	-0.09 ± 0.46	-0.09 ± 0.48
BMI	-0.36 ± 0.67[a]	-0.16 ± 0.65[c]	-0.24 ± 0.67[e]	-0.29 ± 0.66[g]

[†]Mean ± SD. BMI, body mass index. In both sexes, weight, height and body mass index Z scores were significantly lower at follow-up than at baseline. [a]p≤0.0001, [b]p = 0.007, [c]p = 0.029. An exception was in height Z scores in girls which was no significant difference between baseline and follow-up. Change in weight and BMI Z scores over the 2 year period were different between boys and girls. Weight: [1]p = 0.044, BMI: [2]p = 0.051. In both the supplemented and placebo groups, weight and body mass index Z scores were significantly lower at follow-up than at baseline. [d]p = 0.003, [e]p = 0.0006, [f]p = 0.002, [g]p = 0002.

7.6.Pubertal status

7.6.1 Pubertal status at baseline

Table 7.16 shows the percent of children at Tanner stage 1 and II - IV in each group and by sex at baseline. Using a Chi-squared test, there was no significant difference either between the sexes or between the supplemented and placebo groups in the percentage of children who had entered puberty at baseline. However, the trend was for more girls than boys to be in Tanner stage II - IV.

Table 7.16 Pubertal status at baseline.

	Total		Supplemented		Placebo		Boys		Girls	
	(n = 160)		(n = 80)		(n = 80)		(n = 80)		(n = 80)	
	N	%	N	%	N	%	N	%	N	%
Stage										
I	130	81.2	65	81.2	65	81.2	68	85.0	62	77.5
II - IV	30	18.8	15	18.8	15	18.8	12	15.0	18	22.5

N, number of subjects. There was no significant difference between either the supplemented and placebo groups or between boys and girls in percentage of children reaching puberty.

7.7. Pubertal status at outcome

Table 7.17 Pubertal status at outcome

	Total		Supplement		Placebo		Boys		Girls	
	(n = 80)		(n = 80)		(n = 80)		(n = 80)		(n = 80)	
	N	%	N	%	N	%	N	%	N	%
Stage										
I	111	69.4	55	68.8	56	70.0	54	67.5	57	71.2
II - IV	49	30.6	25	31.2	24	30.0	26	32.5	23	28.8

N, number of subjects. There was no significant difference between either the supplemented and placebo groups or between boys and girls in percentage of children reaching puberty.

Table 7.17 gives the percent of children at Tanner stage I and II - IV in each group and by sex at outcome. There was no significant difference either between boys and girls or between the supplemented and placebo groups in the percentage of children who had entered puberty at outcome (Chi-squared test). The percentage of children who had entered puberty had increased over the 12 month period in both the supplemented and placebo groups and in boys and girls.

7.7.1 Comparison of pubertal status at baseline and outcome by sex (%)

Fifteen percent of boys were in Tanner stage II- IV at baseline, 33% at outcome. In girls, pubertal stages II -IV was seen in 23% at baseline and 29% at outcome. When subjects were grouped into sexes, there was no significant difference in pubertal status between supplement and placebo groups in either sex at outcome.

7.7.2 Menarche

At baseline, none of the girls had started menstruation. At outcome, 2 girls had started menstruation and the rest of the girls had not had any periods.

7.7.3 Pubertal status at follow-up

Table 7.18 Pubertal status at follow-up

	Total (n = 80)		Supplement (n = 80)		Placebo (n = 80)		Boys (n = 80)		Girls (n = 80)	
	N	%	N	%	N	%	N	%	N	%
Stage										
I	87	54.5	38	47.5	49	61.2	42	52.5	45	56.2
II - IV	73	45.6	42	52.5	31	38.8	38	47.5	35	43.8

N, number of subjects. There was no significant difference between either the supplemented and placebo groups or between boys and girls in percentage of children reaching puberty.

Table 7.18 shows the percent of children at Tanner stage I and II - IV in each group and by sex at follow-up. There was no significant difference between either boys and girls or between the supplemented and placebo groups in percentage of children who progressed into puberty at follow-up.

The percentage of children who had entered puberty had increased over the 2 year period in both the supplemented and placebo groups and in boys and girls. At follow-up, puberty was seen in 46% of children as opposed to 19% at baseline. The total number of children who had entered puberty at follow-up was significantly greater than that at baseline ($P \leq 0.0001$, Chi-squared test). Forty-eight percent of boys and 44% of girls (baseline 15% and 23% respectively) were pubertal at follow-up.

7.7.4 Pubertal status in percentage at follow-up by sex

None of the boys had reached advanced puberty at follow up. At follow-up, six percent of girls had advanced puberty. The number of girls who had started menstruation had increased to 4 at follow-up.

8 Results: Bone mineral status and ultrasound measurements

8.1 Bone mineral status at baseline

Bone mineral status of the subjects was assessed at baseline to examine whether there was any difference between the supplemented and placebo groups, or between boys and girls which could account for any differences that might emerge at outcome or follow-up and to provide information about bone mineral accretion rate of pre- and peripubertal rural Gambian children.

The baseline bone mineral content, bone mineral density and bone width for all subjects, for supplemented and placebo groups, and for boys and girls separately are shown in Table 8.1. There were no significant differences either between the supplemented and placebo groups or between boys and girls in any of the bone variables at baseline.

8.2 Comparative data

Table 8.2 gives the comparative data obtained in British children of the same age. Using regression analysis, the Gambian children were shown to have 13% lower BMC (SE 2.5), P<0.0001, Figure 8.1), 4% lower BW (SE 1.4), p=0.003) and 9% lower BMD (SE 1.8), p<0.0001, Figure 8.2) than the British children. After adjusting for weight, height, age and sex, the difference in BMC was reduced to 4% which was no longer significant (SE 2.6, p=0.10). The Gambians also had lower height 6 (SE 0.6)%, p<0.0001) and weight 33 (SE 2.1)%, p<0.0001) than the British children (Figures 8.3 and 8.4).

The results indicated that there were significant differences in growth and bone variables between Gambian and British children and that the Gambian children were significantly lighter and shorter and had lower bone mineral status

Table 8.1 BMC, BW, and BMD of the mid-shaft and distal radius for all subjects, and by group and sex at baseline†

	Total (n = 160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n= 80)
Mid-shaft BMC (g/cm)	0.443 ± 0.086	0.452 ± 0.087	0.434 ± 0.085	0.450 ± 0.082	0.437 ± 0.090
Mid-shaft BW (cm)	0.968 ± 0.115	0.976 ± 0.117	0.960 ± 0.114	0.981 ± 0.113	0.954 ± 0.117
Mid-shaft BMD (g/cm^2)	0.456 ± 0.060	0.461 ± 0.058	0.451 ± 0.062	0.457 ± 0.057	0.456 ± 0.063
Distal BMC (g/cm)	0.403 ± 0.100	0.419 ± 0.096	0.388 ± 0.103	0.409 ± 0.097	0.398 ± 0.104
Distal BW (cm)	1.803 ± 0.192	1.828 ± 0.183	1.777 ± 0.198	1.805 ± 0.193	1.801 ± 0.192
Distal BMD (g/cm^2)	0.222 ± 0.042	0.227 ± 0.040	0.217 ± 0.044	0.225 ± 0.042	0.220 ± 0.043

†Mean ± SD. BMC, bone mineral content, BW, bone width, BMD, bone mineral density. No significant difference were observed between either the supplemented and placebo groups or between boys and girls in any of the variables.

than the age and sex matched British children. However, after adjusting for differences in bone and body size, differences between the British and Gambian children in BMC and BMD were no longer significant.

Table 8.2 Comparison of anthropometric and mid-shaft radius bone variables between Gambian and British children[†]

	Gambian (n = 160)	British (n = 85)
Weight (kg)	25.2 ± 4.0	36.7 ± 8.5[a]
Height (cm)	132.1 ± 7.2	142.2 ± 8.0[a]
Mid-shaft radius BMC (g/cm)	0.443 ± 0.086	0.493 ± 0.083[a]
Mid-shaft radius BW (cm)	0.968 ± 0.115	1.045 ± 0.121[b]
Mid-shaft radius BMD (g/cm^2)	0.456 ± 0.060	0.471 ± 0.054[a]

[†] Mean ± SD BMC = bone mineral content, BW, bone width, BMD, bone mineral density. British values significantly greater than Gambian., [a] $p \leq 0.0001$, [b] $p = 0.003$.

Data from Cambridge children aged 8.9 - 11.97 years (see methods).

Fig.8.1

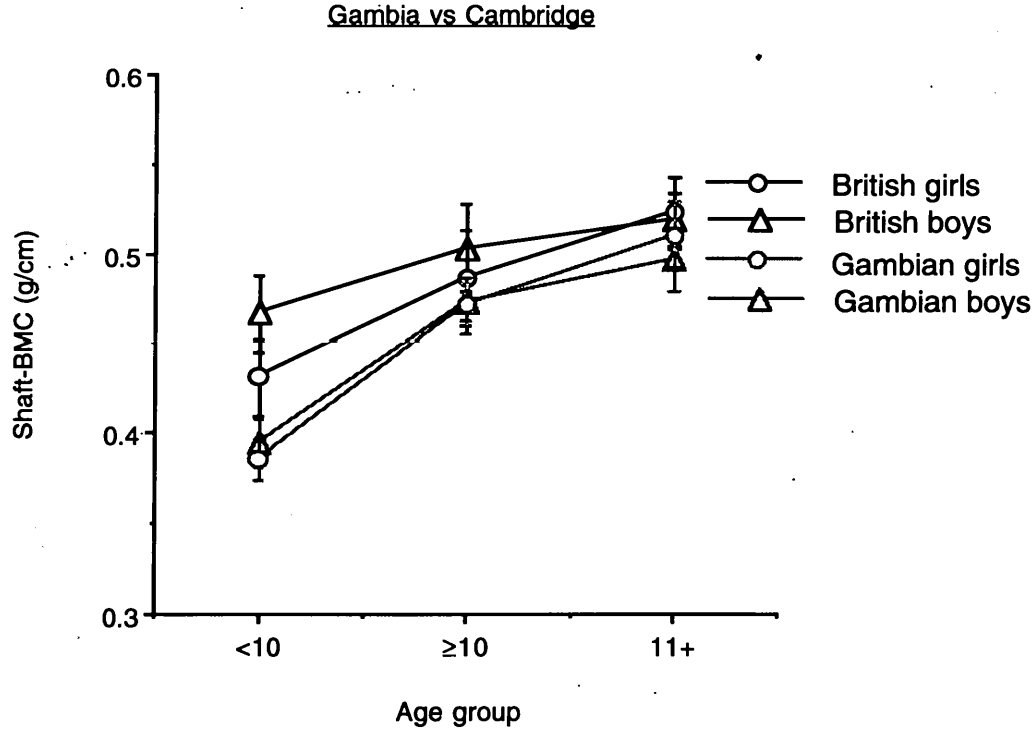


Figure 8.2

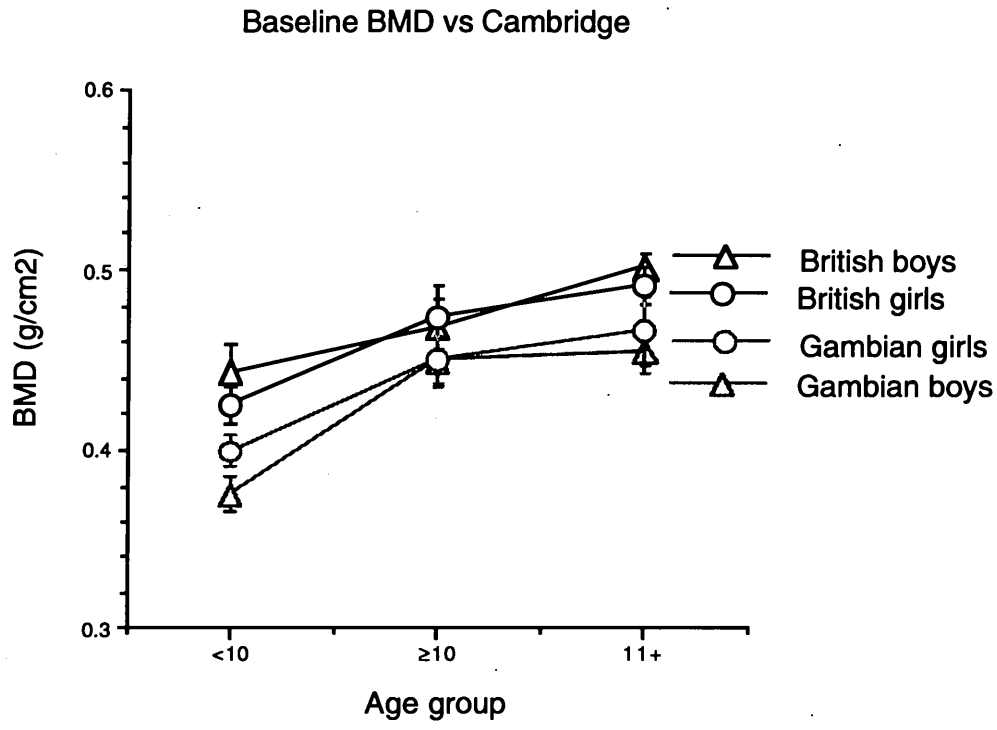


Fig.8.3

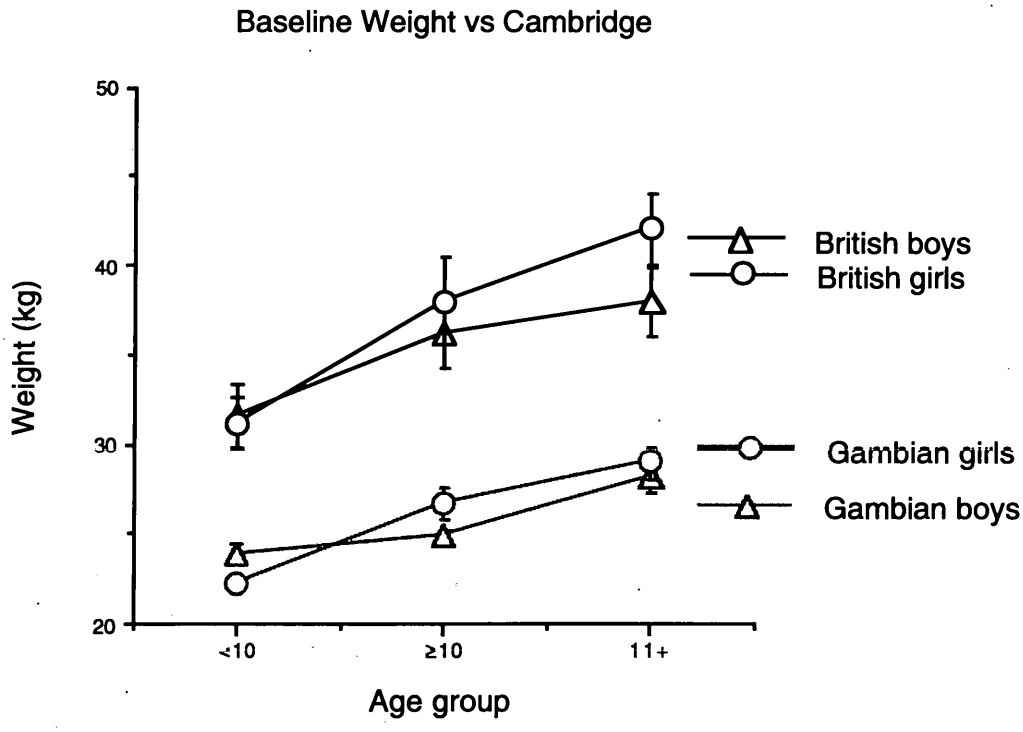
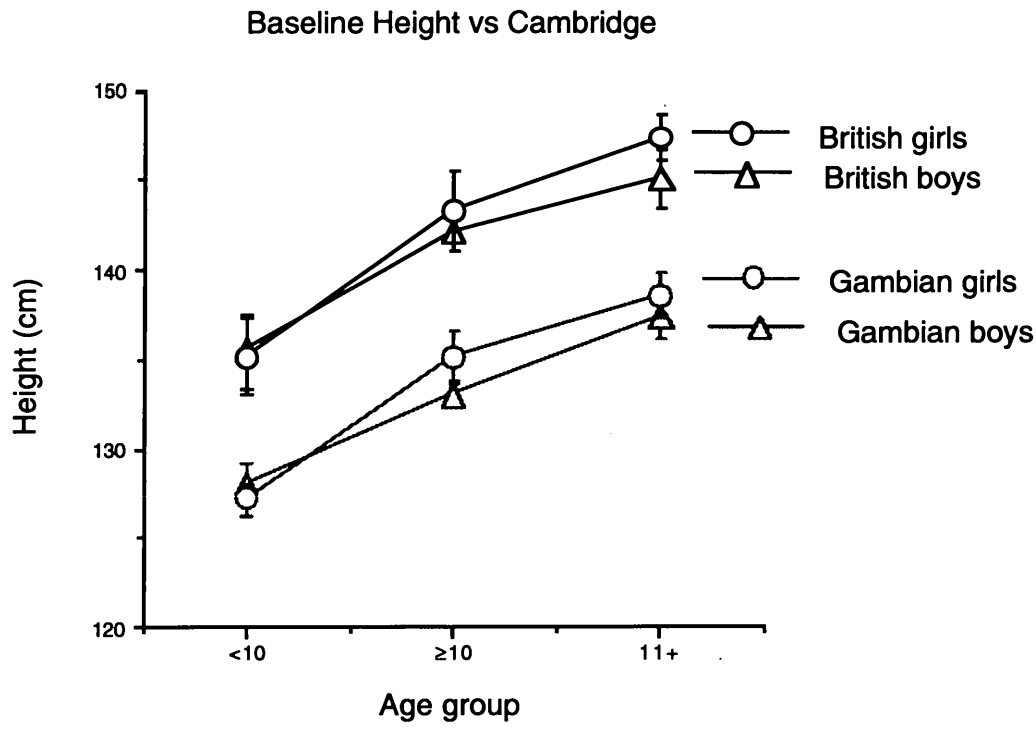


Fig.8.4



8.3 Bone mineral status at outcome

8.3.1 Differences between boys and girls

There was no significant difference between boys and girls in any of the bone variables at outcome (Table 8.3).

8.3.2 Differences between supplemented and placebo groups

Bone mineral content and bone mineral density at the mid-shaft radius were significantly greater in the supplemented compared with placebo group at outcome (Table 8.3). There was a trend towards a smaller bone width at the mid-shaft radius in the supplemented group compared with the placebo group at outcome but the difference between the two was not significant (Table 8.3). At the distal radius, the supplemented group had significantly greater bone mineral content (BMC), bone width (BW) and bone mineral density (BMD) than placebo group at outcome (Table 8.3).

Table 8.3 Bone variables of the supplemented and placebo groups and by sex at outcome[†]

	Total (n = 80)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n= 80)
Mid-shaft BMC (g/cm)	0.521 ± 0.099	0.493 ± 0.078	0.467 ± 0.090[a]	0.487 ± 0.085	0.474 ± 0.085
Mid-shaft BW (cm)	1.029 ± 0.116	0.988 ± 0.101	0.991 ± 0.118	1.006 ± 0.113	0.972 ± 0.110
Mid-shaft BMD (g/cm^2)	0.504 ± 0.061	0.498 ± 0.050	0.470 ± 0.060[b]	0.483 ± 0.057	0.486 ± 0.057
Distal radius BMC (g/cm)	0.499 ± 0.124	0.487 ± 0.132	0.423 ± 0.111[c]	0.452 ± 0.131	0.458 ± 0.121
Distal radius BW (cm)	1.990 ± 0.215	1.903 ± 0.199	1.827 ± 0.190[d]	1.868 ± 0.195	1.862 ± 0.202
Distal radius BMD (g/cm^2)	0.249 ± 0.046	0.253 ± 0.050	0.231 ± 0.050[e]	0.239 ± 0.052	0.245 ± 0.051

[†]Mean ± SD. The supplemented group had significantly greater BMC and BMD at the mid-shaft radius and in all cases at distal radius than placebo group at outcome. Differences between the supplemented and placebo groups in BW at the mid-shaft radius were not significant but were greater in the placebo group than the supplemented group. [a] $p = 0.049$, [b] $p = 0.013$, [c] $p = 0.001$, [d] $p = 0.014$, [e] $p = 0.005$. There was no significant difference between boys and girls in any of the bone variables at outcome.

8.4 Change in bone variables over 12 months

8.4.1 Differences between boys and girls

Tables 8.4 and 8.5 show changes in bone mineral over the 12 month period for the supplemented and placebo groups and for boys and girls. After adjustment for baseline value, there was no significant difference between boys and girls in any of the bone variables at outcome (Table 8.4).

Table 8.4 Differences in bone measures between boys and girls after 12 months of the trial[†]

	Outcome - Baseline[‡]		#Δ%difference at outcome Boys vs girls		
	Boys (n = 80)	Girls (n = 80)			
			mean	SE	significance
Mid-shaft BMC (g/cm)	0.037 ± 0.040	0.037 ± 0.049	+0.1	1.4	0.96 (ns)
Mid-shaft BW (cm)	0.025 ± 0.053	0.018 ± 0.066	+1.1	0.9	0.23 (ns)
Mid-shaft BMD (g/cm^2)	0.025 ± 0.029	0.030 ± 0.031	-1.1	1.0	0.27 (ns)
Distal BMC (g/cm)	0.043 ± 0.109	0.060 ± 0.093	-4.3	3.2	0.18 (ns)
Distal BW (cm)	0.063 ± 0.148	0.061 ± 0.138	+0.2	1.2	0.84 (ns)
Distal BMD (g/cm^2)	0.014 ± 0.047	0.025 ± 0.046	-4.1	2.7	0.14 (ns)

[†] Mean ± SD. BMC, bone mineral content; BW, bone width. #Δ% = percentage difference at outcome, after correcting for baseline value. Data transformed to natural logarithms before analysis (regression). Changes in bone variables were not significantly different between boys and girls. After correcting for baseline value, there was no significant difference between boys and girls in any of the bone variables at outcome. ns, not significant.

8.4.2 Effect of the calcium supplement

The gain in BMD at the mid-shaft and distal radius BMC over the 12 month period was significantly greater in the supplemented than placebo group (Table 8.5). The increment in BW at the mid-shaft radius was significantly smaller in the supplemented than the placebo group, an effect that was not seen in distal BW.

After correcting for baseline value there was a significantly higher BMC and BMD at the mid-shaft and distal radius in the calcium supplemented group compared with placebo (Table 8.5). There was a trend towards a smaller BW in the supplemented group at the mid-shaft radius but the difference did not reach statistical significance ($p = 0.06$).

After adjusting for differences in BW, statural height and body weight at baseline, size-adjusted BMC at the mid-shaft and distal radius was significantly greater in the supplemented than placebo group (mid-shaft, +4.8 SE 0.9 %, $p \leq 0.0001$; distal radius, +5.5 SE 2.7%, $p=0.042$). There was no interaction of supplement effect with age, sex, dietary calcium intake, or pubertal status. This demonstrates a significant effect of calcium supplementation on bone mineral accretion of Gambian children accustomed to a low calcium intake. Since the calcium supplementation for 12 months had no significant effect on height (S - P, -0.0 SE 0.0%, $p = 0.88$), body weight (+0.8 SE 0.8%, $p = 0.32$) (see section 7.3.3) or BW at either the mid-shaft radius (-1.6 SE 0.9%, $p = 0.064$) or distal radius (+2.0 SE 1.2%, $p = 0.086$), the effect of calcium supplement appears to have been on bone mineralisation rather than on skeletal growth.

Table 8.5 Differences in bone measures between the supplemented and placebo groups after 12 months of the trial[†]

	Outcome - Baseline[‡]		[#]Δ%difference at outcome Supplement vs placebo		
	Supplement (n = 80)	Placebo (n = 80)			
			mean	SE	significance
Mid-shaft BMC (g/cm)	0.041 ± 0.044	0.033 ± 0.045	+3.0	1.4	0.034
Mid-shaft BW (cm)	0.012 ± 0.062	0.031 ± 0.056[c]	-1.6	0.9	0.064(ns)
Mid-shaft BMD (g/cm^2)	0.037 ± 0.027[a]	0.019 ± 0.030	+4.5	0.9	≤0.0001
Distal BMC (g/cm)	0.068 ± 0.102[b]	0.035 ± 0.099	+8.4	3.2	0.009
Distal BW (cm)	0.075 ± 0.154	0.049 ± 0.131	+2.0	1.2	0.086(ns)
Distal BMD (g/cm^2)	0.026 ± 0.045	0.014 ± 0.048	+7.0	2.7	0.011

[†] Mean ± SD. BMC, bone mineral content; BW, bone width. [#]Δ% = percentage difference at outcome, after correcting for baseline value. Data transformed to natural logarithms before analysis (regression). Changes in shaft BMD, distal radius BMC over the 12 month period were significantly greater in the supplemented than placebo group. Change over the 12 month period in shaft BW was significantly greater in the placebo group compared with supplemented group but became non significant after correcting for baseline value. [a]p≤0.0001; [b]p=0.040; [c]p=0.044. ns, not significant.

8.5 Size correction

To examine the effect of calcium supplement and other variables on bone mineral status independently of bone and body size, bone mineral content (BMC) was corrected for bone width (BW), body weight and height, using multiple regression analysis (see methods). The baseline value was entered as an independent variable in all regression analysis to adjust for regression towards the mean (method section for detail). All continuous variables were converted to natural logarithms prior to analysis. Example of the full model and after backwards elimination (parsimonious model) are given below.

8.5.1 Example

Fig.8.5 Effect of supplement on BMC at the mid-shaft radius at outcome. Full model

Dependent variable is: 100ΔlnsbmC*
No Selector
R squared = 73.0% R squared (adjusted) = 71.0%
s = 5.342 with 160 - 12 = 148 degrees of freedom

Source	Sum of Squares	df	Mean Square	F-ratio
Regression	11446.5	11	1040.59	36.5
Residual	4224.18	148	28.5417	

Variable	Coefficient	s.e. of Coeff	t-ratio	prob
Constant	-140.644	80.52	-1.75	0.0828
100* ln1sbmc	-0.2388	0.0364	-6.56	≤ 0.0001
100Δlnsbw	1.0564	0.0801	13.2	≤ 0.0001
100lnmBW	0.2358	0.0610	3.86	0.0002
100Δlnwt	-0.0476	0.0942	-0.505	0.6143
100lnwtm	0.0635	0.0706	0.898	0.3705
100Δlnht	0.9318	0.3031	3.07	0.0025
100lnhtm	0.2286	0.1986	1.15	0.2514
Sex	0.4489	0.9209	0.487	0.6266
pub2	-0.8189	1.201	-0.682	0.4964
Agem	-1.0002	0.6718	-1.49	0.1387
S/P	4.5955	0.8718	5.27	≤ 0.0001

All variables are in natural logathrims, except sex, pubertal status and age mean. 100ΔlnsBMC, change in mid-shaft BMC multiplied by 100; at baseline 100ln1SBMC, mid-shaft radius BMC at baseline multiplied by 100; 100Δlnsbw, change from baseline to outcome in BW multiplied by 100; 100lnmBW, mean BW multiplied by 100; 100Δlnwt, change in weight multiplied by 100; 100lnwtm, mean weight multiplied by 100; 100Δlnht, change in height multiplied by 100; 100lnhtm, mean height multiplied by 100; Sex, boys = 0, girls = 1; S/P refers to supplemented and placebo groups respectively, where s = 1, p = 0; pub2 is pubertal stage at outcome (year2), Tanner stage 1 = 0, stage 2 - 4 = 1; Agem, mean age of subjects at baseline plus outcome.

Figure 8.6 Effect of supplement on BMC at the mid-shaft radius at outcome. After backward elimination.

Dependent variable is: 100ΔlnsbmC*
No Selector
R squared = 72.3% R squared (adjusted) = 71.2%
s = 5.330 with 160 - 7 = 153 degrees of freedom

Source	Sum of Squares	df	Mean Square	F-ratio
Regression	11323.9	6	1887.32	66.4
Residual	4346.77	153	28.4103	

Variable	Coefficient	s.e. of Coeff	t-ratio	prob
Constant	-145.364	51.98	-2.80	0.0058
100*lnsbmc1*	-0.2575	0.0342	-7.52	≤ 0.0001
100Δlnsbw*	1.0799	0.0780	13.8	≤ 0.0001
100lnmBW*	0.2586	0.0581	4.45	≤ 0.0001
100Δlnht	1.0347	0.2768	3.74	0.0003
100lnhtm	0.2534	0.1040	2.44	0.0160
S/P	4.8268	0.8547	5.65	≤ 0.0001

All variables are in natural logathrims, except sex, pubertal status and age mean. 100ΔlnsBMC, change in mid-shaft BMC multiplied by 100; 100*lnSBMC1*, mid-shaft radius BMC at baseline multiplied by 100; 100Δlnsbw, change from baseline to outcome in BW multiplied by 100; 100lnmBW, mean BW multiplied by 100; 100Δlnht, change in height multiplied by 100; 100lnhtm, mean height multiplied by 100; S/P refers to supplemented and placebo groups respectively, where s = 1, p = 0.

8.6 Differences in bone mineral status between the supplemented and placebo groups, divided by sex

In both sexes, many of the changes in bone variables over the 12 month period were similar in the supplemented and placebo groups (Table 8.6).

After correcting for baseline value, the supplemented group in boys had significantly greater radial bone mineral density at mid-shaft and distal radius than placebo group at outcome (mid-shaft BMD, +4.6 (SE 1.3)%, p = 0.0007; distal radius BMD, +10.1 (SE 3.9)%, p = 0.012, Table 8.6). In girls, the increase in BMD at the mid-shaft and BW at distal radius was significantly higher in the supplemented than placebo group at outcome, after adjustment for baseline

value (mid-shaft BMD, +4.3 (SE 1.3)%, p = 0.001; distal radius BW, +3.8 (SE 1.6)%, p = 0.018, Table 8.6).

However, the magnitude of the effects at the mid-shaft was similar in both boys and girls. No interactions were observed between supplement group and age, sex, dietary calcium intake or pubertal status, indicating that the response to the supplement was not influenced by any of these variables.

Table 8.6 Differences in bone variables of the supplemented and placebo groups, by sex over 12 months[†]

	Outcome - Baseline		[#]Δ%difference at outcome supplemented vs placebo		
	supplemented (40 M, 40 F)	placebo (40 M, 40 F)			
			mean	SE	significance
Boys					
Mid-shaft BMC (g/cm)	0.041 ± 0.040	0.033 ± 0.040	+3.0	1.9	p=0.11 (ns)
Mid-shaft BW (cm)	0.015 ± 0.054	0.035 ± 0.050	-1.6	1.1	p=0.16 (ns)
Mid-shaft BMD (g/cm^2)	0.035 ± 0.025 [a]	0.016 ± 0.030	+4.6	1.3	p=0.0007
Distal BMC (g/cm)	0.062 ± 0.106	0.023 ± 0.108	+9.4	4.8	p=0.056(ns)
Distal BW (cm)	0.062 ± 0.166	0.065 ± 0.130	-0.2	1.7	p=0.91 (ns)
Distal BMD (g/cm^2)	0.025 ± 0.043[b]	0.004 ± 0.050	+10.1	3.9	p=0.012
Girls					
Mid-shaft BMC (g/cm)	0.042 ± 0.048	0.032 ± 0.050	+2.8	2.1	p=0.18 (ns)
Mid-shaft BW (cm)	0.010 ± 0.069	0.027 ± 0.062	-1.5	1.4	p=0.27 (ns)
Mid-shaft BMD (g/cm^2)	0.039 ± 0.029[c]	0.021 ± 0.031	+4.3	1.3	p=0.001
Distal BMC (g/cm)	0.073 ± 0.100	0.047 ± 0.088	+7.4	4.1	p=0.077(ns)
Distal BW (cm)	0.088 ± 0.141	0.034 ± 0.131	+3.8	1.6	p=0.018
Distal BMD (g/cm^2)	0.027 ± 0.049	0.023 ± 0.044	+3.8	3.7	p=0.31 (ns)

[†] Mean ± SD. BMC, bone mineral content; BW, bone width. [#]Δ% = percentage difference at outcome, after correcting for baseline value. Data transformed to natural logarithms before analysis (regression). Change over the 12 month period in mid-shaft radius BMD and distal BMD in boys were significantly greater in the supplemented compared with placebo group.[a] p=0.002,[b] p=0.045. In girls, gain in mid-shaft radius BMD was significantly greater in the supplemented than placebo group, [c] p=0.009. There were no significant differences between the supplemented and placebo group in either sex in the incremental gain in other bone variables. ns, not significant

8.7 Bone mineral status at follow-up

Bone variables at follow-up for all subjects, for boys and girls and for supplemented and placebo groups separately are summarised in Tables 8.7 - 8.10.

8.7.1 Differences between boys and girls

There were no significant differences between boys and girls in any of the bone variables at follow-up.

8.7.2 Differences between supplemented and placebo groups

Unadjusted bone mineral content (BMC) and bone mineral density (BMD) at the mid-shaft radius and distal radius were significantly higher in the supplemented than placebo group at follow-up (Table 8.7). There was no significant difference in BW at either the mid-shaft or distal radius between the groups at follow-up.

Table 8.7 Bone variables of the supplemented and placebo group and of each sex, at the mid-shaft and distal radius at follow-up[†]

	All subjects (n = 160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
Mid-shaft BMC (g/cm)	0.521 ± 0.099	0.539 ± 0.095[a]	0.502 ± 0.100	0.517 ± 0.098	0.525 ± 0.100
Mid-shaft BW (cm)	1.029 ± 0.116	1.034 ± 0.114	1.025 ± 0.117	1.039 ± 0.118	1.020 ± 0.113
Mid-shaft BMD (g/cm^2)	0.504 ± 0.061	0.520 ± 0.053[b]	0.488 ± 0.066	0.495± 0.058	0.513 ± 0.064
Distal radius BMC (g/cm)	0.499 ± 0.124	0.519 ± 0.121[c]	0.478 ± 0.124	0.495 ± 0.132	0.502 ± 0.116
Distal radius BW (cm)	1.990 ± 0.215	2.017 ± 0.207	1.963 ± 0.220	1.985 ± 0.227	1.995 ± 0.204
Distal radius BMD (g/cm^2)	0.249 ± 0.046	0.256 ± 0.043[d]	0.242 ± 0.048	0.248 ± 0.048	0.250 ± 0.045

[†]Mean ± SD. BMC, bone mineral content, BW, bone width, BMD, bone mineral density. The supplemented group had a significantly greater BMC and BMD at both the mid-shaft and distal radius than placebo group at follow-up. BW at either the mid-shaft radius or distal radius was not significantly different between the groups at follow-up. There was no significant different between boys and girls in any of the bone variables at follow-up.[a] $p = 0.017$, [b] $p = 0.0009$, [c] $p = 0.035$, [d] $p = 0.047$.

8.8.1 Change in bone mineral status over the 24 month period

8.8.1.1 Differences between boys and girls

Both boys and girls had significant gains in BMC, BW and BMD at the mid-shaft and distal radius over the 24 month period (Table 8.8). The increases in mid-shaft radius BMC and BMD were significantly greater in girls than boys (Table 8.8). Girls had greater gains in BMC and BMD at distal radius than boys, but differences did not reach statistical significance. Gain in BW at distal radius over 24 months was similar in boys and girls (Table 8.8).

After adjustment for baseline value, the incremental gain in BMC and BMD at the mid-shaft were significantly greater in girls than boys at follow up (Table 8.8). These differences remained even after correcting for supplement effect (see 8.8.3.2). BW adjusted for baseline at mid-shaft was not significantly different between the sexes at follow-up. After correcting for baseline value there was no significant difference in the distal radius bone variables between boys and girls at follow-up, but there were trends towards greater BMC and BMD in girls.

Table 8.8 Difference between boys and girls in the incremental gain in mid-shaft radius and distal radius variables at follow-up[†]

	Follow-up - Baseline		#Δ%difference at follow-up		
	Boys (n = 80)	Girls (n = 80)	Boys vs Girls		
			mean	SE	significance
Mid-shaft BMC (g/cm)	0.067 ± 0.051[a]	0.089 ± 0.056[a]	-4.4	1.7	p=0.010
Mid-shaft BW (cm)	0.058 ± 0.054[a]	0.066 ± 0.061[a]	-0.6	0.8	p=0.51 (ns)
Mid-shaft BMD (g/cm^2)	0.038 ± 0.035[a]	0.057 ± 0.039[a]	-3.8	1.2	p=0.002
Distal radius BMC (g/cm)	0.086 ± 0.113[a]	0.104 ± 0.088[a]	-4.0	3.0	p=0.20 (ns)
Distal radius BW (cm)	0.180 ± 0.208[a]	0.194 ± 0.137[a]	-0.8	1.3	p=0.54 (ns)
Distal radius BMD (g/cm^2)	0.023 ± 0.047[a]	0.031 ± 0.043[a]	-2.4	2.5	p=0.33 (ns)

[†] Mean ± SD. BMC, bone mineral content; BW, bone width. Data transformed to natural logarithms prior to analysis. Follow-up significantly different from baseline. [a] p≤0.0001 (paired t- test). Change in mid-shaft BMC, and BMD over the 24 month period were significantly greater in girls than boys, p=0.011, p=0.001 respectively. #Δ% = percentage difference at follow-up after correcting for baseline. After adjusting for baseline value, girls had a significantly greater BMC and BMD at mid-shaft radius than boys at follow-up. The BW at mid-shaft radius was not significantly different between the boys and girls. After correcting for the initial value, there was no significant difference between boys and girls in any of the distal radius bone variables at follow-up. ns, not significant.

8.8.1.2 Differences between supplemented and placebo groups at follow-up

Bone mineral status of the supplemented and placebo group were compared at follow-up to examine whether the effect of calcium supplementation on bone mineral accretion observed at outcome remain after supplement withdrawal. The comparisons were made after the variables at follow-up were adjusted for baseline value, sex and pubertal status.

8.8.2.1 Gain in bone mineral at mid-shaft radius

Both the supplemented and placebo groups showed significant gains in BMC, BW and BMD at the mid-shaft and distal radius over the 2 year period (Table 8.9). Gains in BMC and BMD at mid-shaft radius were significantly greater in the supplemented than placebo group (Table 8.9). Change at the mid-shaft BW was not significantly different between the groups at follow-up.

After correcting for baseline value and sex, the gain in BMC and BMD at the mid-shaft were significantly greater in the supplemented compared with placebo group (Table 8.9). The percentage differences (mean SE) at follow-up between supplemented and placebo groups after correcting for baseline were, mid-shaft radius, BMC = + 4.5 SE 1.7%, p = 0.007, BMD = + 5.0 SE 1.2%, p ≤0.0001. There was no interaction of supplement effect with age, sex, dietary calcium intake or pubertal status.

Table 8.9 Comparison of gain in bone variables over 24 months between the supplemented and placebo groups[†]

	Follow-up - Baseline		#Δ%difference at follow-up supplement vs placebo		
	supplement (n = 80)	Placebo (n = 80)			
			mean	SE	significance
Mid-shaft BMC g/cm)	0.087 ± 0.055[a]	0.068 ± 0.051[a]	+4.5	1.7	p=0.007
Mid-shaft BW (cm)	0.059 ± 0.062[a]	0.065 ± 0.052[a]	-0.4	0.8	p=0.66 (ns)
Mid-shaft BMD (g/cm^2)	0.058 ± 0.036[a]	0.037 ± 0.038[a]	+5.0	1.2	p≤0.0001
Distal radius BMC (g/cm)	0.101 ± 0.098[a]	0.090 ± 0.105[a]	+4.1	3.0	p=0.18 (ns)
Distal radius BW (cm)	0.189 ± 0.157[a]	0.185 ± 0.194[a]	+0.9	1.3	p=0.47 (ns)
Distal radius BMD (g/cm^2)	0.029 ± 0.043[a]	0.025 ± 0.047[a]	+4.0	2.5	p=0.11 (ns)

[†] Mean ± SD. BMC, bone mineral content; BW, bone width. #Δ% = percentage difference at follow-up after correcting for baseline value and sex. Data transformed to natural logarithms prior to analysis. Follow-up significantly different from baseline (paired t- test), [a] p≤0.0001. Changes in mid-shaft radius BMC and BMD were significantly greater in the supplemented than placebo groups, p=0.021, p=0.0003 respectively. Changes in distal radius variables were not significantly different between the groups. ns, not significant.

After adjusting for differences at baseline, and for body and bone size (size-adjustment), BMC at the mid-shaft was significantly greater in the supplemented than placebo group at follow-up (+5.0 (SE = 1.1)%, p≤0.0001). There were no interactions between the supplement effect with age, sex, dietary calcium intake or pubertal status, indicating that the effect of supplement was not influenced by any of these variables.

8.8.3.1 Change at distal radius

There was an incremental gain in BMC, BW and BMD at the distal radius over the 24 month period (Table 8.9). There were no significant differences between the supplemented and placebo group in gain in BMC, BW, and BMD at distal radius at follow-up (Table 8.9). After adjustment for baseline value, the supplemented group had greater BMC and BMD than placebo group at follow-up which was similar to the differences observed at mid-shaft radius but was not statistically significant. The percentage difference between the supplemented and placebo groups after adjusting for baseline value, were, BMC, + 4.1 SE 3.0%, p = 0.18 (ns); BMD, +4.0 SE 2.5%, p = 0.11 (ns), (Table 8.9). After correcting for baseline value, there was no significant difference between the supplemented and placebo groups in change in BW at distal radius over the 2 year period (S -P, +0.9 SE 1.3%, p = 0.47).

After adjustment for differences at baseline, and for bone and body size size-adjusted BMC at the distal radius was not significantly different between the supplemented and placebo group at follow-up (+2.7 SE = 2.4%, p = 0.27).

The results at follow-up indicate that the effect of calcium supplementation on bone mineral accretion of rural Gambian children was sustained 12 months after supplement withdrawal especially at mid-shaft radius.

8.8.3.2 Differences between the supplemented and placebo groups by sex

Table 8.10 shows supplement effect in each sex. After correcting for the initial value, effects of supplement were still visible in boys and girls when each sex was looked at separately. After correcting for baseline value, differences in the incremental gain in BMC, BW or BMD at either the mid-shaft or distal radius between the supplemented and placebo groups were not statistically significant in any of the sexes (Table 8.10).

The results provided no evidence that the supplement effect was different in boys and girls and the magnitude of the effects were similar. The lack of significance probably reflects the decrease in power associated with splitting the data by sex. This analysis confirms the lack of a sex interaction in the regression models examining the effect of supplement in the total group (see section 8.5).

Table 8.10 Changes in bone variables over 24 months of the supplemented and placebo groups, by sex[†]

	Follow-up - Baseline		#Δ%difference at follow-up		
	supplemented (40 M, 40 F)	placebo (40 M, 40 F)	supplement vs placebo		
			mean	SE	significance
Boys					
Mid-shaft BMC (g/cm)	0.072 ± 0.048	0.061 ± 0.053	+0.5	2.3	p=0.81 (ns)
Mid-shaft BW (cm)	0.053 ± 0.054	0.063 ± 0.053	-1.2	1.2	p=0.32 (ns)
Mid-shaft BMD (g/cm^2)	0.046 ± 0.033[a]	0.030 ± 0.037	+2.2	1.7	p=0.20 (ns)
Distal BMC (g/cm)	0.089 ± 0.104	0.083 ± 0.123	+2.6	4.8	p=0.58 (ns)
Distal BW (cm)	0.184 ± 0.167	0.175 ± 0.245	+1.2	2.2	p=0.58 (ns)
Distal BMD (g/cm^2)	0.025 ± 0.041	0.021 ± 0.052	+2.9	3.7	p=0.43 (ns)
Girls					
Mid-shaft BMC (g/cm)	0.103 ± 0.058[b]	0.075 ± 0.049	+3.3	2.6	p=0.20 (ns)
Mid-shaft BW (cm)	0.065 ± 0.069	0.067 ± 0.052	-0.0	1.3	p=1.00 (ns)
Mid-shaft BMD (g/cm^2)	0.071 ± 0.035[c]	0.044 ± 0.039	+3.8	2.0	p=0.06 (ns)
Distal BMC (g/cm)	0.112 ± 0.090	0.096 ± 0.085	+4.4	3.9	p=0.26 (ns)
Distal BW (cm)	0.194 ± 0.148	0.195 ± 0.127	-0.4	1.5	p=0.77 (ns)
Distal BMD (g/cm^2)	0.033 ± 0.044	0.029 ± 0.041	+3.9	3.5	p=0.27 (ns)

[†] Mean ± SD. BMC, bone mineral content; BW, bone width, BMD, bone mineral density. #Δ% = percentage difference at follow-up, after correcting for baseline value and sex at mid-shaft radius and after variables at distal radius were adjusted for the initial value. Data transformed to natural logarithms before analysis. Change over the 24 month period at shaft BMD in boys were significantly greater in the supplemented than placebo group [a] p=0.0408. In girls, gain at mid-shaft BMC and BMD were significantly greater in the supplemented than placebo group, [b] p=0.023, [c] p=0.002. There were no significant differences between the groups in the incremental gain in other bone variables in either sex. ns, not significant.

8.9 Ultrasound at baseline

8.9.1 Effect of age and other characteristics on ultrasound variables

Normalised bone ultrasound attenuation (nBUA, dB/MHZ) was positively related to age at baseline. There was an increase with age (p≤0.0001). There was no association between velocity of sound (VOS, m/second) and age at baseline.

There was no effect of weight or height on VOS at baseline (weight, p = 0.18; height, p = 0.054). After adjusting for age and pubertal status, there were significantly positive effects of weight and height on nBUA at baseline ($p \leq 0.0001$, p = 0.001 respectively). When boys and girls were considered separately, weight and height were associated with nBUA in girls (weight, $p \leq 0.0001$; height, p = 0.0007) but not in boys (weight, p = 0.19; height, p = 0.31).

There was a significantly positive correlation between nBUA and pubertal status at baseline ($p \leq 0.0001$) but no association between VOS and pubertal status (p = 0.26).

Table 8.11 gives mean ± SD of baseline VOS and BUA. After correcting for age, weight, height and pubertal status, there was no significant difference between either the supplemented and placebo groups or between boys and girls in any of the ultrasound variables at baseline.

Table 8.11 Baseline ultrasound VOS and nBUA for all subjects, by supplemented and placebo group, and by sex†.

	Total (n = 160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
VOS (dB/MHZ)	1744 ± 51	1741 ± 49	1745 ± 52	1737 ± 48	1750 ± 52
nBUA(m/s)	60.3 ± 13.1	60.2 ± 11.1	60.4 ± 14.9	61.3 ± 11.7	59.3 ± 14.4

† Mean ± SD. VOS, velocity of sound, nBUA, broadband ultrasound attenuation. After correcting for age, there was no significant difference between either the supplemented and placebo groups or between boys and girls in any of the ultrasound variables at baseline.

8.9.2 Correlations between ultrasound and SPA variables

Correlations between ultrasound and SPA variables were tested using Pearson Product-moment. nBUA measurement was significantly correlated with

mid-shaft radius BMC (r = 0.40, p = 0.032) and BW (r = 0.38, p = 0.009) but not BMD (r = 0.27, p = 0.40) using SPA. There were also significant correlations between nBUA and BMC (r = 0.42, p = 0.001), BW (r = 0.35, p = 0.025) and BMD (r = 0.36, p = 0.003) at distal radius. No significant correlations were found between VOS and SPA measurements.

8.10 Ultrasound measurements at outcome

After correcting for age and pubertal status, there were no significant differences in ultrasound variables either between the supplemented and placebo groups or between boys and girls at outcome (Table 8.12).

Table 8.12 Velocity of sound and broadband ultrasound attenuation of supplemented and placebo groups, and by sex at outcome[†]

	Total (n=160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
VOS (dB/MHZ)	1716 ± 82	1716 ± 79	1717 ± 85	1710 ± 76	1723 ± 87
nBUA (m/s)	60.6 ± 10.7	60.4 ± 9.3	60.8 ± 12.0	59.3 ± 9.2	62.0 ± 11.9

[†]Mean ± SD. VOS, velocity of sound, nBUA, broadband ultrasound attenuation. After adjusting for age and pubertal status, there was no significant difference between either the supplemented and placebo groups or between boys and girls at outcome.

8.10.1 Gain in ultrasound measures

8.10.1.1 Differences between boys and girls

Girls had a significant change in VOS and nBUA over the 12 month period (Table 8.13). Only the VOS at outcome in boys was significantly different from baseline (Table 8.13).

Table 8.13 Changes in ultrasound variables in boys and girls over 12 months[†]

	Baseline		Outcome	
	Boys (n = 80)	Girls (n = 80)	Boys (n = 80)	Girls (n = 80)
VOS (dB/MHZ)	1737 ± 48	1750 ± 52	1710 ± 76[a]	1723 ± 87[b]
nBUA (m/s)	61.3 ± 11.7	59.3 ± 14.4	59.3 ± 9.2	62.0 ± 11.9[c]

[†]Mean ± SD. VOS, velocity of sound; nBUA, broadband ultrasound attenuation. Baseline not significantly different between the sexes. Change in VOS and nBUA over 12 months were significantly greater in girls. Change over 12 month in VOS but not nBUA was significant in boys. [a] p = 0.001, [b] p = 0.008, [c] p = 0.012.

Changes in ultrasound variables over 12 month were significantly different between boys and girls (Table 8.14). After adjustment for age and pubertal status, change in VOS and nBUA in girls were 1.1% lower and 7.4% greater than boys respectively (p = 0.032 and p = 0.0006). After further correction for baseline value, age and pubertal status, girls had significantly lower VOS and higher nBUA than boys (Table 8.14).

Table 8.14 Gains in ultrasound measures, of boys and girls after 12 months of the study[†]

	Outcome - Baseline		[#]Δ%difference at outcome Boys vs Girls		
	Boys (n = 80)	Girls (n = 80)			
			Mean	SE	significance
VOS (dB/MHZ)	-27.5 ± 72.0	-26.5 ± 86.6[a]	+1.0	0.5	p=0.043
nBUA (m/s)	-2.0 ± 11.2	2.6 ± 9.3[b]	-7.7	2.1	p=0.0004

[†] Mean ± SD. [#]Δ% = percentage difference at outcome after correcting for baseline value, age and pubertal status. Data transformed to natural logarithms before analysis. VOS, velocity of sound; nBUA, broadband ultrasound attenuation. Change in ultrasound variables over 12 month was significantly different between boys and girls. [a] p = 0.032, [b] p = 0.0006.

8.10.1.2 Differences between supplemented and placebo groups

Both the supplemented and placebo groups had a significant decrease in VOS over the 12 months (Table 8.15). After correcting for baseline value, age, sex and pubertal status, there were no significant differences between the supplemented and placebo groups in percentage change in VOS and nBUA at outcome (Table 8.15). There were no interactions between the supplemented group and age, sex or pubertal status.

Table 8.15 Gains in ultrasound measures of the supplemented and placebo groups after 12 months of the study[†]

	Outcome - Baseline		#Δ%difference at outcome Supplement vs Placebo		
	Supplemented (n = 80)	Placebo (n = 80)	Mean	SE	significance
VOS (dB/MHZ)	-25.9 ± 74.7[a]	-28.1 ± 84.2[b]	+0.6	0.7	p=0.95 (ns)
nBUA (m/s)	0.24 ± 9.2	0.44 ± 11.7	-0.6	2.2	p=0.78 (ns)

[†] Mean ± SD. Data transformed to natural logarithms before analysis. VOS, velocity of sound; nBUA, broadband ultrasound attenuation. VOS at outcome was significantly different from baseline. [a]p = 0.003, [b]p = 0.004. There was no significant difference between the supplemented and placebo group in change in ultrasound variables over the 12 month period. ns, not significant.

8.11 Ultrasound measures at follow-up

8.11.1 Differences between the supplemented and placebo groups and between boys and girls in ultrasound variables at follow-up

After correcting for age and pubertal status, there was no significant difference between either the supplemented and placebo groups, or boys and girls in any of the ultrasound variables at follow-up (Table 8.16).

Table 8.16 Ultrasound VOS and nBUA for all subjects, divided by supplemented and placebo groups and by sex at follow-up†.

	Total (n = 160)	Supplement (n = 80)	Placebo (n = 80)	Boys (n = 80)	Girls (n = 80)
VOS (dB/MHZ)	1730 ± 42	1725 ± 41	1734 ± 43	1727 ± 42	1732 ± 43
nBUA(m/s)	63.1 ± 11.4	62.3 ± 11.2	64.0 ± 11.7	64.0 ±11.4	62.4 ± 11.5

† Mean ± SD. VOS, velocity of sound, BUA, broadband ultrasound attenuation. After correcting for age, height and pubertal status, there was no significant difference between either the supplemented and placebo groups, or boys and girls in any of the ultrasound variables at follow-up.

8.11.2 Change in ultrasound variables over 24 months

8.11.2.1 Differences between the supplemented and placebo groups and between boys and girls

After adjustment for baseline value, age and pubertal status, there were no significant differences in percentage VOS and nBUA between boys and girls at follow-up (Table 8.17). After correcting for baseline value, age, height and pubertal status, changes in nBUA and VOS over the 2 year period were not significantly different between the supplemented and placebo groups (Table 8.18).

Table 8.17. Difference between boys and girls in the incremental gain in ultrasound variables at follow-up†

	Follow-up - Baseline		#Δ%difference at follow-up Boys vs Girls		
	Boys (n = 80)	Girls (n = 80)			
			mean	SE	significance
VOS (dB/MHZ)	-9.9 ± 51.0	-17.9 ± 54.3[a]	+0.2	0.4	p=0.68 (ns)
nBUA (m/s)	2.5 ± 12.8	3.0 ± 13.1[b]	-0.1	2.7	p=0.98 (ns)

† Mean ± SD. vos, velocity of sound, BUA, broadband ultrasound attenuation. Data transformed to natural logarithms prior to analysis. Change in ultrasound variables over 24 months were not significantly different between the sexes. #Δ% = percentage difference at follow-up after correcting for baseline value, age and pubertal status. Follow-up significantly different from that at baseline. [a]p = 0.042, [b]p = 0.004. After adjusting for baseline value, age and pubertal status, there was no significant difference between boys and girls in any of the variables at follow-up. ns, not significant.

Table 8.18 Difference between the supplemented and placebo group in the incremental gain in ultrasound variables at follow-up[†]

	Follow-up - Baseline		#Δ%difference at follow-up		
	Supplemented (n = 80)	Placebo (n = 80)	Supplement vs Placebo		
			mean	SE	significance
VOS (dB/MHZ)	-16.8 ± 55.0[a]	-11.1 ± 50.4[b]	-0.4	0.4	p=0.26 (ns)
nBUA (m/s)	2.1 ± 10.9	3.5 ± 14.7[c]	-4.0	2.7	p=0.14 (ns)

[†] Mean ± SD. VOS, velocity of sound, BUA, broadband ultrasound attenuation. Data transformed to natural logarithms prior to analysis. Follow-up significantly different from baseline, [a] p=0.008, [b] p=0.053, [c] p=0.040. Change in ultrasound variables over 24 months were not significantly different between the groups. #Δ% = percentage difference at follow-up after correcting for baseline value, age, sex and pubertal status. ns, not significant.

9. Results: Biochemical markers of calcium and bone metabolism

9.1 Skewed distributions

The distributions of most of the markers of bone metabolism in blood and urine were positively skewed. Data were, therefore, transformed to natural logarithms prior to analysis. Summary statistics for all biochemical markers for this study, are presented as geometric mean unless otherwise stated.

9.2. Effect of age and other characteristics on biochemical markers in blood

There were associations between age, pubertal status and number of biochemical markers measured in blood. A positive association between plasma osteocalcin concentration and age at follow-up ($p = 0.014$) but not at baseline ($p = 0.57$) or outcome ($p = 0.85$) was seen. There was a positive effect of age on alkaline phosphatase at baseline ($p = 0.036$) and follow-up ($p \leq 0.0001$) but not at outcome. Plasma concentration of calcium was negatively related to age at baseline ($p = 0.036$) and follow-up ($p = 0.030$) but not at outcome ($p = 0.062$). There was an age effect on calcium adjusted for albumin at follow-up ($p = 0.025$) but not at other timepoints. There was a positive correlation between plasma concentration of phosphorus and age at follow-up ($p = 0.004$) but not at baseline ($p = 0.39$) or outcome ($p = 0.092$). There was a positive effect of age on PTH at outcome ($P = 0.0006$) but not at baseline ($p = 0.12$) or at follow-up ($p = 0.614$). A negative effect of age on albumin and a positive effect on creatinine concentration at outcome were also observed ($p = 0.0006$ and $p \leq 0.0001$) but not at baseline or follow-up.

After adjusting for age, a positive effect of puberty was seen on the following: plasma osteocalcin concentration at baseline ($p = 0.006$) and outcome ($p = 0.020$) but not at follow-up, PTH at follow-up only ($p = 0.040$), alkaline phosphatase at outcome ($p \leq 0.0001$) and follow-up ($p = 0.0008$), phosphorus at outcome only ($p = 0.010$).

On the basis of the effect of age and pubertal status on some of the blood markers and the differences between boys and girls in some of the variables (see section 9.3), the effect of supplement on blood markers of calcium and bone metabolism was examined after values at outcome and follow-up were appropriately adjusted for these confounding variables.

9.3 Differences between boys and girls

9.3.1 Differences between boys and girls in bone markers at baseline

There were no significant differences between boys and girls in plasma osteocalcin concentration or parathyroid hormone concentration at baseline but boys had significantly lower total plasma alkaline phosphatase, bone-specific alkaline phosphatase and calcium corrected for albumin than girls (Table 9.1).

9.3.2 Differences between boys and girls in bone markers at outcome

There were significant changes in some of the blood markers over the 12 month period. Plasma osteocalcin concentration in both boys and girls was significantly reduced (Tables 9.2, 9.3). Other markers had increased significantly in both boys and girls: total alkaline phosphatase, bone specific alkaline phosphatase and creatinine (Table 9.3). Plasma PTH concentration was significantly increased in girls over the 12 month period (Table 9.3).

After correcting for baseline value, the change in plasma osteocalcin concentration was more negative in boys than girls (-18.1 SE 6.6%, p = 0.008, Table 9.3) and in those who were prepubertal (prepubertal versus pubertal, -15.8 SE 7.2%, p = 0.003), but was influenced independently by age after correcting for puberty. After correcting for baseline and for confounders, boys had significantly lower increases in plasma concentration of total alkaline phosphatase, bone-specific alkaline phosphatase and parathyroid hormone than girls at outcome (Table 9.3).

9.3.3 Differences between boys and girls in bone markers at follow-up

Both boys and girls had significant increases in plasma osteocalcin, total alkaline phosphatase, bone specific alkaline phosphatase concentration over the 24 month period (Tables 9.4, 9.5). PTH concentration was significantly increased in girls over the 2 year period (Table 9.5). After adjusting for baseline value, age, pubertal status and supplement group, the change in plasma osteocalcin concentration at follow-up was significantly lower in boys than girls (-18.3 SE 6.9%, p = 0.009, Table 9.5). Plasma concentrations of total alkaline phosphatase and bone-specific alkaline phosphatase were significantly lower in boys than girls at follow-up (Table 9.4). After correcting for the initial values and other confounders, the incremental gains in plasma total and bone specific alkaline phosphatase were significantly lower in boys than girls at follow-up (Table 9.5).

9.3.4 Differences between boys and girls in other circulating biochemical markers

Both boys and girls had significant increases in creatinine concentration over the 24 month period (Table 9.5). Plasma concentration of albumin was significantly reduced in girls over the 2 year period (Table 9.5). Plasma concentration of albumin was significantly lower in boys than girls at baseline (Table 9.1). Otherwise, there was no significant difference between the sexes in albumin concentration at either outcome or follow-up (Tables 9.2 and 9.4). There were no significant differences between boys and girls in other biochemical markers measured in blood at baseline, outcome or at follow-up before or after adjusting for baseline value and other confounders (Tables 9.1, 9.2, 9.4). An exception was in plasma creatinine concentration which was higher in boys than girls at outcome and at follow-up (Tables 9.2 and 9.4). After adjusting for baseline value, age, pubertal status and supplement group, the change was larger in boys by +8.2% ($p = 0.0002$) compared with girls at follow-up (Table 9.5).

9.4 Effect of supplement on blood markers

9.4.1 Differences between supplemented and placebo groups in bone markers at baseline

On the basis of the effect of sex, age and pubertal status on biochemical markers in blood, the effect of calcium supplement on biochemical markers was explored after appropriate adjustments were made.

Plasma concentrations of osteocalcin, PTH or any other bone marker did not differ significantly between the supplemented and placebo groups at baseline (Table 9.1).

9.4.2 Differences between the supplemented and placebo groups in bone markers at outcome

At outcome, the supplemented group had a significantly lower plasma osteocalcin concentration than at baseline and exhibited a greater decrease over 12 month period compared with the placebo group (supplemented, -26.4 SE 5.01% vs placebo, -3.9 SE 6.44%, Tables 9.2 and 9.6). In contrast, the osteocalcin concentration of the placebo group at outcome was not significantly different from the baseline value (Tables 9.1 and 9.6). After adjusting for baseline value, sex and pubertal status plasma osteocalcin concentration in the supplemented group was significantly lower than in the placebo group by -21.9 SE 6.5%, $p = 0.001$ (Table 9.6). There was no evidence of any interaction between the supplement effect and age, sex or pubertal status.

Plasma concentration of PTH at outcome was significantly greater than baseline in the placebo group (Table 9.6). In contrast, PTH concentration decreased over the 12 month period in the supplemented group but the difference between outcome and baseline was not statistically significant (Table 9.6). At outcome, the plasma concentration of parathyroid hormone was significantly lower in the supplemented group compared with the placebo group (Table 9.2). After correcting for the initial value and other confounders, plasma concentration of parathyroid hormone in the calcium supplemented group was significantly lower than the placebo group (-20.1 SE 5.8%, $p = 0.0008$, Table 9.6). There were significant increases in total alkaline phosphatase, bone specific alkaline phosphatase and creatinine concentration in the supplemented and placebo groups over the 12 month period (Table 9.6). There were no significant differences between the supplemented and placebo group in plasma

concentration of either total alkaline phosphatase or bone-specific alkaline phosphatase at outcome before or after adjustment.

9.4.3 Differences between supplemented and placebo groups in bone markers at follow-up.

Plasma concentration of osteocalcin, total alkaline phosphatase and bone specific alkaline phosphatase in the supplemented and placebo groups at follow-up were significantly greater than at baseline (Tables 9.7). After correcting for baseline value, age and puberty, there was no significant difference between the supplemented and placebo group in osteocalcin concentration at follow-up (supplement : placebo, -0.5 SE 6.5%, p = 0.94, Table 9.7). By follow-up PTH had increased above baseline in the placebo group but not in the supplemented group (Table 9.7). After adjusting for baseline value and other confounders, the supplemented group had significantly lower parathyroid hormone than placebo group at follow-up (-19.3 SE 5.4%, p = 0.0006, Table 9.7). Testing of a calcium by sex interaction term in PTH at follow-up did not achieve statistical significance. There were no significant differences between the supplemented and placebo group in other bone formation markers at follow-up before and after adjustment for baseline and other confounders (Tables 9.4, 9.7).

9.4.4 Differences between supplemented and placebo groups in other markers in blood.

Among other markers in the blood, concentration of plasma creatinine at follow-up was significantly different from baseline in the two groups (Table 9.7). Phosphorus concentration in the placebo group was significantly increased and albumin concentration in the supplemented group was significantly reduced over the 2 year period. Concentrations of other markers in blood were not significantly

Table 9.1 Circulating biochemical markers in the supplemented and placebo groups, and divided by boys and girls at baseline[†]

	Supplement			Placebo			Boys			Girls		
	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N
Osteocalcin (µg/l)	22.4	3.11 ± 0.46	66	22.7	3.12 ± 0.44	67	21.1	3.05 ± 0.50	73	24.3	3.19 ± 0.36	60
Total alk phos (U/L)	395	5.98 ± 0.35	71	372	5.92 ±0.40	75	361	5.89 ±0.41	80	412	6.02 ±0.31[a]	66
Bone alk phos (U/L)	228	5.43 ± 0.42	71	217	5.38 ±0.42	75	204	5.32 ±0.45	80	245	5.50 ± 0.36[b]	66
PTH (pg/ml)	26.8	3.29 ± 0.35	50	24.5	3.20 ±0.34	50	25.8	3.25 ±0.33	49	25.8	3.25 ± 0.36	51
Calcium (mmol/L)	2.27	0.82 ± 0.10	71	2.32	0.84 ±0.08	74	2.27	0.82 ±0.10	79	2.34	0.85 ± 0.07	66
Ca-corr alb(mmol/L)	2.36	0.86 ± 0.12	71	2.39	0.87 ±0.10	72	2.34	0.85 ±0.13	79	2.44	0.89 ±0.08[c]	64
Phosphorus (mmol/L)	1.63	0.49 ± 0.10	71	1.63	0.49 ± 0.12	76	1.65	0.50 ±0.11	80	2.00	0.47 ±0.11	67
Albumin (mmol/L)	39.7	3.68 ± 0.09	71	39.3	3.67 ±0.10	74	38.9	3.66 ±0.11	80	40.0	3.69 ±0.08[d]	65
Creatinine (µmol/L)	43.5	3.77 ± 0.22	71	43.9	3.78 ± 0.27	76	44.4	3.79 ± 0.23	80	42.9	3.76 ± 0.27	67

[†] Mean ± standard deviation of variables after being transformed to natural logarithms. Gmean, geometric mean. Total alk phos, total alkaline phosphatase, Bone alk phos, bone alkaline phosphatase, PTH, parathyroid hormone, Ca - corr alb, calcium corrected for albumin, N, number of subjects in each data set. Data transformed to natural logarithms prior to analysis. Boys - girls, [a] $p = 0.0397$, [b] $p = 0.0097$, [c] $p = 0.0156$, [d] $p = 0.0159$. No other difference between supplement and placebo or between boys and girls was significant.

Table 9.2 Circulating biochemical markers in the supplemented and placebo groups, and divided by boys and girls at outcome[†]

	Supplement			Placebo			Boys			Girls		
	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N
Osteocalcin (µg/l)	16.8	2.82 ± 0.36	56	21.3	3.06 ± 0.44[a]	57	16.4	2.80 ± 0.41	57	21.5	3.07 ± 0.38[b]	56
Total alk phos (U/L)	446	6.10 ± 0.31	74	455	6.12 ± 0.28	75	407	6.01 ± 0.28	80	508	6.23 ± 0.27[c]	69
Bone alk phos (U/L)	279	5.63 ± 0.45	72	284	5.65 ± 0.37	75	245	5.50 ± 0.37	79	334	5.81 ± 0.39[c]	68
PTH (pg/ml)	25.0	3.22 ± 0.36	49	29.1	3.37 ± 0.39[d]	50	24.8	3.21 ± 0.33	49	29.4	3.38 ± 0.41[e]	50
Calcium (mmol/L)	2.29	0.83 ± 0.10	73	2.32	0.84 ± 0.05	74	2.29	0.83 ± 0.10	80	2.32	0.84 ± 0.05	67
Ca-corr alb (mmol/L)	2.39	0.87 ± 0.09	71	2.41	0.88 ± 0.07	72	2.36	0.86 ± 0.10	80	2.41	0.88 ± 0.05	63
Phosphorus (mmol/L)	1.58	0.46 ± 0.12	74	1.67	0.51 ± 0.12[f]	76	1.65	0.50 ± 0.12	80	2.00	0.47 ± 0.12	69
Albumin (mmol/L)	39.3	3.67 ± 0.08	72	39.3	3.67 ± 0.10	72	38.9	3.66 ± 0.10	80	39.7	3.68 ± 0.08	64
Creatinine (µmol/L)	52.1	3.95 ± 0.15	74	50.8	3.93 ± 0.19	75	53.8	3.99 ± 0.17	80	48.9	3.89 ± 0.16[g]	69

[†]mean ± SD of variables after being transformed to natural logarithms. Gmean, geometric mean. Total alk phos, total alkaline phosphatase, Bone alk phos, bone alkaline phosphatase, PTH, parathyroid hormone, Ca-corr alb, calcium corrected for albumin, N, number of subjects in each data set. Data transformed to natural logarithms prior to analysis. Supplement - placebo or boys - girls, [a] $p = 0.002$, [b] $p = 0.0005$, [c] $p \leq 0.0001$, [d] $p = 0.046$, [e] $p = 0.032$, [f] $p = 0.0104$, [g] $p = 0.0004$. No other differences were significant.

Table 9.3 Differences in circulating biochemical markers between outcome and baseline in boys and girls[†]

	Boys		Girls		#Δ%difference at outcome		
	#Δ%	N	#Δ%	N	Boys vs Girls		
					Mean	SE	significance
Osteocalcin (µg/l)	-18.8 ± 6.60[a]	50	-11.3 ± 5.20[b]	48	-18.1	6.6	p=0.008
Total alk phos (U/L)	+11.3 ± 4.07[c]	80	+21.5 ± 4.19[a]	65	-18.8	3.9	p≤0.0001
Bone alk phos (U/L)	+17.0 ± 4.72[a]	79	+30.3 ± 5.61[a]	64	-25.7	5.4	p≤0.0001
PTH (pg/ml)	-3.24 ± 4.95	49	+13.1 ± 4.99[d]	50	-21.3	5.9	p=0.0005
Calcium (mmol/L)	+1.36 ± 1.39	79	-0.23 ± 1.16	63	-1.1	1.3	p=0.38 (ns)
Ca-corr alb (mmol/L)	+1.82 ± 1.46	79	-0.75 ± 1.08	61	-1.0	1.4	p=0.48 (ns)
Phosphorus (mmol/L)	+0.00 ± 1.17	80	+0.19 ± 1.61	66	+1.5	1.7	p=0.37 (ns)
Albumin (mmol/L)	+0.84 ± 1.09	80	-1.21 ± 1.15	63	+0.0	1.3	p=0.99 (ns)
Creatinine (mmol/L)	+19.3 ± 2.75[a]	80	+12.7 ± 3.31[e]	66	+8.1	2.6	p=0.002

[†] Mean ± SE. #Δ% = percentage difference outcome - baseline (100ln[outcome]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at outcome, after adjusting for baseline value, age, pubertal status and supplement group. Data transformed to natural logarithms before analysis. Differences between outcome and baseline significantly different from nought (paired t- test): [a] p≤0.0001, [b] p = 0.037, [c] p = 0.007, [d] p = 0.011, [e] p = 0.0003. Total alk phos, total alkaline phosphatase, Bone alk phos, bone alkaline phosphatase, PTH, parathyroid hormone, Ca-corr albumin, calcium corrected for albumin, N, number of subjects in each data set. ns, not significant. Differences between outcome and baseline for other markers were not significantly different.

Table 9.4 Circulating biochemical markers in the supplemented and placebo groups, and in boys and girls at follow-up[†]

	Supplement			Placebo			Boys			Girls		
	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†]	N
Osteocalcin (µg/l)	29.7	3.39 ± 0.41	51	29.4	3.38 ± 0.41	50	26.3	3.27 ± 0.41	50	33.1	3.50 ± 0.38[a]	51
Total alk phos (U/L)	545	6.30 ± 0.34	51	550	6.31 ± 0.28	50	503	6.22 ± 0.28	50	590	6.38 ± 0.32[b]	51
Bone alk phos (U/L)	361	5.89 ± 0.46	51	369	5.91 ± 0.35	50	330	5.80 ± 0.37	50	403	6.00 ± 0.43[c]	51
PTH (pg/ml)	26.6	3.28 ± 0.40	49	30.3	3.41 ± 0.31	50	27.4	3.31 ± 0.36	48	29.1	3.37 ± 0.37	51
Calcium (mmol/L)	2.36	0.86 ± 0.04	51	2.36	0.86 ± 0.04	51	2.34	0.85 ± 0.04	50	2.36	0.86 ± 0.04	52
Ca-corr alb (mmol/L)	2.41	0.88 ± 0.06	50	2.41	0.88 ± 0.06	50	2.41	0.88 ± 0.07	50	2.44	0.89 ± 0.05	50
Phosphorus (mmol/L)	1.63	0.49 ± 0.12	51	1.67	0.51 ± 0.10	50	1.65	0.50 ± 0.12	50	1.65	0.50 ± 0.10	51
Albumin (mmol/L)	38.1	3.64 ± 0.09	50	38.1	3.64 ± 0.08	50	37.7	3.63 ± 0.10	50	38.1	3.64 ± 0.08	50
Creatinine (µmol/L)	54.9	4.01 ± 0.12	51	53.1	3.97 ± 0.11	50	56.3	4.03 ± 0.11	50	51.9	3.95 ± 0.11[d]	51

[†]mean ± SD of variables after being transformed to natural logarithms. Gmean, geometric mean. Total alk phos, total alkaline phosphatase, Bone alk phos, bone alkaline phosphatase, PTH, parathyroid hormone, Ca-corr alb, calcium corrected for albumin, N, number of subjects in each data set. Data transformed to natural logarithms prior to analysis. Boys - girls: [a] $p = 0.003$, [b] $p = 0.007$, [c] $p = 0.016$, [d] $p = 0.0002$. No other differences between supplement and placebo groups or between boys and girls were significant.

Table 9.5 Differences in circulating biochemical markers between follow-up and baseline in boys and girls at follow-up†

	Boys		Girls		#Δ%difference at follow-up		
	*Δ%	N	*Δ%	N	Boys vs Girls		
					Mean	SE	significance
Osteocalcin (µg/l)	+27.4 ± 5.97 [a]	50	+31.1 ±5.29 [a]	50	-18.3	6.9	p=0.009
Total alk phos (U/L)	+42.4 ± 4.42 [a]	50	+41.6 ± 5.19 [a]	49	-9.1	5.5	p=0.035
Bone alk phos (U/L)	+58.8 ± 6.00 [a]	50	+51.3 ± 7.30 [a]	49	-15.4	7.6	p=0.046
PTH (pg/ml)	+7.30 ± 4.12	48	+12.4 ± 4.65 [b]	51	-6.9	5.5	p=0.21 (ns)
Calcium (mmol/L)	+1.51 ± 1.01	50	+0.55 ± 1.16	50	+0.2	0.9	p=0.86 (ns)
Ca-corr alb (mmol/L)	+1.15 ± 1.21	50	-1.14 ± 1.31	48	+0.7	1.2	p=0.56 (ns)
Phosphorus (mmol/L)	+1.53 ± 1.55	50	+1.41 ± 1.55	50	-1.1	1.9	p=0.56 (ns)
Albumin (mmol/L)	-0.83 ± 1.30	50	-4.81 ± 1.22 [c]	49	+1.7	1.7	p=0.31 (ns)
Creatinine (mmol/L)	+24.2 ± 3.73 [a]	50	+16.7 ± 2.47 [a]	50	+8.2	2.1	p=0.0002

† Mean ± SE. *Δ% = percentage difference follow-up - baseline (100ln[follow-up]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at follow-up, after adjusting for baseline value, age, pubertal status and supplement group. Data transformed to natural logarithms before analysis. Differences between follow-up and baseline significantly different from nought (paired t- test): [a] p≤0.0001, [b] p = 0.010 and [c] p = 0.0002. Differences between follow-up and baseline for other markers were not significantly different. Total alk phos, total alkaline phosphatase, Bone alk phos, bone alkaline phosphatase, PTH, parathyroid hormone, Ca-corr alb, calcium corrected for albumin, N, number of subjects in each data set. ns, not significant.

Table 9.6 Differences in circulating biochemical markers between outcome and baseline in supplemented and placebo groups[†]

	Supplement		Placebo		#Δ%difference at outcome		
	#Δ%	N	#Δ%	N	Supplement vs Placebo		
					Mean	SE	significance
Osteocalcin (µg/l)	-26.4 ± 5.01[a]	49	-3.90 ± 6.44	49	-21.9	6.5	p=0.001
Total alk phos (U/L)	+11.8 ± 3.67[b]	71	+19.8 ± 4.56[a]	74	-4.0	3.8	p=0.30 (ns)
Bone alk phos (U/L)	+18.5 ± 4.94[c]	69	+27.1 ± 5.32[a]	74	-5.3	5.3	p=0.32 (ns)
PTH (pg/ml)	-6.85 ± 4.86	49	+16.7 ± 4.78[d]	50	-20.1	5.8	p=0.0008
Calcium (mmol/L)	+0.65 ± 1.48	70	+0.66 ± 0.90	72	-1.1	1.3	p=0.38 (ns)
Ca-corr alb (mmol/L)	+0.48 ± 1.52	70	+0.92 ± 1.16	70	-1.0	1.3	p=0.46 (ns)
Phosphorus (mmol/L)	-2.37 ± 1.35	71	+2.41 ± 1.33	75	-4.8	1.6	p=0.004
Albumin (mmol/L)	-0.52 ± 1.04	71	+0.39 ± 1.21	72	-0.3	1.3	p=0.83 (ns)
Creatinine (mmol/L)	+18.1 ± 3.01[a]	71	+14.5 ± 3.03[a]	75	+2.3	2.5	p=0.37 (ns)

[†] Mean ± SE. #Δ% = percentage difference outcome - baseline (100ln[outcome]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at outcome, after correcting for baseline value, age, sex and pubertal status. Data transformed to natural logarithms before analysis. Differences between outcome and baseline significantly different from nought (paired t- test): [a] p≤0.0001, [b] p = 0.002, [c] p = 0.0004; [d] p = 0.001. No significant differences between outcome and baseline in other markers. Total alk phos, total alkaline phosphatase, Bone alk phos, bone alkaline phosphatase, PTH, parathyroid hormone, Ca-corr alb, calcium corrected for albumin, N, number of subjects in each data set. ns, not significant.

Table 9.7 Differences in circulating biochemical markers between follow-up and baseline in supplemented and placebo groups at follow-up[†]

	Supplement		Placebo		#Δ%difference at follow-up		
	*Δ%	N	*Δ%	N	Supplement vs Placebo		
					Mean	SE	significance
Osteocalcin (µg/l)	+28.3 ± 5.45[a]	50	+30.0± 5.83[a]	50	-0.5	6.5	p=0.94 (ns)
Total alk phos (U/L)	+39.3 ± 5.29[a]	50	+44.7 ± 4.22[a]	49	-3.2	5.4	p=0.56 (ns)
Bone alk phos (U/L)	+51.1 ± 7.41[a]	50	+59.1 ± 5.80[a]	49	-4.5	7.2	p=0.53 (ns)
PTH (pg/ml)	-0.80 ± 4.47	49	+20.4 ± 3.83[a]	50	-19.3	5.4	p=0.0006
Calcium (mmol/L)	+1.70 ± 1.16	50	+0.36 ± 1.01	50	+0.0	0.8	p=0.98 (ns)
Ca-corr alb (mmol/L)	+0.66 ± 1.25	49	-0.61 ± 1.27	49	+0.0	1.1	p=0.97 (ns)
Phosphorus (mmol/L)	+0.18 ± 1.83	71	+2.76 ± 1.17[b]	50	-2.4	1.9	p=0.20 (ns)
Albumin (mmol/L)	-3.01 ± 1.18[c]	49	-2.60 ± 1.39	50	-0.4	1.6	p=0.78 (ns)
Creatinine (mmol/L)	+23.4 ± 3.61[a]	50	+17.5 ± 2.68[a]	50	+3.8	2.1	p=0.08 (ns)

[†] Mean ± SE. *Δ% = percentage difference follow-up - baseline (100ln[follow-up]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at follow-up, after correcting for baseline value, age, sex and pubertal status. Data transformed to natural logarithms before analysis. Differences between follow-up and baseline significantly different from nought (paired t- test): [a] p≤0.0001, [b] p = 0.023 and [c] p = 0.014. No significant differences between follow-up and baseline in other markers. Total alk phos, total alkaline phosphatase, Bone alk phos, bone alkaline phosphatase, PTH, parathyroid hormone, Ca-corr alb, calcium corrected for albumin, N, number of subjects in each data set. ns, not significant.

different between the supplemented and placebo groups at either baseline, outcome or follow-up (Tables 9.1, 9.2, 9.4, 9.6, 9.7). An exception was the adjusted plasma concentration of phosphorus which was significantly lower in the supplement compared with placebo group at outcome (After adjustment: -4.8 SE 1.6%, p = 0.004, Table 9.6) but not at follow-up. There was no evidence that plasma calcium was affected by the supplement (Tables 9.6, 9.7).

9.4.5 Differences in blood markers between the supplemented and placebo groups, by sex.

Although no significant interaction between the effect of supplement and sex was observed (see section 9.4.2), the effect of calcium supplementation on plasma concentration of some of the biochemical markers was examined in each sex separately to investigate whether there was any evidence that the effect of supplement on blood markers differed between boys and girls. Only the blood markers that were affected by calcium supplementation when sexes were combined are presented here. Similar analyses on the remaining analytes did not reveal any effect of supplement in either sex.

Plasma concentration of osteocalcin in the supplemented group in boys and girls, and PTH in placebo group in girls at outcome were significantly different from baseline (Table 9.8). Changes over the 1 year period of supplementation in plasma osteocalcin, PTH and phosphorus concentrations were not significantly different between the groups in boys. In all variables, changes over 12 months were significantly different between the supplemented and placebo groups in girls (Table 9.8). The magnitude of the change in PTH in the placebo group in boys was lower than that of the placebo group in girls but the difference was not significant as indicated by a lack of supplement x sex

interaction. The effect of the supplement on osteocalcin and phosphorus was of similar magnitude in the two sexes.

After correcting for baseline value, age and pubertal status, there were no significant differences between the supplemented and placebo groups in boys in the incremental gain in plasma concentration of parathyroid hormone and phosphorus at outcome. The decrease in plasma osteocalcin concentration in boys over the 12 month period was greater in the supplemented than placebo group but the difference did not reach statistical significance. After correcting for baseline value and other confounders, the supplemented group in girls had significantly lower plasma concentration of parathyroid hormone (-31.9 SE 8.1%, $p = 0.0003$) and phosphorus (-5.2 SE 2.6%, $p = 0.048$) than placebo group at outcome. After correcting for baseline, age and pubertal status, the supplement effect on plasma PTH concentration was still apparent at follow-up in both sexes (supplement - placebo, boys: -15.8 SE 7.7%, $p = 0.047$; girls: -23.6 SE 7.6%, $p = 0.003$). Interaction terms between calcium supplement and age, and between calcium supplement and pubertal status in girls were not statistically significant.

Table 9.8 Changes in blood variables over 12 months of the supplemented and placebo groups, by sex[†]

	Supplement		Placebo		#Δ%difference at outcome		
	#Δ%	N	#Δ%	N	supplement vs placebo		
					mean	SE	significance
Boys							
Osteocalcin (µg/l)	-28.1 ± 8.24[a]	25	-9.5 ± 10.12	25	-20.8	9.9	p=0.050
PTH (pg/ml)	-8.6 ± 7.37	24	+1.9 ± 6.61	25	-10.0	8.0	p=0.18 (ns)
Phosphorus (mmol/l)	-1.6 ± 1.63	40	+1.6 ± 1.65	40	-3.8	2.2	p=0.092(ns)
Girls							
Osteocalcin (µg/l)	-25.8 ± 5.62[b]	25	+2.7 ± 7.62	25	-25.4	8.6	p=0.005
PTH (pg/ml)	-5.1 ± 6.50	25	+31.4 ± 5.60[c]	25	-31.9	8.1	p=0.0003
Phosphorus (mmol/l)	-3.4 ± 2.27	31	+3.4 ± 2.16	35	-5.2	2.6	p=0.048

[†] Mean ± SE. #Δ% = percentage difference at outcome, after correcting for baseline value and age. Data transformed to natural logarithms before analysis. Variables at outcome corrected for baseline value, pubertal status and age. Differences between outcome and baseline significantly different from nought (paired t - test): [a] p = 0.0012, [b] p = 0.0002, [c] p≤0.0001. PTH , parathyroid hormone, N, number of subjects in each data set. ns, not significant.

9.5 Effect of age and other characteristics on urinary biochemical markers

There was a significant effect of age on the daily outputs of potassium, creatinine, deoxypyridinoline and deoxypyridinoline-to-creatinine ratio at baseline, with output higher in the older children (p = 0.0006, p≤0.0001, p = 0.0002 and p = 0.042 respectively). There was a negative association between the deoxypyridinoline-to-creatinine ratio and age (p = 0.036). This was due to the highly positive correlation of creatinine and less positive correlation of deoxypyridinoline with age. For similar reasons, urinary phosphorus-to-creatinine ratio at baseline was negatively related to age (p≤0.0001). At outcome, the following were negatively related to age: calcium, sodium, calcium-

to-creatinine, sodium-to-creatinine and potassium-to-creatinine ratio ($p = 0.036$, $p = 0.019$, $p = 0.016$, $p = 0.017$, and $p = 0.008$ respectively).

After adjusting for age, urinary outputs of creatinine, potassium, sodium, deoxypyridinoline, sodium-to-creatinine and deoxypyridinoline-to-creatinine ratio were positively related to pubertal status ($p = 0.039$, $p = 0.036$, $p = 0.0005$, $p = 0.023$, $p = 0.034$ and $p = 0.005$ respectively). In contrast, the phosphorus-to-creatinine ratio was significantly negatively related to pubertal status ($p = 0.013$).

On the basis of the effect of age and pubertal status on some of the urinary markers, and the pronounced differences between boys and girls in many of the urinary variables (see section 9.6), the effect of supplement on the urinary markers of calcium and bone metabolism was examined, after values at outcome and follow-up were appropriately adjusted for confounding variables.

9.6 Differences between boys and girls in urine markers

9.6.1 Differences between boys and girls at baseline

The outputs of biochemical markers measured in urine were similar in boys and girls at baseline (Table 9.9).

9.6.2 Differences between boys and girls at outcome

Both boys and girls had significant increases in urinary outputs of calcium, calcium-to-creatinine and calcium-to-sodium ratio and a significant decreases in potassium, deoxypyridinoline-to-creatinine, phosphorus-to-creatinine and potassium-to-creatinine ratios over the 12 month period (Tables 9.10, 9.11). Urinary output of sodium-to-potassium in girls at outcome was significantly greater than baseline value (Table 9.11). Boys had significant increase in

creatinine and a significant decrease in sodium-to-creatinine ratio over the 1 year period. Adjusted calcium and creatinine outputs were significantly greater in boys than girls at outcome (Table 9.11) reflecting differences between sexes at baseline. After adjusting for baseline value, age, pubertal status and supplement group there was no significant difference between boys and girls in change in urinary output of deoxypyridinoline (Table 9.11). The change in sodium-to-creatinine ratio over the 12 month period was significantly lower in boys than girls before and after adjusting for baseline value and other confounders (Tables 9.10, 9.11). Boys had significantly greater increases in calcium-to-sodium ratio than girls before and after correcting for baseline value, age, pubertal status and supplement group (Table 9.10, 9.11) again, reflecting the baseline difference in urinary calcium between the sexes.

9.6.3 Differences between boys and girls at follow-up

There were significant increases in urinary outputs of calcium, creatinine, calcium-to-creatinine and calcium-to-sodium ratios and significant decreases in potassium, potassium-to-creatinine and sodium-to-creatinine ratios in boys and girls over the 24 month period (Tables 9.12, 9.13). A significant increase in phosphorus and deoxypyridinoline output and a significant reduction in deoxypyridinoline-to-creatinine ratio over the 2 year period were seen in the boys but not in girls. Similarly girls but not the boys had significant decrease in phosphorus-to-creatinine ratio and a significant increase in sodium-to-potassium ratio over the 24 month period (Table 9.13). Boys had significantly lower daily urinary output of sodium and potassium, sodium-to-creatinine ratio, potassium-to-creatinine ratio and deoxypyridinoline-to-creatinine ratio than girls before and

after appropriate adjustment (Table 9.12, 9.13). The change in unadjusted calcium-to-sodium ratio was higher in boys compared with girls at follow-up (Table 9.13). After adjusting for baseline value and other confounders, the change in calcium-to-sodium ratio was significantly greater in boys than girls at follow-up (Table 9.13).

9.7 Effect of supplement on urine markers

9.7.1 Differences between supplemented and placebo groups at baseline

There were no significant differences between the supplemented and placebo group in daily output of minerals or deoxypyridinoline measured in urine at baseline (Table 9.9).

9.7.2 Differences between supplemented and placebo groups at outcome

After correcting for baseline and other confounders, change in deoxypyridinoline over the 12 month period was lower in the supplemented (-12%) than placebo group but the difference did not reach statistical significance (Table 9.14). At outcome, the supplemented group had significantly greater daily output of calcium than the placebo group before and after adjusting for baseline value, age, puberty and sex (+64.5 SE 13.0%, $p \leq 0.0001$, Table 9.11, 9.14), but the creatinine output did not differ between the groups (-2.7 SE 4.8%, $p = 0.58$), resulting in a higher calcium-to-creatinine ratio in the supplemented group compared with placebo (Table 9.14). Daily outputs of phosphorus and phosphorus-to-creatinine ratio were significantly lower in the supplemented compared with placebo group before and after adjusting for, pubertal status, sex and age (-41.8 SE 8.3%, $p \leq 0.0001$, Table 9.10, 9.14). The supplemented group had significantly greater calcium-to-sodium ratio than

placebo group at outcome before and after adjustment for the initial value, puberty, sex and age, (Table 9.14).

Daily outputs of calcium, creatinine, calcium-to-creatinine and calcium-to-sodium ratio at outcome were significantly greater than at baseline in the supplemented and placebo groups. There was a significant increase in urinary output of sodium-to-potassium ratio and a significant decrease in deoxypyridinoline in the supplemented group over the 12 month period (Table 9.14). There was a decrease in daily outputs of potassium, potassium-to-creatinine and deoxypyridinoline-to-creatinine in both the supplemented and placebo group over the 12 month period (Table 9.14). There was a significant decrease in urinary output of phosphorus in the supplemented group and a significant increase in daily output of phosphorus in the placebo group over the 1 year period (Table 9.14). A significant decrease in urinary output of phosphorus-to-creatinine was seen in the supplemented group over the 1 year period (Table 14). The placebo group had significant reduction in sodium-to-creatinine ratio over the 1 year period. There was no change in urinary sodium output in either the supplemented or placebo group, resulting in a significantly greater sodium-to-potassium ratio in the supplemented than placebo group at outcome (Table 9.14). There was no interaction between the supplement effect and age, sex or pubertal status, indicating that the effect of supplement was not affected by any of these variables.

9.7.3 Differences between supplemented and placebo groups at follow-up

There were significant changes over the 24 month period in urinary output of calcium, creatinine, potassium, calcium-to-creatinine, sodium-to-creatinine, potassium-to-creatinine and calcium-to-sodium ratios in both the supplemented and placebo groups (Table 9.15). Concentrations of phosphorus and deoxypyridinoline in the placebo group at follow-up were significantly greater than baseline value. Only in the supplemented group did sodium-to-potassium ratio at follow-up was significantly greater than baseline value (Table 9.15). Changes over 24 months in urinary outputs of biochemical markers were not significantly different between the groups. There were no significant differences between the supplemented and placebo groups in the daily output of minerals in urine at follow-up before and after value at follow-up was adjusted for baseline and other confounders (Tables 9.13 and 9.15).

9.7.4 Differences between the supplemented and placebo groups in urinary markers by sex

Although statistical analysis did not reveal any interaction between sex and supplement, the effect of supplement on the concentration of some of the urinary markers were evaluated in boys and girls separately to examine whether there was any suggestion of a different response between the groups in boys and girls. Only the variables that were significantly affected by the calcium supplement when sexes were combined are presented here. Similar analyses of remaining analytes revealed no effect of supplement in either sex.

Plasma concentration of calcium, calcium-to-creatinine and calcium-to-sodium ratios in boys at outcome were significantly greater than that at baseline. Phosphorus and phosphorus-to-creatinine ratio in the supplemented group in boys at outcome were significantly lower than that at baseline (Table 9.16). In

contrast, phosphorus concentration in the placebo group in boys at outcome was significantly greater than baseline value (Table 9.16). In girls, urinary outputs of calcium, calcium-to-creatinine, calcium-to-sodium and sodium-to-potassium ratios at outcome were all significantly greater than that at baseline (Table 9.16). Outputs of phosphorus and phosphorus-to-creatinine ratio in the supplemented group were significantly lower at outcome than at baseline (Table 9.16). In contrast, phosphorus output in the placebo group in girls at outcome was not significantly greater than baseline value (Table 9.16). In both sexes, many of the changes in urinary markers over the 12 month period were significantly different between the supplemented and placebo groups after appropriate adjustment (Table 9.16). Conversely, after baseline adjustment, changes in phosphorus and phosphorus-to-creatinine in girls over 12 months were significantly lower in the supplemented compared with the placebo group (Table 9.16). The magnitude of the increases in calcium and calcium-to-creatinine ratio in the supplemented group over the 12 month period were similar in both sexes. In both sexes, other markers were not significantly different between the supplemented and placebo groups after approriate adjustment (data not shown).

There was no evidence that the effect of the supplement was different between boys and girls as indicated by a lack of sex x supplement interaction when sexes were combined (see section 9.7.2).

Table 9.9 Urinary biochemical markers in the supplemented and placebo groups, and in boys and girls at baseline†

	Supplement		Placebo		Boys		Girls	
	Gmean	mean ± SD† (n = 79)	Gmean	mean ± SD† (n = 80)	Gmean	mean ± SD† (n = 79)	Gmean	mean ± SD† (n = 80)
Calcium (mmol/d)	0.33	-1.10 ± 1.08	0.29	-1.23 ± 1.06	0.35	-1.06 ± 1.13	0.28	-1.26 ± 1.00
Ca/Cr (mmol/mmo/)	0.10	-2.29 ±1.03	0.10	-2.34 ± 0.90	0.11	-2.21 ± 1.00	0.09	-2.42 ± 0.93
Phosphorus (mmol/d)	6.75	1.91 ± 0.52	6.36	1.85 ± 0.58	6.42	1.86 ± 0.61	6.75	1.91 ± 0.50
P/Cr (mmol/mmol)	2.05	0.72 ± 0.38	2.10	0.74 ± 0.44	2.03	0.71 ± 0.43	2.10	0.74 ± 0.40
Creatinine (mmol/d)	3.32	1.20 ± 0.32	3.30	1.11 ± 0.47	3.16	1.15 ± 0.44	3.19	1.16 ± 0.36
Sodium (mmol/d)	87.4	4.47 ± 0.53	82.3	4.41 ± 0.56	83.9	4.43 ± 0.50	86.5	4.46 ± 0.59
Na/K (mmol/mmol)	6.82	1.92 ± 0.70	6.96	1.94 ± 0.68	7.39	2.00 ± 0.72	6.36	1.85 ± 0.65
Na/Cr (mmol/mmol)	26.6	3.28 ± 0.46	27.1	3.30 ± 0.46	26.6	3.28 ± 0.46	27.1	3.30 ± 0.45
Potassium (mmol/d)	12.8	2.55 ± 0.63	11.9	2.48 ± 0.64	11.3	2.42 ± 0.62	13.6	2.61 ± 0.63
K/Cr (mmol/mmol)	3.90	1.36 ± 0.58	3.94	1.37 ± 0.61	3.60	1.28 ± 0.62	4.22	1.44 ± 0.57
Dpd (nmol/d)	75.9	4.33 ± 0.43	72.2	4.28 ± 0.47	72.2	4.28 ± 0.45	75.9	4.33 ± 0.45
Dpy/Cr (nmol/mmol)	23.1	3.14 ± 0.32	23.8	3.17 ± 0.32	23.1	3.14 ± 0.33	23.6	3.16 ± 0.31
Ca/Na (mol/mmol)	3.82	1.34 ± 0.95	3.56	1.27 ± 0.87	4.14	1.42 ± 0.92	3.29	1.19 ± 0.89

†mean ± SD of variables transformed to natural logarithms. Gmean, geometric mean. There was no significant differences between either the supplemented and placebo groups or between boys and girls in any of the variables measured in the urine at baseline. Ca, calcium, Cr, creatinine, K, potassium, Na, sodium, Dpd, deoxypyridinoline, N, number of subjects in each data set. Data transformed to natural logarithms prior to analysis.

Table 9.10 Urinary biochemical markers in the supplemented and placebo groups, and in boys and girls at outcome[†]

	Supplement		Placebo		Boys		Girls	
	Gmean	mean ± SD[†] (n = 80)	Gmean	mean ± SD[†] (n= 80)	Gmean	mean ± SD[†] (n = 80)	Gmean	mean ± SD[†] (n = 80)
Calcium (mmol/d)	1.03	0.03 ± 0.86	0.52	-0.66 ± 0.94[a]	0.86	-0.15 ± 0.87	0.62	-0.48 ± 1.02[b]
Ca/Cr (mmol/mmo/)	0.28	-1.27 ± 0.76	0.14	-1.95 ± 0.88[a]	0.22	-1.50 ± 0.87	0.18	-1.72 ± 0.90
Phosphorus (mmol/d)	5.05	1.62 ± 0.58	7.54	2.02 ± 0.47[a]	1.67	0.51 ± 0.51	1.73	0.55 ± 0.39
P/Cr (mmol/mmol)	1.39	0.33 ± 0.42	2.08	0.73 ± 0.40[a]	1.67	0.51 ± 0.51	1.73	0.55 ± 0.39
Creatinine (mmol/d)	3.63	1.29 ± 0.37	2.63	1.29 ± 0.32	3.86	1.35 ± 0.33	3.42	1.23 ± 0.35[c]
Sodium (mmol/d)	85.6	4.45 ± 0.52	79.8	4.38 ± 0.61	78.3	4.36 ± 0.55	87.4	4.47 ± 0.58
Na/K (mmol/mmol)	10.2	2.32 ± 0.60	8.25	2.11 ± 0.71[d]	8.58	2.15 ± 0.67	9.68	2.27 ± 0.65
Na/Cr (mmol/mmol)	23.8	3.16 ± 0.44	22.0	3.09 ± 0.58	20.3	3.01 ± 0.55	25.5	3.24 ± 0.46[e]
Potassium (mmol/d)	8.50	2.14 ± 0.61	9.68	2.27 ± 0.50	9.12	2.21 ± 0.60	9.03	2.20 ± 0.52
K/Cr (mmol/mmol)	2.34	0.85 ± 0.55	2.66	0.98 ± 0.52	2.36	0.86 ± 0.54	2.64	0.97 ± 0.53
Dpd (nmol/d)	63.4	4.15 ± 0.65	70.1	4.25 ± 0.71	66.7	4.20 ± 0.66	66.7	4.20 ± 0.70
Dpy/Cr (nmol/mmol)	19.9	2.99 ± 0.36	21.5	3.07 ± 0.31	19.9	2.99 ± 0.34	21.5	3.07 ± 0.34
Ca/Na (mol/mmol)	11.9	2.48 ± 0.81	6.49	1.87 ± 0.88[a]	11.02	2.40 ± 0.91	7.03	1.95 ± 0.83[f]

[†]mean ± SD of variables transformed to natural logarithms. Gmean, geometric mean; Ca, calcium, Cr, creatinine, K, potassium, Na, sodium, Dpd, deoxypyridinoline, N, number of subjects in each data set. Data transformed to natural logarithms prior to analysis. The supplemented groups had significantly higher urinary output of calcium, calcium-to-creatinine ratio and calcium-to-sodium ratio than the placebo group at out come. Conversely, the supplemented group had significantly higher phosphorus and ratio of phosphorus-to-creatinine out put compared with the placebo group. Boys had significantly greater daily output of calcium, creatinine and calcium-to-sodium ratio than girls at outcome. The change in daily output of sodium-to-creatinine ratio was more positive in girls than boys. [a] $p \leq 0.0001$, [b] $p = 0.0265$, [c] $p = 0.0307$, [d] $p = 0.0452$, [e] $p = 0.0048$, [f] $p = 0.0013$ (regression, Data desk 4.1)

Table 9.11 Differences in urinary biochemical markers between outcome and baseline in boys and girls[†]

	Boys		Girls		#Δ%difference at outcome		
	*Δ%	N	*Δ%	N	Boys vs Girls		
					Mean	SE	significance
Calcium (mmol/d)	+91.7 ± 12.6[a]	79	+77.7 ± 13.0[a]	80	+33.7	13.3	p=0.013
Ca/Cr (mmol/mmol)	+71.6 ± 11.4[a]	79	+70.7 ± 11.7[a]	80	+22.4	12.0	p=0.64 (ns)
Phosphorus (mmol/d)	-0.33 ± 9.48	79	-12.0 ± 7.20	80	+7.3	8.4	p=0.39 (ns)
P/Cr (mmol/mmol)	-20.4 ± 7.06[b]	79	-19.0 ±5.59[c]	80	-4.3	6.4	p=0.51 (ns)
Creatinine (mmol/d)	+20.1 ± 4.85[a]	79	+7.00 ± 3.97	80	+11.6	4.8	p=0.016
Sodium (mmol/d)	-7.60 ±7.26	79	+1.04 ± 8.69	80	-8.3	8.8	p=0.35 (ns)
Na/K (mmol/mmol)	+14.8 ± 9.68	79	+41.4 ± 9.31[a]	80	-14.7	10.2	p=0.15 (ns)
Na/Cr (mmol/mmol)	-27.7 ± 7.05[d]	79	-5.96 ± 6.90	80	-19.0	8.0	p=0.019
Potassium (mmol/d)	-22.4 ± 8.8[e]	79	-40.3 ± 7.56[a]	80	+7.9	8.8	p=0.37 (ns)
K/Cr (mmol/mmol)	-42.5 ± 8.01[a]	79	-47.3 ± 7.29[a]	80	-3.0	8.4	p=0.72 (ns)
Dpd (nmol/d)	-7.90 ± 6.68	79	-13.2 ± 7.62	79	+2.8	9.7	p=0.78 (ns)
Dpd/Cr (nmol/mmol)	-15.2 ± 3.29[a]	80	-8.95 ± 3.99[f]	79	-5.4	4.6	p=0.24 (ns)
Ca/Na (mol/mmol)	+99.3 ± 11.6[a]	79	+76.6 ± 10.2[a]	80	+37.6	11.9	p=0.002

[†] Mean ± SE. *Δ% = percentage difference outcome - baseline (100ln[outcome]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at outcome, after correcting for baseline value, age, pubertal status and supplement group. Data transformed to natural logarithms before. Differences between outcome and baseline significantly different from nought (paired t- test): [a] p≤0.0001, [b] p = 0.005, [c] p = 0.001, [d] p = 0002, [e] p = 0.013, [f] p = 0.028. Ca, calcium, Cr, creatinine, P, phosphorus, Na, sodium, K, potassium, Dpd, deoxypyridinoline, N, number of subjects in each data set. ns, not significant.

Table 9.12 Urinary biochemical markers in the supplemented and placebo groups, and in boys and girls at follow-up[†]

	Supplement			Placebo		Boys		Girls	
	Gmean	mean ± SD[†]	N	Gmean	mean ± SD[†] (n = 80)	Gmean	mean ± SD[†] (n + 79)	Gmean	mean ± SD[†] (n = 79)
Calcium (mmol/d)	0.70	-0.36 ± 0.94	79	0.58	-0.55 ± 0.84	0.68	-0.39 ± 0.94	0.59	-0.52 ± 0.84
Ca/Cr (mmol/mmo/)	0.18	-1.73 ± 0.89	79	0.15	-1.93 ± 0.78	0.18	-1.74 ± 0.89	0.15	-1.90 ± 0.78
Phosphorus (mmol/d)	7.32	1.99 ± 0.47	79	7.61	2.03 ± 0.39	7.39	2.00 ± 0.45	7.54	2.02 ± 0.41
P/Cr (mmol/mmol)	1.86	0.62 ± 0.33	80	1.92	0.65 ± 0.30	1.88	0.63 ± 0.30	1.90	0.64 ± 0.33
Creatinine (mmo/d)	3.94	1.37 ± 0.36	79	3.97	1.38 ± 0.38	3.94	1.37 ± 0.41	3.97	1.38 ± 0.33
Sodium (mmo/d)	88.2	4.48 ± 0.52	77	76.7	4.34 ± 0.79	73.0	4.29 ± 0.81	91.8	4.52 ± 0.48[a]
Na/K (mmo/mmol)	9.78	2.28 ± 0.54	77	8.33	2.12 ± 0.74	8.94	2.19 ± 0.74	9.12	2.21 ± 0.55
Na/Cr (mmol/mmol)	22.2	3.10 ± 0.43	77	19.3	2.96 ± 0.72	18.5	2.92 ± 0.74	23.1	3.14 ± 0.39[b]
Potassium (mmol/d)	8.94	2.19 ± 0.56	77	9.21	2.22 ± 0.56	8.17	2.10 ± 0.57	10.07	2.31 ± 0.52[c]
K/Cr (mmol/mmol)	2.27	0.82 ± 0.47	77	2.32	0.84 ± 0.48	2.08	0.73 ± 0.46	2.53	0.93 ± 0.46[d]
Dpd (nmol/d)	83.9	4.43 ± 0.72	76	82.3	4.41 ± 0.63	83.9	4.43 ± 0.71	83.1	4.42 ± 0.65
Dpy/Cr (nmol/mmol)	22.0	3.09 ± 0.31	77	22.0	3.09 ± 0.29	21.1	3.05 ± 0.28	23.1	3.14 ± 0.31[e]
Ca/Na (mol/mmol)	7.92	2.07 ± 0.92	77	7.46	2.01 ± 0.98	9.21	2.22 ± 1.07	6.42	1.86 ± 0.78[f]

[†]mean ± SD of variables after being transformed to natural logarithms. Gmean, geometric mean; Ca, calcium, Cr, creatinine, K, potassium, Na, sodium, Dpd, deoxypyridinoline, N, number of subjects in each data set. Data transformed to natural logarithms prior to analysis. Girls had significantly greater urinary output of sodium, sodium-to-creatinine, potassium, potassium-to-creatinine ratio and deoxypyridinoline-to-creatinine ratio than boys at follow-up. Conversely, calcium-to-sodium ratio was significantly higher in boys than girls at follow-up. Deoxypyridinoline output did not differ between the sexes at follow-up. There was no significant different between the supplemented and placebo groups in either deoxypyridinoline output or in any of the other variables in urine at follow-up. [a] $p = 0.0342$, [b] $p = 0.0204$, [c] $p = 0.0182$, [d] $p = 0.0069$, [e] $p = 0.0462$, [f] $p = 0.0165$.

Table 9.13 Differences in urinary biochemical markers between follow-up and baseline in boys and girls[†]

	Boys		Girls		#Δ%difference at follow-up		
	#Δ%	N	#Δ%	N	Boys vs Girls		
					Mean	SE	significance
Calcium (mmol/d)	+67.9 ± 13.9[a]	78	+73.6 ± 12.3[a]	80	+9.9	13.6	p=0.47 (ns)
Ca/Cr (mmol/mmol)	+44.5 ± 12.0[a]	78	+52.2 ± 10.8[a]	80	+8.2	12.4	p=0.51 (ns)
Phosphorus (mmol/d)	+15.0 ± 7.37[b]	78	+11.1 ± 6.43	80	-0.3	6.6	p=0.97 (ns)
P/Cr (mmol/mmol)	-8.40 ± 5.16	78	-10.2 ± 4.87[c]	80	-0.6	4.9	p=0.90 (ns)
Creatinine (mmol/d)	+23.4 ± 5.59[a]	78	+21.4 ± 4.20[a]	80	+0.3	5.3	p=0.95 (ns)
Sodium (mmol/d)	-13.0 ± 9.31	78	+5.39 ± 8.09	78	-22.0	10.3	p=0.034
Na/K (mmol/mmol)	+19.5 ± 10.2	78	+36.1 ± 9.59[a]	78	-3.5	10.4	p=0.74 (ns)
Na/Cr (mmol/mmol)	-36.4 ± 8.38[a]	78	-15.2 ± 6.07[d]	78	-22.0	9.2	p=0.017
Potassium (mmol/d)	-32.5 ± 8.11[a]	78	-30.7 ± 8.35[a]	78	-18.7	8.5	p=0.028
K/Cr (mmol/mmol)	-55.9 ± 7.87[a]	78	-51.3 ± 7.64[a]	78	-19.0	7.4	p=0.010
Dpd (nmol/d)	+14.3 ± 7.10[e]	78	+9.00 ± 6.92	77	+3.9	9.7	p=0.69 (ns)
Dpd/Cr (nmol/mmol)	-9.55 ± 3.55[f]	79	-1.19 ± 4.26	77	-9.6	4.4	p=0.030
Ca/Na (mol/mmol)	+80.9 ± 13.7[a]	78	+68.3 ± 10.6[a]	78	+30.5	14.2	p=0.034

[†]Mean ± SE. #Δ% = percentage difference follow-up - baseline (100ln[follow-up]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at follow-up, after correcting for baseline value, age, pubertal status and supplement group. Data transformed to natural logarithms before analysis. Differences between follow-up and baseline significantly different from nought (paired t- test): [a] p≤0.0001, [b] p = 0.045, [c] p = 0.039, [d] p = 0.014, [e] p = 0.048, [f] p = 0.009, Ca, calcium, Cr, creatinine, P, phosphorus, Na, sodium, K, potassium, Dpd, deoxypyridinoline, N, number of subjects in each data set. ns, not significant.

Table 9.14 Differences in urinary biochemical markers between outcome and baseline in supplemented and placebo groups[†]

	Supplement		Placebo		#Δ%difference at outcome		
	#Δ%	N	#Δ%	N	Supplement vs Placebo		
					Mean	SE	significance
Calcium (mmol/d)	+112.6 ± 11.8[a]	79	+57.0 ± 13.0[a]	80	+64.5	13.0	p≤0.0001
Ca/Cr (mmol/mmol)	+103.4 ± 10.3[a]	79	+39.3 ± 11.6[a]	80	+66.9	11.8	p≤0.0001
Phosphorus (mmol/d)	-30.0 ± 8.21[b]	79	+17.3 ± 7.77[c]	80	-41.8	8.3	p≤0.0001
P/Cr (mmol/mmol)	-39.2 ± 6.07[a]	79	-0.48 ± 5.87	80	-40.7	6.4	p≤0.0001
Creatinine (mmol/d)	+9.22 ±4.20[d]	79	+17.7 ± 4.71[a]	80	-2.7	4.8	p=0.58 (ns)
Sodium (mmol/d)	-2.87 ± 7.35	79	-3.63 ± 8.64	80	+5.7	8.6	p=0.51 (ns)
Na/K (mmol/mmol)	+39.5 ± 9.61[a]	79	+17.1 ± 9.45	80	+21.1	10.2	p=0.040
Na/Cr (mmol/mmol)	-12.1 ± 6.22	79	-21.4 ± 7.80[e]	80	+6.9	7.8	p=0.38 (ns)
Potassium (mmol/d)	-42.3 ± 8.60[a]	79	-20.7 ± 7.72[f]	80	-15.9	8.5	p=0.062 (ns)
K/Cr (mmol/mmol)	-51.5 ± 8.09[a]	79	-38.4 ± 7.14[a]	80	-14.0	8.0	p=0.082 (ns)
Dpd (nmol/d)	-17.5 ± 7.20[g]	79	-3.64 ± 7.06	79	-11.7	9.7	p=0.23 (ns)
Dpd/Cr (nmol/mmol)	-14.2 ± 3.64[a]	80	-10.0 ± 3.69[h]	79	-6.2	4.5	p=0.17 (ns)
Ca/Na (mol/mmol)	+115.5 ± 10.4[a]	79	+60.6 ± 10.7[a]	80	+59.6	11.9	p≤0.0001

[†]Mean ± SE. #Δ% = percentage difference outcome - baseline (100ln[outcome]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at outcome, after correcting for baseline value, age, sex, and pubertal status. Data transformed to natural logarithms before analysis. Differences between outcome and baseline significantly different from nought. [a] p≤0.0001, [b] p = 0.005, [c] p = 0.029, [d] p = 0.031, [e] p = 0.008, [f] p = 0.009, [g] p = 0.018, [h] p = 0.009 (paired t- test). Ca, calcium, Cr, creatinine, P, phosphorus, Na, sodium, K, potassium, Dpd, deoxypyridinoline, N, number of subjects in each data set. ns, not significant.

Table 9.15 Differences in urinary biochemical markers between follow-up and baseline in supplemented and placebo groups[†]

	Supplement		Placebo		#Δ%difference at follow-up		
	*Δ%	N	*Δ%	N	Supplement vs Placebo		
					Mean	SE	significance
Calcium (mmol/d)	+74.3 ± 13.8[a]	78	+67.4 ± 12.4[a]	80	+17.5	13.5	p=0.20 (ns)
Ca/Cr (mmol/mmol)	+56.2 ± 12.2[a]	78	+40.8 ± 10.5[a]	80	+22.4	12.3	p=0.069 (ns)
Phosphorus (mmol/d)	+8.46 ± 6.71	78	+17.5 ± 7.05[b]	80	-6.0	6.6	p=0.37 (ns)
P/Cr (mmol/mmol)	-9.65 ± 5.04	78	-9.00 ± 4.99	80	-2.6	4.8	p=0.58 (ns)
Creatinine (mmol/d)	+18.1 ± 4.32[a]	78	+26.5 ± 5.40[a]	80	-4.6	5.3	p=0.38 (ns)
Sodium (mmol/d)	+0.04 ± 7.99	76	-7.45 ± 9.41	80	+9.5	10.4	p=0.36 (ns)
Na/K (mmol/mmol)	+37.4 ± 8.68[a]	76	+18.7 ± 10.8	80	+17.3	10.3	p=0.094 (ns)
Na/Cr (mmol/mmol)	-17.2 ± 6.25[c]	76	-34.0 ± 8.22[a]	80	+14.9	9.1	p=0.11 (ns)
Potassium (mmol/d)	-37.3 ± 8.28[a]	76	-26.1 ± 8.14[d]	80	-7.6	8.4	p=0.36 (ns)
K/Cr (mmol/mmol)	-54.6 ± 7.28[a]	76	-52.6 ± 8.17[a]	80	-3.0	7.3	p=0.68 (ns)
Dpd (nmol/d)	+9.35 ± 7.71	76	+13.9 ± 6.30[e]	79	-2.9	9.7	p=0.77 (ns)
Dpd/Cr (nmol/mmol)	-3.78 ± 3.55	77	-7.02 ± 4.26	79	+0.6	4.4	p=0.88 (ns)
Ca/Na (mol/mmol)	+74.4 ± 12.0[a]	76	+74.8 ± 12.5[a]	80	+8.7	14.3	p=0.54 (ns)

[†]Mean ± SE. *Δ% = percentage difference follow-up - baseline (100ln[follow-up]-100ln[baseline], equivalent to 100* difference/mean). #Δ% = percentage difference at follow-up, after correcting for baseline value, age and sex. Data transformed to natural logarithms before analysis. Differenes between follow-up and baseline significantly different from nought (paired t- test): [a] p≤0.0001, [b] p = 0.015, [c] p = 0.007, [d] p = 0.002, [e] p = 0.030. Ca, calcium, Cr, creatinine, P, phosphorus, Na, sodium, K, potassium, Dpd, deoxypyridinoline, N, number of subjects in each data set. ns, not significant.

Table 9.16 Changes in urinary outputs over 12 months of the supplemented and placebo groups, by sex[†]

	Supplement		Placebo		#Δ%difference at outcome		
	#Δ%	N	#Δ%	N	supplement vs placebo		
					mean	SE	significance
Boys							
Calcium(mmol/dl)	+121.3 ± 16.8[a]	39	+62.8 ± 17.6[b]	40	+69.1	16.6	p≤0.0001
Ca/Cr (mmo/mmol)	+107.1 ± 14.4[a]	39	+36.9 ± 15.9[c]	40	+72.4	16.0	p≤0.0001
Phosphorus (mmol/d)	-30.6 ± 11.7[d]	39	+29.1 ± 13.5[e]	40	-51.0	13.2	p=0.0002
P/Cr (mmol/mmol)	-44.7 ± 8.51[a]	39	+3.3 ± 9.98	40	-16.5	13.7	p =0.23 (ns)
Na/K (mmol/mmol)	+25.3 ± 12.9	39	+4.65 ± 14.4	40	+17.0	14.8	p = 0.25 (ns)
Ca/Na (mmol/mmol)	+133.7 ± 14.9[a]	39	+65.7 ± 16.2[a]	40	+73.9	17.4	p≤0.0001
Girls							
Calcium(mmol/dl)	+104.1 ± 16.6[a]	40	+51.2 ± 19.2[f]	40	+60.5	20.6	p=0.004
Ca/Cr (mmo/mmol)	+99.8 ± 14.8[a]	40	+41.6 ± 17.2[g]	40	+62.1	17.8	p=0.0008
Phosphorus (mmol/d)	-29.4 ± 11.7[h]	40	+5.4 ± 7.54	40	-33.1	10.5	p=0.002
P/Cr (mmol/mmol)	-33.8 ± 8.66[a]	40	-4.2 ± 6.35	40	-31.9	8.0	p = 0.0001
Na/K (mmol/mmol)	+53.2 ± 14.01[I]	40	+29.5 ± 12.1[J]	40	+25.3	14.2	p=0.079 (ns)
Ca/Na (mol/mmol)	+97.7 ± 14.2[a]	40	+55.5 + 14.1[a]	40	+45.5	16.2	p = 0.006

[†] Mean ± SE. #Δ% = percentage difference at outcome, after correcting for baseline value, age and puberty. Data transformed to natural logarithms before analysis. Phoshorus and phosphorus-to-creatinine at outcome were adjusted for the initial value, age and puberty. Differences between outcome and baseline were significantly different from nought (paired t-test): [a] p≤0.0001, [b] p = 0.001, [c] p = 0.025, [d] p = 0.013, [e] p = 0.037, [f] p = 0.011, [g] p = 0.021, [h] p = 0.016, [I] p = 0.0005, [J] p = 0.020. Ca, calcium, Cr, creatinine, K, potassium, Na, sodium, N, number of subjects in each data set. ns, not significant

Table 9.17 Titratable acid output and urinary pH of the boys and girls (by time point)[†]

	Baseline		Outcome		Follow-up	
	Boys (n = 79)	Girls (n = 80)	Boys (n = 80)	Girls (n = 80)	Boys (n = 79)	Girls (n = 80)
Titratable acid out (mmol/l)	12.07 ± 8.33[1]	8.38 ± 5.74	10.61 ± 9.03[2]	6.77 ± 5.41	14.78 ± 8.16[3]	9.41 ± 7.04
pH	6.24 ± 0.39	6.36 ± 0.46	6.18 ± 0.38	6.35 ± 0.44[4]	6.00 ± 0.42	6.22 ± 0.42[5]

[†] Mean ± SD. Titratable acid output significantly greater and urinary pH significantly lower in boys than girls. [1] p = 0.002, [2] p = 0.023, [3] p = 0.002, [4] p = 0.027, [5] p = 0.00

Table 9.18 Titratable acid output and urinary pH of the supplemented and placebo groups, divided by time point[†]

	Baseline		Outcome		Follow-up	
	supplement (n =79)	placebo (n =80)	supplement (n = 80)	placebo (n =80)	supplement (n =79)	placebo (n = 80)
Titratable acid out (mmol/l)	10.16 ± 6.68	10.27 ± 8.01	6.82 ± 5.50[1]	10.61 ± 9.03	12.07 ± 9.08	12.09 ± 6.69
pH	6.32 ± 0.46	6.28 ± 0.40	6.31 ± 0.44	6.22 ± 0.39	6.15 ± 0.47	6.08 ± 0.39

[†] Mean ± SD. 1 significantly different from placebo after correcting for baseline value and sex, p = 0.001

Table 9.19 Titratable acid output and urinary pH of the supplemented and placebo groups and by sex, divided by time point[†]

	Baseline		Outcome		Follow-up	
	supplement (39M, 39F)	placebo (39M, 40F)	supplement (40M, 39F)	placebo (40M, 39F)	supplement (39M, 40F)	placebo (40M, 40F)
Boys						
Titratable acid out (mmol/l)	11.67 ± 6.71	12.47 ± 9.75	7.95 ± 5.82	13.28 ± 10.81[1]	14.17 ± 9.40	15.38 ± 6.81
pH	6.26 ± 0.42	6.22 ± 0.37	6.21 ± 0.42	6.14 ± 0.32	6.07 ± 0.43	5.94 ± 0.40
Girls						
Titratable acid out (mmol/l)	8.64 ± 6.36	8.13 ± 5.12	5.67 ± 4.97	7.87 ± 5.68	10.02 ± 8.37	8.80 ± 5.44
pH	6.39 ± 0.50	6.34 ± 0.42	6.41 ± 0.43	6.30 ± 0.44	6.23 ± 0.50	6.22 ± 0.33

[†] Mean ± SD. Titratable acid output but not pH was significantly greater in the placebo group than supplemented groyp in boys. [1] p = 0.006. There were no significant differences between the groups in any of the two variables in girls.

9.8 Titratable acid output and urinary pH

9.8.1 Differences between boys and girls

Titratable acid output was significantly greater in boys than in girls at baseline, outcome and follow-up (p = 0.002, p = 0.023, and p = 0.002 respectively, Table 9.17). There was a decrease in titratable acid output over the 12 month period. After correcting for baseline value, supplement group, age and puberty, boys had significantly lower decreases in titratable acid output than girls at outcome and follow-up (outcome, +26.5 SE 13%, p = 0.043; follow-up, +34.1 SE 12.2%, p = 0.006, Tables 9.17, 9.20) reflecting the difference at baseline, and over 24 months there was general increase with values for boys greater than that for girls.

There was a significant difference in urinary pH between boys and girls at outcome and follow-up (Table 9.17) with lower values in boys than girls (outcome, p = 0.027; follow-up, p = 0.004, Table 9.17).

Table 9.20 Differences in titrable acid output between boys and girls after 12 months and 24 months of the study[†]

	Boys	Girls	#Δ%difference at outcome		
12 months	#Δ% (n = 80)	#Δ% (n = 80)	Boys vs girls		
			mean	SE	significance
Titratable acid output (mmol/l)	-22.4 ± 10.03[a]	-22.2 ± 6.56[a]	+26.5	13.0	0.043
	Boys	Girls	#Δ%difference at follow-up		
24 months	#Δ% (n = 80)	#Δ% (n = 80)	Boys vs girls		
			mean	SE	significance
Titratable acid output (mmol/l)	+22.8 ± 9.84[b]	+12.7 ± 10.15[b]	+34.1	12.2	0.006

[†] Mean ± SE. #Δ% = percentage difference at outcome and follow-up respectively, after correcting for baseline value, age, pubertal status and supplement group. Data transformed to natural logarithms before analysis. Differences between outcome and baseline, and between follow-up and baseline were significantly different from nought: [a] p = 0.046 and [b] p = 0.006 respectively.

9.8.2 Differences between supplemented and placebo groups

There was a significant decrease in titratable acid output in both the supplemented and placebo groups over the 12 month period and a significant increase over the 24 months (Table 9.21). The magnitude of the decrease at outcome was greater in the supplemented than placebo group (Table 9.21). After correcting for baseline value, age, pubertal status and sex, titratable acid output was significantly lower in the supplemented group compared with the placebo group at outcome (-43.7 SE 12.1%, p = 0.0004, Table 9.21). There was no significant difference in titratable acid output between the supplemented and placebo groups either at baseline or follow-up (Table 9.18, 9.21). There was no evidence of any interaction between supplement effect and sex.

When the effect of supplement on titratable acid output was examined in each sex separately, only in boys was the titratable acid output significantly different between the groups and was lower in the supplemented group compared with placebo group (Table 9.19). Otherwise, there were no significant differences between the supplemented and placebo groups in titratable acid output or pH in either sex after correcting for baseline value.

Urinary pH was not different between the groups at baseline and was not affected by supplement at any study timepoint (Table 9.18).

Table 9.21 Differences in titrable acid output between the supplemented and placebo groups after 12 months and 24 months of the study[†]

12 months	Supplement $^{\#}\Delta\%$ (n = 80)	Placebo $^{\#}\Delta\%$ (n = 80)	$^{\#}\Delta\%$difference at outcome Supplement vs Placebo		
			mean	SE	significance
Titratable acid output (mmol/l)	-42.6 ± 10.33[a]	-2.28 ± 10.16[a]	-43.7	12.1	0.0004

24 months	Supplement $^{\#}\Delta\%$ (n = 80)	Placebo $^{\#}\Delta\%$ (n = 80)	$^{\#}\Delta\%$difference at follow-up Supplement vs Placebo		
			mean	SE	significance
Titratable acid output (mmol/l)	+12.3 ± 11.52[b]	+22.0 ± 8.29[b]	+12.2	11.8	0.303

[†] Mean ± SE. $^{\#}\Delta\%$ = percentage difference at outcome and follow-up respectively, after correcting for baseline value, age, pubertal status. Data transformed to natural logarithms before analysis. Differences between outcome and baseline, and between follow-up and baseline were significantly different from nought: [a] p = 0.046 and [b] p = 0.006 respectively.

Summary

9.9 Biochemical markers

9.9.1 Differences between the supplemented and placebo groups in biochemical markers

Plasma concentration of osteocalcin at outcome in the supplemented group was significantly lower than at baseline. In contrast, the osteocalcin concentration of the placebo group at outcome was not significantly different from that at baseline. There were significant increases in total and bone specific alkaline phosphatase over the 12 month period in the two groups. PTH was significantly increased in placebo group but not in the supplemented group over the 1 year period. There were significant increases in osteocalcin, total alkaline phosphatase, bone specific alkaline phosphatase and creatinine concentration in the supplemented and placebo groups over the 24 month period. PTH concentration in the placebo group remained significantly increased at follow-up compared with baseline value. Daily urinary outputs of calcium, creatinine, calcium-to-creatinine and calcium-to-sodium ratios were significantly increased and potassium, titratable acid and deoxypyridinoline ratio were significantly reduced in the supplemented and placebo groups over the 12 month period. There was a significant increase in urinary phosphorus, and a significant decrease in sodium-to-creatinine ratio in the placebo group over the 1 year period. Urinary phosphorus and deoxypyridinoline were reduced and sodium-to-potassium significantly increased in the supplemented group over the 12 month period. Both the supplemented and placebo groups had significant gains in daily outputs of calcium, creatinine, calcium-to-creatinine and calcium-to-sodium ratio and significant decreases in potassium, sodium-to-creatinine and potassium-to-creatinine ratios over the 2 year period. Urinary phosphorus and

deoxypyridinoline outputs were significantly increased in the placebo group over the 24 month period.

This study indicated a significant effect of calcium supplementation on plasma osteocalcin concentration. There was a 22% reduction in osteocalcin concentration in the supplemented compared with placebo group at outcome. Plasma concentration of osteocalcin is a marker of bone formation and bone turnover.

Calcium supplementation had no significant effect on either total or bone-specific alkaline phosphatase since changes over the 12 month period were not significantly different between the two groups. Similarly changes in deoxypyridinoline, a bone resorption marker in urine were not significantly different between the supplemented and placebo groups at outcome although the magnitude was -12% suggestive of a trend.

The supplemented group had a significantly lower parathyroid hormone, titratable acid output and a significantly higher urinary output of calcium than the placebo group at outcome, after correcting for baseline value. These are effects that would be expected given the large increase in calcium and carbonate intake. In addition, after adjusting for baseline value, the supplemented group had a significantly lower plasma phosphate and lower urinary output of phosphorus at outcome compared with placebo group.

At follow-up, the effect on plasma osteocalcin had disappeared but parathyroid hormone remained significantly reduced in the supplemented group compared with placebo group. There were no significant differences between

the supplemented and placebo group in any of the other biochemical markers measured at follow-up.

9.9.2 Differences between boys and girls

Both boys and girls had significant decreases in plasma osteocalcin concentration over the 12 month period and significant increases in total alkaline phosphatase, bone specific alkaline phosphatase and creatinine over this time period. Only girls had significant increase in PTH over the 1 year period. Plasma osteocalcin, total alkaline phosphatase, bone specific alkaline phosphatase and creatinine concentration in boys and girls at follow-up were significantly greater than at baseline. Plasma concentration of PTH was significantly increased and albumin concentration significantly reduced in girls over the 24 month period. Daily urinary outputs of calcium, calcium-to-creatinine and calcium-to-sodium ratio were significantly greater and potassium, titratable acid, potassium-to-creatinine and deoxypyridinoline-to-creatinine ratio significantly lower in boys and girls at outcome compared with baseline value. Only in boys was creatinine output at outcome was significantly greater than at baseline. There were significant changes in urinary outputs of calcium, creatinine, potassium, calcium-to-creatinine, sodium-to-creatinine, potassium-to-creatinine ratio and titratable acid in boys and girls over the 2 year period. Outputs of urinary phosphorus and deoxypyridinoline at follow-up in boys were significantly greater than that at baseline.

Both plasma total and bone specific alkaline phosphatase concentration were significantly lower in boys than girls at baseline. Similarly, both total and bone-specific alkaline phosphatase were significantly lower in boys than girls at

12 months and 24 months. Boys had significantly lower plasma concentration of osteocalcin than girls at outcome and follow-up after appropriate adjustment. After adjusting for the initial value, plasma concentration of total and bone-specific alkaline phosphatase were significantly lower in boys compared with girls at follow-up. Since alkaline phosphatase is a marker of bone formation, the result indicates that bone mineral accretion rate was greater in girls than boys throughout the 2 years.

After adjusting for baseline value, boys had significantly greater urinary output of calcium than girls at outcome but not at follow-up. Urinary output of potassium was significantly higher in boys than girls at outcome. Changes in urinary deoxypyridinoline were not significantly different between boys and girls at either timepoint. Interaction term between supplement effect and sex was not significant and there was no evidence to suggest that the response to supplement was different between boys and girls. No interaction of supplement effect with age or pubertal status was found.

Table 9.22 Effect of supplement on biochemical markers in blood

Maker	Baseline	Outcome	Follow-up
Osteocalcin	0	-	0
Total alkaline phosphatase	0	0	0
Bone-specific alkaline phosphatase	0	0	0
Parathyroid hormone	0	-	-
Calcium	0	0	0
Ionised calcium	0	0	0
Phosphate	0	-	0
Albumin	0	0	0
Creatinine	0	0	0

Plus (+) = positive effect, minus (-) = negative effect, 0 = no effect.

Table 9.23 Effect of supplement on biochemical markers in urine

Maker	Baseline	Outcome	Follow-up
Calcium output	0	+	0
Calcium/creatinine output	0	+	0
Phosphorus output	0	-	0
Phosphorus/Creatinine output	0	-	0
Creatinine output	0	0	0
Sodium output	0	0	0
Sodium/creatinine output	0	0	0
Sodium/potassium output	0	+	0
Potassium output	0	0	0
Potassium/creatinine output	0	0	0
Deoxypyridinoline output	0	0	0
Deoxypyridinoline/creatinine output	0	0	0
Calcium/Sodium output	0	+	0
Titratable acid output	0	-	0
pH	0	0	0

Plus (+) = positive effect, minus (-) = negative effect, 0 = no effect.

10 Discussion

This study was carried out in a rural area of The Gambia where the growth of children is poor, puberty is delayed and bone mineral is low compared with British and American children (Lo *et al.*, 1990; Prentice *et al.*, 1990). In addition, the calcium intake is low, averaging 200 - 400 mg Ca/d (Prentice & Paul, 1990, Prentice *et al.*, 1993; Dibba *et al.*, in press). This study was conducted in order to gain insight into the calcium requirements for growth and bone mineral accretion of children accustomed to a low calcium diet.

Until recently, there have been few direct studies of the benefits of an increase in calcium intakes during growth in countries where the habitual calcium intake is low. Early supplementation studies which used linear growth as the main outcome showed little effect of calcium (Pettifor *et al.*, 1981; Luyken *et al.*, 1967; Malan & Ockerse, 1941). An exception was two Indian studies in which significant differences in height and weight gain were reported between those supplemented with relatively low doses of calcium and control children (Aykroyd & Krishnan, 1938; Aykroyd & Krishnan, 1939). However, a number of recent, detailed calcium supplementation and balance studies have demonstrated that an increase in calcium intake is associated with an increase in bone mineral in children, even in those whose calcium intakes are close to current recommended levels (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lee *et al.*, 1994; Matkovic *et al.*, 1990). These data suggested that the dietary calcium requirement of children may be greater than previously thought.

10.1 Calcium intake

Increasing awareness of the importance of a high calcium intake for the maintenance of bone health has stimulated recommendations in United States and elsewhere to increase calcium intake. Calcium intake varies greatly worldwide, ranging from 800 mg per day or more in developed countries to 200 to 300 mg in some developing countries (FAO, 1991, Lee *et al.*, 1994; Prentice *et al.*, 1994). The differences are mainly due to variations in the intake of dairy products, which are sources of calcium. In countries where milk consumption is rare, notably Africa, calcium may be obtained from cereals and leaf sauces such as millets and baobab but the overall consumption of calcium from these sources is low.

The measured calcium intake of children who took part in this study was extremely low and averaged 338 mg/d. This intake is considerably below current recommendations for children (National Academy Science, 1989, National Academy Science, 1997; Department of Health, Committee on Medical Aspects of Food Policy (COMA), 1992). A very low calcium intake in rural areas of The Gambia has been recorded in previous studies in Keneba involving other age groups where calcium intakes averaged 300 - 400 mg/d (Prentice *et al.*, 1990; Prentice *et al.*, 1993; Prentice *et al.*, 1994). A similarly low level of dietary calcium intake of 300 mg/d has been reported in 11 year old Chinese children (Lee *et al.*, 1994).

The diet of rural Gambia is based on cereals, and the major sources of calcium are dark green leafy vegetables, rice, fish and groundnuts. Dried and fresh leaf sauces were shown to be the principal sources of calcium for the children in the study and contributed 29 percent of their mean calcium intake. In

contrast, milk contributed only 6 percent. As in The Gambia, the diet of Chinese children is based on rice and the main sources of calcium are dark green leafy vegetables, beans, tofu, cereals and fish (Lee *et al.*, 1997). Calcium intakes of the Chinese and Gambian children are well below the current recommendations by US-AI and UK-RNI of 1300 and 1000 mg/d calcium respectively (National Academy of Science, 1997, Department of Health, COMA, 1992). The possibility that low calcium intakes may affect growth and bone mineral development of rural Gambian children was suggested by a previous study that demonstrated that Gambian infants have low size-adjusted bone mineral mass and are short compared to British reference children of the same age (Prentice *et al.*, 1990). The comparative study presented here demonstrated differences in bone mineral status between older Gambian children and British which could largely be accounted for by the smaller size of Gambian children but showed that there were still marked differentials in overall bone mineral content and in height at 8 - 12 years of age.

The calcium and phosphorus intakes of the study children were not affected by season in contrast to the marked seasonal variation in the intakes of most of the other nutrients measured. The wet season, traditionally known as hungry season, is the time of the year when people of this community are involved in strenuous agricultural activities and food stuffs from previous years' harvest are depleted. The lower energy and protein intakes of the children paralleled seasonal variations seen in adults in this community (Prentice *et al.*, 1981).

The diet of rural Gambia is based on cereals and groundnuts which mean that calcium in the diet may be poorly absorbed due to phytate and oxalate content of the diet. Other components of the diet that are considered to affect bone accretion such as protein and sodium were also evaluated in this study.

Low intakes of protein are associated with low calcium absorption (Kerstetter *et al.*, 1997), while very high intakes of animal protein are associated with higher rates of hip fracture (Abelow *et al.*, 1992). High intakes of animal protein are thought to result in metabolic acidosis which leads to bone resorption independent of PTH and osteocalcin activation (Breslau *et al.*,1988, Abelow *et al.*, 1992). However, the exact association between animal protein and bone health is unclear. Some (Breslau *et al.*,1988; Schuette *et al.*, 1980; Allen, 1982) but not all (Spencer *et al.*, 1978) human metabolic studies suggest diet containing high levels of animal protein can increase urinary calcium excretion and result in a negative calcium balance. The main sources of protein in the diet of rural Gambia are groundnuts while meat and fish protein are rarely consumed. However, the average protein intake of boys and girls was 58 and 53 g/d respectively, greater than the reference range of 41 - 42 g/d for a 11 - 15 year old in Britain (Committee on Medical Aspects of Food Policy (COMA), 1992). Therefore, the protein intake of the subjects in this study appeared adequate.

High sodium intakes are thought to increase the amount of calcium excreted in the urine and thereby increase the body's need for calcium (Massey & Whiting, 1996). This is due to the intimate association between calcium and sodium re-absorption in the proximal tubule and loop of Henle (Goulding & Lim, 1983; Devine *et al.*, 1995; Matkovic *et al.*, 1995). The mean sodium intake of the

subjects as indicated by their 24h urine excretion was high (97 mmol/d) compared with the British recommendation of 25 mmol/d for 11 - 14 year olds (Department of Health, Committee on Medical Aspects of Food Policy (COMA), 1992). This was a surprising finding given the influence that high sodium intake is thought to have on bone health.

Although the impact of low calcium intake on bone health of populations accustomed to low calcium intake is still an area of great controversy it could be argued that such low intakes by children in the developing countries could limit their skeletal development, reduce peak bone mass and influence osteoporotic risk in old age. The hypothesis is extremely difficult to prove since the diet of the world's poorer countries are marginal in other essential nutrients and not just calcium. Despite the lower calcium intake, there is no evidence that fractures due to low bone mineral is more common in developing than in industrialised countries, rather the opposite (Adebajo *et al.*, 1991; Solomon, 1979). It has been suggested that this is due to adaptation, made possible by low protein intakes (Heaney, 1997, Food and Agricultural Organization, 1962). It is this paradox and the concept of adaptation that led to a recommendation by FAO/WHO Expert Committee in 1960 for a calcium allowance of 500 mg/d (Food and Agricultural Organization, 1962). However, bone mineral measurements in Keneba suggest that poor bone mineral status is common (Aspray *et al.*, 1996) and lack of fractures may be related to number of factors which if changed (e.g., lifestyle) could lead to an emerging problem.

Racial differences in calcium intakes are well documented. American Blacks consume only 70 to 80 percent as much calcium as Whites (Park *et al.*, 1997). The ethnic differences in calcium intake generally reflect low consumption of milk and milk products by black people. However some suggest that the low dairy consumption by blacks could be partially due to a higher prevalence of lactose intolerance among blacks (Federation of American Societies for Experimental Biology, 1995). The situation may be different in The Gambia, where lactose intolerance is rare. In The Gambia, the overwhelming reason for people not to consume milk and milk products is more to do with the small dairy industry and small amounts of milk available than not being able to tolerate milk.

10.2 Growth

The Z scores for weight-and height-for-age of Gambian children who took part in the study were ≥1SD below the British reference population and Gambian children showed no improvement in growth relative to British children throughout the two year period. The results of this study support previous reports which indicated that Gambian children have poor growth and delayed puberty compared with age matched British and American children (Prentice *et al.*, 1990; Lo *et al.*, 1990). This study also supports many previous observations that Gambian children show little sign of catch up until adolescence once a growth deficit has occurred (Whitehead, 1979, Rowland *et al.*, 1977).

Gambian children who took part in the study were shorter than their age and sex matched reference children in the United Kingdom (Prentice & Bates, 1993). Girls had a slightly greater fat mass than boys at study entry (P = 0.0001),

and fat deposition as assessed by change in triceps skinfold thickness, measured over 12 months, was greater in girls than boys (triceps, girls : boys, +0.94 mm/year vs 0.39 mm/ year, p ≤0.0001).

Calcium supplementation had no effect on longitudinal growth of the study children. Neither their height Z scores nor the weight Z scores were altered over the 12 month period. Similar findings have been observed in other supplementation studies using calcium salts (Johnston *et al.*, 1992; Lloyd *et al.*, 1996; *Lee et al.*, 1994; Lloyd *et al.*, 1993). The lack of an effect of supplement on skeletal growth of the rural Gambian children is consistent with a number of early calcium supplementation studies in children where calcium salts failed to have any effect on weight and height velocity of children from poor social backgrounds (Malan & Ockerse, 1941; Pettifor *et al.*, 1981). However, the results of these studies contrast with those of supplementation studies using calcium phosphate extracted from milk (Bonjour *et al.*, 1997) or milk (Cadogan *et al.*, 1997) where increases in BMD in girls was accompanied by increased gains in body size and statural height. This suggests a possible beneficial effect of milk on longitudinal growth as opposed to calcium itself.

10.3 Pubertal status

Early puberty has been shown as a significant predictor of bone mass acquisition in several studies (Bonjour *et al.*, 1991; Glastre *et al.*, 1990; Lloyd *et al.*, 1992; Turner *et al.*, 1992; Lee *et al.*, 1996). The onset of puberty in girls is usually earlier than in boys, and hormonal changes during early puberty bring about the differences in growth spurt and bone mineral development between boys and girls. The age-adjusted Z scores for the Gambian children were

considerably below 0 and decreased with age suggestive of delayed puberty relative to British children. In this study, puberty was defined as Tanner stage 2 - 5. The majority of the children were prepubertal at study entry and remained so throughout the study. In Britain, about fifty percent of 12 year old would be Tanner stage 2 or more. However, some of the older children had entered puberty before the study began (19%). The number of children who had entered puberty increased to 31% at outcome and 46% by the end of the 2 years. Again, puberty would be expected in almost all the 12 - 14 year olds in Britain.

This study is in agreement with a previous report that puberty is delayed in rural Gambian children by about 1 - 2 years compared with age matched British and American children (Lo *et al.*, 1990). The same study demonstrated that Gambian females attained the same bone mineral content by age 19 years as British girls did at age 14. Unlike the previous study, this study showed that size adjusted bone mineral content of the Gambian children was similar to the age and sex-matched British children.

There was no influence of the calcium supplement on stage of puberty. Both the supplemented and placebo groups made similar progress through puberty. In addition, the response to the supplement was similar in prepubertal and those who were either pubertal or went into puberty during the study. This finding was similar to that found in some other calcium supplementation studies where the effect of calcium supplement on bone mineral status was similar in prepubertal and pubertal children (Nowson *et al.*, 1997; Lloyd *et al.*, 1995). In contrast, Johnston et al (1992). found no evidence of a supplement effect in twin

pairs who were post-puberty (4 pairs) at the study entry, or who passed through puberty during the study (19 pairs).

Studies in Caucasian populations have demonstrated that bone mineral in the forearm is similar in prepubertal boys and girls, but increases rapidly with the onset of puberty. This pubertal increase is thought to start 2 years later in boys, at about age 13, and to last longer than girls, resulting in boys having a greater bone mineral mass by the end of puberty (Krabbe & Christenson, 1984; Peacock, 1991). This study is in agreement with previous observations in that the British boys in the comparative group had greater bone mass at mid-shaft radius than girls at age ≥11 years (see section 8.2, Figure 8.2). The same pattern of skeletal growth was not found in Gambian children, in fact the opposite was true since bone mineral mass at age ≥11 years was greater in girls than boys (see section 8.2, Figure 8.2).

10.4.1 Effect of supplement on bone mineral status

Table 10.1 Effect of calcium supplement on bone mineral status

	Baseline	Outcome	Follow-up
Mid-shaft radius BMC	0	↑	↑
Mid-shaft radius BW	0	(↓)	0
Mid-shaft radius BMD	0	↑	↑
Mid-shaft size-adjusted BMC	0	↑	↑
Distal radius BMC	0	↑	(↑)
Distal radius BW	0	0	0
Distal radius BMD	0	↑	(↑)
Distal size-adjusted BMC	0	↑	(↑)

↑ = positive effect; zero (0) = no effect, (↑) = positive effect but not significant, (↓) = negative effect but not significant. Year1 = baseline; year2 = outcome; year3 = follow-up
Bone mineral status = BMC adjusted for BW, weight, height and baseline value.

The results of this study, summarised in Table 10.1, demonstrated that an increased calcium consumption over a 12 month period by Gambian children resulted in a greater bone mineral acquisition. The difference in bone mineral accretion between the supplemented and placebo groups at the end of the supplementation period was 3% at mid-shaft radius and 8% at distal radius. Boys and girls had similar gains in BMC at the mid-shaft and distal radius over the 12 month period and these were not different in children who entered puberty and those who were prepubertal. The effect of supplement was not affected by pubertal status and did not differ between boys and girls. The results of this study is in agreement with the hypothesis that calcium supplementation increases bone mineral accretion (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lee *et al.*, 1994, Lee *et al.*, 1995; Nowson *et al.*, 1997) which if sustained throughout the pubertal growth period and early adulthood, could positively modify peak bone mass.

Dairy supplementation of girls in early puberty has been shown to have a positive effect on bone mineral acquisition (Chan *et al.*, 1995; Cadogan *et al.*, 1997). There is evidence to suggest that a high calcium intake, generally in the form of dairy products, in early life is positively associated with greater peak bone mineral mass in adult life (Chan, 1991; Fehily *et al.*, 1992; Hu *et al.*, 1993). Supplementation with dairy products has been shown to protect bone loss in premenopausal and postmenopausal women (Polley *et al.*, 1987; Smith *et al.*, 1989; Elders *et al.*, 1994; Dawson-Hughes *et al.*, 1990; Prince *et al.*, 1995).

Calcium supplementation of rural Gambian children appeared to have no effect on the size of the growing skeleton. Neither the width of bone at the 2 measurement sites, nor statural height were altered. The increase was similar whether expressed as BMC, BMD or size-adjusted BMC suggesting that the amount of mineral within the bone envelope was increased rather than that the size of the bones had increased. Similar findings have been observed in other supplementation studies (Johnston *et al.*, 1992; Lloyd *et al.*, 1993; Lee *et al.*, 1994, Lee *et al.*, 1995). However, the result is in contrast to studies using calcium extracted from milk or using milk where an increase in BMD in girls was accompanied by increased gains in body size and statural height (Bonjour *et al.*, 1997; Cadogan *et al.*, 1997). This suggests that the effects of calcium supplements and milk on the skeleton may differ.

In this study, the magnitude of the supplement effect was similar to that reported in supplementation studies of children accustomed to a high calcium diet (Table 10.2). Supplementation study in Chinese school children with mean dietary calcium intake of 280 mg/d (7.0 mmol/d) ((Lee *et al.*, 1994) who were

supplemented with 300 mg/d (7.5 mmol/d) calcium produced a similar effect on bone mineral density to that reported in both this study and that of the American study (Johnston *et al.*, 1992). This is an interesting finding, considering the large difference in habitual calcium intake and in supplemented dose between these studies. This suggests that the effect may not necessarily reflect correction of a calcium deficiency but may indicate an effect of calcium supplement on bone turnover. A reduction in bone turnover results in a decreased reversible calcium space, and this can lead to a temporary increase in BMC as measured by absorptiometry (Lee *et al.*, 1994; Slemenda *et al.*, 1997; Prentice *et al.*, 1997). This is supported by the marked decrease noted in plasma osteocalcin concentration in the supplemented group (22%). The same effect was noted in prepubertal twins (Johnston *et al.*, 1992) but not in peri and postpubertal children (Slemenda *et al*, 1996). Reduced rates of skeletal remodelling in children have been associated with increased bone mineral density and high peak bone mass (Slemenda *et al.*, 1997; Department of Health, Committee on Medical Aspects of Food Policy (COMA), Report of the Subgroup on Bone Health, 1998).

10.4.2 Controlled calcium supplementation studies of children

Table 10.2 Calcium supplementation studies of children

variable	This study	Johnston *et al.*[1]	Lloyd *et al.*	Lee *et al.*	Chan *et al.*	Bonjour *et al.*	Cadogan *et al.*
Number	160	44	94	162	48	144	82
sex	M+F	M+F	F	M+F	F	F	F
Age at entry (y)	10	7	12	7	11	8	12
Ca. intake, controls #	334	908	935	280	728	879	703
Diet + suppplement #	1056	1612	1370	580	1437	1723	1125
Duration (months)	12	36	18	18	12	12	18
% increase in BMD[2]							
Midshaft radius	+4.5	+5.1	-	-	-	+1.6	-
Distal radius	+7.0	+3.8	-	+3.1	Ns[3]	+2.4[4]	-
Lumbar Spine	-	+2.8	+2.9	-	+9.9	NS	-
Femoral neck	-	+1.2	-	-	NS	NS	-
Trochanter	-	+3.5	-	-	-	+1.9	-
Total body	-	-	+1.3	-	-	-	+1.1[6]
% in ↑TBBMC[5]	-	-	-	-	+6.6	-	+2.9[6]

[1]Data represented are for prepubertal pairs of twins only. [2]Percent increase in BMD in supplemented group minus percent increase in control group. [3]No significant difference between supplement and placebo groups. [4]Site measured was radial metaphysis. [5]Percentage increase in total body BMC in supplemented group minus percent increase in control group. [6]Percent increase in total body BMC, BMD in supplemented group minus percent increase in control group. TBBMC, total body bone mineral content. [#] Intakes are in mg/d.

10.5 Follow-up

Results from calcium supplementation studies in children are inconclusive with regard to maximising peak bone mass. A number of studies have shown an association between high calcium intake in early life and attainment of greater peak bone mass (Matkovic, 1996; Theintz *et al.*, 1992; Sandler *et al.*, 1985; Chan, 1991; Fehily *et al.*, 1992; Anderson., 1996b) but others failed to show any effect of calcium intake during childhood on bone mineral mass attained in old age (Katzman *et al.*, 1991, Carrié & Bonjour, 1995). For calcium supplementation to be beneficial, its effect on bone mineral accretion should be sustained after the cessation of supplement, effective in maximising peak bone mass, and should have long-term effect in preventing osteoporotic fracture from occurring in later life. So far there have been only three supplementation studies in children in which follow-up data were collected and all these studies have indicated that gains in bone mineral acquired as a result of calcium supplementation were not sustained after the withdrawal of calcium supplement (Lee *et al.,* 1996; Lee *et al.*, 1997; Slemenda *et al.*, 1997). This suggests that the effect of calcium supplement on bone mass is a transient effect, which disappears following supplement withdrawal.

In this study however, differences in mean BMC and BMD between the supplemented and placebo groups were still visible at mid-shaft and distal radius 12 months after the supplement withdrawal. At mid-shaft the magnitude was similar to that seen at outcome. At distal radius the magnitude of increase in BMC and BMD was reduced and was not statistically significant. Differential gains in BMC, BMD and size-adjusted BMC at mid-shaft by the supplemented

group were +4.5 (SE = 1.7)%, p=0.007) and +5.0 (SE = 1.2)%, p≤0.0001) and +5.0 (SE 1.1)%, p≤0.0001) respectively. This suggests a possible long-term effect of calcium supplementation on bone mineralisation in this group of rural Gambian children accustomed to a low calcium intake. A similar observation has been reported in supplementation studies of girls using milk or milk extracts in which the effect of supplement on bone mineral accretion was sustained 1 year after supplement withdrawal (Bonjour *et al.*, 1997; Cadogan *et al.*, 1997).

The mean differences in BMC, BMD and size-adjusted BMC at distal radius between the supplemented and placebo groups were reduced during the 12 month follow-up (BMC, +4.1 (SE = 3.0)%, p=0.177), BMD, +4.0 (SE = 2.5)%, p=0.112), size-adjusted BMC, +2.7 (SE = 2.4)%, p = 0.27) and were no longer statistically significant at the end of the follow-up. However, the magnitude of the differences between supplemented and placebo group in BMC and BMD at the distal site at follow-up was similar to that seen at mid-shaft radius. Measurement of bone mineral mass at the distal radius using single photon absorptiometry is difficult and has a large positioning error compared with mid-shaft radius. It is possible that measurement of distal site with a more sensitive instrument such as dual energy x-ray absorptiometry (DXA) may give a better measure of changes occurring in bone mineral at the distal radius than single photon absorptiometry. This possibility is currently being explored in a second follow-up in which bone mineral status is being assessed by single photon absorptiometry and dual energy x-ray absorptiometry (DXA) 2 years after withdrawal of supplement. It is equally plausible that the effect of supplement on bone mineral status at mid-

shaft at follow-up could be explained by differences in bone turnover rate between different skeletal sites, and that bone turnover rate may be slower at sites with higher proportion of cortical bone such as the mid-shaft radius compared with trabecular bone sites, example, the distal radius. Reduced bone turnover in blacks compared with whites has been associated with increased bone mineral mass (Weinstein & Bell, 1988; Johnston *et al.*, 1992), so the effect seen at the mid-shaft radius of rural black Gambian children at follow-up could also mean that it takes longer for bone turnover rate to get back to normal in black people once changes in bone remodelling have occurred.

Whether the transient effect of calcium supplementation on bone mineral accretion in children should be regarded a beneficial to bone health or be seen as a temporal change in a normal bone physiology that reverts to a normal steady state once the supplementation is stopped is not clear. Evidence from recent literature on the subject of bone remodelling transient suggests the latter (Heaney, 1994; Slemenda *et al.*, 1997; Slemenda *et al.*, 1996). A bone remodelling transient is described as an imbalance between the amount of bone being removed and the amount being added in favour of bone formation as a result of reduction in bone remodelling rate. The alteration in remodelling rate is thought to last for one remodelling cycle (Heaney, 1994) and does not affect the fine balance between bone formation and resorption in other remodelling sites. How much of the change in bone reported by calcium supplementation studies represents a remodelling transient or a change in modelling is not clear. It is not clear whether reduced rates of skeletal remodelling are beneficial during growth in children whose habitual calcium intake is low.

10.6 Ultrasound

In this study, calcium supplementation had no effect on either broadband ultrasound attenuation (BUA) or velocity of sound (VOS) at the heel. Our result contrasts with a supplementation study in healthy adults using clodronate as an anti-resorptive therapy where a significant change in both the BUA and VOS were reported in the supplemented group compared with placebo group (Detakats *et al.*, 1998). Whether calcium supplementation has a similar effect on ultrasound variables in normal adults is not clear. There have been no reported studies on the effect of calcium supplementation on ultrasound variables in children. In this study change in nBUA (mean% ± SE) was significantly greater in girls than boys at outcome (+5.8 ± 2.2% vs -2.7 ± 2.0%, p = 0.0006) but there was no effect of the supplement on nBUA.

Amongst the girls, those who had received supplement had a significant reduction in nBUA compared with placebo at follow-up (-8.9 (SE 3.6)%, p = 0.015). No difference between the supplemented and placebo group in boys was found at follow-up and there were no interactions between the supplement effect and sex indicating that the response to the supplement was not different in boys and girls.

The VOS measurements were negatively related to age in both sexes ($p \leq 0.0001$). BUA was also found to be age dependent and increased with age. A recent Swedish study of children 11 - 16 years old found a similar increase of BUA with age (Sundberg *et al.*, 1998).

There was no direct relationship between VOS with either height or weight. After adjustment for age, weight and height were positively related to nBUA. However, when sexes were considered separately, nBUA was related to weight and height in girls but not in boys. Others found a similar relationship between adjusted BUA and weight but not height in girls (Lee & Stevenson, 1993; Schott *et al.*, 1993; Moris *et al.*, 1995). These studies found no association between nBUA with either weight or height in boys.

It should be noted that most of the studies on the relationship of ultrasound parameters and bone status conducted so far were on adults and normal elderly and osteoporotic patients. Little is known about the accuracy of the technique in determining bone mineral and bone strength in children. Therefore more research is needed to determine the performance of ultrasound in measuring bone status at the heel in children. A similar observation was reported by Aloia and associates, where ultrasound of the calcaneus failed to predict bone mineral density in Black and White women (Aloia *et al.*, 1998). In addition, measurement of young children with ultrasound is extremely difficult. This was the case in this study particularly with the younger children with small feet. It is equally possible that calcium supplementation had no effect on ultrasound variables.

In this study, BUA was correlated with BMC, BW and BMD at both the mid-shaft and distal radius. An exception was at mid-shaft radius where the correlation between nBUA and BMD was not significant. The correlation between nBUA and BMD at distal radius was similar to that previously reported (Waud *et al.*, 1992; Gluer & Genant, 1994; Graafmans *et al.*, 1994; Salamone *et al.*, 1994). However, lower correlations were reported in other recent reports

(Massie *et al.*, 1993; Van Daele *et al.*, 1994; Young *et al.*, 1993). VOS was not related to either BMD or BMC at any of the sites measured, a similar poor correlation of VOS with BMD was evident in a study by Brooke-Wavell et al (1995) but contrasted with some previous findings (Faulkner *et al.*, 1994). The result of this study suggests that bone mineral content and bone mineral density could be predicted by ultrasound measurement. The other advantage of the use of quantitative ultrasound techniques (QUS) is that QUS is thought to allow assessment of bone microstructure which currently is unachievable by bone densitometry techniques (Gluer *et al.*, 1994).

In addition to daily calibration of the instrument, the stability of the ultrasound was assessed by measuring range of phantoms supplied by the manufacturer and the coefficient of variation ranged from 0.4 - 1.3%. The stability of the SPA was also assessed with phantoms provided by the manufacturer and the instrument was stable throughout the study period. The reproducibility of ultrasound measurements was assessed by repeated measurement of an adult's foot weekly for the 2 year period and the CV for BUA and VOS were 2.1% and 0.7% respectively which was comparable with values reported in adults (Hans *et al.*, 1993; Stewart *et al.*, 1994; Truscott *et al.*, 1992; Schott *et al.*, 1993; Herd *et al.*, 1992; Herd *et al.*, 1993).

10.7 Biochemistry

In this study, the increase in bone mineral in the calcium supplemented group was associated with a significant decrease in osteocalcin concentration compared with the placebo group. This suggests that the increase in bone

mineral observed in the Gambian children consuming the 714 mg/d calcium supplement may have been associated with a decrease in bone formation, suggesting that the supplement may have acted by altering bone remodelling. There are only two supplementation studies of adolescence where osteocalcin concentration was evaluated (Johnston *et al.*, 1992; Cadogan *et al.*, 1997) and the results are conflicting. One study showed an effect of a calcium supplement on osteocalcin concentration in blood (Johnston *et al.*, 1992) while the other failed to show any change in osteocalcin concentration following supplementation (Cadogan *et al.*, 1997). This difference could be due to the fact that one study, like the Gambian study used a calcium salt as the supplement (Johnston *et al.*, 1992) while the other supplementation was based on milk (Cadogan *et al.*, 1997). This supports the possibility that calcium salts and milk may have different effects on the growing skeleton. In the Gambian study, the supplemented group had a significantly lower plasma parathyroid hormone concentration than the placebo group at outcome indicating that the sustained increase in calcium intake had had the expected effect on calcium homeostasis. A similar reduction in parathyroid hormone secretion has been documented in postmenopausal women supplemented with calcium (Heaney, 1994).

There was no influence of the calcium supplement on other biochemical markers of bone turnover. Neither bone-specific nor total alkaline phosphatase were significantly different between the supplemented and placebo group at outcome. The urinary output of deoxypyridinoline was slightly but not significantly lower in the calcium-supplement group (-11.7 (SE 9.7)%, $p = 0.23$). In this study, it is plausible that the calcium supplementation of Gambian children

reduced bone turnover by reducing bone formation as reflected in the plasma osteocalcin concentration, resulting in reduced bone remodelling space. Urinary output of deoxypyridinoline has been regarded as the most specific marker of bone resorption in the urine. Deoxypyridinoline output did not differ significantly between the supplemented and placebo group at outcome although there was a trend towards lower excretion in the supplemented group. The lack of a statistically significant result could have been due to the fact that there was a large intra-individual variation in the urinary concentration of deoxypyridinoline in these children which could have made it difficult to detect subtle changes in bone metabolism.

Calcium supplementation was associated with decreased urinary titratable acid and phosphate outputs, a common effect of calcium carbonate loading (Adams *et al.*, 1979; Prentice *et al.*, 1995) and indicative of good compliance.

As expected, the supplemented group had a significantly greater urinary calcium output (+65% (SE 13.0), $p \leq 0.0001$) than placebo group. The difference averaged 28 mg/d (0.7 mmol/d), equivalent to 3.9% of the ingested dose. The effect of the calcium supplement on urinary calcium output is similar to that reported in other studies in which the influence of dietary change or the ingestion of calcium salts on urinary calcium output was assessed (Reid *et al*, 1993; Dawson-Hughes *et al.*, 1990; Need *et al.*, 1991), but smaller than the 6.6% reported in previous calcium supplementation study of lactating Gambian women (Prentice *et al.*, 1995). Conversely, phosphorus excretion was significantly lower in the supplemented group (-42% (SE 8.3), $p \leq 0.0001$) compared with the placebo group at outcome. Similar results have been reported in a number of

studies in adults where an increase in calcium intake resulted in elevated level of urinary calcium excretion (Prentice *et al.*, 1995; Heaney, 1994). This study was not designed to measure calcium absorption so it is not known whether the amount of calcium absorbed differed between the supplemented and placebo group. However, judging by the higher urinary calcium output in the supplemented group compared with placebo group at outcome and the increase in bone mineral status, the net absorption appeared higher in those children who had supplement than the placebo group.

At follow-up, only the parathyroid hormone concentration remained significantly reduced in the supplemented group compared with placebo group. This finding is in line with the residual effect of the calcium supplement on bone mineral and may suggest a long-term response. There were however no significant differences observable between the supplemented and placebo group in any of the other biochemical markers measured at follow-up. Urinary output of calcium and phosphorus were not significantly different between the supplemented and placebo groups at follow-up, and the deoxypyridinoline was similar in the two groups at follow-up.

10.8 Differences between boys and girls

Girls had a higher growth velocity than boys indicated by a significant gain in weight, height and triceps skinfold thickness in girls over the 12 month period compared with boys, and although not statistically significant weight and height gains were greater in girls than boys at follow-up. The incremental gains in bone variables over the 12 month period were not statistically different between boys and girls. However, there were trends towards a greater BMC and BMD at the

distal radius in girls. The increases in both mid-shaft and distal radius BMC and BMD over the 2 year period were greater in girls than boys but only differences at mid-shaft radius BMC and BMD reached statistical significance. A similar sex difference has been reported by Lee and coworkers (Lee *et al.*, 1996).

Bone formation markers were higher while bone resorption markers were somewhat lower in girls than boys. This is demonstrated by a significantly higher concentration of osteocalcin, total and bone-specific alkaline phosphatase and a slight reduction in deoxypyridinoline (ns) output in girls than boys. The higher plasma alkaline phosphatase concentration in girls compared with boys suggests that rural Gambian girls may be reaching their growth spurt earlier than boys, an assumption which is supported by a previous study in Keneba (Lo *et al.*, 1990). This study is consistent with the observation by Husain and associates who found no significant sex difference in urinary output of deoxypyridinoline in 4 to 10 years old children (Husain *et al.*, 1999). In contrast, Marowska et al found a greater urinary excretion of deoxypyridinoline in girls compared with boys (Marowska *et al.*, 1996). Girls had a similar urinary sodium but a significantly lower calcium output than boys at outcome.

The concentration of both total and bone-specific alkaline phosphatase, osteocalcin and urinary output of potassium remained elevated while urinary output of titratable acid output was significantly lower in girls compared with boys at follow-up. There was no significant sex difference in urinary output of calcium at follow-up. In contrast, urinary output of sodium was significantly greater in girls than boys at follow-up while the opposite was true at outcome. Calcium-to-sodium ratio remained significantly elevated in boys compared with girls at

follow-up, suggesting that the renal handling of calcium may be different between boys and girls in this study.

10.9 Suggestions for future research

The above discussion indicates that the long-term effects of high calcium intake on growth and bone mineral accretion rate of Gambian children accustomed to a low calcium diet would merit further investigation and to determine whether such effects are beneficial. In particular, a long-term controlled calcium supplementation studies in children, coupled with the studies of bone turnover and calcium retention are required to examine whether greater gains in bone mineral mass resulting from high calcium intake in childhood will persist through to peak bone mass, or whether the effect of high calcium intake on bone merely reflects temporary changes in bone turnover. In view of the conflicting reports on the effect of supplementation on longitudinal growth, the need for adequate and long-term supplementation of children will be even more important in evaluating the long term effect of high calcium on growth and peak bone mass.

This study is unique among all other calcium supplementation studies in a number of ways. In particular, calcium supplementation studies to date have failed to demonstrate a sustained effect of supplementation with a calcium salt on bone mineral status following supplement withdrawal. An other interesting finding in this study which deserves further investigation is the finding that parathyroid hormone concentration in the supplemented group was significantly lower than in the placebo group at outcome and remained so at follow-up. There is so far no explanation for the low parathyroid hormone concentration in children

who were supplemented one year after withdrawing the supplement. A further study investigating calcium metabolism in this group of rural Gambian children is needed to address this issue. Furthermore, the significant reduction in osteocalcin concentration in the calcium supplemented group at outcome compared with placebo group and the subsequent return to a similar level to the control group after the supplement was withdrawn cannot be ignored. Understanding the bone metabolism fully may help us to understand better what is happening at cellular level, and more importantly it will help in our understanding about the mechanism involved in bone remodelling transient, which could help to establish whether reduced rate of skeletal remodelling are beneficial during growth. Finally, a full understanding of these key areas could provide a valuable information for nutrition advice and policy making in an area of the world such as The Gambia where improving child health and nutrition are of paramount importance.

10.10 Summary

In conclusion, this study has demonstrated that calcium intake of rural Gambian children is very low and that the children's growth is poor and puberty delayed if compared with international standards. Calcium supplementation of rural Gambian children on low calcium diet resulted in an increase in bone mineral status. The magnitude of change was similar to that in other supplementation studies of children on higher calcium intakes. If the gain in bone mineral accretion is sustained, it could result in an increase in peak bone mass and hence reduce the risk of osteoporotic fractures later in life. However, calcium supplementation did not have any effect on the size of the skeleton, body weight and height, and the increase in bone mineral acquisition of Gambian children was marked by a significant decrease in osteocalcin concentration. This raises the possibility that the calcium supplement may have acted by altering bone turnover rather than by promoting bone growth. The follow-up study demonstrated a significantly greater bone mineral status at the mid-shaft in those children who received the calcium supplement compared with the placebo group. Similar results were found at the distal radius bone mineral, where the supplemented children had greater BMC, BW and BMD than the placebo group, but differences between the two groups were not statistically significant. However, BMC and BMD at the distal radius remained higher in the supplemented than placebo group at follow-up. The result of the follow-up study suggested that rural Gambian children accustomed to a low calcium diet may benefit from a higher calcium intake by promoting skeletal mineralisation.

A further follow-up study is required to determine whether the effect of calcium supplement on bone mineral accretion is sustained over the long-term.

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Appendices

Appendix 1

To be read to each prospective participant and the parent/guardian in their own language.
Two copies of the form to be signed: one to be given to the parent/guardian, the other to be kept by MRC.

Calcium Requirements of Children in The Gambia

Purpose of the study

We are interested in finding out whether the growth and bone development of Gambian children can be improved by the sort of food they eat. This work will benefit children throughout The Gambia and in other places in the world where the diet is similar. We are asking your child to take part in a study in which he/she will be given tablets to eat for one year. Half of the boys and girls in the study will be given tablets which contain calcium, an ingredient of many of the foods you normally eat and which is needed by the body to make strong bones and teeth. The other children in the study will be given tablets which are very similar but which will provide no extra calcium. The tablets will be kept at the supplement centre in your village and we will ask your child to visit the centre once a day, five days a week, to eat the tablets. If your child cannot come to the centre on a particular day for any reason, a fieldworker will bring the tablets to your home or arrange for your child to eat them over the weekend. The effect of the calcium on your child's bones will be measured at the Keneba clinic before your child starts receiving the tablets, 12 months later when he/she stops taking the tablets and 12 months after that. At these visits we will make measurements of the bones in your child's arms and feet, measure his/her height, weight and size, and take a blood sample. During the same week, fieldworkers will visit your home on 2-3 days to make measurements of the child's food intake and to help him/her collect urine.

Your child's involvement in this study is entirely voluntary and if you, or your child, decide that he/she prefers not take part, it will not affect the medical treatment you, your child or your family receive from the MRC in any way. If you decide to allow your child to join the study, your child is free to leave the study at any time, or to withdraw from parts of it, without explanation.

Consent

This explanation of the study was given by .....

Date .....

Name of participating child .....

Clinic number of child .....

Name of parent/guardian .....

I agree to this child taking part in this study and confirm that the child has been fully informed about the study and is happy to participate. We understand that participation is voluntary, and will not affect the medical treatment received by the child or the family in any way.

Signature (or thumbprint) of parent/guardian .....

MRC Dunn Nutrition Unit

Subject Characteristics

Child M/F Date |__|__|__|__|__|__|

Study ..... Study time point |__|__|

Study Number |__|__|__|__|__|__|

Subject's ID |__|__|__|__|__|

Subject's name .....

Procedure explained by .....

Family History

1. Are you currently living with your born father? (Y/N) |__|

   If not, who are you living with?.....

2. Are you currently living with your born mother? (Y/N) |__|

   If not, who are you living with?

3. How many people are currently in your sinkiro? |__|__|

4. How many other children are currently in your sinkiro? |__|__|

5. How many born brothers and sisters have you? |__|__|

.....

6. How many of them are currently in your sinkiro? |__|__|

.....

7. How many born elder brothers/sisters have you |__|__|

.....

Fracture and Medical History

1. Are you left or right handed? (L/R/A) ⌊_⌋

2. Have you had a serious medical problem in the last year? ⌊_⌋

.....

.....

3. Have you had a serious medical problem at a younger age?

.....

.....

4. Have you ever broken a bone? (Y/N) ⌊_⌋

Age	Bone	Side	Cause

.....

.....

5. Are you presently on medication from MRC? (Y/N) ⌊_⌋

Type	Reason

.....

.....

.....

6. Are you presently on any other medication? (Y/N) ⌊_⌋

Type	Reason

.....

.....

.....

Occupation

1. What is/was your born father's occupation? (F/E/T/A) |__|

.....

2. What is your born mother's occupation? (F/H/E/T) |__|

.....

3. If approriate, what is/was your step-father's occupation? (F/E/T/A) |__|

.....

4. If appropriate, what is/was your step-mother's occupation? (F/H/E/T) |__|

5. Is there a wage earner in the family other than parents or step-parents? (Y/N) |__|

If yes, how many? |__|

Key: A = Animal herdsman F = Farmer E = Employee T = teacher H = House-wife

Socio-Economic Status

1. How many tin houses are currently in the compound? |__|

2. How many thatch houses are currently in the compound? |__|

3. How many radio/bicycle/car/cassette player are currently in the family? |__|

.....

.....

.....

.....

4. Is the family regarded as poor, average or well-off for Keneba (P/A/W) |__|

Appendix 3

Results of the characteristics questionnaire at baseline[†]

Questionnaire	Subjects	%
Wage earner in the family	71	44
Thatch house	50	31
Children from house wife mothers	152	95
Children from employed mothers	8	5
Children whose fathers were farmers	109	68
Children whose fathers were herdsmen	8	5
Employed fathers	43	27
Radio in the family	126	79
Cassette player	99	62

[†]160 subjects

Appendix 4

Activity Questionnaires

M/F Date |__|__|__|__|__|

Study ..... Study timepoint |__

Study number |__|__|__|__|__|

Subject's ID |__|__|__|__|

Subject's name .....

Questions asked by .....

Questions answered by Subject / Mother / Other .....

Education

Are you currently attending school

Which school ..... English/Ara

How many years have you been attending school |__

Which class are you currently in |__

Have you ever attended another school

Which school ..... English/Ara

How many years |__

Sport

Do you play any extra sport organized by the school (not PE)

If yes, what activity and how often .....

Do you play any sport not involving school

If yes, what activity and how often .....

Dally activities

Are you responsible for looking after any younger child

If yes, who and what age .....

If yes, how often and in which season .....

Do you regularly pound

Do you regularly cook

Do you regularly fetch water from well

Do you regularly pump water from well

Are you responsible for looking after sheep/goats in the bush

Doyou drink goat or sheep milk

If yes, how much and how often .....

Appendix 5

CALCIUM FOOD FREQUENCY

M/F Date |__|__|__|__|__|

Study ..... Study timepoint |__

Study number |__|__|__|__|__|

Subject's ID |__|__|__|__|

Subject's name .....

Questions asked by .....

Questions answered by Subject / Mother / Other .....

How often does the subject eat the following foods?

A. FOODS AVAILABLE ALL YEAR *Think about now and the preceding y*

Fish - fresh or smoked, as a separate item

Fish - dry, fresh or smoked, as part of a recipe

Milk - fresh, sour or tinned as a drink Usual portion .....

Milk - fresh, sour or tinned added to porridge/tea

Kolanut (kuruo) Usual portion .....

B. FOODS AVAILABLE IN SEASON *Think about the most recent season for this f*

Baobab fruit - raw (sito) Usual portion .....

Baobab fruit - as a drink or ice lolly (sita nono/iso)

Baobab fruit - added to porridge

Kucha fruit - as a drink/ice lolly (wonjo/iso)

Kucha leaf - in sauces

Baobab leaf - added to sanyo/kinto during steaming

Leaves - in sauces (eg morongo, jambanduro, baobab but not kucha)

Locust bean - fermented (nete tuo)

Locust bean - prepared as a porridge

COMMENTS

Never = 0 Occasionally = 1 Once a week = 2 2-3 times a week = 3 Once a day = 4 Several times a day = 5

Appendix 6

Genital (penis) development:

Stage 1	Pre- adolescent, testes, scrotum and penis are of the same size and proportion as in early childhood
Stage 2	Enlargement of testes and scrotum. Skin of scrotum reddens and changes in texture. Little or no enlargement of penis at this stage
Stage 3	Enlargement of penis, which occurs at first mainly in length. Further growth of testes and scrotum.
Stage 4	Increased size of penis with growth in breadth and development of glans. Testes and scrotum larger: scrotal skin darkened.
stage 5	Genitalia adult in size and shape.

Pubic hair

Stage 1	Pre- adolescent. no pubic hair.
Stage 2	Sparse growth of long, slightly pigmented downy hair, straight or slightly curled, chiefly at the base of the penis.
Stage 3	Considerably darker, coarser and more curled. The hair spreads sparsely over the junction of the penis.
Stage 4	Hair now adult in type, but area covered is still considerably smaller than in the adult. No spread to the medial surface of thighs.
Stage 5	Adult in quantity and type with distribution of the horizontal pattern. Spread to medial surface of thighs

Stage of Breast Development (B)

B1	Preadolescent, elevation of papilla only.
B2	Breast bud stage, elevation of breast and papilla as a small mound, and enlargement of areolar diameter.
B3	Further enlargement of breast and areola, with no separation of their contour.
B4	Projection of areola and papilla to form a secondary mound above the level of the breast
B5	Mature stage, projection of papilla only, owing to recession of the areola to the general contour of the breast.

Appendix 7

Table 12.1 Calcium and phosphorus contents of typical Gambian foods.

Food name	Ca mg/100g	Phosphorus mg/100g
Staples		
mani Fajiringo	6	34
Mani Nyankatango	11	69
Mani Mono	5	12
Mani Mono plus sour milk	10	35
Tiakere Churo	6	29
Sanyo Futo	57	162
Sanyo Nyelengo	31	95
Sanyo Mono	5	13
Findi Nyelengo	38	51
Findi Mono	6	13
Kinti Futo	56	219
Kinti Nyelengo	25	84
Kinti Mono	3	29
Tubanyo Futo	56	169
Tubanyo Nyelengo	30	88
Groundnuts		
Tio	45	260
Sauces		
Tia Durango	21	69
Jambo, plus tio	133	91
Kucha/Domoda	99	51
Bukolo	39	62
Leaves		
Baobab leaves, fresh	319	89
Baobab leaves, dried	1750	193
Kucha leaves fresh	354	49
Morongo leaves, fresh	433	144
Jambanduro leaves, fresh	705	124
Vegetables		
Locust bean seed	449	282
Locust bean pod power	127	160
Bitter Tomatoes	10	12
Okra	70	60
Fruits		
Mango, unripe	10	13
Mangoes, ripe	10	13
Orange	30	18
Baobab, fruit	390	35
Fish		
Chalo, boiled	90	217
large fresh water fish (Kujalo)	30	75
Furo/Furindingo, boiled	49	138
Chalo dried	395	728
Kujalo, dried	177	409
Furo/Furindingo, dried	4000	2700
Other foods		
Meat, boiled	10	190
Milk, cow	128	93
Water	1	0
Salt, local	275	5

Mani, rice; Fajiringo, boiled; Mono, porridge; Sanyo, millet; Futo, steamed cereal with dried baobab; Nyelengo, boiled millet/sorghum; Findi, wild grass; Tubanyo, maize; tio, groundnuts. Source: McCrae & Paul, 1996