Vitamin D Supplementation In Adolescent Female Ballet Dancers And Gymnasts in a 12 Month Randomised Controlled Trial In Auckland, New Zealand

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Wendy Jessup

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Abstract

Aim: To examine the effects of vitamin D supplementation on the bone health of female adolescent ballet dancers and gymnasts.

Method: Adolescent female ballet dancers and gymnasts from Auckland, New Zealand were recruited to a 12 month randomised double-blind trial. Participants were supplemented with cholecalciferol 50,000 IU per month or a placebo. At baseline detailed dietary intake was collected by a four day food record; at baseline and 12 months bone mineral density (BMD) and content were recorded by DXA as well as bone-free, fat-free, lean body mass, percentage body fat, height and weight. At baseline, six months and 12 months serum markers for vitamin D (oestradiol and parathyroid hormone) were collected.

Results: A total of 61 adolescent girls were recruited at baseline, BMD and content by DXA was completed in 45 girls and 41 provided vitamin D serum samples. Serum vitamin D concentration was recorded for 41 female ballet dancers and gymnasts aged 12 to 18 years was 72 nmol/L and remained adequate (>50 nmol/L) in both intervention and control groups for the 12 month duration. There was no significant difference between intervention and control groups in bone mineral density and content at any bone site at 12 months. The significant predictors of increased bone mineral density at baseline were older age (P=0.002) higher bone-free, fat-free, lean body mass (P=0.001) and higher calcium intake (P=0.005). For higher bone mineral content the significant predictors at baseline were older age (P=0.01) and higher bone-free, fat-free, lean body mass (P=0.001). In all participants (n=48) bone mineral density and content increased significantly at 12 months (total body BMD and content, areal BMD, total hip BMD and content, femoral neck BMD and content and lumbar spine BMD and content).

Discussion: More than adequate baseline serum vitamin D levels in this adolescent group may explain the lack of significant difference in any of the bone measures between intervention and control groups. As the age range of the adolescent girls varied markedly and older age predicted both an increase in BMD and content, it is likely that there was also bone accrual due to growth. The nil effect of vitamin D supplementation on bone measures was also limited by the small sample size.

Conclusion: In this study vitamin D supplementation had no effect on the bone mineral density and content of female adolescent ballet dancers and gymnasts.

Further investigations are needed to examine vitamin D supplementation on bone measures in a large group of adolescent girls.

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Finally, to my friends and family, thanks for your love and support during this project.

Contributions

Masters student and author: Wendy Jessup

Responsible for half of this study in conjunction with Masters student Sarah Mitchell and Research assistant Owen Mugridge. Responsible for serum preparation at endpoint and DXA scans at endpoint and assisted with monitoring of training diaries. Compiled and documented bone mineral measures, anthropometric values and serum data and performed all statistical analysis.

Masters student: Sarah Mitchell

Sarah was involved in the application for ethical approval and recruitment of participants. She collected baseline and six months anthropometric data and monitored supplement compliance.

Research assistant: Owen Mugridge

Coordinated data collection appointments and performed phlebotomy.

DXA management and operation: Dr Pamela von Hurst and Wendy Jessup

Dr von Hurst provided training and supervision throughout.

Laboratory manager: PC Tong

Prepared the serum for analysis of intact PTH, oestradiol and 25(OH)D.

Performed DXA QC for scans and conducted bone mineral analysis from scan reports.

Table of Contents

Abstract ..... **I**

Acknowledgements ..... **III**

Contributions ..... **IV**

List of tables ..... **VIII**

List of figures ..... **IX**

Abbreviations ..... **XI**

Terms ..... **XII**

1.0 Introduction **1**

1.1 Background **2**

1.2 Justification **3**

1.3 Aim and Objectives **3**

1.3.1 Aim 3

1.3.2 Objectives 3

1.4 Hypotheses **4**

2.0 Review of the literature **5**

2.1 ..... **6**

Introduction **6**

2.1 Food sources of vitamin D **7**

2.2 Chemistry and physiology **8**

2.2.1 Metabolism of vitamin D 8

2.3 Vitamin D in New Zealand **9**

2.3.1 Vitamin D recommendations 9

2.3.2 Vitamin D status 10

2.3.3 Seasonal variation of vitamin D 10

2.3.4 The effect of latitude on vitamin D status 12

2.4 The relationship between serum vitamin D, parathyroid hormone and calcium **12**

2 ..... **14**

2.5.1 Protein 15

2.5.2 Calcium 17

2.5.3 Energy intake 19

2.6 Peak bone mass **20**

2.6.1 The achievement of peak bone mass 20

2.7 Bone mineral measures **22**

2.8 Physical activity and bone mineral density including loading **22**

2.9 Sex hormones and bone mineralisation **27**

2.9.1 Oligomenorrhoea 32

2.9.2 Gynaecological age (GA) 32

2.10 Characteristics of ballet dancers and gymnasts **32**

2.11 Dancers, gymnasts and diet **34**

2.12 Vitamin D status of ballet dancers **35**

2.13 Sex steroids and issues in this population **36**

2.14 Summary **37**

3.0 Methods **38**
3.1 Primary Aim **39**
3.2 Ethical Approval **39**
3.3 Study Design **39**
3.4 Subject Recruitment **40**
3.5 Screening **40**
3.6 Procedures **41**
3.7 Funding **42**
3.8 Vitamin D supplementation protocol **42**
3.9 Compliance **42**
3.10 Laboratory measurements **42**
3.11 Bone measures **43**
3.12 Anthropometric measurements **45**
3.13 Questionnaires **46**
3.14 Dietary survey procedure **46**
3.15 Analysis of the 4-day estimated food records **46**
3.16 Assessment of physical activity **46**
3.17 Statistical methods **47**
3.18 Data storage and handling **48**

4.0 Results **49**

4.1 Study participants **50**
4.1.1 Attrition rates 50
4.2 Results Section 1 Baseline Characteristics of participants in the randomised controlled trial **52**
4.2.1 Serum vitamin D and other biomarkers 52
4.2.2 Bone mineral measures at baseline 53
4.2.3 Anthropometric characteristics at baseline 54
4.2.4 Analysis of the 4-day estimated food records 55
4.3 Change in parameters over 12 months – differences between intervention and control groups **56**
4.3.1 Change in vitamin D and other serum markers over 12 months 56
4.3.2 Intact PTH 57
4.3.3 Oestradiol 57
4.3.4 Change in bone mineral measures over 12 months 58
4.3.5 Changes in anthropometry 60
4.4 Results Section 2 Baseline Data Investigations **62**
4.4.1 Predictors of bone mineral at baseline 62
4.4.2 Total body bone mineral density 62
4.4.3 Total body bone mineral content 63
4.4.4 Age groups and bone 64
4.4.5 Age of menarche 64
4.4.6 Gynaecological Age 65
4.4.7 Oligomenorrhoea 66
4.4.8 Physical training volume 66
4.4.9 Change by age group 67

5.0 Discussion **68**
5.1 Summary of outcomes **69**
Section 1 Outcomes from the randomised controlled trial 69
Section 2 Outcomes from the baseline data 69
5.2 Primary outcome **70**
5.2.1 Vitamin D status at baseline 70
5.2.2 Vitamin D supplement dosage 73

5.2.3 Vitamin D and leanness 75
5.2.4 Supplement compliance 76
5.3 Further investigation of baseline data 78
5.3.1 Skeletal development and growth 78
5.3.2 Predictors of bone mineral density at baseline 78
5.3.3 Lean mass 78
5.3.4 Calcium 79
5.4 Change in bone mineral over 12 months 80
5.5 Weight-bearing and non-weight bearing sports 80
5.6 Age-related bone mineral accrual 80
5.7 Bone mineral density at the femoral neck 81
5.8 Menstrual function 81
5.8.1 Age of menarche 81
5.8.2 Oligomenorrhoea 82
5.9 Strengths and limitations 82
5.9.1 Population 82
5.9.2 Study design 82
5.9.3 Range of ages 83
6.1 Key findings 85
6.1.1 Bone 85
6.1.2 Vitamin D 85
6.1.3 Age-related changes in bone 86
6.1.4 Menstruation and bone 86
6.1.5 Summary 87
6.2 Recommendations 88
6.2.1 Future research 88
7.0 References 89
8.0 Appendices 107
Appendix A 107
Appendix B 107
Appendix C 107
Appendix A 108
Appendix B 109
Appendix C 115

List of tables

Chapter 3	Description	Page
Table 1.	Bone measures analysed by DXA scan at baseline and 12 months.	45
Chapter 4		
Table 2.	Serum blood results at baseline for gymnasts and dancers.	54
Table 3.	Bone mineral measurements at baseline for dancers and gymnasts.	55
Table 4	Baseline demographic and anthropometric characteristics of ballet dancers and gymnasts	56
Table 5.	Change in serum measures from baseline, six mons and endpoint, between and within intervention an d control groups.	58
Table 6.	Change in bone mineral measures from baseline to endpoint within and between intervention and control groups	60
Table 7.	Changes from baseline to endpoint in anthropometry within and between intervention and control groups	62
Table 8.	Predictors of total body bone mineral density at baseline	64
Table 9.	Predictors of total body bone mineral content at baseline	64
Table 10.	Differences between variables in dancers and gymnasts who had experienced menarche and those who had not experienced menarche at baseline	66
Table 11.	Gynaecological age of participants at baseline.	66
Table 12.	Regular vs irregular periods and bone measures at baseline.	67
Table 13.	Differences in mean change over 12 months in bone variables and height.	68

List of figures

Chapter 2	Description	Page
Figure 1.	Vitamin D metabolism.	9
Figure 2.	The hormonal control of blood calcium in the body by parathyroid hormone and calcitonin.	14
Figure 3.	Dietary protein intakes, IGF-I, and calcium-phosphate homeostasis. IGF-I influences bone growth, bone mass accumulation and mineral homeostasis	28
Figure 4.	The interaction of energy availability, menstrual dysfunction and bone mineral density noted in The Female Athlete Triad	29
Chapter 3		
Figure 5.	Diagrammatic representation of phases of the study design.	39
Figure 6.	An example of a DXA scan of the lumbar spine, showing the vertebrae L1-L4.	44
Figure 7.	An example of a DXA scan of the hip showing the femoral neck.	44
Chapter 4		
Figure 8.	Diagram of sporting codes included in this study	52
Figure 9.	Diagram of selection process from assessment for eligibility in study through to analysis of results.	52
Figure 10.	Change in serum 25(OH)D in the intervention and control groups over 12 months.	57
Figure 11.	Differences in mean bone mineral density at 4 age groups. The age groups were 12-13 years, 14 years, 15 years and 16-18 years.	65

Figure 12 Serum 25(OH)D in relation to BMI (kg/m2) showing response to vitamin D supplementation 75

Abbreviations

$1,25(OH)_2D_3$	α,25-dihydroxyvitami D_3 or calcitriol
$25(OH)D_3$	25-hydroxyvitamin D_3
aBMD	Areal bone mineral density
BFFFLBM	Bone free, fat free, lean body mass
BMC	Bone mineral content
BMCLS	Bone mineral content lumbar spine
BMCTH	Bone mineral content total hip
BMDLS	Bone mineral density lumbar spine
BMDTH	Bone mineral density total hip
GA	Gynaecological age
GH	Growth hormone
IGF-1	Insulin like growth factor 1
PBM	Peak bone mass
PBMAS	Saskatchewan Paediatric Bone Mineral Accrual Study
PHV	Peak height velocity

Terms

The following terms are used in this thesis:

Amenorrhoea: no menstrual cycles for >90 days, in women of reproductive age (Birmingham, 2004).

Anthropometrics: refers to the measurement of the human individual, in this thesis including bodyweight (kg), height (m) and BMI /m^2.

Areal bone mineral density: bone mineral density per surface area (Zemel et al., 2011).

Bone free, fat free, lean body mass (kg): Lean mass measured by DXA less bone mineral content.

Bone mineral density (g/cm^2): refers to grams of bone mineral per unit of bone area scanned (Kalkwarf et al., 2007).

Bone mineral content (g): refers to grams of bone within a specific area (Zemel et al., 2011).

Body mass index: the ratio of weight to height squared (BMI= kg/m^2).

Body composition: includes lean body mass body fat percentage and bone.

Dual Energy X-Ray Absorptiometry: based on the decrease in photon energy of the photon beam as it passes through bone and non-mineralized soft tissue (Bachrach, 2000).

Eumenhorrea: Normal cycles of menstruation.

Hypogonadism: hypothalamic disruption due to insufficient energy intake relative to energy expenditure (Rothman & Wierman, 2008)).

Gynaecological age: (GA) can be defined as the difference between chronologic age and menarchal age. It is the reference criterion for biological maturity (Stevens-Simon, Forbes, Kreipe, & McAnarney, 1986).

Oligomenorrhoea: irregular menstrual periods, <9 menstrual periods over 12 months (Birmingham, 2004; Thein-Nissenbaum & Carr, 2011).

Osteoporosis: a skeletal disease characterised by low density and general deterioration of bone tissue.

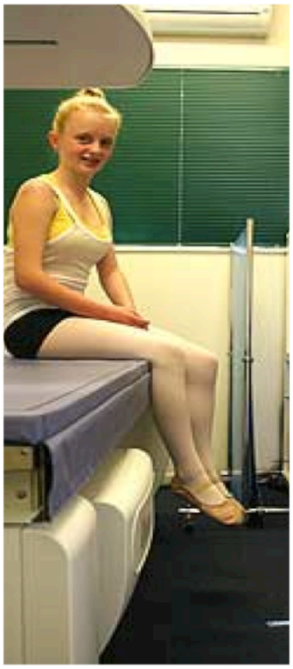
Peak bone mass: highest bone mineral content during adulthood (Heaney et al., 2000).

Peak height velocity: Period in adolescence when growth is at maximum rate.

Primary amenorrhoea: a delay in menarche past 15 years due to the late commencement of puberty (Barrack, Rauh, & Nichols, 2008; Birmingham, 2004).

Secondary amenorrhoea: the absence of menstruation, post-menarche, lasting three months (Birmingham, 2004).

1.0 Introduction



1.1 Background

Bone mass and size develops during childhood and adolescence to peak in the second decade. The amount of bone mineral accrued is a predictor of bone mass and osteoporosis in later life (Baxter-Jones, Faulkner, Forwood, Mirwald, & Bailey, 2011; Rizzoli, Bonjour, & Chevalley, 2010; Zhu & Prince, 2012).

The determinants of peak bone mass have become the focus of worldwide research. The goal is to identify interventions which will reduce osteoporosis in the ageing process (Holick & Chen, 2008). The financial and clinical burden associated with osteoporosis and fracture is considerable (Gutiérrez et al., 2012) and is set to increase with the aging population.

Low vitamin D status is a known determinant in the development of osteoporosis and now recognised as a worldwide health problem(Cashman, 2007; Cashman et al., 2008; Deluca, 2004; Georgopoulos et al., 2004; Holick & Chen, 2008; Pekkinen, Viljakainen, Saarnio, Lamberg-Allardt, & Makitie, 2012). Findings from observation studies that have investigated the association between bone mineral and vitamin D status have been inconsistent. A recent meta-analysis by Reid et al. (2013) of 23 randomised studies of vitamin D supplementation reported a small positive benefit on bone mineral density at the femoral neck, but no benefit in any other bone site. In contrast, an earlier meta-analysis of 12 randomised controlled trials (Bischoff-Ferrari et al., 2009) reported that a dose of vitamin D greater than 400 IU per day was associated with a reduction in non-vertebral fracture risk of 20%.

However, the critical time for development of peak bone mass to prevent osteoporosis is in childhood and adolescence (Mora & Gilsanz, 2003). The question, to be answered, is: does ensuring adequate vitamin D during adolescence have a significant impact on the development of peak bone mass?

To answer this question, good quality randomised controlled trials are essential to provide the evidence-base to make public health recommendations.

1.2 Justification

Dancers and gymnasts are known to have low vitamin D (Chen et al., 2007; Lovell, 2008; Wolman et al., 2013). They also may be at risk of poor bone health (Burckhardt, Wynn, Krieg, Bagutti, & Faouzi, 2011; Hinrichs, Chae, Lehmann, Allolio, & Platen, 2010) due to insufficient serum vitamin D levels (Wolman et al., 2013), low energy intake (Klentrou & Plyley, 2003), low micronutrient intake(Munoz, de la Piedra, Barrios, Garrido, & Argente, 2004) and menstrual dysfunction (Valentino et al., 2001).

Dancers and gymnasts train many hours each week; this physical activity may offset poor bone health in this group (Lehtonen-Veromaa et al., 2000). Physical activity has a protective effect on bone and is a potentially confounding factor in randomised controlled trials (Constantini, Arieli, Chodick, & Dubnov-Raz, 2010; Constantini, Dubnov-Raz, et al., 2010). By selecting a population who are as homogenous as possible with regard to age, type, intensity and volume of exercise, the effect of vitamin D supplementation can be more clearly identified.

1.3 Aim and Objectives

1.3.1 Aim

To examine the effects of vitamin D supplementation on the bone health of adolescent female ballet dancers and gymnasts.

1.3.2 Objectives

To recruit a cohort of young female dancers and gymnasts aged between 12-18 years, who train at least 5 hours each week in their chosen sport.

To administer an oral dose of vitamin D_3 (cholecalciferol) 50,000 IU or placebo once each month for 12 months.

To measure the total bone mineral density and anthropometry (ie. Measure height, weight, bone-free, fat-free, lean body mass and percentage body fat at baseline and at 12 months by DXA to determine the effects of vitamin D supplementation on bone whilst controlling for physical activity and calcium intake in a cohort of dancers and gymnasts.

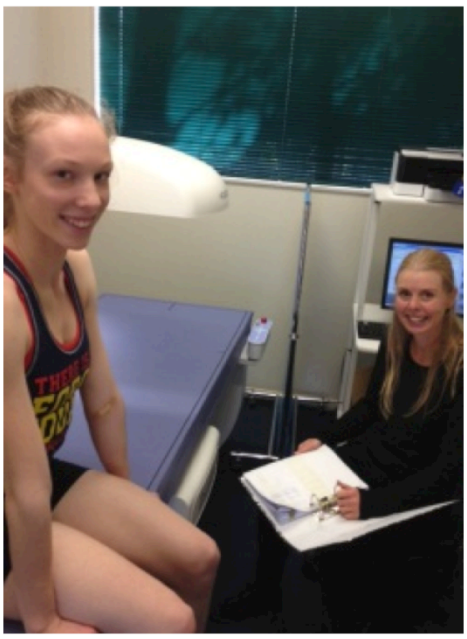
1.4 Hypotheses

Hypothesis 1. That the chosen research population will have a less than adequate vitamin D status,

Hypothesis 2. That all participants will have improved BMD as measured by DXA at the end of the 12 months period, due to increased growth,

Hypothesis 3. That there will be a difference in change in BMD in any of the bone sites between the control and intervention groups.

2.0 Review of the literature



2.1 Introduction

In 1914 Edward Mellanby, a British researcher investigated a cure for rickets which was prevalent in infants and young children in Britain at this time. Rickets were occurring in children as the smog from industrial chimney obliterated the sun' rays (Harrison, 1966). Mellenby fed Scottish oats to dogs and deprived them of UV rays. The dogs developed rickets. He then fed cod liver oil to the dogs and cured the rickets. Mellanby concluded that a dietary substance within the cod liver oil could prevent the disease. Later, Elmer McCollum fed dogs cod liver oil and identified a new substance which prevented rickets, he named this new substance vitamin D (McLean & Budy, 1964).

Analyses of the physiological function of vitamin D emerged during the 1950s with Steenbock and Herting (1955), who investigated the relationship between growth and vitamin D using rats. They reported that growth in tissue and bone in young rats could be maximised when vitamin D supplemented their diet. Interestingly, the effects of vitamin D supplementation on growth were greater than when any manipulation of phosphorus or calcium concentration was made with the diet. They hypothesised the increase in the growth of tissue and bone suggested that other metabolic processes may be directly affected by the vitamin D. Building on this work was a series of elegant studies by DeLuca et al. (1962) and Ponchon (1969), which identified that vitamin D was the major site of hydroxylation of (cholecalciferol) into 25-hydroxycholecalciferol. Investigation into the relationship between parathyroid hormone (PTH) and vitamin D by DeLuca (1963) noted that the effect of vitamin D was not limited by parathyroid hormone (PTH) and PTH was not effective without vitamin D.

These works played out in harmony with other investigations, which linked together liver disease, vitamin D and bone disease. In one study, Kobayashi and colleagues (1974) noted that 23 out of 39 (59%) child or infant patients who had biliary atresia (liver disease where the common bile duct is blocked or absent) also had rickets and osteoporosis. They recommended a dose of 2000 IU daily of vitamin D be given

prophylactically to children with biliary atresia to reduce the prevalence and outcome of rickets. The mechanism which linked rickets with hepatobiliary disorders was identified shortly after as intestinal malabsorption of vitamin D rather than poor metabolic function of the liver (Kooh, Jones, Reilly, & Fraser, 1979). In the same year, the connection between vitamin D metabolism and bone health was illustrated further by Gallagher and colleagues (1979), who reported that if the metabolism of vitamin D was impaired, calcium absorption was affected, both in control groups which were not osteoporotic and osteoporotic subjects.

Throughout the 1970s and 80s investigations into the role of vitamin D in relation to human health were continued. Studies sought to identify the role and importance of vitamin D nutritional status in the elderly (MacLennan & Hamilton, 1977), in cardiovascular health (Olson, 1973), cancer (Garland & Garland, 1980) muscle (Ritz, Boland, & Kreusser, 1980) metabolism (Brown, Spanos, & MacIntyre, 1980). By the 2000s, vitamin D deficiency was a recognised problem worldwide (Holick, 2007). Debate as to the definition of insufficiency and dose to correct insufficiency were in full swing (Bouillon, Norman, & Lips, 2007).

More recently, a Cochrane review (Winzenberg, Powell, Shaw & Jones, 2010) of randomised controlled trials (RCT)s of vitamin D supplementation, did not support vitamin D supplementation in those who had adequate vitamin D. The inclusion criteria were that the trials were at least three months duration in a cohort of healthy individuals less than 20 years of age. Adequate vitamin D status in this group mean that bone mineral density (BMD) did not improve at the hip, lumbar spine (LS), forearm or total body. However, in those who were vitamin D deficient, vitamin D supplementation could improve bone density.

2.1 Food sources of vitamin D

It is very difficult to achieve adequate vitamin D levels from food sources alone (Ministry of Health, 2012b), which is why safe exposure to the sun is so important to achieve sufficient vitamin D status (Ministry of Health, 2012a). Food sources of vitamin

D are fatty fish such as salmon and mackerel, and cod liver oil, eggs, liver (Chen et al., 2007)and milk products. The fortification of milk products depends upon the country and fortification policy of the country.

In New Zealand some foods, such as margarine and yoghurt may be fortified with vitamin D, although this is not mandatory as it is in Australia. Analysis has shown that vitamin D concentration in margarine ranges between 10-18 µg per 100g (Thomson, 2006). It would be difficult to achieve vitamin D sufficiency through the intake of margarine and milk sources in New Zealand as the amount of vitamin D in these milk products is relatively low.

Both D_2 and D_3 are used for food fortification and the manufacture of supplements throughout the world. Food sources of ergocalciferol, or vitamin D_2, are irradiated mushrooms and fungi and some invertebrates. This sterol is produced by UV irradiation also, similar to vitamin D_3 (Holick et al., 2011).

2.2 Chemistry and physiology

2.2.1 Metabolism of vitamin D

Vitamin D exists in two forms; vitamin D_3 or cholecalciferol, which is formed in the skin of animals and birds; and vitamin D_2, ergocalciferol , which is synthesised by UV irradiation of ergosterol and found in plants and invertebrates (Al-Shaar et al., 2013). The main source of vitamin D is sunlight, and specifically ultra violet B rays (290–315 nm)(Holick, 2012).

These two forms of vitamin D must undergo activation. When skin is exposed to adequate irradiation from sunlight, UVB rays convert 7-dehydrocholesterol (7-DHC) in the skin to pre-vitamin D (Chen et al., 2007) , which then isomerises to form vitamin D_3 (Holick et al., 1980). The vitamin D is stored in adipose tissue or transported to the liver for activation, hydroxylation to 25-hydroxyvitamin D, that is 25(OH)D (Holick, 2013).

The main form of vitamin D in the body is 25(OH)D, and the circulating level is measured to assess vitamin D status. The kidneys metabolise 25(OH)D to 1,25-dihydroxyvitamin D (Holick, 2013; Jones, 2006) see Figure 1. Recently, new evidence has brought to light other human tissue capable of metabolising vitamin D (Christakos, Ajibade, Dhawan, Fechner, & Mady, 2010). The placenta and decidua (Zehnder et al., 2002) , synovial fluid (Hayes, Denton, Freemont, & Mawer, 1989) and extra-renal sites of metabolism occur under the influence of cytokines (Lips et al., 2006).

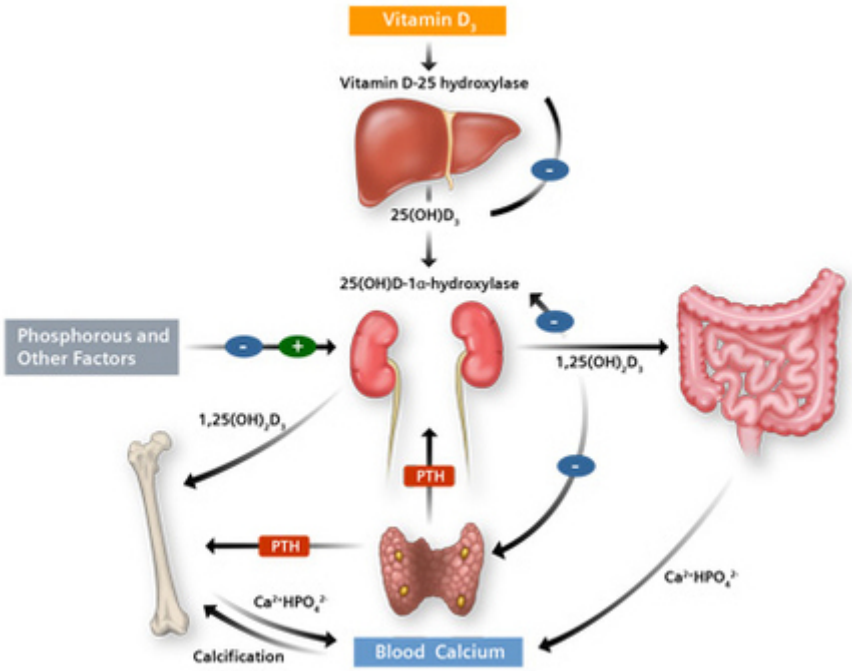


Figure 1. Vitamin D metabolism (Bringhurst, Demay, Krane, & Kronenberg, 2005)(Copyright free).

2.3 Vitamin D in New Zealand

2.3.1 Vitamin D recommendations

The Ministry of Health (2012a) recommends the following serum vitamin D cut-offs in New Zealand:

- high levels of vitamin D – serum 25(OH)D concentrations of 125+ nmol/L.
- Vitamin D sufficiency - equal to or above the recommended level – serum 25(OH)D concentrations of 50 + nmol/L

- below recommended level but not deficient – serum 25(OH)D concentrations of 25.0–49.9 nmol/L
- vitamin D deficiency – serum 25(OH)D concentrations less than 25 nmol/L
- mild to moderate deficiency – serum 25(OH)D concentrations of 12.5–24.9 nmol/L
- severe vitamin D deficiency – serum 25(OH)D concentrations less than 12.5 nmol/L

2.3.2 Vitamin D status

Data from the 2008/09 New Zealand Adult Nutrition Survey (2012b) reported that the prevalence of vitamin D deficiency was 5% (vitamin D concentrations of <25nmol/L) in adults over 15 years of age and 27% had vitamin D levels that were below the recommended level but not deficient (≥25 nmol/L and < 50 nmol/L). In 2008/09 the annual mean level of vitamin D for New Zealand adults was 63 nmol/L and the majority of adults (68.1%) had equal to or above the recommended level of vitamin D (≥50 nmol/L) (2012b).

2.3.3 Seasonal variation of vitamin D

There is a seasonal variation in vitamin D levels in New Zealand, which reflects the number of sunlight hours. Vitamin D insufficiency peaked over the winter months (August through to October) and vitamin D levels were at their highest in the summer months (Bolland et al., 2007). Vitamin D has a half-life of one-two months (Vieth, 1999) though this has not been adequately determined in literature and is still being investigated.

Supplementation of vitamin D has been recommended by the Ministry of Health for individuals who are at risk of low vitamin D status (2012a). Individuals include people who have a dark skin or those who wear a veil or clothing which totally covers their body. It also includes people who deliberately avoid sun exposure and people who are

housebound. This at-risk group includes sportspeople who spend a lot of time training indoors, such as ballet dancers and gymnasts.

Research within New Zealand by Bolland et al (2007), investigated the 25(OH)D status of almost 22,000 adults over an 18 month period. The prevalence of vitamin D insufficiency (<50nmol/L) was 48% (range of 30-35%) of this cohort between January-March and 61-63% over the winter period July to September. Vitamin D deficiency < 25 nmol/L in the same cohort was 15% ranging between 7-8% over the summer period with a larger range of 21-23% over winter. They estimated that vitamin D levels over summer must be at least 60-75 nmol/L in order to prevent insufficiency over winter (Bolland et al., 2007). The vitamin D stored in adipose tissue can be metabolised to maintain levels over winter when adequate exposure to sunlight for vitamin D synthesis is reduced. However, the half-life of vitamin D means that it will not sustain the individual sufficiently for the winter months (Pearce & Cheetham, 2010).

Rockell and colleagues (2005) investigated 25(OH)D concentration in New Zealand children and adolescents aged 5-14 years in a nationwide nutrition survey. They found that 25(OH)D concentration declined by 50% between March and August (the winter months). The strongest determinants of serum 25(OH)D levels in New Zealand children were season and ethnicity.

These findings have been supported in a similar study by Van der Mei and colleagues (2007) in a large population-based study in Australia. Serum vitamin D concentration samples were collected from three areas, Geelong, South East Queensland and Tasmania. This work found vitamin D insufficiency was common across Australia. The winter season appeared to have more effect on vitamin D status than latitude.

Similarly, research investigating vitamin D levels in Australian ballet dancers collected by Wolman (2013) observed that all of the 19 elite ballet dancers in the study had insufficient vitamin D levels of <50 nmol/L in winter.

2.3.4 The effect of latitude on vitamin D status

Testing of a random sample of children aged 6-23 months from Auckland City, New Zealand, where the latitude is 36.52°S indicated that New Zealand children were vulnerable to vitamin D deficiency, as 13% of the group were deficient (Grant, Wall, Crengle, & Scragg, 2009). Later these results were backed up by another study investigating the vitamin D status of a sample of children aged 12-22 months from Dunedin, New Zealand (latitude 45°S). They reported that almost 80% of the children who had their blood samples taken in winter had 25(OH)D levels <50 nmol/L (Houghton et al., 2010).

Similarly, a cross-sectional study of Year 3 children from the Waikato area (Graham, Kira, Conaglen, McLennan, & Rush, 2009) reported that children who received vitamin D supplemented milk at school had serum vitamin D levels significantly higher than children from control schools that did not receive milk. Both groups had vitamin D levels less than the recommended range (<50 nmol/L) (Graham et al., 2009).

Research shows that Maori and Pacific children are particularly at risk of low vitamin D status. A large sample of children from across New Zealand (latitude 35-46°S) aged 5-14 years showed that the prevalence of insufficiency was 41% in Maori children, 59% in Pacific Island children and 25% in New Zealand European children (J. E. Rockell et al., 2005); this is thought to be due to skin colour and sun exposure. It seems that children in New Zealand are at risk of vitamin D insufficiency, particularly in winter or in low latitudes, which is likely to be due to reduced sun exposure.

2.4 The relationship between serum vitamin D, parathyroid hormone and calcium

Calcium is involved in functional processes in the body. Calcium ions in the blood have a key role in muscle contraction. They are also involved in voltage-gated ion channels in cell membranes (Guillemant, Cabrol, Allemandou, Peres, & Guillemant, 1995). The level of calcium in the blood is tightly regulated to within a narrow range; PTH and

calcitonin are released and return the serum levels to homeostasis (Reece, Cain, Wasserman, Minorsky, & Jackson, 2010).

Parathyroid (PTH) and calcitonin are the two hormones that regulate calcium levels in the blood. They regulate serum Ca^{2+} concentrations by working with opposite effects, see Figure 2 (Steingrimsdottir, Gunnarsson, Indridason, Franzson, & Sigurdsson, 2005). Parathyroid hormone is secreted by the parathyroid gland which stimulates the production of 1, 25(OH)D_2. It does this by promotion of the transcription of 1α(OH)ase (Zierold, Nehring, & DeLuca, 2007). This causes increased absorption of dietary calcium and raises the level of calcium ions in the blood. Parathyroid hormone also causes osteoclasts to resorb bone, which increases serum Ca^{2+} concentrations. To offset this rise in Ca^{2+}, calcitonin is produced by the thyroid gland, which then causes Ca^{2+} to be deposited in the bones. Calcitonin also causes a reduction in Ca^{2+} uptake in the renal tubules, however the role of calcitonin in bone is small compared to those of PTH and vitamin D (Aloia, Talwar, Pollack, Feuerman, & Yeh, 2006).

Steingrimsdottir (2005) and colleagues reported a strongly negative association between adequate serum 25-hydroxyvitamin D and PTH. In their study, sufficient vitamin D enabled PTH to maintain homeostasis even when calcium intake was < 800 mg per day. They also saw that a high calcium intake, >1200 mg per day, was not sufficient to maintain serum PTH if vitamin D status was insufficient, suggesting that adequate vitamin D may be more important for maintaining healthy PTH levels than adequate calcium intake. Secondary hyperparathyroidism has been linked with bone disease (Cunningham, Locatelli, & Rodriguez, 2011), and low vitamin D status is a key factor in the progression of this disease.

Figure 2 below, shows how homeostasis of serum calcium is maintained in the body. When Ca^{2+} levels are high calcitonin is released which increases deposition of calcium into bone, decreases Ca^{2+} absorption from the intestines and in the renal tubules, which causes serum calcium levels to fall. If Ca^{2+} levels are low, resorption of Ca^{2+} occurs in the bones and Ca^{2+} absorption is increased in the intestines and the kidneys.

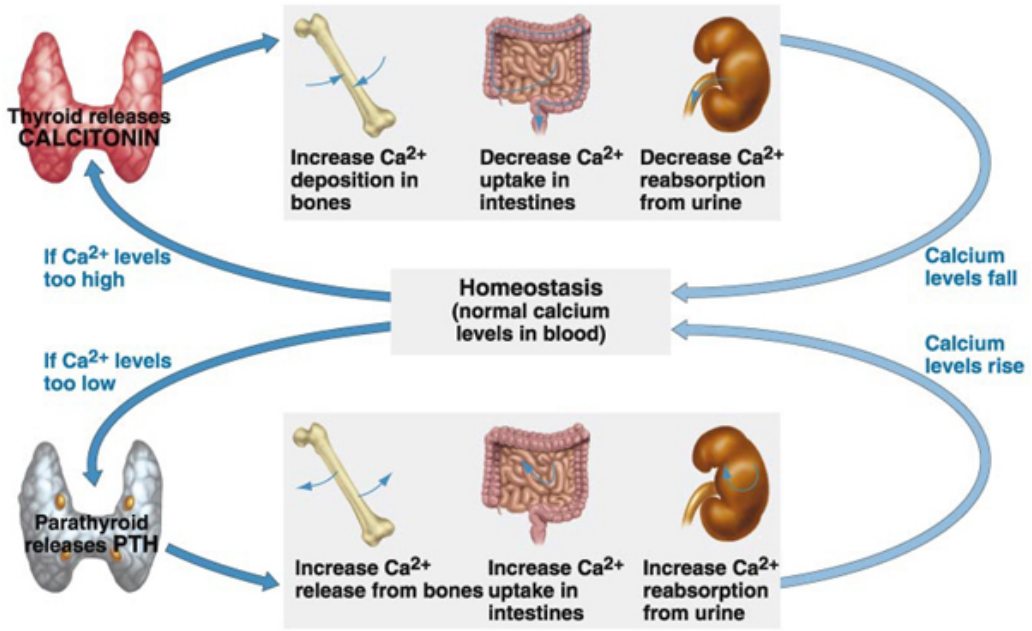


Figure 2. The hormonal control of blood calcium in the body by parathyroid hormone and calcitonin (from (Raven, Johnson, Losos, & Singer, 2005) (Copyright free).

2.5 Diet and bone including protein, energy and calcium

Dancers and gymnasts are at risk of low protein intakes because their dietary intake is often low in total energy (Rizzoli et al., 2013). However it must be noted that energy requirements are based on both physical activity, age and body size. Gymnasts and dancers are often smaller and lighter than the public and due to their size, caloric demands and macronutrient amounts may be less than are recommended by national guidelines.

The evidence of low protein intake in dancers is mixed. One study, examined the protein intake of 127 pre-professional ballet dancers with a mean age of 16.7 years. The study reported that this group consumed less than the recommended food intake in all food groups. However, they consumed more than twice the amount of recommended non-dairy protein, while dairy product consumption was low. The dancers and gymnasts consumed only 1.8 servings of dairy products compared to the recommended 3-4 servings (Burckhardt et al., 2011).

Similarly, elite dancers with a mean age of 24.3 ± 1.3 years were pair-matched with non-dancing controls. The dancers consumed more protein than matched controls although total energy intake was less in the dancers than the controls (Doyle-Lucas, Akers, & Davy, 2010). Protein intake as a percentage of total energy was 17% in the dancers compared with 16% in the control group. It is unknown if this different was statistically significant. Although energy intake is often less in dancers than non-dancing groups, protein intake may be adequate (Yannakoulia, Keramopoulos, & Matalas, 2004).

2.5.1 Protein

The recommended daily intake of protein for a female 13–18 years old is 35-45g per day and the upper limit of protein intake is 25% of total energy intake (Ministry of Health and National Health and Medical Research Council, 2006). Findings from the 08/09 national nutritional survey (Ministry of Health, 2011) show that the mean protein intake for adolescent females aged 15–18 years was 69g per day. Protein intake was a mean of 15.2% of total energy intake (Ministry of Health, 2011).

Epidemiological studies have shown that inadequate protein intake is associated with low bone mineral density (Hannan et al., 2000). Protein intake is positively associated with the accrual of bone mineral content (BMC) in adolescents and the elderly (Alexy, Remer, Manz, Neu, & Schoenau, 2005). During puberty, adequate dietary protein intake is critical for growth and maintenance of bone and protein has a direct effect on insulin-like growth factor 1 (IGF-1) (Mann & Truswell, 2007).

Protein has another role in bone turnover as it stimulates the production of IGF-1 (Rizzoli, Bonjour, & Chevalley, 2007). IGF-1 influences longitudinal bone growth by regulating chondrocytes in the epiphyseal growth plates (Pass, MacRae, Ahmed, & Farquharson, 2009). It does this by stimulating the production of proliferating chondrocytes (Isaksson, Lindahl, Nilsson, & Isgaard, 1987).

Levels of IGF-I increase through the lifecycle to peak during puberty (Rizzoli et al., 2007), in line with peak growth velocity, and gradually decline with age (Adami et al., 2010). There is a strong correlation between protein intake in children and adolescents and levels of IGF-1 (Hoppe et al., 2004; Kerver, Gardiner, Dorgan, Rosen, & Velie, 2010). Caloric restriction or a low dietary protein intake can affect levels of IGF-1 in the body, which may affect growth in adolescence (Hoppe et al., 2004; Smith, Underwood, & Clemmons, 1995).

Many studies have investigated bone response to protein in populations of post-menopausal women. A one-year randomised control trial of caloric restriction in post-menopausal women (Sukumar et al., 2011) found that the high protein intake group (protein intake 24% of total energy intake) had higher levels of IGF-1 compared to the normal protein group (protein intake 18% of total energy intake). The high protein diet during caloric restriction reduced the loss of bone mineral from the radius, lumbar spine, total hip, trabecular BMD and bone mineral content of the tibia.

This confirmed earlier findings by Schurch et al. (1998) who found that protein supplementation in aged patients increased IGF-1 levels and attenuated bone loss after a hip fracture. There has been concern in recent decades that a high protein intake causes an increase in urinary calcium excretion, as some studies have shown (Barzel & Massey, 1998; Fieddy, Wang, KhashayarSakhaee, & Linda Brinkley, 2002; Zhang et al., 2010). However, this evidence is now questionable in the light of recent research (Beasley et al., 2010; Bonjour, Ammann, Chevalley, & Rizzoli, 2001; Rizzoli & Bonjour, 2004).

Furthermore, a cross-sectional study of dietary protein intake and bone mineral density in females, by Beasley and colleagues (2010) examined baseline dietary protein consumption and BMD among 560 females aged 14–40 years. The low protein intake group consisted of those in the lowest tertile, whose protein intake was between 5.7% and 14.3% of energy intake as protein. The group in the middle tertile had a protein intake of between 14.4% and 17.1% of energy intake, and those in the high protein

group had a protein intake of 17.2–27.6% of energy intake. Beasley et al. found that a higher protein intake was associated with a higher BMD in women younger than 30 years. The evidence was a statistically significant increase in BMD at the lumbar spine in 14–19 year olds as examined by Dual-energy X-ray absorptiometry (DXA).

The association of protein and the positive effects on bone were also reported in a meta-analysis by Darling et al. (2009). A selection of 15 cross-sectional studies demonstrated a significantly positive relationship between protein intake and the skeleton as measured by DXA.

2.5.2 Calcium

Calcium intake is positively associated with increases in BMD at radius, lumbar spine and femoral neck (FN) in a large body of evidence (Rizzoli, Bianchi, Garabédian, McKay, & Moreno, 2010; Rizzoli & Bonjour, 2004; Rizzoli et al., 2007), including a meta-analysis in 2008 (Huncharek, Muscat, & Kupelnick), which reported that increases in BMC at total body and lumbar spine were noted when the diet was supplemented with dietary calcium and dairy products.

In New Zealand, the recommended daily intake (RDI) of calcium for females aged 9-13 years is 1100-1300 mg per day, and for females 14-18 years the RDI is 1300 mg per day. The most recent data reporting calcium intakes in adolescents from New Zealand are the 2008/09 New Zealand Adult Nutrition Survey (2011), which reported that females aged 15-18 years had a calcium intake of 724 mg per day, and that 88% of this age group had an inadequate calcium intake. Comparative data of calcium intakes from children from the 2002 National Children's Survey (2003) reported that female children 11-14 years had a prevalence of inadequate intake of calcium of 29.6 %, with a daily calcium intake of 733 mg. Similar results have been seen in international studies (R. L. Bailey et al., 2010).

To investigate the association between calcium intake and BMD, Bonjour and colleagues (1997) completed a double-blind, placebo-controlled study in pubertal girls.

The participants received either food products containing 850 mg of calcium, or, food products without the calcium, for one year. They reported that BMD gains in the intervention group were greatest in those who had a baseline calcium intake below the median of 880 mg per day. However, there was a significant increase in bone mass for the entire intervention group when compared to controls.

A Cochrane review in 2006 (Winzenberg, Shaw, Fryer & Jones, 2006a) of randomised controlled trials supplementing calcium, concluded that they could not recommend calcium supplementation to the public. The inclusion criteria for the review included that the intervention must have supplemented calcium in tablet form combined with food sources. The intervention group was compared with control groups who were given placebo tablets. The review reported a slight increase in BMD in the upper body in the intervention group, but it was considered unlikely that this increase would translate to a reduction in the risk of fracture. Because of this review, prophylactic calcium supplementation could not be recommended in the public.
However, calcium supplementation is still practised in some population groups to reduce the risk of developing osteoporosis (Rizzoli et al., 2013).

As mentioned earlier, dancers and gymnasts are known to have low total energy intake (Rizzoli et al., 2013). This may result in an increased risk of an inadequate of micronutrients as seen by Hassapidou and colleagues (2001), who reported calcium intakes of 878 ± 25 mg per day in a cohort of dancers aged 22.5 ± 2.3 years. This was considered adequate according to the local recommended daily allowance RDA. However, this would be considered inadequate by New Zealand recommendations.

Research by Burckhardt (2011) reported a less than recommended number of portions of dairy products each day for ballet dancers. Their intake of dairy products per day was 1.8 while the recommended number of products was 3-4 per day. Similarly, Sorich (2013) reported inadequate calcium intakes in 9-13 year old ballet dancers, rhythmic gymnasts, artistic gymnasts and non-dancing controls.

In contrast, Yang et al. (2010) reported that calcium intakes in 16-year-old ballet dancers were significantly higher ($P<0.05$) than the non-dancing control group. This was thought to be because of high milk consumption.

2.5.3 Energy intake

The aesthetic nature of sports such as ballet and gymnastics requires a very lean build. To achieve this desired look many dancers resort to calorie restriction. Energy intake of ballet dancers aged 10–13 years was investigated in a three-year longitudinal study (Matthews et al., 2006). Energy intakes ranged between 1700 and 2000 kcal per day, which appears to be low when compared to the New Zealand and Australian recommended energy Intake for females aged 10-13 years with a moderate level of activity (Ministry of Health and National Health and Medical Research Council, 2006). The RDI for this age group was 2000–2400kcal per day. In this same study, mean training hours ranged from 6–10 hours per week, which equates to a moderate to heavy level of physical activity, their energy needs are actually 2500-2900 kcal per day. However, energy requirements are dependent on body size and dancers, who are often small and light, may have less energy requirement compared to the public.

Constantini (2010) described low energy intakes in 15-year-old female ballet dancers who had mean energy intakes between 1600 and 1800 kcal per day, and whose training ranged from a mean of 10–13 hours per week. Others have seen similar energy intakes in adult dancers (Glace, Kremenic, & Liederbach, 2006; Valentino et al., 2001).

The aesthetic requirements of dancing and gymnastics increases the likelihood of disordered eating behaviours (Braisted, Mellin, Gong, & Irwin Jr, 1985). This was evidenced by Thomas et al. (2011) , who described a cohort of 15-year-old ballet dancers with 9.25 years mean years of training. The prevalence of eating disorders in this group was 21% and the prevalence two or more eating disorders was 10%. This compares to other research in the general population of 14-15 year old girls where the prevalence of and eating disorder was 17.9% (Kjelsås, Bjørnstrøm, & Götestam, 2004).

Unfortunately, meeting the aesthetic demands of the sport have serious health implications such as delayed pubertal development (Markou et al., 2004), low bone mineral density (Warren et al., 2002) and menstrual dysfunction (Quintas, Ortega, López-Sobaler, Garrido, & Requejo, 2003).

2.6 Peak bone mass

2.6.1 The achievement of peak bone mass

Peak bone mass can be defined as the total amount of bone present in the skeleton when the skeleton is fully matured (Heaney et al., 2000). During puberty, bone mass accrual accelerated and then slows in late adolescence to plateau somewhere between the end of the second decade (Bonjour, Theintz, Law, Slosman, & Rizzoli, 1994; Heaney et al., 2000). It is important to achieve an adequate BMD during adolescent growth to offset the subsequent loss of bone during the aging process (Boot et al., 2010; Heaney et al., 2000).

Bone mineral accrual is affected by modifiable and non-modifiable factors. Non-modifiable risk factors for bone health are age, gender, genetically inherited or disease related, while modifiable aspects include environmental factors such as dietary intake and physical activity patterns (Tylavsky et al., 2004; Whiting et al., 2004). Lifestyle patterns of diet and exercise begun in childhood often continue throughout life and may determine bone health as an adult (Kalkwarf et al., 2010).

Low vitamin D status in childhood and adolescence is a risk factor for the inadequate development of peak bone mass (PBM) (Georgopoulos et al., 2004). Research has shown that low BMD in childhood may continue into later life, increasing the risk of osteoporosis and fracture (Heaney et al., 2000; Kalkwarf et al., 2010).

Longitudinal studies of bone health in childhood and adolescence help us to understand the determinants of poor bone health in adulthood. A longitudinal study of 1554 girls and boys aged between 6 and 16 years over three years found a statistically significant correlation ($P<0.001$) between baseline BMD as a child and BMD in

adolescence (Kalkwarf et al., 2010). The implication is that if bone mineral density is low in youth, it will continue to be low throughout the life-cycle, increasing risk of osteoporosis and fracture in the aging process (Bachrach, 2005).

The age at which PBM is achieved appears to be variable. This is mainly due to the types of studies, which have contributed evidence to date, many studies of which are cross-sectional.

One such study of 300 women aged 18-32 years, reported that peak BMD at the lumbar spine was achieved at 23 years and peak BMD at the femoral neck was achieved at 18.5 years (Lin et al., 2003).

These findings were supported by evidence in a large longitudinal study: The Saskatchewan Paediatric Bone Mineral Accrual Study (PBMAS) examined BMD by DXA every six months in a cohort of 228 8–15 year olds. They reported that PBM had been achieved seven years post peak height velocity (PHV) in both males and females. Peak height velocity is the period in adolescence when growth is at its maximum rate (Philippaerts et al., 2006).

Peak height velocity has been identified earlier in a longitudinal study as mean PHV of 11.8 years in females and 13.5 years for males (Baxter-Jones, Mirwald, McKay, & Bailey, 2003). The study also observed that female dancers reached PHV at 11.9 years (Matthews et al., 2006). This differs slightly from other research which observed that PHV was achieved at 12.50 ± 0.86 years in females (McKay, Bailey, Mirwald, Davison, & Faulkner, 1998). Peak bone mass at the lumbar spine and total hip was reached at a chronological age of 16.8 years in females (Baxter-Jones et al., 2011). Interestingly, the two bone sites were found to be at their peak mineral density at the same age, in contrast to Henry and colleagues (2004) who concluded that peak mineralisation occurs in the femoral neck before the lumbar spine. This may be explained by stress on the bone in the femoral neck, which affects the modelling. In this study, it was observed that 79% of bone mineral density at the lumbar spine and 90% of femoral neck BMC was accrued by 16 years.

A longitudinal study followed the BMC and BMD of 395 females aged between 10 and 24 years (Sabatier, Guaydier-Souquières, Benmalek, & Marcelli, 1999), who used DXA scans to analyse BMD and BMC at baseline and after a two-year time period. The four years preceding menarche were crucial for bone mineral accrual, as 46% of adult BMC at the LS (L2-L4) was achieved during this period. Up to two years after menarche 85% of BMC of the adult value was accrued (Sabatier et al., 1999).

Studies which have examined the timing of increases in bone mineralisation have observed that the greatest increases occur pre-menarche (Heinonen et al., 2000) or in the early pubertal stages (MacKelvie, Khan, & McKay, 2002).

2.7 Bone mineral measures

Some authors have commented that bone mineral content is the preferred measure for use in growth studies, as BMC factors-in the accrual of bone associated with an increase in bone size (Heaney, 2003). More recently there has been a move to account for growth in some way when measuring changes in bone in pediatrics (Budek, Mark, Michaelsen, & Mølgaard, 2010; Zemel et al., 2011; Zemel et al., 2010). However, Zemel (2013; 2013; 2011; 2010) and others (Budek et al., 2010) have continued to employ both BMC and BMD when assessing bone in children and adolescents.

2.8 Physical activity and bone mineral density including loading

Physical activity plays a central role in the dynamics of BMD. When a mechanical load is placed onto the body during exercise, the body makes changes to withstand this load, according to Wolff's Law (Chamay & Tschantz, 1972). The cross-sectional area of the muscle increases, pulling on the bone at the attachment points which stresses the bone, and mineralisation is increased to make the bone stronger (Daly, Stenevi-Lundgren, Linden, & Karlsson, 2008).

Exercise exerts a variety of forces onto bone, depending on what sort of forces are applied. Bone mineralisation occurs accordingly as the forces applied cause deposition

of minerals to strengthen the bone (Daly et al., 2008). Identifying which exercise causes beneficial responses in bone mineralisation is a significant step towards being able to make recommendations to reduce the risk of fracture and osteoporosis.

In the early stages of osteoporosis research, many studies were conducted in post-menopausal women with exercise interventions (Bravo & Gauthier, 1997; Wolff, Van Croonenborg, Kemper, Kostense, & Twisk, 1999) because this was the population group most affected by osteoporosis. It was observed that exercise interventions in this cohort could increase bone mineralisation and reduce fracture risk, even in the very elderly (Clemson et al., 2012; Kemmler, von Stengel, Engelke, Haberle, & Kalender, 2010). A body of evidence established the link between bone mineralisation and physical activity in older-aged women; these outcomes were then tested in younger age groups to see if outcomes were similar.

Winters-Stone (2006) and colleagues ran a physical activity intervention consisting of upper body and lower body exercises in a group of pre-menopausal women. They demonstrated that women who exercised the lower body only increased bone mineralisation at the hip, while women who added upper body resistance training increased bone mineralisation of both the hip and spine. These changes to bone mineralisation are desirable as they improve bone health in both the short and long term (Karinkanta et al., 2009; Kelley, 1998).

These findings were applied to youth studies to positively affect bone health earlier in the lifecycle to prevent osteoporosis (Henderson, White, & Eisman, 1998). The effect of exercise on the BMD of young people was first investigated in the late 1980s, and observations of the effect of physical activity on the mineralisation of bone was first reported in the 1990s (McCulloch, Bailey, Houston, & Dodd, 1990).

These early studies were followed by a succession of others confirming the hypothesis that exercise increased BMD in youth (Fuchs, Bauer, & Snow, 2001; Heinonen et al., 2000; Morris, Naughton, Gibbs, Carlson, & Wark, 1997). Increases in BMD through

physical activity in childhood lasted into adolescence and young adulthood, which was understood to increase the level of peak bone mass achieved and reduce the risk of osteoporosis in later years (Harvey et al., 2012; Rizzoli, Bianchi, et al., 2010).

A number of studies have shown that physical activity interventions increased BMD significantly in girls in the early pubertal stage (Detter, Rosengren, Dencker, Nilsson, & Karlsson, 2013; Lehtonen-Veromaa et al., 2000; Valtueña et al., 2012). To ascertain which types of physical activity achieved the maximum benefit to bone health, Völgyi and colleagues (2010) examined the different intensity and volume of physical activity in childhood and observed the effect on bone mineral content and BMD in adulthood. Their prospective study of seven years involved more than 200 girls aged 10-12 years at baseline. They reported that girls who exercised consistently throughout the adolescent period had a significant increase in bone accretion in total body, total femur and lumbar spine as assessed by DXA at aged 18 years. This illustrates that exercise in childhood improves bone health as an adult, a valuable finding to add to the knowledge of ways to reduce osteoporosis.

Another large longitudinal study (Detter et al., 2013) of 362 girls in a controlled physical activity intervention group and 780 girls in a control group, had their BMD assessed yearly for five years beginning at aged 6–9 years. The intervention group received 40 minutes of physical education each school day, while the control group received 60 minutes each week. The intervention group had increased bone mineralisation at femoral neck, spine BMD and tibial BMC at endpoint. The physical activity improved their BMD over this time; it would be even more useful to know how the BMD of the physical activity group was affected in later life.

A similar study with similar results was conducted by Meyer and colleagues (2013) who assessed BMC and BMD three years after an intervention of daily physical activity in 297 children (aged 6 -10 years). Comparisons in bone measures were made between the intervention group and the control group of 205 children of the same age. The assessment by DXA showed the physical activity intervention group had significantly

greater BMC for total body, femoral neck and hip and were independent of pubertal stage.

This study demonstrates that increases in BMD through exercise will persist for at least three years after the exercise intervention concluded. The limitation of this study is that after the study was finished physical activity may have continued, which may have influenced increases in BMD. The question surrounding the positive effects on bone health in later life is left unanswered by this piece of research.

A meta-analysis (Nikander et al., 2010) , which examined the effects of exercise on BMD in children, adolescents and pre-menopausal women, examined RCTs from 2001-2008. The analysis highlighted the variation between study size, exercise duration and intensity and sample size. They were unable to draw a definitive conclusion about the effect of exercise on BMD, and the study highlighted the need for quality, well-powered RCTs to enable robust conclusions to be drawn. Much of the research in this area has employed different study designs, different interventions and different measurement techniques. There is still a need for studies using standard methodology and analysis methods so that consensus statements can be made from pooled data.

To make public health recommendations about the type of exercise which best benefits bone health, the duration, intensity and timing and type of activity that will affect BMD must be pinpointed; this is yet to be clearly identified (C. A. Bailey & Brooke-Wavell, 2008). Competitive dancers and gymnasts tend to spend many hours training each week; the effect of this training on BMD is of interest as it helps to define the site-specific effects of exercise on bone health. Dancing and gymnastics tend to attract young females; this is an ideal cohort to observe the effects of exercise on BMD both pre and post puberty. A meta-analysis by Burt et al. (2013) showed that pre-pubertal gymnasts had greater BMD in the lumbar spine and lower body and greater BMC in the upper body, as assessed by DXA than non-gymnasts. A limitation of this analysis was that many of the included studies did not take into account the anthropometric differences between gymnasts and controls, as gymnasts were often

smaller and lighter. Nutritional, social and ethnic differences were also not included in the data analysis.

The positive effects of gymnastics on bone health have been observed 24 years after retirement from competitive gymnastics (Pollock, Laing, Modlesky, O'Connor, & Lewis, 2006). Former artistic gymnasts had significantly greater BMD than control groups at the LS, hip and total body, while energy intake, physical activity levels and fat mass were not significantly different between groups. The implication is that bone health in adulthood can be improved significantly by high impact loading as a young person. There is evidence to support that increased BMD in youth reduces the risk of osteoporosis in old age. Greater accrual of bone mineral at peak bone mass reduces the risk of bone mineral dropping to a critical level in old age (Heaney et al., 2000).

Ballet dancers as a group have also provided relevant information about the effects of bone mineralisation from exercise. A longitudinal study of 82 dancers and 61 controls between the ages of 9-14 years were examined over three years (Matthews et al., 2006). Bone mineral content was assessed biannually at the hip and lumbar spine by DXA. The dancers had significantly greater BMC for total body, hip and lumbar spine than the controls. The differences in bone health were apparent pre-puberty and were maintained in the pubertal years.

Similarly, adolescent Chinese dancers aged 15-17 years had greater BMD and BMC of total body and hip when compared to controls (Yang et al., 2010). In contrast, Doyle-Lucas and colleagues (2010) observed lower BMD in elite ballet dancers mean age of 23 years when compared to matched controls. This may have been due to their restricted energy intake, which was significantly less than the controls. Some studies have seen improved bone health in spite of a low energy intake (Yang et al., 2010), which may be due to protein intake, calcium intake and vitamin D status. A three-year study of dancers and bone health observed that the age of PHV was similar between dancers and controls (11.9 -11.6 years), respectively. Differences in BMC were observed between dancers and controls at one year post-PHV, which was estimated to

be in the peri-pubertal period, aged 11-14 years. Studies with gymnasts have also reported increases in BMC accrual in gymnasts compared with controls (Recall & Trochanter, 2002). The timing of PHV and bone accrual is consistent with other studies in healthy young females (Heinonen et al., 2000; MacKelvie et al., 2002).

An earlier retrospective study (Khan et al., 1998), which investigated the link between ballet and bone health in later life (mean age 51 years), found that ballet classes taken at age 10–12 years was associated with a significantly greater BMD at the hip when compared with the control group (Yang et al., 2010). In a slightly older cohort of dancers, aged 18-25 years, BMD was also greater in dancers than in the controls (Friesen et al., 2011).

In a study by Young et al. (1994), the benefit of physical activity was seen in the femoral neck of the dancers who achieved a BMD similar to the non-dancing control group. The effect of physical activity had ameliorated the effect of hypogonadism on weight-bearing sites such as the femoral neck, but the lumbar spine was similar in BMD to the girls who had anorexia nervosa and were oestrogen deficient. The definition of hypogonadism is the disruption of the hypothalamic-pituitary-ovarian axis which usually results in a deficiency of oestrogen and irregular menstrual cycles (Rothman & Wierman, 2008).

Ackerman and colleagues (2012) reported similar findings in an investigation into the differences in BMD between female adolescents ranging from 14-21 years. The amenorrheic athletes had significantly lower BMD of the lumbar spine, total hip and femoral neck ($P<0.05$). They concluded that amenorrhea and associated oestrogen deficiency reduces the effect of weight-bearing exercise in adolescent athletes.

2.9 Sex hormones and bone mineralisation

The sex steroids responsible for the changes that occur in puberty are the androgens, mainly testosterone, and oestrogens such as oestradiol. The main determinants of bone mass accrual during puberty are the sex steroids, growth hormone (GH) and

insulin-like growth factor (IGF-1) (Havens et al., 2012). The mechanism by which pubertal changes occur begins with pulsatile secretions of the gonadotropin-releasing hormone (GnRH) from the hypothalamus. This hormone stimulates the production of gonadotropins from the hypothalamus, which in turn stimulates the synthesis of sex steroids and growth factors (Thackray, Mellon, & Coss, 2010). This sequence of events sets in motion the changes that occur in females during puberty, including the deposition of adipose tissue, breast development and the onset of menarche.

Insulin-like growth factor (IGF-1) is known to increase mineralisation of bone by recruiting osteoblast cells (Yakar et al., 2002), and there is a known link between BMD and levels of IGF-1 (Langlois et al., 1998). Although deficiency of GH in childhood causes short limb bones, the bones are adequately mineralised (Högler & Shaw, 2010), which challenges the common perception that GH deficiency causes low BMD, fractures and osteoporosis. The factors that influence the production of IGF-1 in humans and effects on bone are presented in Figure 3 below.

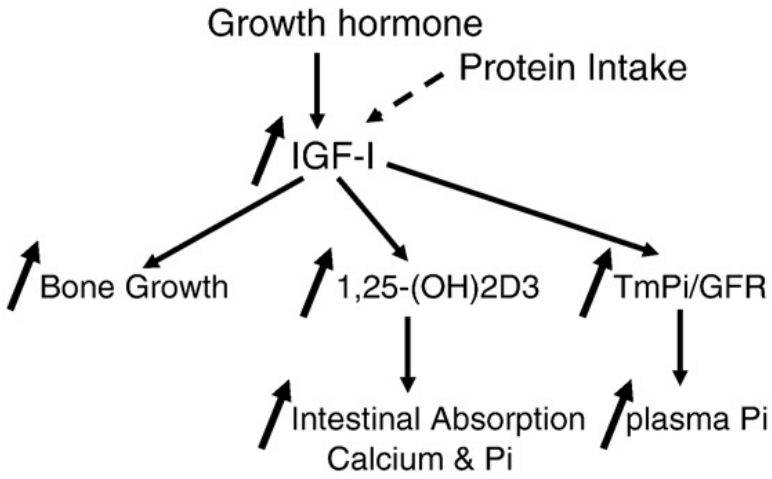


Figure 3. Dietary protein intakes, IGF-I, and calcium-phosphate homeostasis. IGF-I influences bone growth, bone mass accumulation and mineral homeostasis (Rizzoli, 2007)(used with permission).

A low dietary energy intake suppresses IGF-1, which in turn represses the accretion of bone (Fontana, Weiss, Villareal, Klein, & Holloszy, 2008; Grinspoon, Baum, Peterson, & Klibanski, 1995). If dietary energy intake is very low, oestrogen is suppressed.

Oestrogen is stimulates bone formation allowing osteoblast activity and stimulating apoptosis of osteoclasts. Low oestrogen levels increases bone resorption and leads to bone loss in later life (Abrams, Hawthorne, & Chen, 2013; Ferrari et al., 2012; Lips et al., 1988).

Oestrogen levels have been noticeably low in dancers and gymnasts (Weimann, 2002), possibly due to the disruption of the hypothalamic axis as mentioned earlier. Primary amenorrhoea (the absence of menarche at age 15), secondary amenorrhoea (the disappearance of menstrual periods for more than three months), menstrual irregularities and low energy intake are a set of characteristics, which appear together. This set of characteristics is known as the Female Athlete Triad and can be defined as an eating disorder, amenorrhoea and osteoporosis (Rodriguez, DiMarco & Langley, 2009). The Female Athlete Triad, is known to disrupt regulation of the hypothalamus and affect pubertal development (Laughlin, Dominguez, & Yen, 1998). An individual who is affected by the female athlete triad may experience any or all of the clinical conditions associated with the syndrome. The severity of the clinical conditions may be experienced anywhere on the spectrum shown below in Figure 4 (Nattiv et al., 2007) At one end of the spectrum the individual may experience a restricted energy intake with low energy availability, with or without an eating disorder. They may experience menstrual dysfunction due to disruption of the hypothalamic–pituitary–ovarian axis and may have low bone mineral density or be at risk of osteoporosis.

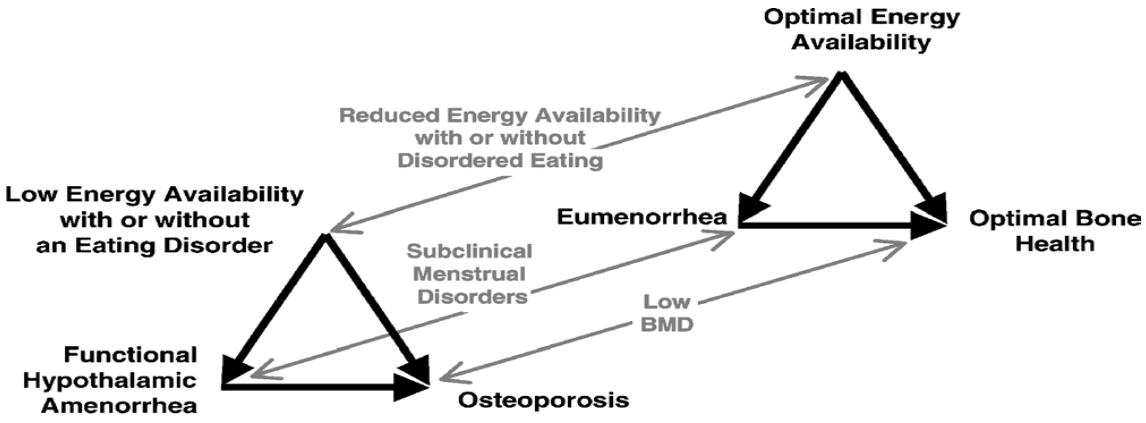


Figure 4. The interaction of energy availability, menstrual dysfunction and bone mineral density noted in The Female Athlete Triad (Nattiv, 2007) (used with permission).

Females who have anorexia may have physical and psychological similarities to dancers and gymnasts. Anorexia nervosa is a multi-dimensional condition which usually involves severely restricted calorie intake, a very low body weight and amenorrhoea (Garner, Olmstead, & Polivy, 1983). This group have been compared to dancers in gymnasts in some studies due to their similarities and to control for their exercise demands (Garner et al., 1983).

The focus of one study was to identify the different effects of physical activity and low oestrogen on bone sites in adolescent dancers. Young and colleagues (Young et al., 1994) observed normal or elevated BMD at weight-bearing sites, including the femoral neck, in ballet dancers aged 17 years, who had regular menstrual cycles. In contrast, the same group had significantly reduced BMD at non-weight-bearing sites, including the arms, ribs and skull. When compared with a control group of girls with anorexia nervosa and an age-matched group of girls with regular menstrual cycles, the ballet dancers' non-weight-bearing sites, including the lumbar spine, were similar to the group with anorexia nervosa. Fat mass was similar between the dancers and the girls with anorexia nervosa and significantly less than the girls with regular menstrual cycles. The effects on BMD in the ballet dancers were attributed to hypogonadism, excessive leanness and vigorous exercise (Bachrach, Guido, Katzman, Litt, & Marcus, 1990).

Low BMD has also been observed in adolescents with anorexia nervosa (Bachrach et al., 1990) who exhibit similar characteristics of negative energy balance, such as primary or secondary amenorrhea as in the female athlete triad.

A 10-year longitudinal study (Petit et al., 2004) was of 112 participants selected from the general population. The first DXA was completed at age 12 at baseline, and participants were DXA scanned biannually for the first four years and annually thereafter for 10 years. The purpose was to analyse BMD, bone width and cortical thickness and the bone cross-sectional area. Femoral neck, shaft cortical thickness and

BMD were predicted by levels of oestradiol and by sports score/lean mass. Conclusions were that bone strength changes in relation to mechanical loading and levels of oestradiol are associated with positive changes in BMD. The femoral neck has an exterior composed of cortical bone.

This compact bone is a dense layer of calcium and minerals that supports the weight of the body (Henry et al., 2004) . Bone at weight-bearing sites, such as the femoral neck, responds positively to physical activity by increased BMD (Lu, Cowell, Lloyd-Jones, Briody, & Howman-Giles, 1996). Trabecular bone is the main bone, which takes the compression forces in the lumbar spine, whereas cortical bone is a thin layer (Boot, de Ridder, Pols, Krenning, & de Muinck Keizer-Schrama, 1997; Van der Sluis, De Ridder, Boot, Krenning, & de Muinck Keizer-Schrama, 2002). The lumbar spine is largely trabecular bone and can be loaded less directly; it appears to be more affected by hypogonadism than the femoral neck (Young et al., 1994).

In the study by Young (1994), the benefit of physical activity was seen in the femoral neck of the dancers who achieved a BMD similar to the non-dancing control group. The effect of physical activity had ameliorated the effect of hypogonadism on weight-bearing sites such as the femoral neck, but the lumbar spine was similar in BMD to the girls who had anorexia nervosa and were oestrogen deficient.

Ackerman (2012) reported similar findings in an investigation into the differences in BMD between female adolescents ranging from 14-21 years. The amenorrheic athletes had significantly lower BMD of the lumbar spine, total hip and femoral neck ($P<0.05$). They concluded that amenorrhea and associated oestrogen deficiency reduces the effect of weight-bearing exercise in adolescent athletes.

Stage of maturation appears to be the strongest influence on bone mineral accrual in the lumbar spine for females (Aloia et al., 2010; Heaney & Weaver, 2003) (Baxter-Jones et al., 2011; Baxter-Jones et al., 2003; McKay et al., 1998; Slemenda et al., 1994). This is thought to be due to the effects of oestrogen, while BMD at the femoral neck may be influenced by load (Khan et al., 1998).

2.9.1 Oligomenorrhoea

Oligomenorrhoea can be defined as irregular menstrual periods, and has been described as nine or fewer menstrual periods over 12 months (Birmingham, 2004; Thein-Nissenbaum & Carr, 2011). Menstrual irregularity following menarche may be erratic and anovulatory. Oligomenorroea may persist for up to 3 years following menarche (Van Hooff et al., 1998). This has been reported in 2.5% of adolescents in the six months post menarche (Chiazze et al., 1968).

Secondary amenorrhoea is the absence of menstruation, post-menarche, lasting three months (Birmingham, 2004). A delay in menarche past 15 years due to the late commencement of puberty is known as primary amenorrhoea (Barrack et al., 2008; Birmingham, 2004).

2.9.2 Gynaecological age (GA)

Gynaecologic age (GA) can be defined as the difference between chronologic age and menarchal age. It is the reference criterion for biological maturity (Stevens-Simon et al., 1986). It can be useful to determine biological maturity in the absence data from a Tanner Maturity Rating scale (Klein & Litt, 1981).

2.10 Characteristics of ballet dancers and gymnasts

Dancers and gymnasts are at increased risk of inadequate calorie and nutrient intakes which may negate the positive effects of exercise on bone health (Kuennen, 2007; Valentino et al., 2001; Yannakoulia et al., 2004).

One observational study compared nine rhythmic gymnasts, 12 ballet dancers and 14 controls, all aged 16 years (Munoz et al., 2004). Their weight and height, menstrual history, nutritional intake, bone age and BMD were assessed by DXA at lumbar spine, hip and radius. The dancers and gymnasts were training at least 20 hours a week while the controls exercised less than three hours each week. The study found a statistically

significant difference in the mean age at menarche between the groups: mean ± SD of 15 ± 0.9 years in the rhythmic gymnasts and 13.7 ± 1 year in the ballet dancers, compared with 12.5 ± 1 year in the controls. Interestingly, BMD in the hip was significantly greater in the rhythmic gymnasts compared to the dancers and controls, despite their later age in menarche. This may be due to increased bone loading in the gymnasts, as it cannot be explained by differences in age, weight, height, bone age or calcium intake, as there was no significant difference between the groups. Energy intake was 1828 ± 500 kcal per day for the gymnasts and 1946 ± 639 kcal per day for the dancers; this is vastly inadequate for the volume of training these girls were undertaking each week. This may have created a negative energy balance and the girls may have been losing weight.

In a similar observational study (Klentrou & Plyley, 2003), 45 elite rhythmic gymnasts, aged around 14 years, were compared with 68 controls of a similar age who were not athletes. The mean age of menarche for the gymnasts was significantly delayed compared to that of the controls. Oligomenorrhoea and amenorrhoea were reported in 61% and 17% of the 2 groups respectively. The gymnasts also had lower body fat, BMI and weight.

The same physical characteristics have been reported in a number of studies in young ballet dancers and gymnasts (Burckhardt et al., 2011; Doyle-Lucas et al., 2010; Yang et al., 2010).

The height and weight of elite ballet dancers are significantly less than dancers of other types or control groups (León, Viramontes, García, & Sánchez, 2009; Yang et al., 2010) and they make up a more homogenous group compared to groups of other dancers (León et al., 2009). The age of menarche in dancers is typically later (14 ± 0.9 years) than the control groups (13 ± 1.3) years), and this is thought to increase the risk of osteoporosis due to reduced BMD in the lumbar spine (Keay, Fogelman, & Blake, 1997). The lumbar spine is influenced by oestrogen levels, as observed by Takahashi (1996), who noted that girls who had begun menarche by 12 years of age had greater lumbar spine BMD than those had not begun menarche at 14 years. A protective effect

on BMD has been observed in dancers (Keay et al., 1997), thought to be due to the high intensity weight-bearing exercise (Keay et al., 1997; Yang et al., 2010).

The association between physical activity, 25(OH)D levels and BMD was examined by Constantini and colleagues (2010) with a group of 166 female adolescent ballet dancers who danced more than 15 hours per week and a control group of adolescents. Vitamin D insufficiency was defined as a 25(OH)D serum level below 75 nmol/L whereas vitamin D deficiency was defined as being below 37 nmol/L (Holick & Chen, 2008). All participants were vitamin D-insufficient and 64% were deficient. There was a greater turnover of bone biomarkers in those who had lower vitamin D levels. A positive association was found between BMD and physical activity in the group who were vitamin D deficient. Bone mineral density increased in those who were the most physically active, it was suggested that physical activity may offset the negative effects which deficiency in vitamin D is known to have on bone health.

2.11 Dancers, gymnasts and diet

Nutrition has a central role in pubertal growth and insufficient nutrients can have a significant effect on growth and bone mineralisation (Steinberg et al., 2008; Styne, 2004). Adolescence is a time of peak height velocity, when the demand for nutrients is high to meet the requirements for growth. Their intake must cover their basal metabolic processes and support the demands of intense exercise (Nancy R Rodriguez, Nancy M DiMarco, & Susie Langley, 2009; Sureira, Amancio, & Braga, 2012)

Female adolescent athletes are particularly at risk of pressure to conform as the pubertal years are when adipose tissue is laid down (Kaplowitz, 2008). Krentz and Warschburger (2011) reported a greater body dissatisfaction among adolescents who participate in aesthetic sports at an elite level than those who play ball sports.

An investigation by Schaal et al. (2011) reported that the highest prevalence of eating disorders were seen in endurance and aesthetic sports. In contrast, Martinsen (2010) observed a higher prevalence of disordered eating among ‘controls’ than adolescent athletes. This was thought to be due to under-reporting in the athletes, as the

questionnaires were self-reported. Under-reporting has been observed in dancers by other researchers (Dahlström, Jansson, Nordevang, & Kaijsery, 1990).

A discrepancy between energy intake and expenditure was noted in one study (Hassapidou & Manstrantoni, 2001) which examined the weighed food intake of adult elite ballet dancers for seven days and compared this with energy expenditure. They observed that for most of the time energy balance was not achieved. This may have been affected by methodological issues.

Low energy intakes have also been noted in gymnasts, one study (Weimann, 2002) of elite teenage gymnasts (22 girls mean ± SD age 13.6 ± 1 years) reported very low energy intakes and an average intake of vitamins and minerals less than 50% of the recommended intake for vitamin A, vitamin D, iodine and carbohydrates (Weimann, 2002). In contrast these results were not seen by Soric and colleagues (2008) in an investigation of the dietary intakes of 39 female gymnasts and ballet dancers aged 9-13 years. Their research found that the energy intakes of the athletes were not significantly different from the control group.

2.12 Vitamin D status of ballet dancers

A recent study (Wolman et al., 2013) investigated serum 25-hydroxyvitamin D levels and bone turnover markers in 19 elite ballet dancers aged 26 ± 8.86 years over a six-month period. The group was an international touring ballet company. Measurements were timed to occur in winter and summer, and all data was collected at a latitude of 52.29°N. Wolman and colleagues (2013) observed a significant difference between serum 25(OH)D levels in winter and summer. Vitamin D insufficiency was defined as a serum 25(OH)D levels of 25-75 nmol/L and deficiency as a level below 25 nmol/L (Chen et al., 2007; Lovell, 2008). In winter, all 19 dancers had 25(OH)D levels that were either insufficient or deficient, with levels ranging from 37.9 ± 7.59 nmol/L to 40.8 ± 11.09 nmol/L. In summer, the serum 25(OH) D levels had increased so that three dancers had adequate 25(OH)D levels of >75 nmol/L, 14 remained insufficient and two were still deficient. PTH levels were also measured and noted to be significantly higher in winter

than the summer. The authors reported an inverse relationship between serum vitamin D and serum hormone parathyroid levels (Saggese, Baroncelli, & Bertelloni, 2002).

2.13 Sex steroids and issues in this population

Adolescent dancers and gymnasts often have delayed menarche and menstrual irregularities which result from deficiencies in oestrogen (Bacchi et al, 2013;Roupas & Georgopoulos, 2011). Intense training is thought to have an effect on oestrogen levels, particularly when this is combined with strict body weight control in some aesthetic sports. Intense training in females is associated with hypothalamic dysfunction (Klentrou & Plyley, 2003).

A mounting body of evidence over the past decade points to inadequate dietary energy intake as the cause of the disruption in the hypothalamic-pituatary-ovarian axis (Warren & Perlroth, 2001). The athlete is in a continued state of negative energy balance evidenced by ongoing weightloss common in sports which encourage a lean body type. Low energy intakes and deficiency of sex steroids may limit bone accretion and increase bone resorption (Bachrach, 1997; Zanker, Cooke, Truscott, Oldroyd, & Jacobs, 2004) which may affect long-term bone health.

A fascinating study by Ihle & Loucks (2004) which determined the dose-response relationship between energy availibility and bone turnover in healthy young eumenorhhoeic females, reported that when energy availability was severely restricted BMD resorption and accretion were uncoupled. As energy intake decreased hormonal function was affected. Lutenising hormone function was disrupted at 30 kcal per kg lean body mass per day. Oestradiol concentrations were not affected until the energy restrictions were as low as 10 kcal per kg per LBM per day. At this level the LH disruption was so severe that it repressed ovarian function (Ihle & Loucks, 2004).

2.14 Summary

Bone metabolism is a complex process involving many external and internal factors. Vitamin D is central to bone metabolism, and the effect of insufficient vitamin D status has a marked effect on bone health. Population groups who are at risk of low vitamin D status are at increased risk of poor bone health. The effect of low vitamin D on bone health in childhood and adolescence has not yet been clearly established. This research aims to present observations of the effects on bone health of a vitamin D intervention in a unique population group of adolescent dancers and gymnasts.

The primary aim of this research was to examine the effects of vitamin D supplementation on the bone health of adolescent ballet dancers and gymnasts in a randomised controlled trial of 12 months.

The objectives were:

- To recruit a cohort of young female dancers and gymnasts aged between 13-18 years, who trained at least 5 hours each week.
- To administer an oral dose of vitamin D_3 (cholecalciferol) 50,000 IU or placebo once each month in a double blind protocol every month for 12 months.
- To measure the total bone mineral density and anthropometry at baseline and 12 months by DXA; and
- To determine the effects of vitamin D supplementation on bone whilst controlling for physical activity and calcium intake.

3.0 Methods

3.1 Primary Aim

To examine the effects vitamin D supplementation on the bone health of adolescent female ballet dancers and gymnasts.

3.2 Ethical Approval

Ethics approval was granted for this study by Northern Y Regional Ethics Committee, Health and Disability Ethics Committees (HDEC), NTY/12/02/013 and each participant gave written informed consent for participation in the study. The clinical trial was registered – Registration No. ACTRN1261000031864.

3.3 Study Design

The study design was a 12-month randomised double- blind, placebo-controlled, parallel supplementation trial. The primary outcome measures were changes in bone mineral density at 12 months.

The study consisted of four phases, illustrated in Figure 5 below.

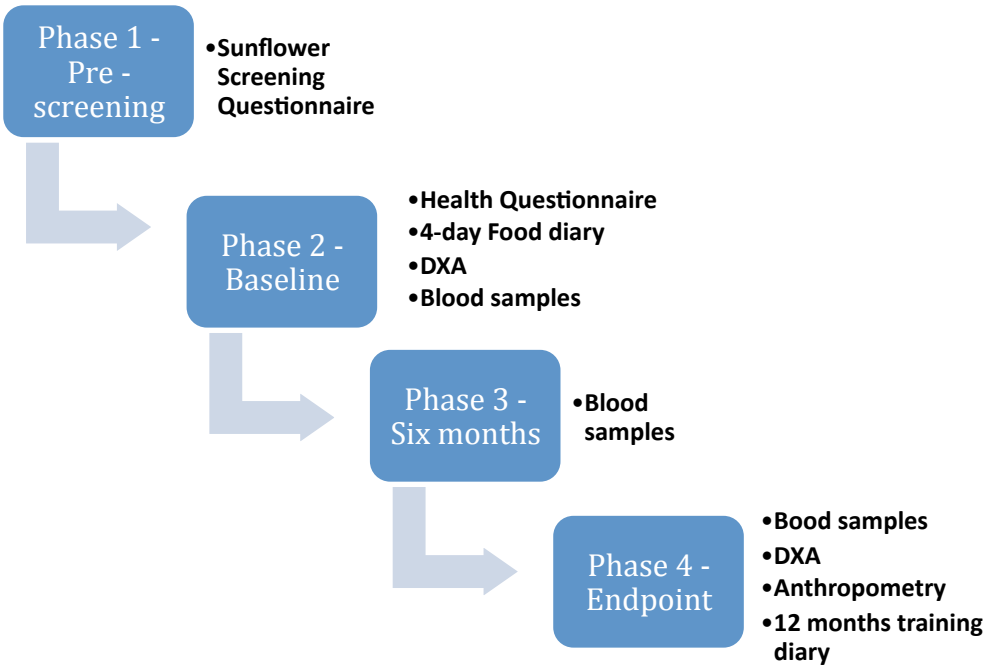


Figure 5. Diagrammatic representation of the study design.

3.4 Subject Recruitment

Participants were healthy females who were dancers or gymnasts, skaters or swimmers aged between 12-18 years. They were recruited from dance schools and through personal contacts. There were media releases in local newspapers and within the Massey University News.

Study poster and Information sheet was placed in dance schools and gymnasiums. The information sheet included a link to the Survey Monkey Screening Questionnaire online, which any interested party could complete. Participants were excluded if they had significant medical health issues or an injury, which prevented on-going training. Participants who were receiving vitamin D supplements greater than 1000 IU per day (equivalent to a prescription dose) were also excluded.

The inclusion criteria for sport were that the individual must have been participating in a minimum of 5 hours of physical training each week. Initially acceptable sports were ballet and gymnastics, both artistic and rhythmic. However, due to difficulties recruiting sufficient numbers to this study, the selection criterion for sporting code was relaxed. The revised criteria allowed girls who were training indoors for more than 5 hours each week to be included. Preference was given to ice skaters, swimmers and gymnasts who all have a demanding training regime. The justification for accepting participants from other sporting codes was the indoor training because of the increased likelihood of low vitamin D status.

The recruitment phase of this study ran between April 2012 and September 2012. The participants were recalled for Phase 3, six months from the date of their Phase two appointment. They were recalled for the fourth and final phase 12 months after their Phase 2 baseline appointment.

3.5 Screening

The first phase was an initial Sunflower Screening Questionnaire, completed online by girls who were interested in volunteering for the study. This screening questionnaire

was an assessment tool to evaluate potential eligibility for participation in the study. The questionnaire enquired about supplement use, general health, chosen sport and training type and volume, see Appendix A for a copy of the Screening Questionnaire.

For Phase 2, eligible volunteers reported to Massey University's Albany campus at a convenient time, they were randomised by age into the intervention or control group, to receive either placebo or vitamin D supplement. Subjects were age-matched for randomisation to ensure that the intervention and control groups had a similar range between groups. Adolescence is a time of growth and change in bone mass and mineralisation and age is an important predictor of bone mass. To prevent bias randomisation was conducted by a researcher who was independent of the study.

3.6 Procedures

Each participant underwent the following procedures:

1. Participants signed a consent form to participate, for those who were aged less than 16 years, consent to participate were given by a parent. Anthropometric measurements: height and weight,
2. DXA scan,
3. An online Health Questionnaire – (refer to appendix A)
4. A venous serum sample
5. Consultation with researcher to provide instructions how to fill out a four-day food diary.
6. Finally, a bottle of cholecalciferol D_3 or placebo was provided with instructions to take one tablet (50,000 IU) each month for 6 months.

At six months (phase 3), a blood sample was taken, and subjects were provided with a new bottle of cholecalciferol D3 and compliance for the first 6 months was checked. The serum biomarkers were collected at six months to check differences between groups and to monitor any seasonal variation in serum vitamin D status over time.

At 12 months (phase 4) as per phase 2, anthropometric, DXA measurements and a venous blood sample was collected.

3.7 Funding

This study was funded by a Massey University Institute of Food, Nutrition and Human Health research grant. Massey University also provided additional funding through a research grant.

3.8 Vitamin D supplementation protocol

The vitamin D_3 supplement was Cal-D-Forte, (calciferol). The active property of Cal-D-Fort was cholecalciferol, a vitamin D compound that possesses the property of preventing or treating rickets. The Cal-D-Fort supplement was chosen because it was a vitamin D_3 form. Some other studies have indicated that supplementation of vitamin D_2 may suppress endogenously formed $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ (Wharton & Bishop, 2003).

The dose administered was 50,000 international units per month or placebo for one year. The active supplement and placebo were purchased from API Consumer Brands, Manukau City, Auckland

3.9 Compliance

The participants were encouraged to develop their own system to remind them to take their vitamin D supplement at the same day each month. Some chose their birthday date as the day to take the supplement and some set reminders into their phone. A Facebook page was set up and monthly reminders to take supplements were posted on the Facebook site. Additionally, reminder emails were sent to participants to prompt supplement compliance.

3.10 Laboratory measurements

Each participant reported to the Massey University IFNHH lab in a non-fasted state on the day of appointment. Venous blood samples were collected by a trained phlebotomist using a sterile Vacutainer Flashback needle, two vials were taken, one

5ml x EDTA and one 10ml x Serum. The samples were left to stand for 30 minutes to allow clotting; the EDTA tube was kept on ice if it could not be processed after 30 minutes. The samples were then centrifuged in a Lobofuge 400R heraeus (Thermo Scientific, USA) centrifuge @ 3500 RPM for 10 minutes at 4°C.
Aliquots from serum and plasma were stored in a -80°C freezer until they were sent for analysis.

All samples were sent for analysis to Waitemata District Health Board Laboratory, Shakespeare, Rd, Takapuna, New Zealand. Analysis of baseline and six months samples were analysed at six months and samples taken at the endpoint were stored until the last sample was collected in September 2013 and then sent for analysis.
Serum 25(OH)D was assessed by Siemans Healthcare Diagnostics (Siemens Australia New Zealand) employing a proprietary monoclonal with minimal 1.1% 3-epi-25(OH) vitamin D_3 cross-reactivity. The precision was a CV of 4.2%-11.9%.
Calcium and albumin were assessed by a Flex reagent cartridge system by Siemens Healthcare Diagnostics (Siemens Australia New Zealand) with a CV of 2.2-3%. Oestradiol and intact PTH were assessed in the laboratory by an ADVIA Centaur® Healthcare Diagnostics (Siemens Australia New Zealand) XP Immunoassay System, the CV ranges from 5-11% for intact PTH and from 6-28 % for oestradiol.

3.11 Bone measures

Each participant underwent three DXA scans to determine bone mineral density and content and body composition. The DXA scans included a full body scan, where the participant lay in a supine position with palms down and beside the torso. The lumbar spine scan was performed with the participant in the supine position with a 50cm foam box placed under the knees. This position enabled a clear view of the vertebra L1-L4 see Figure 6.

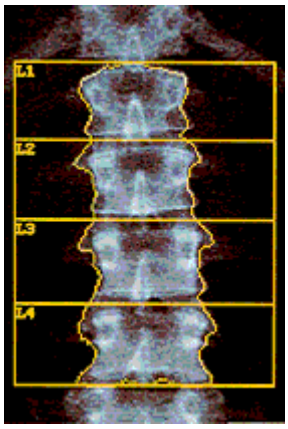


Figure 6. An example of a DXA scan of the lumbar spine, showing the vertebrae L1-L4 (copyright free sourced from Google Advanced Images).

The scan of the femoral hip was conducted with the participant in the same supine position, with right femur rotated medially and the right food held with a Velcro tab on Perspex box placed between the feet. This position enabled a clear scan of the pelvis and neck of the femur see Figure 7.

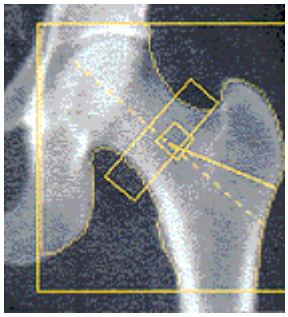


Figure 7. An example of a DXA scan of the hip showing the femoral neck (copyright free, sourced from Google Advanced Images).

Each participant removed metal jewelery or any clothing containing metal prior to the DXA scan. A gown was provided if the participant's outer clothing contained metal elements.

All DXA scans were supervised by a certified DXA technician in accordance with Standard Operating Procedures. The bone mineral density, content, lean mass and fat mass of the whole body was measured by DXA.

Bone mineral density (g/cm^2) and bone mineral content (g) of lumbar spine (LS), total body (TB), femoral neck (FN), total hip (TH) and areal bone mineral density aBMD were

determined by fan beam DXA (Hologic Discovery A; Hologic Inc., Waltham, MA) at baseline and 12 months.

The DXA scan was analysed to find the following variables, see Table 1. The same technician conducted all analyses of bone mineral and body composition.

Table 1. Bone measures analysed by DXA scan at baseline and 12 months

Variable	Abbreviation
Total body bone mineral density	TBBMD
Total body bone mineral content	TBBMC
Areal bone mineral density	aBMD
Bone mineral density of the lumbar spine	BMDLS
Bone mineral content of the lumbar spine	BMCLS
Bone mineral density of the total hip	BMDTH
Bone mineral content of the total hip	BMCTH
Bone mineral density of the femoral neck	BMDFN
Bone mineral content of the femoral neck	BMCFN

Calibration of the measurement was performed using a spine phantom; the interassay CV for the phantom was 0.31%.

No consensus has been formed as to the best measure of bone mineral mass in adolescents, however; both BMD and BMC were measured in this study.

3.12 Anthropometric measurements

Height and weight were measured wearing light indoor clothing and barefoot by trained personnel following standard anthropometric techniques (Ross, 1996). Weight was recorded to the nearest 0.1kg using an electronic scale Tanita THD646 electronic digital scale. Height was recorded to the nearest 0.1cm using a portable S&M 200cm stadiometer. Anthropometric measurements were recorded at baseline and 12 months. Body mass index was calculated by the equation weight (kg)/height (m) squared (kgm^2).

Lean mass and percentage body fat was found by DXA and included bone mineral. The lean mass was then used to find fat free, bone free lean mass (FFBFLM). This was calculated by: lean mass (g) (from DXA) - BMC (from DXA) in grams and was converted to kg.

3.13 Questionnaires

At baseline an online Health Questionnaire was completed by all participants during the first appointment. The Health Questionnaire (see appendix A) asked questions designed to establish menstrual status and frequency, such as age of menarche and regularity of menses and blood loss.

3.14 Dietary survey procedure

At Phase two of the study plan each participant was given a 4-day food food diary to complete, see Appendix C. Detailed instructions how to complete the food record were provided to each participant as well as an instructional DVD. There was an opportunity for each participant to ask questions following the DVD. The participants were asked to record everything they ate or drank over a 4-day period, which included a weekend day. To estimate portion sizes a photographic portion guide and household measuring cups and spoons were also provided. A stamped addressed envelope was included with the food diary information, to post back to Massey IFNHH after completion. Dietary supplements were not included in the dietary analysis. The four-day food record was for food sources of nutrients only.

3.15 Analysis of the 4-day estimated food records

Each participant mailed their food diary to Massey University when they were complete. The four-day food diaries were then reviewed for missing information. Any details, which were not clear, were clarified by contact with the participant.

The 4-day food records were analysed by entering into Foodworks Professional diet analysis programme version 7 (Xyris Software (Australia) Pty Ltd, 2012) by a research technician, under the supervision of a New Zealand Registered Dietitian.

3.16 Assessment of physical activity

The participants recorded their volume of training for each day of the week at Survey Monkey https://www.surveymonkey.com. Regular monitoring and reminders were

sent by email, Facebook or text, to the participants to remind them to continue to fill in the weekly training log. At the conclusion of the study, the training logs were collated and statistically analysed.

3.17 Statistical methods

The sample size calculation was done retrospectively and was based on the mean ± SD (0.93 ± 0.12 g/cm^3) BMD of the lumbar spine baseline data in the current sample. It was calculated that a total sample size of 26 participants (13 participants in the control and 13 in the intervention group) would be needed to provide 80% at a significance level of 0.05 to achieve a difference in lumbar spine BMD of 0.093 g/cm^3. The difference of 0.093 g/cm^3 is based on the difference in increase between the vitamin D supplemented and placebo groups. It is expected that there is an increase in both groups over 1 year period due to growth, the difference due to treatment will be 0.093 g/cm^3.

All data were checked for coding errors prior to the commencement of statistical analysis. Data were analysed with SPSS Version (Armonk, NY; IBM Corporation).
The variables were tested for normality using the Shapiro-Wilks and Kolmogorov-Smirnov test and for homogeneity using the Levene's test. Normally distributed data were expressed as mean ± SD for and non-normally distributed data as median, 25th, and 75th percentile. A *P* value of less than 0.05 was considered significant.

The primary outcome measures were the differences in mean change in bone mineral measures between control and intervention groups over 12 months. Mean change from baseline to 12 months in all variables was compared using the Mann-Whitney test for non-parametric data and dependent Student's *t*-test for parametric data.

Comparisons were made at baseline between intervention and control groups for anthropometric, dietary, bone mineral, physical training volume and biochemical variables by independent Student's *t*-test for parametric data and Mann-Whitney test for non-parametric data.

For statistical purposes, the participants were grouped according to age, to allow comparisons between variables in mean change over 12 months. The age groups were group 12-13 year olds, group 14 year olds, group 15 year olds and group 16-18 year olds. The four age groups were compared using two-way ANOVA and a post-hoc Tukey analysis was done to identify where the differences were located.

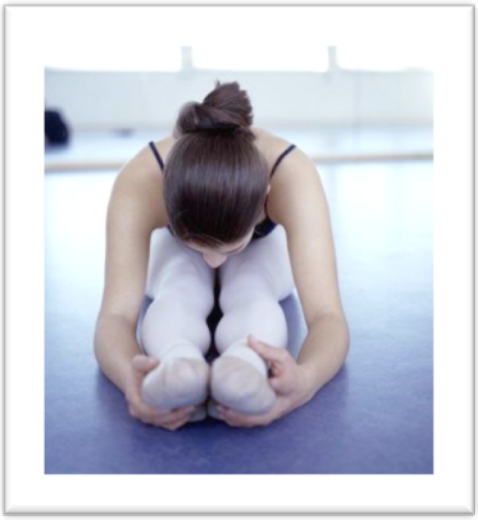
Comparisons were made between mean values for body fat, TBBMD, BMDLS and TBBMC for those who had experienced menarche and those who had not at baseline, by Student's *t*-test. The same test was used to compare mean values for BMDTH, BMDLS and BMCLS at baseline, between those with regular periods and those with irregular periods.

All potentially influencing variables were selected for entry into a multiple regression model to find significant predictors of bone measures at baseline. The regression model holds the effects of other variables constant and the unique contribution of each predictor is calculated. The assumptions for regression analysis were tested using the Kolmogorov-Smirnov test for normality, the residuals were independently tested by the Durbin-Watson test and multi-collinearity was checked. Multiple regression and Pearson's correlations were performed by enter method to find the significant predictors of TBBMD and TBBMC at baseline.

3.18 Data storage and handling

At the end of the study, the list of participants and their study identification number was disposed of. Any raw data on which the results of this project depend will be retained in secure storage for 10 years after participants have turned 16 years of age, after which it will be destroyed. All participants were notified of the project findings at the conclusion of the study.

4.0 Results



4.1 Study participants

4.1.1 Attrition rates

A total of 84 young women completed the online Survey Monkey Screening Questionnaire and were assessed for eligibility for this study, 23 were excluded because they were taking vitamin D supplements, had injuries or suspected stress fractures or medical conditions which prevented their participation, refer to Figure 8.

A total of 61 girls were included into the study and randomised by age into the intervention or placebo group. At six months, 7 of the participants were lost to follow-up, 5 in the intervention group and 2 in the placebo. Three participants stopped training due to injury, 2 in the intervention and 1 in the control group. Two moved overseas, one from each group; one participant stopped taking the supplement and one was too busy to continue, both from the intervention group.

The response rate for this study was 32%. At 12 months, a total of 45 participants were available for the Phase 4 data collection. DXA scans were performed for 45 participants, anthropometry, including height, weight, lean mass, fat mass and BMI was recorded for all of these. Blood samples were given by 41 girls.

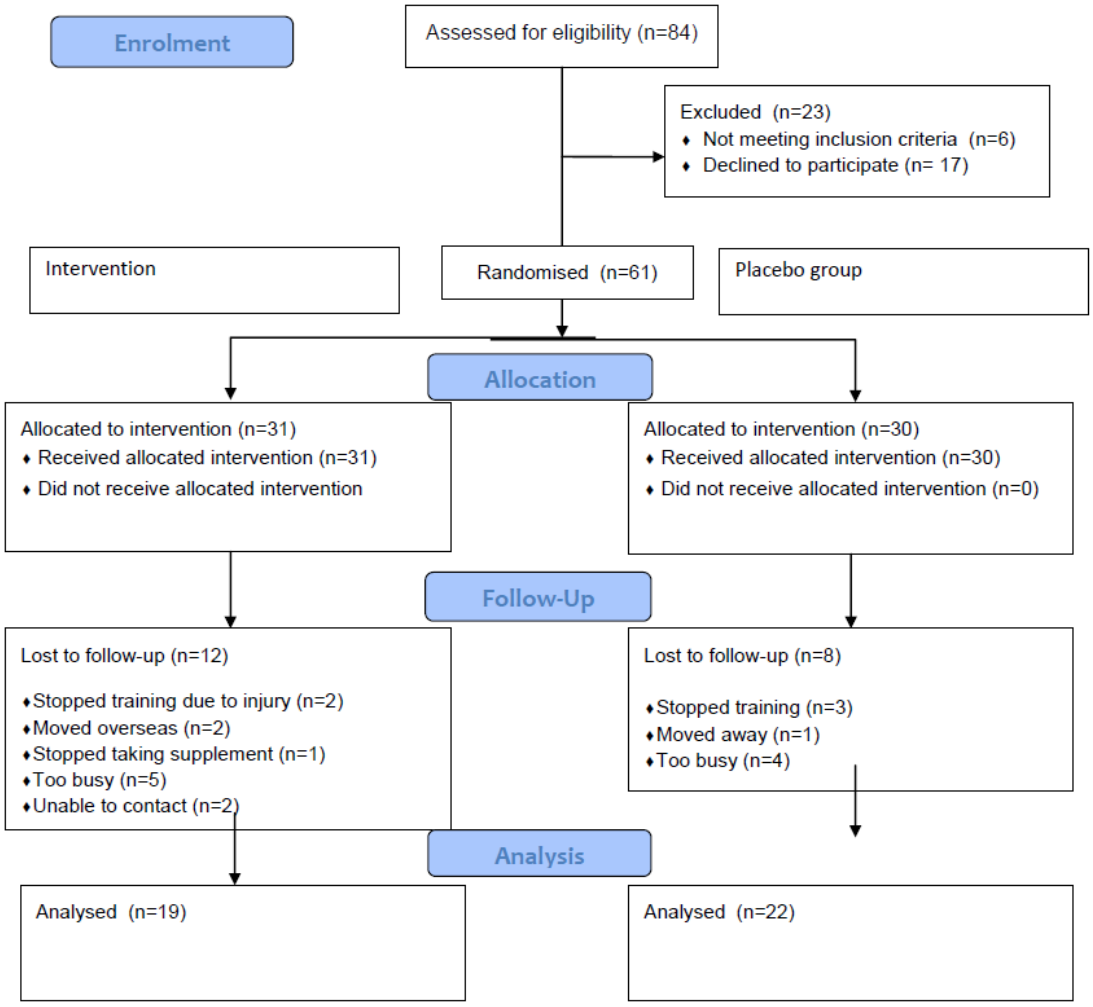


Figure 8. Diagram of selection process from assessment for eligibility to study through to analysis of results.

The ethnicities represented by the cohort at baseline were European 54, Pacific or Maori 2 and Japanese or Chinese 5. At baseline, there were 3 rhythmic gymnasts, 43 ballet dancers, 5 artistic gymnasts, 2 Irish/Highland dancers, 1 ice skater, 3 girls who danced tap/jazz/contemporary and 4 swimmers see Figure 9 for a diagram showing the division of sporting codes within the cohort.

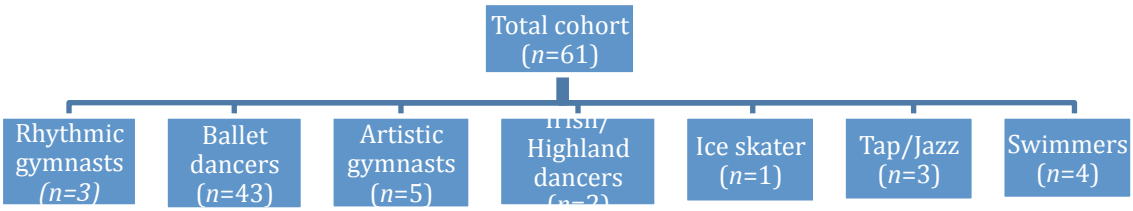


Figure 9. Diagram of participant sporting codes.

4.2 Results Section 1 Baseline Characteristics of participants in the randomised controlled trial

4.2.1 Serum vitamin D and other biomarkers

At baseline, 61 girls presented themselves to the Massey University Institute of Food Nutrition and Human Health for Phase two of the study; all but one girl underwent a DXA scan.

A total of 59 girls agreed to have a venous blood sample taken, one subject requested not to have a sample taken and for one subject no sample could be obtained by the phlebotomist, these participants were both in the control group. The sample size for serum blood samples at baseline were: intervention group (*n*=31) and control group (*n*=28).

At the Phase 3 data collection, venous serum samples were available for 51 girls, 10 girls were lost to follow up. Six of those were in the intervention group and four were in the control group. At Phase 3, the number of participants who provided blood samples in the intervention group (*n*=25) and in the control group (*n*=26). Those lost to follow-up were unavailable due to busy-ness; refusal to undergo blood sampling or the sample was unable to be obtained by the phlebotomist.

At Phase 4 a total of 41 girls were able to provide a serum venous sample. The total number of participants who gave blood samples in the intervention group was (*n*=19) and in the control group (*n*=22). Twenty girls were unable to provide blood samples due to busy-ness, refusal to undergo blood sampling or the phlebotomist could obtain no sample. The participants who were lost to follow up were (*n*= 12) in the intervention group and (*n*=8) in the control group.

All participants who attended the Phase 3 and 4 appointments at Massey University IFNHH laboratory self-reported that they had taken all supplements at the appropriate time.

At baseline there were no significant differences between the control and intervention groups for intact PTH (iPTH), s25(OH)D, oestradiol or calcium adjusted see Table 2.

The mean ± SD serum vitamin D for the total cohort at baseline was 72.9 nmol/L. All of the participants had serum vitamin D status (>25 nmol/L) at baseline. The majority (83%) of the cohort had vitamin D levels that were equal to or above the recommended level (>50nmol/L). There were no participants who had a deficient vitamin D status at baseline (<25 nmol/L). The lowest recorded 25(OH)D value was 36 nmol/L, which was recorded for two participants. There were 9 girls who had serum 25(OH)D concentrations less than recommended (25-49.9 nmol/L) but not deficient. The highest recorded serum vitamin D was 129 nmol/L which falls into the 'high vitamin D levels' according to the Ministry of Health (2012a).

Table 2. Serum blood results at baseline for gymnasts and dancers*.

Variable	Total cohort (n=59) Mean	SD	Intervention group (n=31) Mean	SD	Control group (n=30) Mean	SD	*P* value difference between groups†
Intact Parathyroid (pmol/L)	2.54	1.40	2.53	1.31	2.55	1.48	0.82
25(OH)D (nmol/L)	72.90	19.15	73.87	16.89	71.82	21.64	0.69
	Median	**(25,75)**	**Median**	**(25,75)**	**Median**	**(25,75)**	
Oestradiol (pmol/L)	189	(153,255)	188	(157.75,280)	197	(148.50,251.75)	0.86
Calcium adjusted (nmol/L)	2.3	(2.30,2.40)	2.35	(2.25,2.35)	2.30	(2.30,2.35)	0.86

*Values are reported as mean ± standard deviation or median (25^{th},75^{th} percentiles)

†Significant differences between intervention versus control (*P*<0.05) (Independent Student's *t*-test) .

Abbreviations: pmol/L – picamol per litre; nmol/L – nanomol per litre.

Recommended ranges : for 25(OH)D - deficiency <25 nmol/L, severe deficiency <12.5 nmol/L, mild-moderate deficiency 12.5-24.9 nmol/L, below recommended level but not deficient 25-49.9 nmol/L and equal to or above the recommended level >50 nmol/L. iPTH - 1.5-7.6 pmol/L. Oestradiol – no range specified. Calcium adjusted – 2.1-2.6 mmol/L.

4.2.2 Bone mineral measures at baseline

At Phase 2 a total of 61 DXA scans were performed at baseline.

There were missing data due to a technical difficulty for the hip in two scans, missing data in the spine for three scans and missing data for the total body bone mineral density for one scan. DXA results were not included in the endpoint analysis in the case of the missing scans.

At Phase 4, from the original 60 scans, DXA scans were completed for 49 girls, 12 were unavailable. Bone sites, which did not have baseline values recorded by DXA, were not included in statistical analysis at endpoint.

Table 3 shows all bone mineral measures from analysis of DXA scans at baseline, for the total cohort and the intervention and control groups.

Table 3. Bone mineral measurements at baseline for dancers and gymnasts.

Bone Measurements	Total cohort (*n*=60)	SD	Intervention Group (*n*=31)	SD	Control Group (*n*=29)	SD	*P* value
TBBMD (g/cm^2)	0.98	0.09	0.98	0.07	0.99	0.11	0.40
TBBMC (g)	1820.86	295.65	1793.46	262.88	1836.34	336.65	0.42
aBMD (g)	1845.52	166.45	1834.73	168.24	1856.13	166.75	0.62
BMDLS (g/cm^2)	0.93	0.12	0.91	0.10	0.95	0.15	0.44
BMCLS (g)	49.67	11.54	50.78	10.47	48.55	12.94	0.64
BMDTH (g/cm^2)	0.93	0.12	0.92	0.11	0.94	0.15	0.55
BMCTH (g)	27.76	5.81	26.96	4.78	28.46	6.65	0.28
BMDFN (g/cm^2)	0.88	0.13	0.87	0.11	0.91	0.15	0.85
BMCFN (g)	4.02	0.56	4.02	0.56	4.08	0.71	0.57

*Values are reported as mean ± standard deviation unless otherwise indicated.
†Significant differences between intervention versus control (P<0.05) (Independent Student's t-test) .
Abbreviations: TBBMD-Total body bone mineral density; TBBMC- Total body bone mineral content; aBMD – areal bone mineral density; BMDLS - Bone mineral density lumbar spine; BMCLS - Bone mineral content lumbar spine; BMDTH-Bone mineral density total hip; BMCTH-Bone mineral density total hip; BMCFN- Bone mineral content femoral neck; BMDFN-Bone mineral density femoral neck; g – grams; g/cm^2 – grams per centimetre squared.

4.2.3 Anthropometric characteristics at baseline

Height and weight measures and BMI values were recorded for all participants (n=61) at baseline. Bone free, fat free, lean mass and body fat, are derived from the DXA scan. DXA were recorded for 60 participants, with one subject requesting not to have a scan due to safety concerns.

Baseline anthropometric characteristics of the study population, grouped according to intervention and control groups are presented in Table 4.

There were no significant differences between groups for age or anthropometric measures.

Table 4. Baseline demographic and anthropometric characteristics of ballet dancers and gymnasts*

General characteristics	Total cohort (*n*=61)	SD	Intervention Group (*n*=31)	SD	Control Group (*n*=30)	SD	*P* value†
Age (y)	14.4	1.5	14.4	1.4	14.4	1.5	0.38
Weight (kg)	52.1	6.9	50.7	7.0	53.0	7.0	0.26
Height (m)	1.6	0.1	1.6	0.0	1.6	0.0	0.69
BMI (kg/m^2)	20.0	3.0	19.4	3.4	20.7	3.9	0.38
Body fat (%)	23.4	3.8	19.4	0.6	20.3	0.5	0.50
BFFFLBM (kg)	36.1	4.4	35.6	4.5	36.7	4.4	0.36

Values are reported as mean ± standard deviation
†Significant differences between intervention versus control (P<0.05) (Independent Student's t-test)
Abbreviations: BMI - Body mass index; BFFFLBM – bone-free, fat-free, lean body mass

4.2.4 Analysis of the 4-day estimated food records

Participants returned 52 diaries to Massey University IFNHH for analysis, a response rate of 85 percent.

Statistical analysis revealed there were no significant differences between groups for energy intake, protein intake or calcium intake per day. Energy intake was a mean ± SD of 1956 ± 492 kcal per day, the mean ± SD protein intake was 80 ± 22 g per day and calcium intake per day was a mean 898 ± 329 mg per day. The lowest recorded energy intake was 884 kcal per day. Only 21% of the cohort had an energy intake greater than 2400 kcal per week, which is the recommended energy intake for a moderately active 13 year old. However, the recommended energy intake range does not consider body mass; it is a reference guideline only. The rest of the participants reported energy intakes between 884 kcal per day and 2345 kcal per day.

There were 15% who had an adequate calcium intake of >1300mg per day, but 85% had less than recommended calcium intakes which ranged from 267 mg per day to 1278 mg per day.

Protein intakes were high compared to Ministry of Health (2006) recommendations, of 35-45g/day. Only three girls had less than the recommended daily intake and 63% consumed double or more than the recommended protein intake each day.

4.3 Change in parameters over 12 months – differences between intervention and control groups

4.3.1 Change in vitamin D and other serum markers over 12 months

At baseline there was no significant difference between the control and intervention groups for 25(OH)D. Serum 25(OH)D in the intervention group was a mean ± SD 73.9 ± 16.9 nmol/L and in the control group mean ± 71.8 ± 21.6 nmol/L. At six months the intervention group had increased to a median and range 99 (75.5-115.5) nmol/L and the control group dropped to median and range of 68.5 (57,100). There was a significant difference in the change from baseline to 6 months in s25(OH)D between the control and intervention groups (*P*=0.03).

There was no significant difference within the intervention group from baseline to end point. There was a negative trend in serum 25(OH)D concentration from baseline to endpoint in the control group and a positive upward trend in serum 25(OH)D concentration in the intervention group from baseline to endpoint. There was a non-significant difference in 25(OH)D between groups at end point (*P*=0.05). The change from baseline to endpoint was not significantly different between the two groups. The serum 25(OH)D in the control group and the intervention group remained at a sufficient level from baseline to endpoint (>25 nmol/L) see Table 5.

Graph of the change in serum 25(OH)D for the control and intervention groups is presented in Figure 10 below.

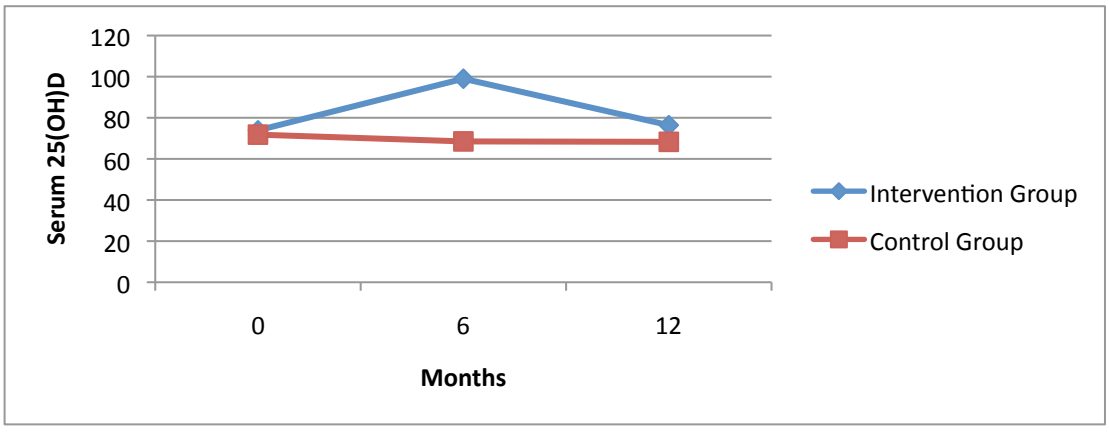


Figure 10. Change in serum 25(OH)D in intervention and control groups over 12 months.

4.3.2 Intact PTH

A t-test of serum intact PTH revealed a significant change from baseline to endpoint in the control group (*P*=0.002), but no significant difference in change between the control and intervention groups, see Table 5.

4.3.3 Oestradiol

There were no significant differences in serum oestradiol from baseline to six months or baseline to endpoint either within groups or between groups, see Table 5.

Table 5. Change in serum measures from baseline, six months and endpoint within and between intervention and control groups *.

Variables	Intervention group Mean or Median	SD or (25,75 quartiles)	*P*-value difference within groups	Control group Mean	SD or (25,75 quartiles)	*P* value difference within groups	*P* value difference between groups
Intact Parathyroid (pmol/L)							
Baseline	2.5	1.3		2.6	1.5		0.82
6 months	2.5	(1.8,3.1)		1.9	(1.3,2.8)		0.08
Endpoint	3.2	1.7		3.4	1.7		0.39
Change 6 months-baseline	-0.4	(-0.8,0.7)	0.73	-0.2	(-2.2,0.4)	0.12	0.53
Change End-Baseline	1.0	2.3	0.13	1.6	1.5	**0.002*‡**	0.85
25(OH)D (nmol/L)							
Baseline	73.9	16.9		71.8	21.6		0.69
6 months	99.0	(75.5,115.5)		68.5	(57.0,100.0)		0.09
Endpoint	76.4	23.0		68.3	23.4		0.05
Change 6 months-baseline	19.0	(-10,47)	**0.03*†**	3.0	(-21.0,16.0)	0.35	**0.02*‡**
Change End-Baseline	0.4	17.3	0.09	-5.4	16.5	0.14	0.27
Oestradiol (pmol/L)							
Baseline	237.6	178.7		223.5	135.3		0.82
6 months	198	(132.5,313.5)		196.5	(116.5,301.5)		0.76
Endpoint	259	18.1		216.2	(-94.0,96)		0.36
Change 6 months-baseline	0	(-113.5,120.5)	0.86	12.5	193.6	0.58	0.85
Change End-Baseline	39.7	241.7	0.52	15.2	277.3	0.52	0.81

*Values are medians (25th, 75th percentiles) and mean and standard deviation as indicated.
. Sample numbers are n=31 and 27 at baseline and n=19 and 23 at 6 months for intervention and control groups respectively.
†Significant differences from baseline to six months (*P* <0.05) (Sign and Wilcoxin Dependent test).
‡Significant differences between intervention *vs.* control (*P* <0.05) (Mann-Whitney Independent test).
§Significant differences in change from baseline to six months (*P* <0.05) (Dependent Student *t*-test).
¶Values are geometric mean (95% CI).
Abbreviations: pmol/L – picamol per litre; nmol/L – nanomol per litre; change at 6 months - 6 months value – baseline value; change at endpoint – endpoint value – baseline value. Recommended ranges : for 25(OH)D - deficiency <25 nmol/L, severe deficiency <12.5 nmol/L, mild-moderate deficiency 12.5-24.9 nmol/L, below recommended level but not deficient 25-49.9 nmol/L and equal to or above the recommended level >50 nmol/L. iPTH - 1.5-7.6 pmol/L. Oestradiol – no range specified. Calcium adjusted – 2.1-2.6 mmol/L.

4.3.4 Change in bone mineral measures over 12 months

No significant differences in bone measures were identified between intervention and control groups at baseline, at endpoint or in change from baseline to endpoint. Growth was observed at all bone sites; bone mineral density, bone mineral content and areal bone mineral density increased in the whole body. There was a significant change in bone mineral content and density at all four bone sites within each group over the 12 months, see Table 6.

Table 6. Change in bone mineral measures from baseline to endpoint within vitamin D and placebo groups, and between groups *.

Bone variable	Intervention Group	SD	*P*-value difference within groups	Group B Baseline	SD	*P*-value difference within groups	*P*-value difference between groups
TBBMD (g/cm^2)							
Baseline	0.98	0.07		0.99	0.11		
Endpoint	0.99	0.06		1.00	0.09		
Change End-Baseline	0.04	0.04	**<0.001****	0.03	0.04	**0.003***	0.33
TBBMC (g)							
Baseline	1793.46	262.88		1836.34	336.65		
Endpoint	1871.59	259.67		1886.59	273.04		
Change End-Baseline	106.25	93.00	**<0.001****	87.88	125.39	**0.003***	0.63
aBMD (g)							
Baseline	1834.70	168.21		1856.98	166.72		
Endpoint	1782.10	186.70		1942.42	152.56		
Change End-Baseline	41.95	67.29	**0.014***	61.08	80.05	**0.001***	0.41
BMDTH (g/cm^2)							
Baseline	0.92	0.10		0.94	0.15		
Endpoint	0.95	0.16		0.96	0.09		
Change End-Baseline	0.05	0.07	**0.005***	0.04	0.06	**0.002***	0.83
BMCTH (g)							
Baseline	29.96	4.78		28.46	6.65		
Endpoint	29.72	4.44		26.97	4.16		
Change End-Baseline	3.28	2.24	**<0.001****	1.95	3.72	**0.02***	0.18
BMDLS (L1–L4) (g/cm^2)							
Baseline	0.91	0.10		0.95	0.15		
Endpoint	0.98	0.09		0.97	0.13		
Change End-Baseline	0.06	0.04	**<0.001****	0.05	0.05	**<0.001****	0.28
BMCLS (L1–L4) (g)							
Baseline	50.78	0.47		48.55	12.94		
Endpoint	55.90	9.56		54.09	11.09		
Change End-Baseline	4.59	6.09	**<0.001****	6.95	7.79	**<0.001****	0.29
BMDFN (g/cm^2)							
Baseline	0.87	0.11		0.91	0.15		
Endpoint	0.89	0.16		0.92	0.13		
Change End-Baseline	0.04	0.08	**0.04***	0.03	0.05	**0.005****	0.82
BMCFN (g)							
Baseline	4.02	0.56		4.08	0.71		
Endpoint	4.09	0.75		4.18	0.64		
Change End-Baseline	0.19	0.40	**0.05***	0.19	0.28	**0.004***	0.97

*Values are mean ± standard deviation unless otherwise indicated.
** Significant differences from baseline to six months (P <0.001) (Dependent Student's *t*-test).
Bold type indicates significant differences. **P* <0.05, ** *P* <0.001,

Abbreviations: TBBMD-Total body bone mineral density; TBBMC- Total body bone mineral content; aBMD – areal bone mineral density; BMDLS - Bone mineral density lumbar spine; BMCLS - Bone mineral content lumbar spine; BMDTH-Bone mineral density total hip; BMCTH-Bone mineral density total hip; BMCFN- Bone mineral content femoral neck; BMDFN-Bone mineral density femoral neck; g – grams; g/cm^2 – grams per centimetre squared

4.3.5 Changes in anthropometry

Adolescence is a time of peak growth, both in height and weight. Comparisons have been made between baseline and endpoint for anthropometric measures to ascertain overall growth and to highlight significant areas of growth in this cohort. At baseline, height and weight were recorded for 61 participants and lean mass and fat mass were recorded for 60. At endpoint all anthropometric measures were recorded for 45 participants, 16 girls were not available.

Baseline to endpoint there was a significant change in weight (*P*=0.001) and height (*P*=0.001) in the intervention group. Similarly, there was a significant change in weight (*P*=0.002) and height (*P*=0.001) from baseline to end-point in the control group. There were no significant differences between groups for height, weight, lean mass, body fat or BMI, see Table 7.

There were no significant increases in body fat over the 12 months, however lean mass increased significantly in the control group (*P*=0.001) but not the intervention group.

Table 7. Changes from baseline to endpoint in anthropometry within and between intervention and control groups*.

	Intervention Group	**SD**	***P* value difference within group**	**Control Group**	**SD**	***P* value difference within group**	***P* value difference between groups**
Weight (kg)							
Baseline	50.68	6.59		52.99	6.95		
Endpoint	54.10	6.84		54.10	6.28		
Change End-Baseline	3.36	2.45	**<0.001****	2.73	3.42	**0.002***	0.69
Height (m)							
Baseline	1.62	0.07		1.62	0.05		
Endpoint	1.64	0.07		1.64	0.05		
Change End-Baseline	0.02	0.02	**<0.001****	0.02	0.02	**<0.001****	0.81
BMI (kg/m^2)							
Baseline	19.39	3.25		20.28	2.58		
Endpoint	20.17	2.31		20.53	2.47		
Change End-Baseline	0.76	3.25	0.34	0.64	2.28	0.24	0.36
Body fat (%)							
Baseline	23.02	3.22		23.69	4.43		
Endpoint	23.36	3.32		23.77	4.43		
Change End-Baseline	0.34	2.02	0.44	0.34	2.02	0.64	0.73
Bone-free, fat-free,lean body mass (kg)							
Baseline	37.78	4.73		38.51	4.64		
Endpoint	39.71	5.15		40.25	5.15		
Change End-Baseline	2.22	1.68	0.86	2.17	1.84	**<0.001****	0.30

*Values are mean ± standard deviation unless otherwise indicated. **P* <0.05, *** P* <0.001,

** Significant differences from baseline to six months (P <0.001) (Dependent Student *t*-test).

†Baseline control group n=30 and intervention group n=31

‡ Six month control group n=20 and control group n=23

Abbreviations: BMI – Body mass index kg/m^2 – weight in kilograms divided by height in metres squared; body fat % - percentage of body fat; kg – kilogram; m – metres; y – years

4.4 Results Section 2 Baseline Data Investigations

4.4.1 Predictors of bone mineral at baseline

Regression models were used to explore the relationship between BMD and BMC of the total body as a dependent variable, see Table 8 and Table 9. The variables BMI, body fat, bone free- fat free- lean mass, protein intake, energy intake, calcium intake, intact PTH concentration, serum 25(OH) D concentration, oestradiol, gynaecological age and training hours were included. The non-significant predictors – BMI, body fat %, protein intake, energy intake, gynaecological age, vitamin D status, oestradiol and intact PTH and training hours were excluded. A regression model was used to determine how much of the variance in BMD and BMC are explained by age, bone-free, fat-free, lean body mass and calcium intake.

4.4.2 Total body bone mineral density

The regression model accounted for 59% of the variance R^2 in TBBMD see Table 8 and 72% of the variance in TBBMC, see Table 9. Among all the independent variables, age, lean mass and calcium intakes were the significant predictors of TBBMD at baseline.

Total body bone mineral density increased by 0.03 g/cm^2 with every unit increase in age (years) (P=0.002); increased 0.01 g/cm^2 for every unit increase in bone-free, fat-free, lean body mass (kg) (P<0.001) and TBBMD also increased by (P<0.001) g/cm^2 for every unit increase in calcium intake (P<0.005). This regression model predicts TBBMD well as 59% of variability in TBBMD is explained by the model (P<0.001).

Table 8. Predictors of total body bone mineral density at baseline (*n*=59)

Model	Coefficient (*B*)	Standard error *B*	95% CI *B*	Standardized *B*	R^2	*P* value
Model 1					0.59	**<0.001****
Intercept	0.19	0.11				
Age (y)	0.03	0.01	-0.03,0.40	0.35		**0.002***
Bone-free, fat-free, lean bodymass (kg)	0.01	0.002	0.006,0.01	0.52		**<0.001****
Calcium intake (mg/day)	<0.001	<0.001	<0.001,<0.001	0.28		**0.005***

**P* <0.05, ** *P* <0.001,
Enter method. F (3, 50) =22.30
Abbreviations: kg – kilogram; mg/day – mg per day

4.4.3 Total body bone mineral content

The significant predictors of TBBMC at baseline were age (*P*=0.01) and bone-free, fat-free, lean mass (*P*<0.001). There was a trend toward calcium intake having an effect on BMC (*P*=0.05) see Table 9. TBBMC increased by 54.5 g when age increased by 1 year (*P*=0.01); TBBMC increased by 45.5 g when bone-free, fat-fee, lean body mass increased by 1kg (*P*<0.001); and TBBMC increased by 1 g when calcium increased by 0.14 mg/day (*P*=0.05). This regression model predicts TBBMC well as 72% of variability in TBBMD is explained by the model (*P*<0.001).

Regression models showed there were no significant predictors of BMD and BMC at total hip, lumbar spine and femoral neck.

Table 9. Predictors of total body bone mineral content at baseline (*n*=59)

Model	Coefficient (*B*)	Standard error *B*	95% CI *B*	Standardised *B*	R^2	*P* value
Model 1					0.72	**<0.001****
Intercept	-41.53	289.02				
Age (y)	54.54	21.13	12.03,97.04	0.22		**0.01***
Bone-free, fat-free, lean body mass (kg)	45.45	5.36	34.66,56.24	0.72		**<0.001****
Calcium intake (mg/day)	0.14	0.68	-0.001,0.27	0.15		0.052

F(3, 50) = 40.88
Bold type indicates a significant predictor (*P*<0.05).
Abbreviations: kg – kilogram; mg/day – milligrams per day

4.4.4 Age groups and bone

Adolescence is a time of peak growth and accretion of bone mineral. To investigate the changes in bone mineral at each age group, the cohort was stratified into age groups, 12 and 13 years together (*n*=18), 14 years (*n*=19), 15 years (*n*=13) and 16-18 years (*n*=11). Comparisons were made between intact PTH, oestradiol, and total bone mineral content and density between age groups.

There were no significant differences between age groups for oestradiol or intact PTH. Significant differences were identified by ANOVA between age groups at total body BMD, BMC and bone sites, and a Tukey post-hoc analysis showed significant differences between 12-13 year olds and 15 year olds for TBBMD and TBBMC and all bone sites ($P<0.05$). There were significant differences between 12-13 year olds and 16-18 year olds at TBBMD, TBBMC, BMDLS, BMCLS and BMCFN. There were also significant differences between 12 and 16-18 year olds for TBBMC see Figure 11.

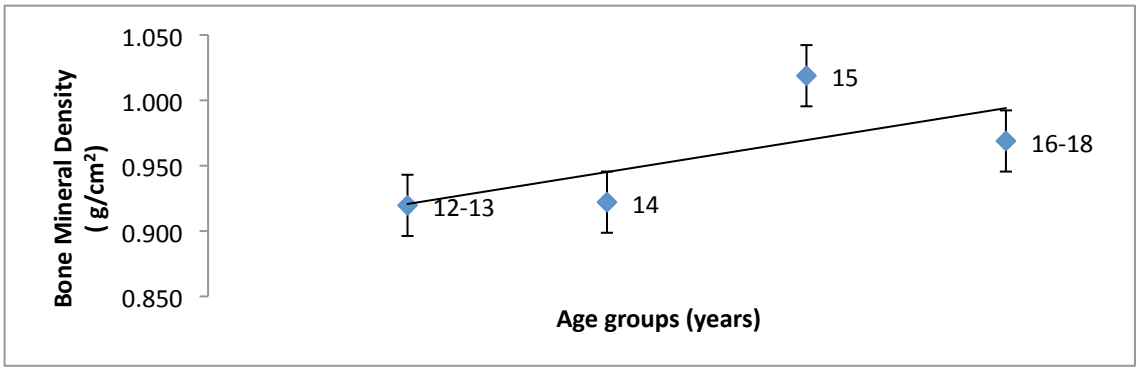


Figure 11. Differences in mean bone mineral density at 4 age groups. The stratification of age groups was 12 and 13 year olds, 14 year olds, 15 year olds and combined 16/17/18 year olds. R^2 = O.45; y=0.02x + 0.90.

4.4.5 Age of menarche

We divided the cohort into those who had experienced menarche and those who had not at baseline to investigate differences in bone mineral and body composition.

One quarter (*n*=14) of our cohort had not yet experienced menarche and three quarters of the cohort (*n*=46) had experienced menarche at baseline. Of those who had experienced menarche, 1/3rd had oligomenorrhoea, while the other 2/3rd

reported eumenorrhoea. There were no significant differences between those who had experienced menarche and those who had not at baseline in age, body fat, TBBMD, TBBMC or BMDLS, see Table 10 below.

Table 10. Differences between variables in dancers and gymnasts who had experienced menarche and those who had not at baseline.*

Variable	*n*=60	SD	Experienced menarche at baseline *n*=46	SD	Have not experienced menarche at baseline *n*=14	SD	*P* value
Age (y)	14.39	1.43	14.24	1.43	14.64	1.15	0.20
Body fat (%)	21.74	6.90	21.72	7.81	23.36	3.22	0.16
TBBMD (g/cm^2)	0.94	0.23	0.91	0.25	0.10	0.11	0.42
BMDLS (g/cm^2)	0.84	0.30	0.82	0.31	0.88	0.30	0.89
TBBMC (g)	1790.51	375.75	1752.25	378.18	1847.77	351.23	0.86

*Values are reported as mean ± standard deviation unless otherwise indicated. (Independent Student *t*-test)
P <0.05, ** *P* <0.001,
BMDLS - Bone mineral density lumbar spine; TBBMD-Total body bone mineral density; TBBMC- Total body bone mineral content; g – grams; g/cm^2 – grams per centimetre squared; body fat % - percentage of body fat; m – metres; y – years

4.4.6 Gynaecological Age

In this study girls who had no yet reached menarche at baseline were allocated a 0. The age of menarche (years) was subtracted from their chronological age at baseline (years) and a number was allocated based on the years since they experienced menarche. Gynaecological age could be determined in 58 participants, see Table 11 below.

Table 11. Gynaecological age of participants at baseline.

Gynaeocological Age	0	1	2	3	4	5
Number of participants (n=58)	24	10	11	6	4	3

Gynaecological age and BMD at baseline were compared by ANOVA at baseline.
There were significant differences in BMD between GA0 and GA2 (*P*=0.009) and GA5 (*P*<0.001). There significant differences in BMD between GA1 and GA5 (*P*=0.02). Gynaecological age was not a significant predictor of bone mass at baseline.

4.4.7 Oligomenorrhoea

Oligomenorrhoea can be defined as <9 menstrual periods over 12 months. Fourteen girls in this study reported irregular periods at baseline, 29 girls reported eumenorrhoea (normal menses). To investigate differences in bone and serum biomarkers between these two groups Student's t-tests were performed.

At baseline there was a trend toward difference in BMDTH (*P*=0.05) BMDLS (*P*=0.07) BMCLS (*P*=0.06) between those who had regular periods and those who had irregular periods at baseline, but it was not significant see Table 12 below.

Table 12. Regular vs. irregular periods and bone measures at baseline (*n* = 44)

	Regular periods at baseline (n=29) Mean	**SD**	**Irregular periods at baseline (n=14) Mean**	**SD**	***P* value**
BMDTH (g/cm^2)	0.98	0.11	0.91	0.12	0.05
BMDLS (g)	0.99	0.10	0.93	0.12	0.07
BMCLS (g)	55.5	8.4	48.9	13.5	0.06

*Values are reported as mean ± standard deviation. (Independent Student *t*-test) (*P*<0.05).
Abbreviations: BMDTH – Bone mineral density of the total hip; BMDLS – Bone mineral density of the lumbar spine; BMCLS – Bone mineral content of the lumbar spine; g/cm2 – grams per centimeter squared; g – grams.

4.4.8 Physical training volume

Each participant completed an online survey each week to record hours of training for that week. The survey recorded the actual hours training each day for a seven-day period. The results of the weekly survey were collated at the endpoint and training volumes were compared between the control and intervention group. Across the whole cohort, the mean number of training hours was 9.2 hours per week. There were no significant differences in physical training volume between the control and intervention groups over the 12-month intervention.

4.4.9 Change by age group

To investigate the changes in bone mineral at each age group, the cohort was stratified into age groups, 12-13 years together (*n*=18), 14 years (*n*=19), 15 years (*n*=13) and 16-18 years (*n*=11).

The change in bone variables over 12 months was compared by ANOVA and a post hoc Tukey analysis was performed to find where the differences between groups existed. There were no significant differences in BMDFN between any age groups. There were significant differences in the mean change between 12-13 year olds and 15 year olds in TBBMD, TBBMC and BMDLS. Similarly there were significant differences between 12-13 year olds and 16-18 year olds in TBBMD, TBBMC, aBMD and BMDLS, see Table 13. There were also significant differences in the mean change between 14 year olds and 16-18 year olds in TBBMD, TBBMC, aBMD and BMDLS. There were no significant differences in any bone site or height between 12-13 year olds and 14 year olds or between 14 and 15 year olds.

Table 13. Differences in mean change over 12 months in bone variables and height

Variable	**12 & 13 years vs. 15 years**	**12 & 13 vs. 16-18 years**	**14 years 16-18 years**
ΔTBBMD (g/cm^2)	**0.04***	**<0.001****	**0.002***
ΔTBBMC (g)	**0.009***	**<0.001****	**< 0.001****
ΔaBMD (g)	0.17	**0.006***	**0.02***
ΔBMDLS (g/cm^2)	**0.008***	**0.001***	**0.02***
ΔBMDFN (g/cm^2)	0.05	0.11	0.42
ΔHeight (m)	0.78	**0.03***	0.95

***P* <0.05, ** *P* <0.001,**

Abbreviations: Δ TBBMD – Change in total body bone mineral density; ΔTBBMC- Change in total body bone mineral content; Δ aBMD – change in areal bone mineral density; Δ BMDLS – Change in bone mineral density lumbar spine; g – grams; g/cm2 – grams per centimetre squared.

g – grams; g/cm^2 – grams per centimetre squared

5.0 Discussion

5.1 Summary of outcomes

Section 1 Outcomes from the randomised controlled trial

All analysis adheres to the CONSORT guidelines for randomised controlled trials (Shulz, Altman, & Moher, 2010).
There were 41 girls who completed the trial at 12 months.

The main outcomes from this research were that there were no significant differences in bone measures at any site between the intervention and control groups at 12 months.

The chosen research cohort had an adequate mean serum 25(OH)D concentration at baseline of 73 nmol/L this was a significant finding. There was no significant difference in vitamin D levels between the control and intervention group at baseline. At six months, vitamin D levels increased significantly in the intervention group and there was no change in the control group. At endpoint, there was no difference in s25(OH)D between the two groups. There was a non-significant difference in the change in s25(OH)D from baseline to endpoint. There was minimal seasonal effect on vitamin D levels observed in the control group.

Section 2 Outcomes from the baseline data

The significant predictors of bone mineral density and content of the total body at baseline were age (P=0.002) bone-free, fat-free, lean body mass (P=0.001) and calcium intake (P=0.005) and the significant predictors of bone mineral content in the total body at baseline were age (P=0.01) and bone-free, fat-free, lean body mass (P=0.001).

There were significant differences in mean change over 12 months between 12-13 year olds, and 15 year olds in TBBMD, TBBMC, and BMDLS but not at BMDFN. There were significant differences in change between 12-13 year olds and 16-18 year olds in TBBMD, TBBMC, aBMD, BMDLS but not in BMDFN. There were also significant

differences in change between 14 year olds and 16-18 year olds at TBBMD, TBBMC, aBMD and BMDLS, but not in BMDFN.

Comparisons between girls with regular menstrual cycles and girls with irregular menstrual cycles revealed a trend in bone mineral density at the total hip (*P*=0.05) and lumbar spine (*P*=0.07) and bone mineral content at the lumbar spine (*P*=0.06).

5.2 Primary outcome

The main aim of this research was to examine the effects of vitamin D supplementation on the bone health of adolescent ballet dancers and gymnasts in a 12 month trial. The outcome was that there was no significant difference in bone mineral density or bone mineral content between the control and intervention groups. Possible explanations for these findings are:
1. Baseline vitamin D levels were adequate, response in bone is inversely proportional to baseline serum 25(OH)D 2. That supplement compliance was poor, 3. The wide range in age of the cohort may have affected the result, 4. The sample size was too small to observe a significant result

5.2.1 Vitamin D status at baseline

The primary hypothesis for this study was that the chosen research population would have a less than adequate vitamin D status at baseline. In fact, the main finding of this study was that the baseline vitamin D levels were more than adequate with a mean of 73 nmol/L. This vitamin D level was greater than the mean vitamin D status of adult New Zealanders, which in 2008/09 was 63 nmol/L (Ministry of Health, 2012b).

It is likely that the high baseline s25(OH)D status of the cohort affected their response to supplementation. As anticipated, there was an increase in s25(OH)D concentrations in the intervention group at six months from mean ± SD of 73.9 ± 16.9 nmol/L to 99 (range 75.6-115.5) nmol/L. However, during the latter 6 months the s25(OH)D

dropped from from 99(range 75.5-115.5) nmol/L to mean ± SD of 76.4 ± 23.0 nmol/L at endpoint. This finding was unexpected.

Over the course of the intervention, there were significant changes in vitamin D levels in the cohort. At six months serum (s) 25(OH)D concentrations in the control group were less than at baseline, dropping from a mean ± SD 71.8± 21.6 nmol/L to 68.5 (range 57.0-100.0) nmol/L, although still not below the recommended level of vitamin D for sufficiency (i.e. s25(OH)D concentration of ≥50 nmol/L). At end point, 25 (OH)D in the control group had not changed, remaining at mean ± SD 68.3 ± 23.4 nmol/L.

One possible explanation for this finding was that supplement compliance was poor in the second half of the study, which would account for the increased s25(OH)D in the first 6 months and the reduced s25(OH)D at endpoint.

The above adequate s25(OH)D concentration in this cohort at baseline, was not anticipated, as other research has identified low levels in dancers and gymnasts (Lehtonen-Veromaa et al., 2002; Lovell, 2008). A potential explanation of these high baseline levels may be related to the sunshine hours in Auckland over the period of data collection.

The baseline s25(OH)D concentrations were recorded between April-September 2012. The seasons which would have negatively impacted the vitamin D levels were autumn and winter (April-Sept) and these were particularly sunny in 2012 (NIWA National Climate Centre, 2013) and may partly explain the high baseline serum 25(OH)D concentration recorded. Very high sunshine hours were recorded in autumn in Auckland 2012 for the period March-May, a total of 530 hours, 69 hours above the normal sunshine hours. The mean number of sunshine hours in Auckland for the period June-August (winter) 2012 was 448 hours; 58 hours more than the normal sunshine hours recorded over winter in Auckland (NIWA National Climate Centre, 2013). The unseasonally sunny weather may have some bearing on the minimal seasonal effect seen in this study.

In Auckland over summer 2012/2013 the recorded sunshine hours were 10% greater than the normal recorded sunshine hours for the period December 2012 to February 2013. Perhaps the high sunlight hours over summer was a factor in the minimal seasonal effect observed serum 25(OH)D concentration at recorded in the control group at endpoint.

Latitude also affects the number of sunlight hours each year. In Auckland, where this study was based, the radiation of the sun during winter in Auckland is adequate during the middle of the day for vitamin D_3 to be produced in the skin. We know this because the synthesis of vitamin D_3 has been recorded in cities at similar latitues during the winter months (Webb, Kline, & Holick, 1988).
However, in spite of this other research has observed vitamin D deficiency over winter in a variety of latitudes (Van der Mei et al., 2007).

Latitude is a factor in serum vitamin D levels around the world as people who live at high latitudes are at greater risk of lower serum vitamin D concentration (Holick & Chen, 2008; Lips, 2010). However, Auckland sits on a latitude of 36.5° South, the risk of low vitamin D status is not as great at this latitude. Cities on similar latitudes in the Northern hemisphere include Malaga, Spain and Monterey, California and Melbourne, Australia (Maps of the World, 2013).

Research based in Australia, by Van der Mei and colleagues (2007) observed that a higher latitude was significantly associated with reduced 25(OH)D concentration. There was an average decrease in 25(OH)D concentration of 1 nmol/L for every degree increase in latitude. For New Zealanders, that means that people who live south of Auckland are at greater risk of low serum vitamin D. Graham and colleagues (2009) observed that children in the Waikato region, which has a latitiude of 38.05° South, had a prevalance of vitamin D insuffiency of 80% over the winter months.

Possible explanations for the elevated serum 25(OH)D concentration at baseline can be speculated upon from anecdotal evidence reported by the participants during data collection appointments. The girls in this cohort attended secondary schools in the Auckland region. Many secondary schools require their students to remain outside during the lunch break period, which is usually in the middle of the day when the sun is at its zenith. In the course of data collection, informal conversation with the girls revealed that they did not apply sunscreen while they were outside during the lunch break. This may have allowed their skin more opportunity to synthesise 25(OH)D. This may explain in part, the high s25(OH)D concentration in this cohort.

There was a small seasonal effect on serum 25(OH)D concentration in the control group at the six month data collection. However, the decrease in vitamin D levels over the winter season was minimal when compared with similar research (Bolland et al., 2007; Graham et al., 2009; Van der Mei et al., 2007; Wolman et al., 2013). The findings cannot be fully explained, but the possible explanations given for high baseline vitamin D levels may also be applied for the minimal seasonal effect observed.

5.2.2 Vitamin D supplement dosage

The dose of vitamin D in this study was 50,000 IU per month,for 12 months which caused a significant increase in serum 25(OH)D concentration in the intervention group from baseline to 6 months. However, the vitamin D levels in the intervention group declined insignificantly at 12 months. Perhaps the dose was not sufficient to maintain the high levels reached at 6 months 99 nmol/L.

Research, which investigated response to vitamin D dose in a population of obese adults, saw a plateau of serum 25(OH)D concentration over 12 months. The randomised 1-year placebo-controlled intervention trial supplemented obese adult participants with 40,000 IU of cholecalciferol each week and stratified them by BMI (Jorde, Sneve, Emaus, Figenschau, & Grimnes, 2010).

The research by Jorde et al. (2010), see Figure 12, found that participants with a BMI <30 kg/m^2 showed the greatest response to supplementation, but vitamin D levels began to drop after 9 months. The serum 25(OH)D in all participants began to plateau off between 6-9 months and the group with BMI <30 kg/m^2 experienced the greatest drop in serum vitamin D levels from 9-12 months despite the constant dose of vitamin D. The plateau of serum 25(OH)D concentration is supported by our research, although the two studies differ in cohort age, type and BMI.

One of the most interesting findings of the study by Jorde et al. (2010) relates to this present research. There was a plateau of vitamin D levels, for the leanest group (BMI <30 kg/m^2), similar to the findings of this study. Perhaps those who have a BMI <30 kg/m^2 respond in this way to supplementation. In our cohort of dancers and gymnasts had a mean BMI< 20 kg/m^2 at baseline.

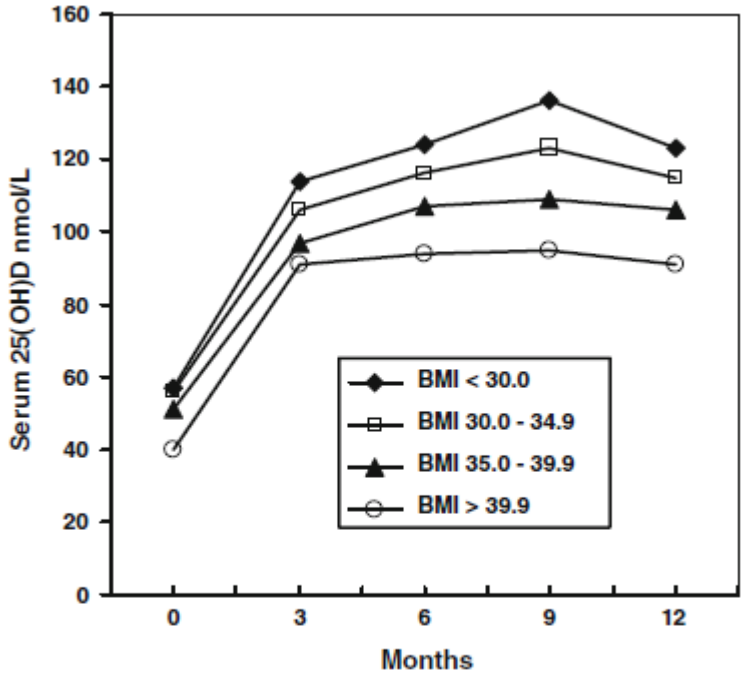


Figure 12. Serum 25(OH)D in relation to BMI (kg/m^2) in 93 participants given 40,000IU vitamin D3 per week for 12 months, showing response to supplementation (Jorde et al., 2010) (used with permission).

In other research in a cohort more similar in age to this present study, variance in vitamin D dose caused increases in serum 25 (OH) D concentrations. The greater the vitamin D dose, the greater the s25(OH)D measured. The study was a double-blinded

randomised trial in which 228 adolescent females (mean age 11.4 ± 0.4 years) were supplemented with 5 and 10 µg (200 and 400 IU) vitamin D_3 per day for 12 months. A positive increase in serum 25(OH)D concentration was seen at 12 months (P<0.001) in both the 5 µg and 10 µg intervention groups, but not the control group (Bischoff-Ferrari et al., 2009). The mean change in vitamin D levels was greater (12.1 ± 13.5 nmol/L) in the intervention group who were supplemented with 10 µg per day, when compared to the change in the intervention group (5.4 nmol ± 15.3 nmol/L), who were supplemented with 5 µg per day.

The serum vitamin D supplement dose in the study by Bischoff-Ferrari (2009) was 5600 IU per month, in the 5µg group and 11200 IU per month in the 10 µg group which is less than the supplementation of 50,000 IU in this present study. However the baseline serum vitamin D levels recorded (a mean range 46-48 nmol/L across all the groups) were markedly less than our recorded values, which may have affected response to supplementation.

Lower baseline vitamin D levels tend to elicit a greater response to vitamin D supplementation, as seen by Viljakainen (2009). Response to vitamin D supplementation is inversely proportional to the s25(OH)D at baseline (Barger-Lux, Heaney, Dowell, Chen, & Holick, 1998). A daily supplement of 800-1000 IU of vitamin D_3 will maintain serum 25(OH)D of 75 nmol/L (Dawson-Hughes et al., 2005). The dose in this present study was approximately 1600 IU per month. According to Dawson-Hughes et al. (2005) that should have maintained the serum 25(OH)D to at least 75 nmol/L.

5.2.3 Vitamin D and leanness

The minimal response to vitamin D supplementation observed in this cohort may be explained by the relatively low mean BMI at baseline 20kg/m^2. Some studies have reported and inverse relationship between 25(OH)D and BMI (Castracane et al., 2012; Holick, 2007).

Vitamin D deficiency has been observed in the obese (Holick, 2007). It has been suggested that sequestration of vitamin D into adipose tissue reduces the bioavailability of vitamin D (Wortsman, Matsuoka, Chen, Lu, & Holick, 2000), so that it is not able to be accessed when required. There have also been suggestions that low serum vitamin D levels in the obese may be due to dilution (Drincic, Armas, Van Diest, & Heaney, 2012).

A large cross-sectional study (Lee, Greenfield, Seibel, Eisman, & Center, 2009) identified a strong negative association between vitamin D levels and BMI. The association became statistically significant over the winter period as vitamin D levels dropped and BMI remained static. In this research, vitamin D_3 supplementation of 10,000 IU per day was provided for 17 hospital patients who had severe vitamin D deficiency. Comparisons were made between obese and lean individuals and the lean group responded to the supplementation to a greater degree than the obese group. The implication is that obese require a higher dose of vitamin D to raise serum concentration than lean individuals (Lee et al., 2009).

5.2.4 Supplement compliance

At the six months appointment the 25(OH)D supplement compliance was checked by viewing the supplement bottle and noting if any supplements had not been consumed. At endpoint the girls were all asked if they had taken all their supplements at the appropriate time and had they missed any. All the participants reported complete compliance at both 6 months and endpoint, however the compliance was not adequately checked.

The participants were reminded on a monthly basis to take their supplement at the appointed time. Monthly doses were more convenient for the participant than daily doses. However, the serum vitamin D levels in the intervention group dropped at endpoint and compliance may not have been as complete as was reported by the participants. Other studies have noted supplement noncompliance in adolescence, mainly due to forgetfulness and busy schedules (Outila, Kärkkäinen, & Lamberg-

Allardt, 2001). The studies which observed good supplement tolerance in adolescents provided check diaries or blister packs for participants which were counted on a monthly basis over a 12 month trial (Bischoff-Ferrari et al., 2009).

5.3 Further investigation of baseline data

5.3.1 Skeletal development and growth

The secondary hypothesis for this study was that all participants would have improved BMD as measured by DXA at the end of the 12 month period, due to growth.

The dancers and gymnasts in this present study all had significant increases in bone mineral density and bone mineral content over the 12 months of this study. The adolescent stage is when we would expect significant increases in bone mineral due to growth (Georgopoulos et al., 2004; Ischander et al., 2007; Rizzoli et al., 2007).

Aside from growth, other predictors of bone mineral accrual are of interest for the development of strategies to reduce the risk of osteoporosis in later life. Our research found that lean mass (P=0.001) and calcium (P=0.004) were the strongest predictors of bone mineral density other than age (P=0.01) at baseline. This finding has important implications for prevention of osteoporosis.

5.3.2 Predictors of bone mineral density at baseline

5.3.3 Lean mass

It is encouraging to compare our findings to those of Matthews and colleagues (2006) who noted that lean mass was an independent predictor of bone mineral content, in a cohort of 8-14 year old dancers. Similar results were reported by Morris et al. (1997) who observed that lean mass was a predictor of total body, femoral neck and lumbar spine bone mineral density. Pekkinen and colleagues (2012) also observed a significant contribution (P=0.002) of lean mass to bone mineral density at the lumbar spine of adolescents aged 7-19 years in a population-based study .

Lean mass is increased by physical activity and growth (Morris et al., 1997). As a key predictor of bone mineral accrual developing lean mass may be important for prevention of osteoporosis. Perhaps physical activity could be advocated to a greater degree in children and adolescence to develop bone mineral density and achieve a health peak bone mass in the second decade.

The lean mass of this cohort of ballet dancers and gymnasts were comparable to other research in adolescent dancers and gymnasts (Doyle-Lucas et al., 2010; Matthews et al., 2006) and reference ranges for lean mass in the country where the study was conducted (Van der Sluis et al., 2002).

5.3.4 Calcium

Another determinant of bone mineral density and content at baseline was dietary calcium intake (*P*=0.004). Calcium intake was associated with positive bone mass accrual in a randomized controlled trial of calcium-enriched food intervention in females aged 7 years (Bonjour et al., 1997). These findings were also observed in four year old children (Harvey et al., 2012) and in a meta-analysis of 21 RCTs (Huncharek et al., 2008). In contrast no positive effect of calcium intake on bone mineral accrual was observed in a meta-analysis of 19 RCTs (Winzenberg, Shaw, Fryer, & Jones, 2006). This present work adds to the evidence that calcium intake is a positive predictor of bone mineral accrual in adolescence, or maybe calcium is more important in adolescents with a high activity level. Calcium intake has a greater effect on bone health when s25(OH)D status is sufficient.

The mean ± SD calcium intake in this cohort of dancers and gymnasts was 892 ± 331 mg per day, this is considerably less than the recommended daily intake of calcium for this age group of 1300 mg/day (Ministry of Health and National Health and Medical Research Council, 2006). However, it is higher than the mean calcium intake for females from New Zealand dietary survey data (Ministry of Health, 2011) in which females aged 11-14 years had a mean calcium intake of 733 mg per day and females aged 15-18 years old had a dietary calcium intake of 749 mg per day.
Other researchers have observed mean calcium intakes of 851 ± 80 mg per day in adult female dancers (Doyle-Lucas et al., 2010) and a range of 730-933 mg per day in adolescent ballet dancers and gymnasts (Munoz et al., 2004).

It has been observed in other research that low calcium intake in adolescents may be caused by the replacement of dairy products in the diet by sugar-sweetened beverages

(Demory-Luce et al., 2004) and the perception that dairy products are high in fat (Malik, Schulze, & Hu, 2006). However, calcium intakes for this cohort were greater than reported for the national mean for this age group. Further research would be required to identify actual sugar sweetened beverage intake in this cohort.

5.4 Change in bone mineral over 12 months

The third hypothesis in this present study was that there would be a difference in change in BMD between the control and intervention groups. There was a significant increase in bone mineral density, content and areal bone density in the total body and bone content and density all bone sites measured over the 12 months, but there were no significant differences in bone measures between groups. The most likely explanation being that both groups had adequate vitamin D status and there was no additional benefit from supplementation.

5.5 Weight-bearing and non-weight bearing sports

Weight-bearing sport causes greater bone mineralisation than non-weight bearing, as was seen in tennis players where the dominant arm had greater BMD than the non-dominant arm (Etherington et al., 1996). The criterion for sporting code was relaxed during subject recruitment to increase the size of the cohort. Six participants were swimmers. Swimmers were considered acceptable to include into the cohort as they spend many hours indoors training and avoid UV exposure, so may have low vitamin D. However, swimming is a non-weight-bearing sport and this may have affected bone mineral measures in these participants. Research has shown that when swimmers were compared to gymnasts they had significantly greater bone-free lean mass ($P<0.001$), while gymnasts had higher BMD and femoral neck BMD than swimmers ($P<0.001$) (Taaffe et al., 1995).

5.6 Age-related bone mineral accrual

The results of this study indicate that bone mineral accrual is greatest at ages 12-13 and slows in later adolescence. At 16-18 years bone mineral accrual continues but at a slower rate, until peak bone mass is achieved somewhere toward the end of the

second decade or the beginning of the third decade (Baxter-Jones et al., 2011). The mean difference in change in TBBMD between 12-13 year olds and 16-18 year olds was a mean ± SD of 0.04 ± 0.03 gcm^2.

5.7 Bone mineral density at the femoral neck

Another interesting finding of this study was that there was no significant difference in bone mineral at the femoral neck between age groups. The femoral neck accrues bone mineral in response to physical activity rather than in response to sex hormones (Khan et al., 1998; Young et al., 1994).

It is possible, therefore that the lack of variation between the age groups is because of a bone mineral accrual at a steady rate in response to physical activity in contrast to the more rapid changes that occur at the lumbar spine during puberty in response to rising oestrogen levels.

5.8 Menstrual function

5.8.1 Age of menarche

Most of the girls who had not yet experienced menarche at baseline were in the normal age range for beginning menarche (<15 years) (Nattiv, 2007). However, there were four girls who were aged 15 years and had not yet experienced menarche. These girls fit the definition of primary amenorrhoea. They had low body weight and two girls had a BMI <18.5 kg/m^2. These two symptoms are also indicative of the female athlete triad, a set of clinical symptoms which are often recognised in female athletes.

To investigate further evidence of the female athlete triad, the TBBMD of these four were compared to the other 15 year old girls with normal menses. Those with primary amenorrhoea had a mean TBBMD only 75% of the mean TBBMD of the other 15 year old girls with normal menses. There is an inverse relationship between TBBMD and menstruation status in this study which has been seen in other research (Borgen, 2002; Martinsen et al., 2010).

In spite of these findings there were no significant differences between those who had experienced menarche and those who had not for age, TBBMD, TBBMC, BMDLS or body fat.

5.8.2 Oligomenorrhoea

All those who reported having irregular periods had experienced menstruation at least once in the past 6 months. Additional information was required to identify more specifically the length of time of absence of menstruation. If this information were available, the group with oligomenorrhoea could then be further classified into those who have irregular menses and those who were experiencing secondary amenorrhoea.

5.9 Strengths and limitations

5.9.1 Population

The strength of this study lies in the unique research population chosen and the range of data collected over a 12 month duration. Although we did not see the expected low baseline serum 25(OH)D, we have made some interesting observations about the predictors of bone mineral in adolescents.

A limitation of the research was the small sample size. The research population had heavy training schedules associated with their sport which may have contributed to the high drop-out rate over the 12 months. Some participants had minimal time available for data collection and may have found the time commitments to the study too much. The number of participants dropped from 61 at baseline to 41 at endpoint a response rate of 32%.

The cohort was made up of a mixture of ethnicities and weight-bearing and non-weight bearing sports. A homogeneous cohort may have revealed more conclusive results.

5.9.2 Study design

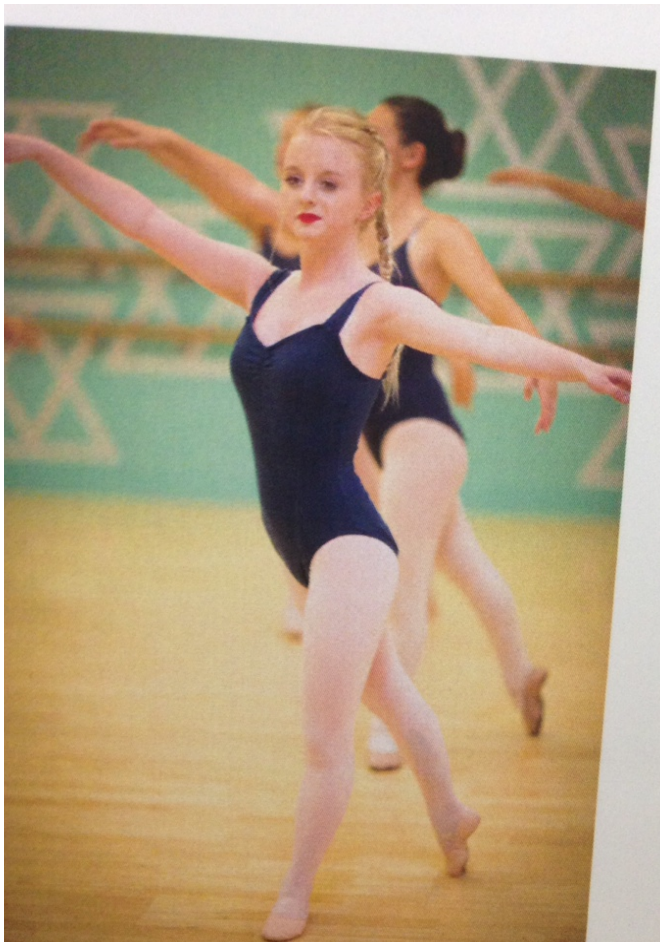
A limitation of the study design was the question of complete compliance. The drop in the serum 25(OH)D in the intervention at 12 months may indicate that compliance was

less than perfect. An infallible method of recording supplement compliance, (for example the participants could have recorded when the supplement was taken, or received the supplement at the laboratory) is necessary to draw definitive conclusions about the responses to vitamin D supplementation.

5.9.3 Range of ages

Regression equations showed that increased age predicted increased BMD and BMC at baseline. TBBMD increased by 0.03 cm^2 for every increase of 1 year of age (P=0.002) and TBBMC increased by 54.5g for every increase of 1 year of age (P=0.01). A limitation of this study was the wide range of ages in this cohort as bone mineral increased significantly with age.

6.0 Conclusion



6.1 Key findings

6.1.1 Bone

There was no significant difference between intervention and control groups in any of the bone site measures. Among the entire group, there were significant increases in total bone mineral density and content, in areal bone mineral density, and bone mineral density and content and all bone sites measured over the 12 months.

Bone mineral is accrued at a maximum rate during adolescence and the amount of bone mass achieved during this period affects peak bone mass. This research makes interesting observations related to the rate of bone mineral accrual at different ages within adolescence, which have been observed in previous literature. The rate of accrual in bone mineral in 12-13 year olds is greater than accrual in later adolescence. This may have implications for the timing of interventions to affect bone mineral accrual and in adolescence and achievement of peak bone mass in order to prevent osteoporosis in later life.

6.1.2 Vitamin D

Baseline serum 25(OH)D concentrations were more than adequate at baseline. There were no significant differences in vitamin D levels between the control and intervention group. At six months vitamin D levels increased significantly in the intervention group and there was only a slight decrease in levels in the control group. Vitamin D levels in the intervention group dropped at endpoint in spite of continued supplementation.

The prevalence of adequacy at baseline was greater than anticipated. We cannot adequately explain the reasons why this particular cohort of dancers and gymnasts had high baseline s25(OH)D concentrations. Nor can we explain the minimal seasonal effect on vitamin D levels over winter. We suggest that there may be adequate sun exposure in Auckland to achieve sufficient vitamin D status, both in summer and winter.

The decision not to screen vitamin D status prior to recruitment was deliberate due to ethical reasons, as deficient vitamin D status would require treatment, therefore excluding the participant from the study. However, due to the effect that the baseline serum 25(OH)D levels may have had on the response to supplementation, this protocol needs further consideration in future research of vitamin D RCTs.

The observed drop in vitamin D levels in the intervention group is difficult to explain. Perhaps their adequate status at baseline is a major factor in their initial increase in s25(OH)D and then return to adequate level of 72nmol/L. Perhaps the low BMI of the group affected the response to supplementation. However, there was no mean in BMI at endpoint in the intervention group. The dose of 50,000 IU and monthly administration of the vitamin D supplementation may have played a role in the response of this cohort to supplementation.

6.1.3 Age-related changes in bone

The significant predictors of bone mineral density and content of the total body at baseline were age (*P*=0.002) bone-free, fat-free, lean body mass (*P*=0.001) and calcium intake (*P*=0.005) and the significant predictors of bone mineral content in the total body at baseline were age (*P*=0.01) and bone-free, fat-free, lean body mass (*P*=0.001).

Lean mass is associated with accrual of bone in adolescence, this may have implications for designing interventions to prevent osteoporosis. Physical activity to develop lean mass may be a key intervention in children and adolescence to increase the accrual of bone mineral and achieve adequate peak bone mass.

Calcium intake is known predictor of bone mass accrual these findings underscore the necessity of an adequate calcium intake during adolescence to achieve a healthy peak bone mass.

6.1.4 Menstruation and bone

Comparisons between girls with regular menstrual cycles and girls with irregular menstrual cycles revealed a trend in difference in bone mineral density at the total hip

(*P*=0.05) and lumbar spine (*P*=0.07) and bone mineral content at the lumbar spine (*P*=0.06).

These findings reveal the association between bone mineral and menstrual function. Girls who have irregular periods may have poorer bone health than those who normal menses. This is supported by a body of evidence in female athletes related to The Female Athlete Triad. The reasons for the menstrual irregularity may be attributed to a low dietary energy availability, which is seen in athletes participating in aesthetic sport. Menstruation may also be irregular in the 1-2 years post menarche.

6.1.5 Summary

The original hypotheses of this research were:

H1: That the chosen research population will have a less than adequate vitamin D status,

H2: That all participants will have improved BMD as measured by DXA at the end of the 12 months period, due to increased growth,

H3: That there will be a difference in change in BMD between the control and intervention groups.

The findings from this research caused us to reject hypothesis 1 and 3, but accept hypothesis 2.

H1: The hypothesis is rejected, as the chosen research population did not have a less than adequate vitamin D status.

H2: The hypothesis is accepted as all participants have improved BMD at the end of the 12 months period due to growth.

H3: The hypothesis is rejected, as there was no difference in change in BMD between any of the bone sites between the control and intervention groups.

The findings described in this thesis further confirms the importance of achieving accrual of bone mineral during adolescence and developing a healthy peak bone mass to reduce the risk of osteoporosis development in the aging process. It also presents

direction for future research to better understand dosage and timing of supplementation in lean cohorts such as this.

6.2 Recommendations

6.2.1 Future research

It is recommended that further research be undertaken to examine vitamin D supplementation in ballet dancers and gymnasts, with the following suggestions:

- Further research is in order to establish the appropriate dose of vitamin D supplement to sustain adequate vitamin D levels in a lean cohort such as this one.

- The timing of administration of the supplement dose requires further research. There may be a relationship between timing of vitamin D supplementation and response. Investigation is warranted to identify differences between monthly and daily supplementation.

- Vitamin D supplementation in a lean cohort necessitates additional study. The response to supplementation may be a factor in the minimal seasonal variation observed in the control group. Leanness may have also had a role in the plateau of vitamin D levels in the intervention group observed at 12 months.

- This research would be improved by repeating in a larger cohort of adolescent females with a smaller range in ages.

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8.0 Appendices

Appendix A

Sunflower Screening Questionnaire

Health Questionnaire

Appendix B

Sunflower Information Sheet

Appendix C

Four-day Estimated Food Diary

Sunflower Training Schedule

Appendix A

Sunflower Screening Questionnaire

We are looking for female dancers and gymnasts between the ages of 13 to 18 years old. If you would like to find out more about the study, please complete this online questionnaire. We will contact you to tell you more about the study but this doesn't mean you have to take part.
*

1. Please complete the following details

Please complete the following details First name

Family name

Email

Contact phone number

2. Does your parent agree for you to take part in the study?

Does your parent agree for you to take part in the study Yes ○ No ○ Don't know

*

3. What is your date of birth?

DD / MM / YYYY

DOB 140

What is your date of birth? DOB Day Month Year

4. Do you currently train in?

Do you currently train in Ballet ○

Modern/contemporary ○

Both ballet and modern ○

Gymnastics ○

Other (please specify)

5. How often do you train?

How often do ○ 1-2 hours per ○ 3-4 hours per ○ 5 hours or more

you train? Less than 1 hour per week | week | week | per week

Other (please specify)

6. Are you able to remove all jewellery and piercings?

Are you able to remove all jewellery and piercings? Yes | No

7. Do you have any medical conditions or injuries which may affect you taking part?

Do you have any medical conditions or injuries which may affect you taking part? Yes

No

Please tell us about your medical condition or injury

8. Are you taking any vitamin D supplements or cod liver oil? (Or you have taken any within the last 2 months please tick not sure)

Are you taking any vitamin D supplements or cod liver oil? (or you have taken any within the last 2 months please tick not sure) Yes | No | Not sure

9. Would you like to be notified about future research studies within the Institute of Food, Nutrition and Human Health?

Would you like to be notified about future research studies within the Institute of Food, Nutrition and Human Health? Yes | No

Thanks for completing the questionnaire
we will be in contact to tell you more about the study
In the meantime check out our page on Facebook

Done

Powered by Survey Monkey
Check out our sample surveys and create your

Appendix B

Sunflower Study Health Questionnaire

1. General Health

We are going to ask you some questions about your eating habits, general health, whether you have ever donated blood and your periods. If you need help at any point please ask one of the researchers.

1. Please enter your Study ID

2. How would you describe your eating pattern?

- Eat a variety of all foods, including animal products
- Eat eggs, dairy, fish and chicken but avoid other meats
- Eat eggs and dairy products but avoid all meats and fish
- Eat eggs but avoid dairy products, all meats and fish
- Eat no animal products
- Other (please specify)

3. Do you follow any diet for cultural or religious reasons?

- Yes
- No

If yes, what type of diet do you follow?

4. Have you dieted strictly in the last year?

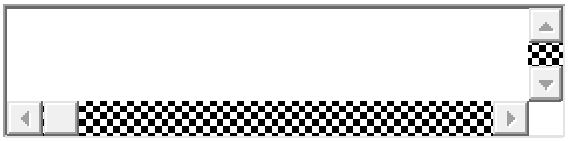
- Yes
- No

If yes please tell us about your dieting

5. Do you smoke?

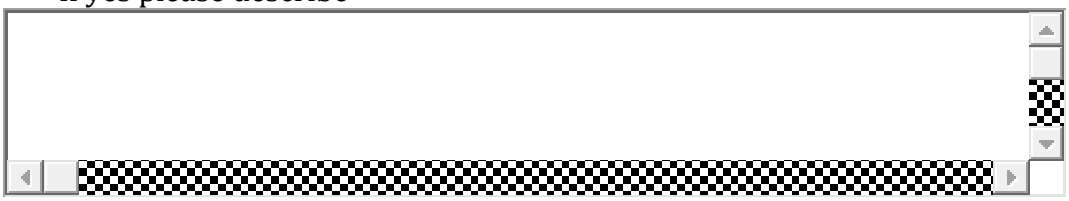
- Yes
- No
- Occasionally

If you smoke please tell us how many cigarettes you usually smoke in a day



6. Do you have or have you had any medical condition which has resulted in blood loss?

- Yes
- No
- If yes please describe



7. Please describe any sources of blood loss such as coughing up blood, blood in stools or urine, blood loss through injury or blisters in the past year (we will ask you about your periods later)

8. If you are over 16 years old, have you ever donated blood?

- Under 16 yrs.
- Yes
- No

2. Blood donation

1. When did you last donate blood

DD MM YYYY

If you cannot remember the day please put 01 and then complete the month and year

DD / MM / YYYY

140

2. If you have donated blood how many times have you donated blood in the past year?

Times in the past year

3. Nose Bleeds

1. Do you get nose bleeds?

- Yes
- No

4. Nose bleeds

1. How often do you get a nose bleed?

Times a month

OR times a year

2. How heavy are your nose bleeds?

- [ ] Light
- [ ] Medium
- [ ] Heavy

5. Your periods

1. Have you started your periods?

- ( ) Yes
- ( ) No

If you want to explain further please provide details here

6. Your periods

1. How old were you when you had your first menstruation period?

Years Old

Months

2. Have you had a period in the last 6 months

- [ ] No
- [ ] Yes

Cancel Copy

7. Current periods

1. Would you say your periods are regular (every month) or irregular?

- ( ) Regular
- ( ) Irregular

2. How best describes your periods and the number of days in your cycle (To count the number of days in your cycle count from the first day of your last period to the day you expect your period to start)

- ( ) I've had my first period but nothing since
- ( ) I have regular periods and my cycle is usually less than 21 days
- ( ) I have regular periods and my cycle is usually between 21 to 35 days

- I have regular periods and my cycle is usually between 36 to 90 days
- I have regular periods and my cycle is usually over 90 days
- My periods are so irregular it's difficult to tell how many days my cycle is
- Other (please specify)

3. When did your last period start?

	DD	MM	YYYY
Day/Month/year	/	/	
140			
OR The week starting Day/Month/Year	/	/	
140			
If you don't know the date please enter 01/01/1111	/	/	
140			

4. On average

	Days
How many days does your period normally last for?	

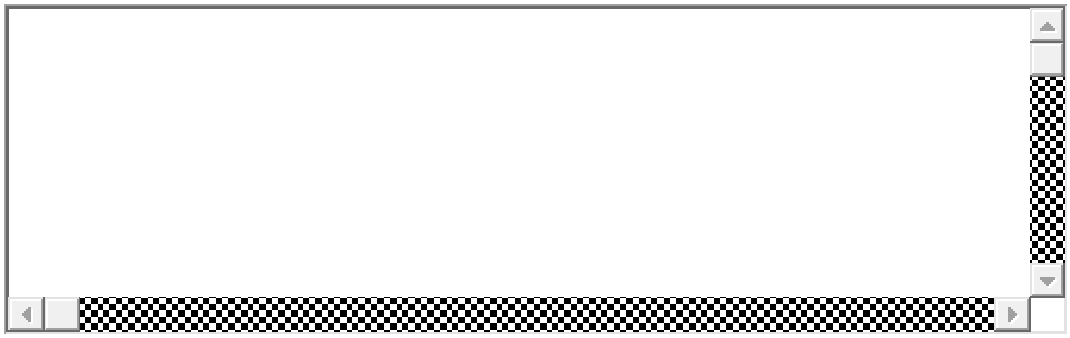
Other (please describe)

5. We need to ask the following questions to help us estimate how heavy your periods are:

How many 'heavy' days do you have during a period?	
How many 'light' days do you have during a period?	

Cancel Copy

6. Any comments



Appendix C

Four day food record

Massey University

Sunflower Study

4 Day Food Record

Thank you very much for taking part in the Sunflower Study. We are extremely grateful for your time, effort and commitment!

If you have any questions, please contact Sarah Mitchell on 021 160 5949 or Pam von Hurst on (09) 414 0800 ext. 41205 or email sunflower@massey.ac.nz

All information in this diary will be treated with the strictest confidence. No one outside the study will have access to this.

Please return this diary to us in the envelope provided

4 day food diary - what to do?

Record all that you eat and drink on the following dates.

_____
_____
_____

If possible record food at the time of eating or just after – try to avoid doing it from memory at the end of the day.

Include all meals, snacks, and drinks, even tap water.

Include anything you have added to foods such as sauces, gravies, spreads, dressings, etc.

Write down any information that might indicate size or weight of the food to identify the portion size eaten.

Use a new line for each food and drink. You can use more than one line for a food or drink. See the examples given.

Use as many pages of the booklet as you need.

Describing Food and Drink

Provide as much detail as possible about the type of food eaten. For example, brand names and varieties / types of food.

General description	Food record description
Breakfast example – cereal, milk, sugar	1 cup Sanitarium Natural Muesli 1 cup Pam's whole milk 1 tsp Chelsea white sugar
Coffee	1 tsp Gregg's instant coffee 1 x 200ml cup of water 2 Tbsp Meadow fresh light green milk
Pasta	1 cup San Remo whole grain pasta spirals (boiled)
Pie	Big Ben Classic Mince and Cheese Pie (170g)

Give details of all the cooking methods used. For example, fried, grilled, baked, poached, boiled…

General description	Food record description
2 eggs	2 size 7 eggs fried in 2tsp canola oil 2 size 6 eggs (soft boiled)
Fish	100g salmon (no skin) poached in 1 cup of water for 10 minutes

When using foods that are cooked (eg. pasta, rice, meat, vegetables, etc), please record the cooked portion of food.

General description	Food record description
Rice	1 cup cooked Jasmine rice (cooked on stove top)
Meat	90g lean T-bone steak (fat and bone removed)
Vegetables	½ cup cooked mixed vegetables (Wattie's peas, corn, carrots)

Please specify the actual amount of food eaten (eg. for leftovers, foods where there is waste)

General description	Food record description
Apple	1 x 120g Granny Smith Apple (peeled, core not eaten – core equated to ¼ of the apple)
Fried chicken drumstick	100g chicken drumstick (100g includes skin and bone); fried in 3 Tbsp Fern leaf semi-soft butter

Because we are especially interested in your calcium intake, please take care to list all the milk you consume, and record what type of milk it was.

General description	Food record description
hot chocolate	I x cup hot chocolate made with Cadbury's powder and 150 mls Calcitrim milk, 100 ml hot water. No sugar

Record recipes of home prepared dishes where possible and the proportion of the dish you ate. There are blank pages for you to add recipes or additional information.

Recording the amounts of food you eat

It is important to also record the quantity of each food and drink consumed. This can be done in several ways.

By using household measures – for example, cups, teaspoons and tablespoons. Eg. 1 cup frozen peas, 1 heaped teaspoon of sugar.

By weight marked on the packages – e.g. a 425g tin of baked beans, a 32g cereal bar, 600ml Coke

Weighing the food – this is an ideal way to get an accurate idea of the quantity of food eaten, in particular for foods such as meat, fruits, vegetables and cheese.

For bread – describe the size of the slices of bread (e.g. sandwich, medium, toast) – also include brand and variety.

Using comparisons – e.g. Meat equal to the size of a pack of cards, a scoop of ice cream equal to the size of a hen's egg.

Use the food record instructions provided to help describe portion sizes.

General description	Food record description
Cheese	1 heaped tablespoon of grated cheese 1 slice cheese (8.5 x 2.5 x 2mm) 1 cube cheese, match box size Grated cheese, size 10B

If you go out for meals, describe the food eaten in as much detail as possible.

Please eat as normally as possible - don't adjust what you would normally eat just because you are keeping a diet record and be honest! Your food record will be identified with a number rather than your name.

Example day

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed (units, measures, weight)
Example 7:55am	Sanitarium weetbix	2 weetbix
" "	Anchor Blue Top milk	150ml
" "	Chelsea white sugar	2 heaped teaspoons
" "	Orange juice (Citrus Tree with added calcium – nutrition label attached)	1 glass (275 ml)
10.00am	Raw Apple (gala)	Ate all of apple except the core, whole apple was 125g (core was ¼ of whole apple)
12.00pm	Homemade pizza (recipe attached)	1 slice (similar size to 1 slice of sandwich bread, 2 Tbsp. tomato paste, 4 olives, 2 rashers bacon (fat removed), 1 Tbsp. chopped spring onion, 3 Tbsp. mozzarella cheese)
1.00pm	Water	500ml plain tap water
3.00pm	Biscuits	6 x chocolate covered Girl Guide biscuits (standard size)
6.00pm	Lasagne	½ cup cooked mince, 1 cup cooked Budget lasagne shaped pasta , ½ cup Wattle's creamy mushroom and herb pasta sauce, ½ cup mixed vegetables (Pam's carrots, peas and corn), 4 Tbsp. grated Edam cheese
6.30pm	Banana cake with chocolate icing (homemade, recipe attached)	1/8 of a cake (22cm diameter, 8 cm high), 2 Tbsp. chocolate icing
" "	Tip Top Cookies and Cream ice cream	1 cup (250g)
7.30pm	Coffee	1 tsp. Gregg's instant coffee 1 x 300ml cup of water 2 Tbsp. Meadow fresh blue top milk 2 tsp. sugar

Recipes (Day 1)

Date_____ DAY 1

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Sunflower Training Diary

About your injury

***5. Did you miss training this week due to an injury?**

- ◯ Yes
- ◯ No

6. What was your injury?

7. Do you think that the injury is as a result of your training?

- ◯ Yes
- ◯ No

Please describe why you think this

8. How long have you had this injury?

Illness

***9. Have you been ill this week?**

- ◯ Yes
- ◯ No

Have you had any symptoms of illness? Did you complete the cold and flu questionnaire each day you were ill?

Please click on Done
(This page will redirect to the cold and flu questionnaire just in case you need to fill it in)