School of Public Health

The Potential Role of Dietary Calcium in Obesity

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge this thesis contains no material previously published by any other person except where due acknowledgement has been made.

Signature:……..
Date:……..

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Acknowledgements of responsibilities of PhD candidate

Unless previously stated I was responsible for all aspects of the experimental protocols described in this thesis including subject recruitment, screening and monitoring of subjects, preparation of test meals and dairy products, collection of blood samples, and execution of laboratory work and its analysis.

Abstract

There is substantial evidence from cellular, animal and epidemiological studies in support of a role for calcium, and in particular, dairy foods in the regulation of weight (McCarron, 1983; Davies *et al.* 2000; Heaney, 2003a; Jacqmain *et al.* 2003; Parikh & Yanovski, 2003; Soares *et al.* 2004a; Azadbakht *et al.* 2005). These studies suggest, but do not provide, a causal link between increased calcium intake and reduced adiposity. In contrast, randomised controlled trails (RCT) are limited and their outcomes, to date, are conflicting in their findings (Zemel *et al.* 2004b; Harvey-Berino *et al.* 2005; Thompson *et al.* 2005; Zemel *et al.* 2005a; 2005b). The primary mechanism involves the control of intracellular calcium by calcitrophic hormones, vitamin D_3 and PTH. It is proposed that a higher calcium intake lowers the calcitrophic hormones, thus reducing intracellular calcium and attenuating lipid storage (Zemel *et al.* 2000). Other flow through effects may include the greater utilisation of fat as a fuel source, increased thermogenesis, increased fat excretion, improved satiety and reduced food intake. (Melanson *et al.* 2003; Sun & Zemel, 2004; Boon *et al.* 2005a; Gunther *et al.* 2005b; Jacobsen *et al.* 2005b; Melanson *et al.* 2006).

In this thesis we demonstrate that the acute ingestion of calcium, increased postprandial fat oxidation in overweight and obese humans. The results were consistent between the two sources of calcium tested (dairy and calcium citrate). Circulating levels of non-esterified fatty acids (NEFA) were less suppressed, while glycerol tended to be higher following both high calcium meals (Cummings *et al.* 2006). There was no evidence of a modulation of subjective feelings of hunger, or satiety, nor immediate food intake (buffet) or 24 hour food intake. A prolongation of the inter-meal interval was however observed in subjects consuming the high calcium meals.

A single-blind 12 week RCT, with a 12 week wash out period, compared two energy restricted (ER) diets either high (HC 1200 mg/d) or low in calcium (LD 600 mg/d). Forty overweight/obese male and female subjects were recruited for the study with 29 subjects completing both arms of the study. Anthropometric data and body

composition from DEXA were measured before, during and following each diet. There was no difference between the diets in the loss of body weight, total fat mass or trunk fat mass. A greater reduction in waist circumference of 1.23 cm was observed when subjects had consumed the HC diet; this however was just short of significance (P=0.052). There was a smaller reduction of resting energy expenditure on the hypocaloric HC diet with a trend for a greater fat oxidation at week 10 of intervention. No differences were observed between the treatment groups for fasting levels of glucose, insulin, Hb_{a1c}, LDL-C, HDL-C or TC. We also found an inverse relationship between resting metabolic rate at the start of ER and body fat lost when subjects consumed the LC diet, but not the HC diet. This is a novel finding in that it would be expected to see an inverse relationship between initial RMR and the amount of fat lost; however, the HC diet seems to achieve the same fat loss as the LC diet by taking away the effect of initial body size/composition.

Overall, the ingestion of a single meal containing 500 mg of dietary and elemental calcium has some benefits for the obese individual. Six hours post-prandially fat oxidation is stimulated following the consumption of the dietary and elemental calcium breakfast meals. During a 12-week weight loss period, a higher calcium intake did not result in a greater weight loss compared to a low calcium diet. The HC diet did result in a trend for a greater reduction in waist circumference; however, this did not transcribe into an increased loss of total or regional body fat.

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Abbreviations

ACE	angiotensin-1-converting enzyme
AMI	acute myocardial infarctions
AMP	Adenosine monophoshate
ATP	adenosine triphosphate
BAT	brown adipose tissue
BCAA	branched chain amino acids
BMI	body mass index
BMR	basal metabolic rate
Ca	Calcium
Ca/d	calcium per day
CaBP	calcium binding protein
CCK	Cholecystokinin
cm	Centimetre
CMP	Caseinomacropeptide
CLA	conjugated linoleic acid
COR	carbohydrate oxidation rate
COX	carbohydrate oxidation
CPT	carnitine palmitoyltransferase
CV	coefficent of variance
DBP	diastolic blood pressure
DEXA	dual energy x-ray absorptometry
DIT	diet induced thermogenesis
ECF	extracellular fluid
EE	energy expenditure
ELISA	enzyme-linked immunosorbent assay
FAS	fatty acid synthase
FFA	free fatty acids
FFM	fat free mass
FFQ	food frequency questionnaire
FM	fat mass
FOR	fat oxidation rate

FOX	fat oxidation
Free T3	3,5,3'-L-triiodothyronine
g	Grams
GI	glycaemic Index
GIP	glucose insulinotrophic polypeptide
GL	glycaemic Load
GLP	glucagon-like peptide
HC	high calcium (meal)
HCB	high calcium breakfast
HD	high dairy calcium (meal)
HDHF	high dairy, high fibre
HDL-C	high density lipoprotein cholesterol
HOMA	homeostatic model assessment
hr	hour
HR	heart rate
HSL	hormone sensitive lipase
IGT	impaired glucose tolerance
iPTH	intact parathyroid hormone
kg	Kilogram
kJ	kilojoule
L	Litre
LCB	low calcium breakfast
LD	low dairy calcium (meal)
LDL	low density lipoprotein
m	Metre
MET	metabolic equivalent
mg	milligram
mVDR	membrane vitamin D receptor
NA	not applicable
NEFA	non esterified fatty acids
NR	not reported
NS	non significant
nVDR	nuclear vitamin D receptor

PDH	pyruvate dehydrogenase
PPAR-γ	peroxisome-proliferator activator receptor γ
PTH	parathyroid hormone
RCT	randomised controlled trial
RDA	Recommeneded dietary allowance
RDI	recommended dietary intake
RMR	resting metabolic rate
ROI	region of interest
RQ	respiratory quotient
RR	relative risk
SAD	sagittal abdominal obesity
SBP	systolic blood pressure
SD	standard deviations
Sd-LDL	small density low density lipoproteins
SEM	standard error of the mean
STPD	standard temperature and pressure
TC	total cholesterol
TG	triaclyglycerol
UCP2	uncoupling protein-2
UHT	ultra heat treated
UR	under reporters
VAS	visual analogue scale
$VC0_2$	carbon dioxide consumed
$V0_2$	oxygen consumed
WHO	world health organisation
yr	year
11-β-HSD-1	11-β-hydro-xysteroid dehydrogenase-1
$1,25(OH)_2D_3$	1,25 dihydroxycholecalciferol or calcitriol

Chapter 1: Literature Review

1.1 Introduction

Australia is in the grip of an epidemic of obesity and the figures are forecast to rise (Ball & Crawford, 2003; The National Obesity Taskforce, 2003). This increase in the prevalence of obesity is leading to an explosion of other significant diseases, namely, Type-2 diabetes, cardiovascular disease, insulin resistance, non-alcoholic fatty liver, hypertension and some cancers (breast, endometrial and colon cancer). These increases in obesity rates are causing considerable financial and functional strain on the public health system. Coincidently, more than 50% of the adult Australian population are not achieving the recommended dietary intake (RDI) for calcium. Whether calcium intake is linked to body weight regulation and hence obesity, is the mainstay of this thesis. Currently, there is strong epidemiological evidence for the role of calcium, and in particular dairy calcium, in the regulation of body weight. However, evidence from clinical trials is still inconclusive. The backbone of this PhD thesis is a novel approach to combating obesity, insulin resistance and dyslipidemia, through the increased use of dairy calcium. While the underlying principles of this program are based on the results of cellular, animal and retrospective epidemiological data, we address our hypothesis through prospective clinical trials.

The observational statements which have provided the foundation for this PhD proposal are that:

(1) The incidence of adult obesity, glucose tolerance and dyslipidemia is high in the Australian population;

(2) simultaneously the dietary intake of calcium is below the RDI;

(3) low calcium intakes elevate circulating concentrations of the calcitrophic hormones, parathyroid hormone and 1,25 Dihydroxyvitamin D_3;

(4) calcitrophic hormones elevate intracellular calcium;

(5) high intracellular calcium (Ca^{2+}) directs the flow of dietary fat towards storage rather than oxidation;

(6) an increase in dairy calcium leads to a lower activity of fatty acid synthase (FAS) and enhances lipolysis;
(7) calcium is thermogenic.

Collectively, the above arguments lead to the following hypothesis.

1.1.1 Hypothesis

Dairy calcium will promote weight loss through increased thermogenesis and increased oxidation of ingested fat. This will result in improved glucose tolerance and decreased fasting and postprandial lipemia.

To address this hypothesis, the specific objectives of this PhD proposal are as follows:

1.1.1.1 Objectives

(1) To study the acute (meal related) effects of dairy calcium and pharmaceutical calcium on postprandial thermogenesis, substrate oxidation and lipemia in overweight and obese adults.

(2) To determine the chronic (dietary) effects of dairy calcium on body composition and fat distribution in overweight and obese adults.

1.2 An Overview of Calcium

1.2.1 The Role of Calcium in the Body

Calcium is a divalent cation with an atomic weight of 40 and an equivalent weight of 20 (Nordin, 1997). Calcium is the fifth ranked element, behind oxygen, carbon, hydrogen, and nitrogen and it makes up 1.9% of the body by weight. Nearly all of the body calcium (99%) is in the skeleton; the remainder is in the teeth (0.6%), the soft tissue (0.6%), the plasma (0.03%) and the extra-vascular fluid (0.06%). Thus body calcium may be regarded as being distributed in three compartments: firstly in the skeleton were it constitutes 25% of dry weight, the extracellular fluid (ECF) where the concentration of ionised calcium is maintained by the parathyroid / vitamin D

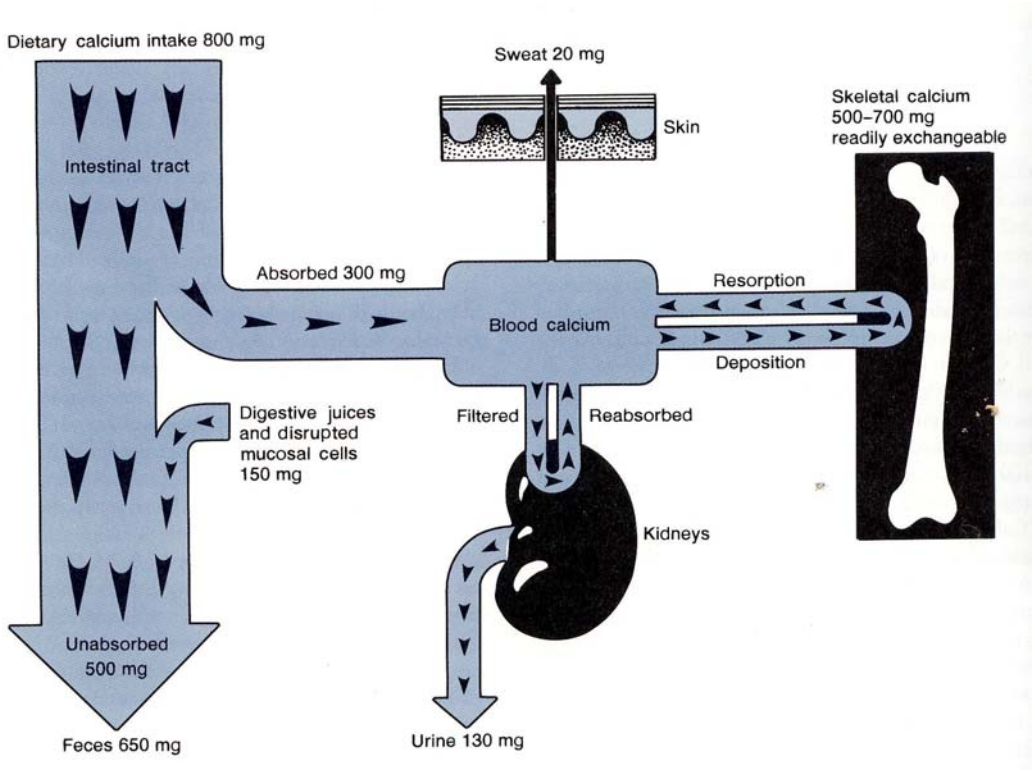
system within the range 1.15-1.25 mmol/L (equivalent to a total plasma calcium of 2.30 to 2.50 mmol/L). Thirdly, in the cellular compartment, where the total calcium concentration is comparable to that in the extracellular fluid but the free calcium concentration is ~10^{-4} mmol/L (Favus, 1999).

1.2.1.1 The Functions of Calcium

The primary function of calcium in the body is to provide human skeleton rigidity, this is provided in the form of calcium phosphate, known generally as hydroxyapatite ($Ca_{10}(OH)_2(PO_4)_6$), which is embedded in the collagen fibrils. The extracellular calcium constitutes a pool into which calcium enters from the gut, by absorption, and from the bone, by resorption. It leaves via the gastrointestinal tract, the kidneys, the skin, and into the bone for bone formation. Calcium balance in the human body is rather complex. Figure 1.1 gives a simplified diagram of the fate of a calcium intake of 800 mg. Only about 300 mg of calcium is absorbed whilst the remaining 500 mg is excreted in the faeces. The calcium that is absorbed into the blood interacts with the current body stores, the result being the excretion of 300 mg through the intestines, kidneys, and sweat to balance the amount originally absorbed. Many neuromuscular and other cellular functions depend on the maintenance of the ionised calcium concentration in the ECF. Intracellular calcium has many functions, from muscle contraction, including the heart, skeletal muscle, and smooth muscle found in the blood vessels. Calcium also activates a number of enzymes; and plays a central role in both the synthesis and breakdown of muscle glycogen and liver glycogen; helps regulate nerve impulse transmission and blood clotting. Calcium fluxes are important mediators of hormonal effects on target organs through the phosphoinositol system and are closely linked with the cyclic AMP systems. The intracellular calcium concentration is regulated by a calcium pump, which eliminates any calcium which may flow in by diffusion (Nordin, 1997).

Figure 1:1 Calcium balance in an adult requiring only 800 mg per day.

See text for discussion (Williams, 2002).



1.2.2 Absorption of calcium

Absorbability or the availability of calcium for absorption by the intestines is often used interchangeably with bioavailability. However, bioavailability depends on absorbability and the incorporation of absorbed calcium into bone. Hence, it also depends on the urinary excretion and faecal loss of endogenous calcium (Guéguen & Pointillart, 2000). As for intestinal absorption of calcium, physiological factors and in particular hormones, play a major role in the incorporation of calcium into bone. Certain types of foods, namely phosphorous increase the likelihood that calcium will be absorbed into bone, whereas, others (sulphate, chloride, chelators, excess sodium or protein) increase its excretion in urine. The bioavailability of calcium may therefore be defined as "*the fraction of dietary calcium that is potentially absorbable by the intestine and can be used for physiological functions, particularly bone mineralisation, or to limit bone loss*" (Guéguen & Pointillart, 2000).

1.2.2.1 Intestinal Absorption

Calcium must be in a soluble form, generally ionised (Ca^{2+}), at least in the upper small intestine or bound to a soluble organic molecule before it can cross the wall of the intestine. Absorption is the result of two processes, active transport across cells, mainly in the duodenum and the upper jejunum, and passive diffusion, which occurs throughout the small intestine, but mainly in the ileum and very little in the large intestine.

1.2.2.1.1 Active transport

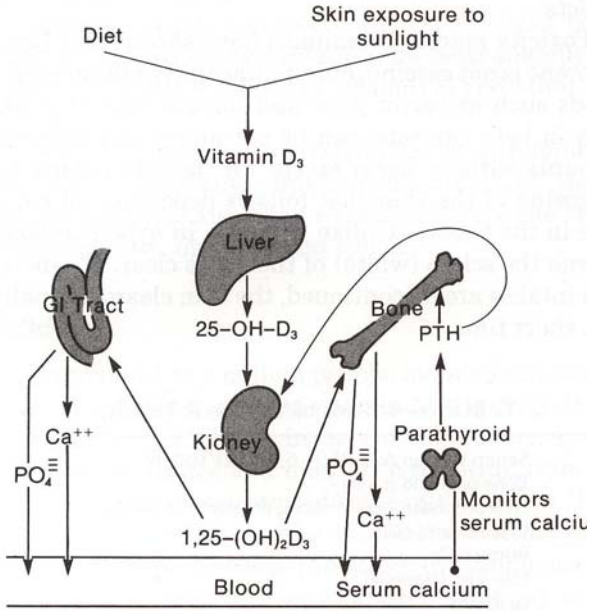
The active transport system for calcium is saturable and regulated by dietary intake and the needs of the body. Active transport involves three stages, which are: entry across the brush border of the enterocyte, diffusion across the cytoplasm and secretion across the basolateral membrane into the extracellular liquid (Bronner, 2003). Calcium enters the cell via a positive electrochemical gradient because the calcium concentration in the cytoplasm is very low. Calcium crosses the membrane via calcium channels and via membrane-binding transport proteins (calmodulin and membrane calcium-binding proteins). Calcium may be stored transiently in Golgi apparatus, endoplasmic reticulum or mitochondria, but then crosses the cytoplasm attached to a calcium binding protein (CaBP or calbindin-D 9K), which is the rate-limiting factor in active calcium transport. Calcium ions are extruded from the cell against an electrochemical gradient by two routes; a small fraction leaves by exchanging 3 Na^+ for 2 Ca^{2+}, but most leaves via a calcium pump, a Ca-ATPase activated by calcium, CaBP and calmodulin.

Vitamin D plays a pivotal part in the active transport of calcium. The active form is 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$) or calcitriol), which is produced by two hydroxylations of vitamin D. The most significant role of calcitriol is its control of the expression of the gene encoding CaBP, causing the synthesis of the protein, thereby regulating the migration of calcium across intestinal cells. Calcitriol also has a liponomic action, increasing membrane permeability and activating the Ca-ATPase (Bronner & Pansu, 1999). Calcitriol acts like a hormone and its renal production is regulated by parathyroid hormone (PTH), the secretion of which is stimulated by a fall in plasma calcium levels. The PTH-calcitriol system is also involved in bone resorption and increases the reabsorption of calcium by the renal tubule. This

hormone system therefore controls all the calcium that enters the extracellular pool of exchangeable calcium and ensures that the plasma calcium concentration varies little from 100 mg/L. Some of the membrane and cystolic proteins involved in calcium transport are not vitamin-D-dependent, one such protein is calmodulin.

Figure 1:2 The Metabolism of Vitamin D_3

Vitamin D_3 (cholecalciferol) is formed in the skin by the action of ultraviolet rays in sunlight on 7-dehydrocholesterol. Vitamin D3 is converted into its biologically active forms, 25-hydroxycholecalciferol in the liver and into its most active form 1,25 dihydroxycholecalciferol, or calcitriol in the kidney (Mahan & Arlin, 1992).



1.2.2.1.2 Passive diffusion

Passive absorption down an electrochemical gradient occurs via intercellular junctions or spaces. It involves the mass movement of water and major solutes, such as glucose and sodium. This diffusion is not saturable and therefore increases with dietary intake, providing that the calcium is in a soluble form. Any dietary component that keeps calcium in a soluble form or keeps it in solution within the ileum would stimulate passive diffusion. Several molecules can do this namely, casein, the amino acids, L-lysine and L-arginine, and lactose. Other dietary factors that make calcium irreversibly insoluble by converting it into forms such as phosphates, oxalates, phytates and soaps, prevent passive absorption in the ileum.

Of great importance is the fact that when dietary calcium intake is low, active transcellular calcium transport in the duodenum is up-regulated and a larger proportion of calcium is absorbed by the active process than by the passive paracellular process, that occurs in the jejunum and ileum (Bronner & Pansu, 2000). In contrast to this, when the dietary intake of calcium is high, passive diffusion is the main transport mechanism, as down-regulation of active transport occurs. Because of the variability of calcium intakes on absorption levels, Heaney developed the "fractional absorption rate". The fractional absorption rate is highly inversely correlated with the logarithm of the calcium load ($P<0.001$). Hence, the equation can be used to predict the actual amount of calcium that will be absorbed from a food product (Heaney *et al.* 1990). For example; a calcium load of 200 mg would result in an absorption rate of 41% and hence, 82 mg of calcium would be absorbed by the body.

Figure 1:3 Absorption Rate Equation

$$\text{Absorptive fraction (\%)} = 1.105 - 0.131 \ln^{\text{load}} \pm 0.095$$

1.2.2.1.3 Dietary & other factors influencing intestinal absorption

It is essential that the relative calcium bioavailability from the diet is studied, as it has been shown that there is a wide range of absorbance levels between individuals. One study by Wolf and associates (2000) indicated that in 142 pre- and perimenopausal women, calcium absorption values averaged 35% and ranged from 17 to 58%. Fractional calcium absorption was positively associated with body mass index, dietary fat intake, serum 1,25 dihydroxyvitamin D concentrations and parathyroid hormone concentrations, whilst being inversely related to dietary fibre intake, total calcium intake, alcohol intake and symptoms of constipation.

More recently, calcium absorptive efficiency has been singled out as being the cause of the variability in calcium balance, rather than actual calcium intake itself. There are a number of factors that can affect the absorption rate in a person. Some components of the diet can influence the availability of calcium for absorption and

the absorption mechanism itself. Some substances can form insoluble complexes with calcium, such as the phosphate ion. However, the dietary phosphorous/calcium ratio has to be very high (>3:1), and the phosphorous has to be in an inorganic form to significantly affect the absorption of calcium. Most dietary phosphorous is in animal protein and hence not available for combination with calcium. Other elements available in the diet may interfere with calcium absorption, such as phytates found in high fibre containing products; bran and most cereals and seeds, oxalates (found in spinach, rhubarb) and tannins (tea) can form insoluble complexes with calcium, thereby reducing its absorbability. However, it has been argued that phytates may not be that harmful to calcium absorption as they may be hydrolysed by phytases in the small intestine (Nordin, 1997). Calcium absorption has been reported to be improved by lactose. The mechanism for this relationship is not well understood, but may relate to calcium availability in the lumen or to transit time.

Vitamin D deficiency arising from the lack of exposure to sunlight or any other cause lowers the serum calcidiol level which ultimately lowers the calcitriol level (despite secondary hyperparathyroidism). This leads to malabsorption of calcium and, if severe and prolonged, to osteomalacia. This is probably from the loss of a direct effect of vitamin D on the calcification process. More recently a number of studies have indicated a relationship between body size and calcium absorption. In cross-sectional data of 315 girls, aged 5-15 years, overall height was significantly related to calcium absorption (corrected for calcium intake, age, Tanner stage and ethnicity) ($P=0.001$). In a similar vein, height-z score was significantly related to calcium absorption ($P<0.007$) (Abrams *et al.* 2005). In fact Barger-Lux & Heaney (2005), state that the magnitude of calcium absorption is determined by body size, such that a women 1.8 m in height would absorb 30+% more calcium from a given intake than a women 1.4 m tall.

Other factors that can affect adult calcium absorption include age, intestinal transition time and oestrogen status. (Heaney *et al.* 1989; Barger-Lux *et al.* 1995b).

1.2.3 Intake of Dairy Products

Dairy products account for over 50% of ingested calcium for men and women in Australia (Table 1:1) (McLennan & Podger, 1998). There appears to be a large variation in calcium intake amongst nations; however, this is dependent largely on their dairy product consumption. In general, it is difficult to achieve dietary calcium recommendations without consuming dairy products. Milk and other dairy products contribute substantially to the intake of other micronutrients, namely, phosphorous, riboflavin, niacin, vitamin B_6 and B_{12}, potassium, zinc, magnesium and vitamin A. Full fat dairy products will also naturally contain some vitamin D; the amount will vary depending on the season of milk production. In the United States there is mandatory fortification of milk and butter/margarine products and voluntary fortification of other foods with vitamin D; however, Australia only has a limited number of fortified milk products.

Table 1:1 Proportion of calcium intake from commonly eaten foods

(McLennan & Podger, 1998).

	Males - 19 years and over (%)	Females - 19 years and over (%)
Milk products and dishes	**51.6**	**53.0**
Dairy milk	**29.2**	**31.5**
Yogurt	**2.0**	**3.7**
Cheese	**12.1**	**11.5**
Frozen milk products	**3.0**	**2.1**
Other dishes where milk or a milk product is a major component	**1.4**	**1.6**
Flavoured milks	**3.3**	**1.7**
Other – cereal and cereals	**13.5**	**12.3**

1.2.3.1 Bioavailability of calcium: Supplements versus Dairy

It appears that there is a wide range of variation between the bioavailability of calcium from different calcium preparations. A randomised crossover study of 25 postmenopausal women demonstrated a greater bioavailability from a single dose of calcium citrate taken with a standard breakfast meal, compared to a calcium carbonate supplement (Heller *et al.* 2000). This finding has been confirmed in a meta-analysis by Sakhaee *et al.* comprising a data set of 15 studies involving 184 subjects, the comparison was made between the absorbance of calcium carbonate and calcium citrate (1999). This study concluded that calcium citrate is better absorbed than calcium carbonate by approximately 22% to 27%, either on an empty stomach or co-administered with meals. Studies seem to imply that it is the greater solubility of calcium citrate that results in a greater availability (Levenson & Bockman, 1994).

However, a number of other studies demonstrate no difference in the bioavailability between calcium citrate and carbonate. One study found an equivalent calcium bioavailability in elderly subjects when 3 high-calcium dietary sources were compared. The sources were orange juice fortified with calcium-citrate malate, skim milk, and a calcium carbonate supplement (Martini & Wood, 2002). The comparison of these calcium preparations was undertaken in 12 subjects (9 women and 3 men, age 70 $\pm$ 3 and 76 $\pm$ 6 years) and the acute response to a breakfast meal measured over a 4-hour period. Heaney *et al*.(2001) study in 24 postmenopausal women, similarly, did not find a difference between the same two supplemental preparations. These two studies used the measures of rise in serum calcium, increase in urinary calcium excretion and suppression of ionised PTH, as a marker of greater bioavailability. Furthermore, Heaney *et al*. in an earlier study using tracer methods, along with calcium supplements at high and low loads, failed to show any difference in absorption when taken with food (Heaney *et al.* 1999). This finding was confirmed in an 8-way crossover designed study in 10 postmenopausal women, given a range of calcium preparations. This study failed to find any difference in absorption, when measured using the gold standard of dual labelled stable isotope technique (Brink *et al.* 2003).

Studies tend to confirm that milk and dairy products have a very similar calcium absorption rate compared to calcium supplements (Sibtain Sheikh *et al.* 1987; Guéguen & Pointillart, 2000). It is generally well accepted that milk and milk products are by far the main source of calcium in our diet. Cow's milk contains on average 1200 mg calcium per one litre. Of this 20% is bound to casein as an inorganic colloid, the remaining 80% is in the mineral form of tri-calcium phosphate and phospho-caseinate. The organic or mineral calcium bound to casein is readily released during digestion, and therefore its bioavailability is regarded as high (Guéguen & Pointillart, 2000). Milk has additional interesting features, when compared to other food sources or supplements. Because it is bound to peptides and proteins, milk calcium is more likely to remain in solution when the pH is unfavorable, such as achlorhydria. Milk calcium may also be absorbed in the absence of vitamin D, but under the influence of lactose. This can occur in the distal small intestine via passive diffusion. Dairy products also lack inhibitors of the absorption of calcium, such as phytates and oxalates.

1.2.4 Intake of Dietary Calcium in Australia

The Australian and New Zealand Nutrient Reference Values (NRVs) (National Health and Medical Research Council, 2004) have set the RDIs for calcium intake at 1000 mg/day for men (19-70 years) and women (19-50 years); and 1300 mg/day for men >70 years and women >50 years. Data based on the 1995 National Nutrition Survey demonstrated that the mean daily calcium intake for males 19-64 years, males 64 years and over, females 19-64, and females 64 years and over was, 946, 796, 749 and 686 mg/day, (McLennan & Podger, 1998). Alarmingly, these figures indicate that over 65% of the adult population is not achieving the recommended intake for calcium. Low calcium intake can be linked to a reduction of milk consumption patterns and an increase in soft drink consumption (Huth *et al.* 2006), along with the belief that milk is fattening (Radak, 2004; Dwyer *et al.* 2005).

1.3 Obesity: Prevalence and Pathogenesis

1.3.1 Introduction

The epidemic of obesity, and its co-morbidities of dyslipidemia, insulin resistance, hypertension and cardiovascular disease, is now a global problem. The prevalence of obesity in Australia is rapidly rising; in males aged 25-64 years the percentage of men who are obese rose from 9 to 17% from 1980 until 2001. Within the same age group the prevalence of women who are obese has doubled, from 8 to 20% (Dixon & Waters, 2003). Alarmingly, at this rate it is predicted that by 2025, 30-40% of the Australian population will be obese (International Obesity Taskforce 2003). The prevalence of overweight is also staggeringly high with 48% of men and 30% of women being overweight (National Health and Medical Research Council, 1997). Obesity is considered to be one of the most significant public health burdens and is estimated to cost the state 1.3 billion dollars annually (The National Obesity Taskforce, 2003). The treatment of obesity-related coronary heart disease and hypertension contribute about 60 per cent of the cost of obesity.

With the growth in the prevalence of obesity is the increased availability of different types of diets and weight loss strategies to overcome this problem. Although the studies are small in number and subject size, it seems that 30-50% of women and ~40% of men, had dieted to lose weight in the previous year (Vogels *et al.* 2005). The most common method employed is increased exercise and reduced energy intake; however, ~20% of female dieters reported to using potentially dangerous techniques (including slimming tablets, laxatives, cigarette smoking). The long-term success of weight loss is poor, and with this lack of long-term success comes an increase in the use of pharmacological preparations, including appetite suppressants and intestinal lipase inhibitors. However, these drugs are not without side-effects and so there is continued effort to find effective and above all safe therapies to tackle the obesity problem.

The role of macronutrients and weight management has been widely studied (Wilkinson & McCargar, 2004; Astrup, 2005; Gumbs *et al.* 2005; Johnston, 2005; Keller & Rastalsky, 2005; Manco & Mingrone, 2005; Schoeller & Buchholz, 2005;

Tangney *et al.* 2005); however, the role of micronutrients has not until recently been investigated. The interest in dietary calcium has arisen from a range of epidemiological, animal and more recently human clinical studies. Heaney (2002) states that the increased consumption of calcium could have a significant effect on the management of obesity; in fact he predicts that an increase of 300 mg to recommended calcium intakes could be associated with a 1 kilogram reduction in body fat in children and 2.5-3 kilogram lower body weight in adults, leading to a 70% reduction in total overweight/obesity. If an increase in dairy and/or calcium intake is successful in its goal of aiding fat loss, it could become one of the safest and simplest ways to combat obesity. Interest is therefore high in the potential role of calcium and /or dairy in the regulation of body weight.

1.3.2 The Regulation of Body Weight in Humans

There is evidence that the obese population have impaired fat oxidation and this may be a primary event in the aetiology of obesity (Blaak & Saris, 2002). As man exists in a post-absorptive state for most of the day, this research is interested in looking at the postprandial events as they pertain to thermogenesis, and the use of different nutrients as a fuel source. Weight and body energy content remains quite stable in most adults for long periods of time, despite fluctuations in energy intake and energy expenditure (Jéquier & Tappy, 1999). To enable this balance to be maintained many regulatory processes must be involved. Some of the proposed models for energy balance include the set point theory, glucostatic controls of feedings, metabolic nutrient-partitioning, adipose tissue signalling and behavioural models (Flatt, 1995a). However, in all these approaches, genetics, the environment and psychosocial factors cannot be ignored.

1.3.3 Postprandial metabolism and nutrient partitioning

The maintenance of a constant body weight and body composition requires that energy and nutrient balances are achieved. The reasoning behind this comes from the fact that the three macronutrients (carbohydrate, protein and fat) are either oxidised or stored in their own compartment. The inter conversion of nutrients for storage does not represent important metabolic pathways (Flatt, 1988).

Energy balance is determined by the macronutrient intake, energy expenditure and partitioning in nutrient storage. Protein and carbohydrate intakes elicit powerful autoregulatory adjustments in protein and carbohydrate oxidation, while fat balance is less accurately regulated and more easily disrupted (Martinez, 2000). Most individuals reach a state of approximate weight maintenance in which the average composition of the fuels they oxidise matches the energy nutrient distribution in their diets. It was found that subjects having a higher 24-hour respiratory quotient (i.e. those tending to burn more glucose than fat) were at a higher risk of weight gain during subsequent years. It would hence follow that obesity could be avoided if one oxidised as much fat as was ingested. As a corollary, obesity may be a feature of those unable to increase their fat oxidation to match fat (Blaak & Saris, 2002). In studies by Marques-Lopez *et al.* (1998) lean and obese subjects were fed two diets of differing macronutrient content. The lean subjects showed a greater glucose oxidation and thermogenesis following the high carbohydrate diet compared to the high fat diet. However, the obese subjects were less efficient in oxidising fat on the high fat diet, and showed *de novo* lipogenesis whilst on the high carbohydrate diet. Thus the postprandial matching of substrate oxidation to the mix of macronutrients in the diet plays a crucial role in the short and long term regulation of weight. Unlike glycogen and protein stores, fat stores are not tightly regulated in the short term, i.e. an increased fat intake does not lead to an equivalent increase in fat oxidation (Swinburn & Ravussin, 1993). Instead, an increased intake of fat can lead to a positive fat balance, expanding fat stores, and leading to increased adiposity over time. An increased capacity to oxidise fatty acids is of significant value as it may help maintain fat balance when excess fat is ingested.

1.3.4 Fat balance: a key process in body weight regulation

Most epidemiological studies show a positive association between fat intake and body weight (Lissner & Heitmann, 1995). The fuel mix oxidised may also influence body weight regulation. In certain groups of sedentary individuals, high insulin sensitivity was associated with subsequent weight gain; these subjects also have a higher mean 24 hour respiratory quotient indicating an increased carbohydrate oxidation and a reduced fat oxidation rate. Thus both the excess intake of fat and the reduced rate of fat oxidation have a profound effect on the development of obesity.

Therefore mechanisms controlling fat oxidation are of great importance in the context of body weight regulation.

The rate of glucose and fatty acid oxidation is dependent on their respective availability. More than 30 years ago Randle *et al.* proposed the concept of the "glucose-fatty acid cycle" (Randle *et al.* 1963; Randle, 1998). According to this concept, the release of fatty acids from adipose tissue triaclyglycerol (or from muscle triacylglycerol) imposes a limitation on glucose metabolism, by decreasing muscle glucose uptake and oxidation, while lipid oxidation is stimulated. When plasma free fatty acid (FFA) levels increase, this mechanism favours muscle FFA uptake, and FFA compete with glucose for oxidation. The enhanced FFA oxidation produces an increased acetyl CoA to CoA-SH ratio and an increase in cytoplasmic citrate concentrations. This elevated concentration of acetyl CoA activates pyruvate dehydrogenase kinase, which phosphorylates and thus inhibits pyruvate dehydrogenase (PDH). Glucose metabolism is inhibited at two important steps, 1) the increase in cytoplasmic citrate concentrations inhibits phosphofructokinase, which results in an increased glucose-6-phosphate concentration, as a consequence, hexokinase is inhibited and finally glucose uptake is impaired. 2) Inhibition of PDH impairs the entry of pyruvate into oxidative metabolism and thus contributes to inhibit glucose oxidation.

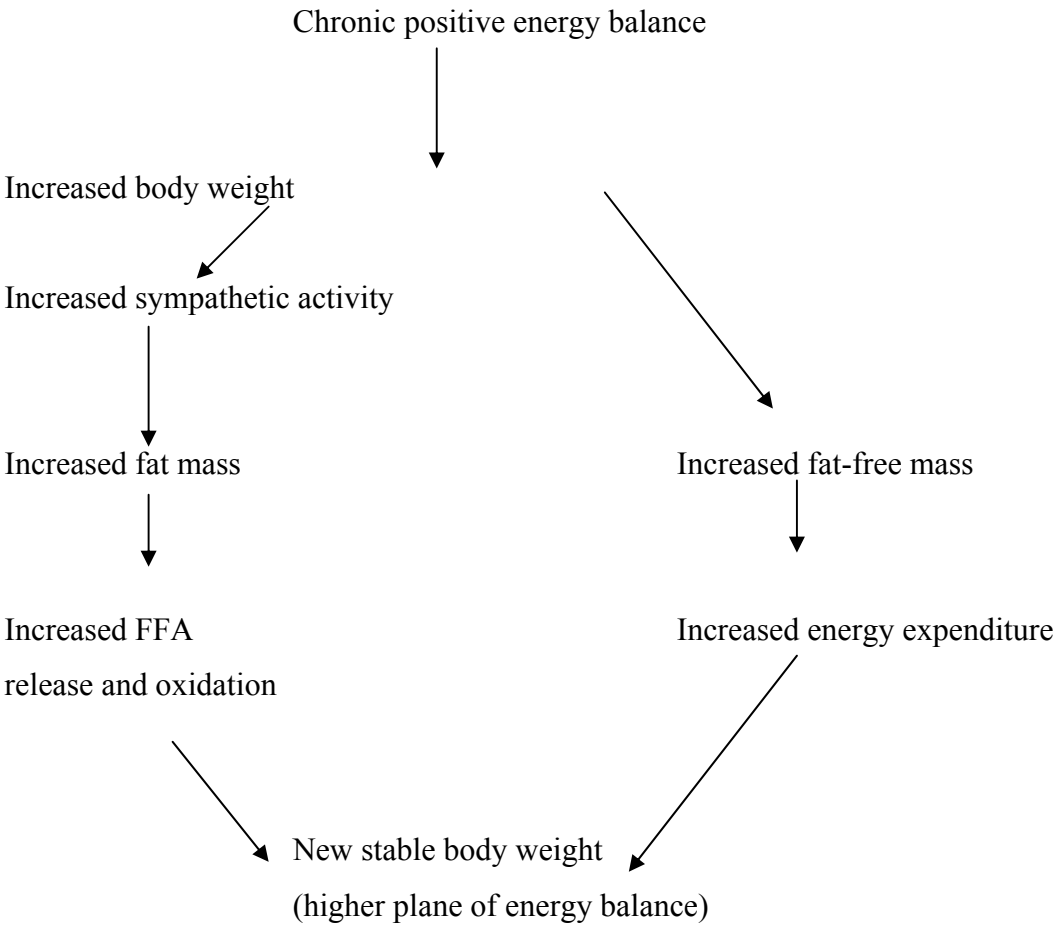
In the whole body, the total rate of fat oxidation is dependent on the concentration of plasma free fatty acids (Randle *et al.* 1963; Randle, 1998). However, the utilisation of triacylglycerol deposits in various tissues, such as skeletal muscle, also influences total body fat oxidation. A mechanism that tends to increase total body fat oxidation is the enlargement of the adipose tissue mass (Schutz *et al.* 1992). The increase in the delivery of free fatty acids into the circulation of obese subjects is not simply related to the quantity of adipose tissue. Levels of plasma free fatty acid concentration have been shown to be more elevated in abdominal obesity (Björntorp, 1991). Abdominal obesity is a condition often associated with insulin resistance and hyperinsulinemia. The issue in this situation is the presence of a systemic elevation of plasma free fatty acids associated with hyperinsulinemia, despite insulin being a very efficient inhibitor of free fatty acid mobilisation. The lipolytic driving forces in patients with abdominal obesity dominate the inhibitory action of insulin. Visceral adipose tissue

has been shown to be more sensitive to lipolytic stimuli than subcutaneous adipose tissue (Rebuffé-Scrive *et al.* 1989). Additionally, cells from visceral adipose tissues are less sensitive to the inhibitory action of insulin on lipolysis than adipose cells from subcutaneous adipose tissue. This seems to be associated with a low density of insulin receptors (Bolinder *et al.* 1996). As a result of the elevated lipolysis in visceral adipose tissue of abdominally obese, the liver is exposed through the portal circulation to excess FFA concentrations. This is known to stimulate gluconeogenesis, which depends on the oxidation of fatty acids in the liver as an energy source; the resulting increased hepatic glucose output reflects insulin resistance in the liver (Ferrannini, 1988). What is of interest is that the high insulin secretion and insulin resistance in various tissues are probably secondary to the obese state, as most data on individuals who have lost weight show a complete reversal of these phenomena.

Body weight in the obese person will eventually reach a near-constant level, in spite of an excess of energy and fat intake (Flatt, 1988). Two mechanisms have been attributed to this weight plateau, 1) The co-enlargement of the fat free mass is accompanied by an increase in BMR and, therefore an enhanced total energy expenditure (Ravussin *et al.* 1982; Prentice *et al.* 1986; Jéquier & Schutz, 1988). 2) The increase in the fat mass is accompanied by an enhanced rate of FFA release into the circulation, which acts to stimulate fat oxidation (Figure 1:4). Thus the enhanced fat oxidation observed in obese individuals in the resting state might serve as a lipostatic mechanism. This metabolic adaptation eventually allows fat oxidation to rise to a level matching fat intake, thus limiting further weight gain. Studies on the relationship between fat mass and fat oxidation shown that a 10-kg increase in fat mass corresponds to a stimulation of fat oxidation of ~20g/day (Schutz *et al.* 1992). Thus the enlargement of body fat stores serves as a mechanism that contributes to equilibrate fat balance in individuals with a chronic excess of fat intake. What needs to be determined is whether the signal for the increased fat oxidation is the rise in plasma FFA levels or a hormonal message related to adipocyte hypertrophy or hyperplasia. A positive energy balance, particularly due to carbohydrate overfeeding, also stimulates sympathetic activity, a mechanism which may contribute to increase energy expenditure (Figure 1:4).

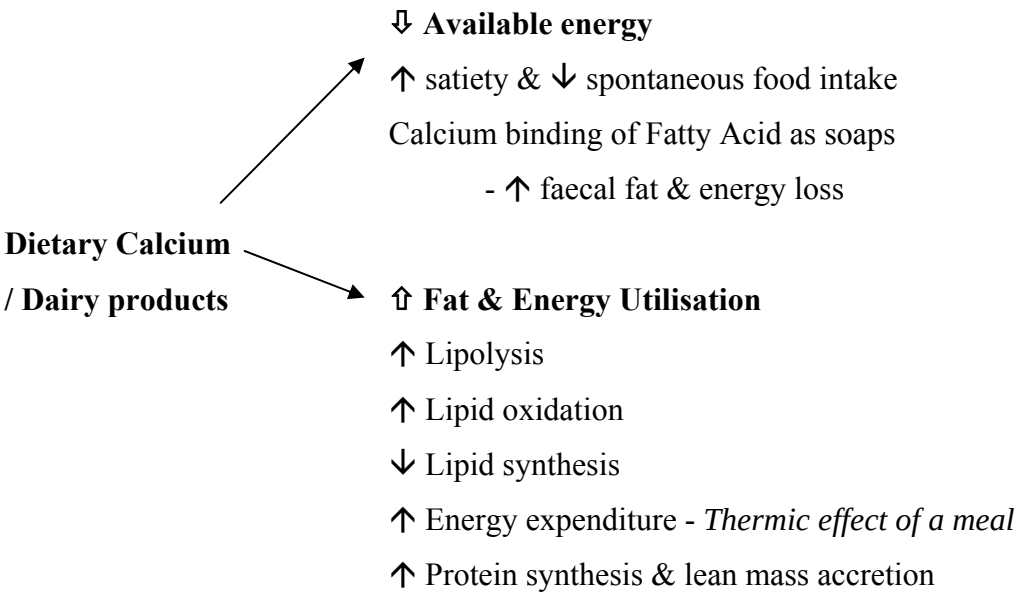
Figure 1:4 Metabolic consequences of a chronic positive energy balance on fat mass and fat-free mass.

Induced increases in free fatty (FFA) release, FFA oxidation, and energy expenditure eventually leads to a new stable body weight, resulting from a new energy balance (Jéquier & Tappy, 1999)



1.3.5 The Role for Dietary Calcium in Weight / Fat Loss

Figure 1:5 Proposed mechanisms contributing to the reduction of fat mass



(adapted from (Teegarden, 2005)

1.3.5.1 Mechanisms mediated by the Calcitrophic hormone

The link between calcium and body weight has only been fairly recently proposed. In 1984 epidemiological data (McCarron *et al.*) reported an inverse link between the intake of calcium and body weight, but it remained an unexplained finding until Zemel *et al.* (2000) reported their research on calcium and body composition. Zemel (2000) hypothesised that intracellular calcium was the key to insulin sensitivity, lipid storage and synthesis. Interestingly Zemel's theory of calcium's role in lipid metabolism came from unrelated clinical trials on hypertension, where it was observed that increasing the intake of calcium from ~400 mg/day to 1000 mg/day resulted in significant weight loss. Zemel speculated that the increasing calcitrophic hormones (PTH, vitamin D_3) secondary to a low calcium intake may stimulated adipocyte calcium influx, which in turn stimulates fatty acid synthase activity and hence lipid storage (2000). If this is true, increasing calcium intake should lower calcitrophic hormones, reduce intracellular calcium and attenuate lipid storage. It

has been hypothesised that high-calcium diets protect against fat gain by creating a balance of lipolysis over lipogenesis in adipocytes.

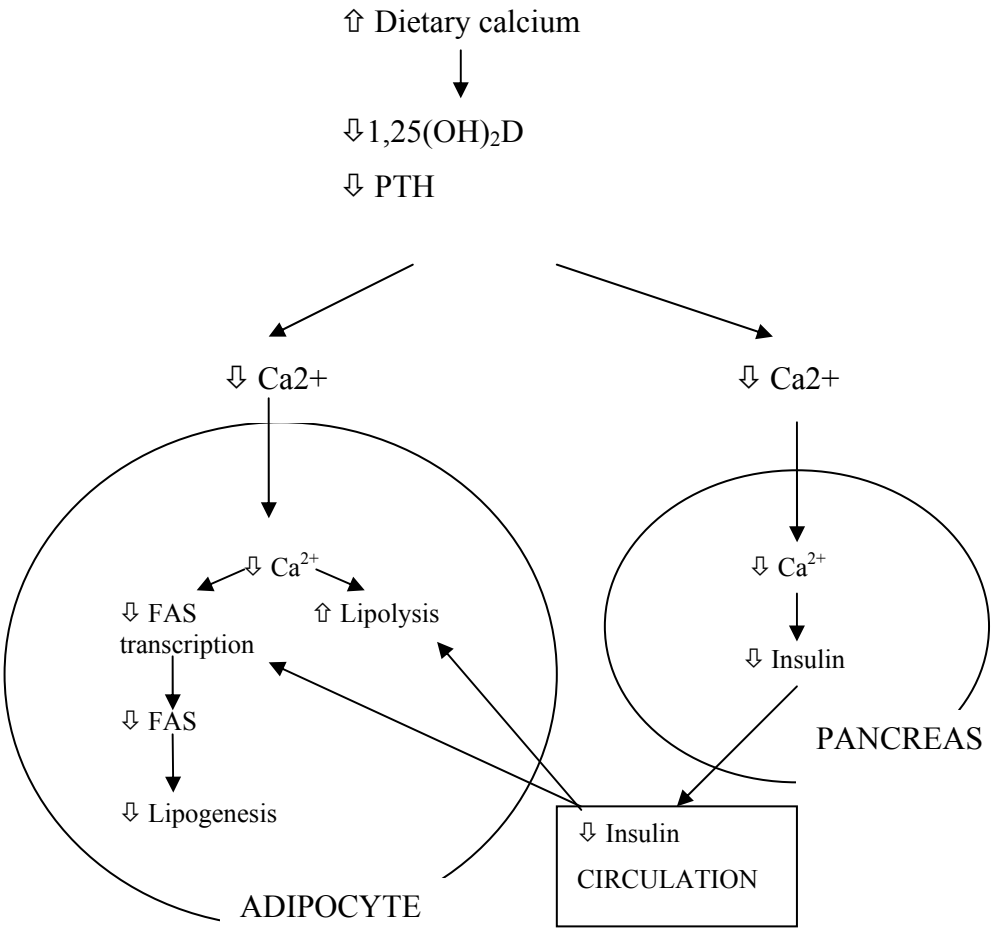
In support of the mechanisms mediated by calcitrophic hormones, data from adipocyte culture studies indicate that alterations in intracellular calcium concentration may regulate fat metabolism. In vitro, the intracellular influx of calcium, mediated either by parathyroid hormone (PTH) or 1,25-dihydroxyvitamin D, was shown to inhibit lipolysis and increase lipogenesis in adipocytes (Zemel *et al.* 2000). Human subcutaneous adipose tissue, in response to 1,25-$(OH)_2$-D or parathyroid hormone, showed an increase in intracellular Ca^{2+}. 1,25-$(OH)_2$-D treatment also resulted in a 83% inhibition of forskolin-stimulated lipolysis ($P<0.001$). Similar observations have been made in the aP2-*agouti* transgenic mouse model. These mice were energy-restricted for 6 weeks where they followed either a high dairy (HD) or a low dairy (LD) diet. They exhibited a 79% decrease in intracellular adipocyte calcium levels (380 $\pm$ 43 (HD) vs 79 $\pm$ 10 (LD) mmol/L). Upon refeeding, the low calcium diets resulted in a 4- to 5-fold increase in adipocyte $[Ca^{2+}]i$, whereas there was no increase in adipocyte $[Ca^{2+}]_i$ among the mice fed the high-calcium diets ($P<0.001$) (Sun & Zemel, 2004).

Conversely, the inhibition of PTH and/or calcitriol caused by a higher calcium intake and hence a lower intracellular calcium level was the explanation for fat loss due to greater lipolysis. This was supported by work from Draznin's group which showed greater intracellular calcium levels in the adipocytes of obese subjects versus lean subjects (1988a). Further support of this mechanism comes from a clinic study whereby patients with primary hyperparathyroidism, a condition in which both serum PTH and calcitriol concentrations are elevated, have increased body weight (Bolland *et al.* 2005).

Figure 1:6 represents an overview of the mechanisms proposed by Zemel *et al.* (2000).

Figure 1:6 Mechanisms whereby Calcium may play a role in reducing fat deposits.

An increase in dietary calcium reduces 1,25-dihydroxyvitamin D [1,25(OH2)D] and PTH concentrations, resulting in down-regulation of calcium transfer into adipose and pancreatic cells. Inside adipocytes, a reduction in intracellular calcium leads to decreased fatty acid synthase (FAS) transcription that results in a lowering of lipogenesis and increased lipolysis. In pancreatic cells, reduced intracellular calcium concentrations decrease insulin output, which results in reduced lipogenesis and enhanced lipolysis in adipocytes. In combination, these processes would help reduce fat deposition into and storage in adipose tissue. (Adapted from (Zemel *et al.* 2000; Zemel, 2003a).

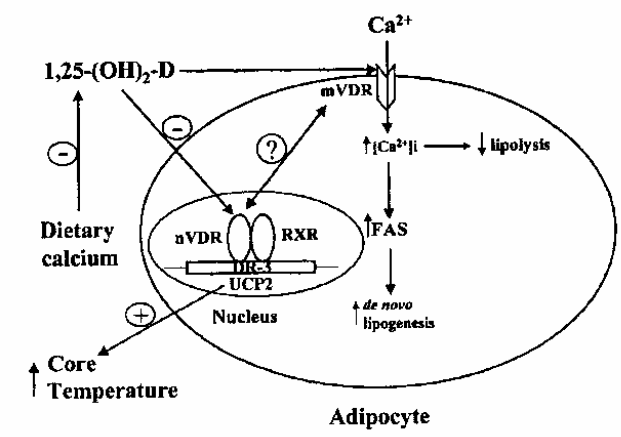


1.3.5.2 A specific role for Vitamin D and its effect on Calcium in Obesity

Serum $1,25(OH)_2D_3$ is an important regulator of a large number of genes (DeLuca & Zierold, 1998), and many of these genes are either related to fat breakdown and storage; namely, hormone sensitive lipase (HSL), glycerol phosphate dehydrogenase and fatty acid synthase (FAS) or adipocyte differentiation (peroxisome-proliferator activator receptor γ (PPAR-γ) (Barbe *et al.* 1998; Large *et al.* 1998; Duque *et al.* 2004). It is well documented that $1\alpha,25\text{-}(OH)_2\text{-}D_3$ generates biological responses via both the genomic and non-genomic pathway. $1\alpha,25\text{-}(OH)_2\text{-}D_3$ generated genomic actions via the binding to a specific nuclear hormone receptor, nuclear vitamin D receptor (nVDR) (Figure). Moreover $1\alpha,25\text{-}(OH)_2\text{-}D_3$ generates rapid, nongenomic signal transduction, including modulation of calcium channels, via a putative membrane mVDR in a wide variety of cells (Shi *et al.* 2002). Zemel's data has previously reported that $1\alpha,25\text{-}(OH)_2\text{-}D_3$ stimulates adipocyte $[Ca^{2+}]_i$, promotes lipogenesis, and inhibits lipolysis via a rapid nongenomic action. Work by Shi *et al.* (2002) further demonstrated that $1\alpha,25\text{-}(OH)_2\text{-}D_3$ exerts an inhibitory effect on adipocyte UCP2 expression via a genomic action. Papakonstantinou's group (2003b) support the work of Zemel *et al.* in that male Wistar rats fed control versus high calcium diets for 85 days resulted in significant reductions in serum $1\alpha,25\text{-}(OH)_2\text{-}D_3$, following the ingestion of the high calcium diets.

Figure 1:7 1α,25-(OH)2-D3 plays an important role in modulating adipocyte lipid metabolism and energy homeostasis via genomic and nongenomic actions

(Shi *et al.* 2002).



The available data suggests that 1α,25-$(OH)_2$-D_3 exerts an inhibitory effect on white adipocyte basal, isoproterenol and fatty acid-stimulated uncoupling protein-2 (UCP2) expression. Thus, suppression of 1α,25-$(OH)_2$-D_3 and consequent up-regulation of UCP2 may contribute to the increased thermogenesis in mice fed high-calcium diets. Furthermore this effect along with a decreased lipogenesis and increased lipolysis secondary to a decreased $[Ca^{2+}]i$ may contribute to the anti-obesity effect of dietary calcium. 1α,25-$(OH)_2$-D_3 has also been shown to promote the expression and secretion of lipoprotein lipase in cultured adipocytes (Querdeld *et al.* 1999), which indicates that 1α,25-$(OH)_2$-D_3 may enhance adipocytes lipid accumulation by increasing fatty acid uptake. A number of studies have also indicated a role for 1α,25-$(OH)_2$-D_3 in human obesity. Ye *et al*. reported that nVDR gene polymorphisms are associated with the susceptibility to obesity in humans with early-onset type II diabetes. Ye *et al*. study states two single polymorphisms in nVDR gene intron 8 and exon 9 account for a 9-kg body weight difference, or 4 kg/m^2 (2001). Likewise, human body weight and body mass index have been shown to be associated with a restriction site polymorphism in nVDR gene (Barger-Lux *et al.* 1995a). Several studies have also demonstrated changes to the vitamin D-endocrine system in obese humans, with an increase in circulating 1α,25-$(OH)_2$-D_3 level (Bell *et al.* 1985; Andersen *et al.* 1988). In addition two studies have indicated that 1α,25-$(OH)_2$-D_3-induced hyperinsulinemia may also be involved in the development of obesity in humans, as 1α,25-$(OH)_2$-D_3 has been shown to stimulate insulin release in pancreatic β-cells (Norman *et al.* 1980; Tanaka *et al.* 1986). However, in a cross-sectional human study by Parikh and colleagues (2004), reported a negative relationship between serum 1,25-vit D and adiposity in obese subjects. These authors concluded that an elevation of 1,25 vit-D is unlikely to be an important hormonal mechanism for maintaining obesity in adults.

In summary, animal studies indicate that antagonism of 1α,25-$(OH)_2$-D_3 on its genomic and/or non-genomic pathways may offer a promising approach for obesity management. Dietary calcium suppression of 1α,25-$(OH)_2$-D_3 levels have been shown in several studies (Sanchez *et al.* 1997; Resnick, 1999). Data from Zemel's animal studies demonstrate that suppression of 1α,25-$(OH)_2$-D_3 by increasing dietary calcium decreased adipocyte intracellular Ca^{2+} concentration, stimulated lipolysis, inhibits lipogenesis and increased adipocyte UCP-2 expression and core temperature

in aP2-*agouti* transgenic mice. Dietary calcium not only attenuated diet-induced obesity but also accelerated weight loss and fat mass reduction secondary to calorie restriction (Zemel *et al.* 2000; Shi *et al.* 2001a).

A clear mechanistic pathway between high calcium intake, intracellular calcium levels and weight loss is however less clear in humans. It has been demonstrated that obese people have greater intracellular Ca^{2+} concentration levels than non-obese, age and sex matched individuals (Draznin *et al.* 1988b). Furthermore intracellular Ca^{2+} concentration levels have been shown to regulate both lipogenesis and lipolysis in human adipocytes (Zemel *et al.* 2000) and the mouse model (Papakonstantinou *et al.* 2003b; Sun & Zemel, 2004), through regulation of the calcitrophic hormones (PTH and vitamin D_3). Patients with hyperparathyroidism (elevated PTH and calcitriol levels) have been shown to be heavier (Bolland *et al.* 2005) and elevated vitamin D levels in humans have been linked with obesity (Norman *et al.* 1980; Bell *et al.* 1985; Barger-Lux *et al.* 1995a; Ye *et al.* 2001). However, there is no whole body evidence that higher intakes of calcium directly influence intra-cellular calcium in humans.

1.3.5.3 Dietary Calcium and its role in Fat Oxidation

Of great interest is whether an increased intake of calcium can affect substrate utilisation towards a greater use of fat as a fuel source. Zemel's animal studies have investigated this by examining liver carnitine palmitoyltransferase (CPT) activity to determine whether the changes in adiposity could be contributable to shifts in liver fat oxidation. In the refeeding phase of a study, aP2-transgenic mice where fed *ad libitum* basal (low calcium) diets, or high (cereal) calcium, high (milk) calcium, high (yoghurt) calcium or a cereal-based control diet. The basal (low calcium) and cereal (low calcium) diet slightly increased liver CPT activity (44 and 56%), whereas the high-calcium diets increased CPT activity by 150% ($P<0.05$) (Sun & Zemel, 2004).

Zemel's group have also demonstrated increases in lipolysis in the animal model, as measured by incubation of perirenal adipose tissue and measuring the ensuing glycerol release into the culture medium (Sun & Zemel, 2004). When the mice were a) energy restricted and fed either a basal (low-calcium) diet versus a high-calcium cereal meal, lipolysis was decreased by 20% ($P<0.05$) and b) in a refeeding situation

the mice exhibited significantly greater lipolysis on the high-milk-calcium diet versus the low-calcium diet ($P<0.03$). Inversely, FAS activity and expression was decreased in the high-calcium diets. In the energy-restricted mice fed the high-calcium cereal diet FAS activity and expression reduced by 91 and 99%, respectively. Upon refeeding, the high-calcium diet fed mice exhibited lower adipose FAS activity and expression, whilst the high-dairy-calcium fed mice had even lower FAS activity and expression than the high-calcium diet fed mice ($P<0.001$).

In a cross-sectional study, Melanson et al. (2003) showed a positive correlation ($r=0.38$, $P=0.03$) between acute calcium intake and fat oxidation using whole-body room calorimetry. Moreover, in a prospective study of energy restriction, these authors showed a greater 24 hour fat oxidation following the short-term consumption of high calcium intakes (Melanson *et al.* 2005). One long-term study has also demonstrated a greater fat oxidation rate in subjects who followed a high calcium diet for a year (Gunther *et al.* 2005b). In contrast, Jacobsen et al. (2005) manipulated calcium and protein intakes over 2 week periods and did not find any change in 24 hour energy expenditure nor fat oxidation between diets. Likewise, in a study of 20 healthy men where diets were manipulated for 7 days no significant difference in fat oxidation rates over 24 hours was obtained, between high dairy/high calcium, high calcium/low dairy and low calcium/low dairy (Boon *et al.* 2005a). Furthermore, Melanson's group have recently published the findings from a short-term study which contradicts their original findings, in that a greater lipolysis and/or fat oxidation was not demonstrated following the 7-day consumption of a high versus a low calcium diet (Melanson *et al.* 2006)(Table 1:9). Clearly, some confirmation of findings is required. In this thesis, we study the acute (single meal) effects of calcium from dairy and non-dairy sources on energy metabolism, as part of the focus.

1.3.5.4 Dietary calcium and Thermogenesis

Since the initial discovery of the brown adipose tissue (BAT) mitochondrial uncoupling protein (UCP), namely UCP2, much interest has gathered and research into the role in energy metabolism. Since lipogenesis is dependent upon mitochondrial ATP production (Kopecky *et al.* 2001), mitochondrial uncoupling in adipocytes may contribute to control of lipid metabolism. Work from Zemel's

laboratory (discussed above) has demonstrated a role for intracellular calcium ($[Ca^{2+}]_i$) in regulating adipocyte energy metabolism (2000). The work in human adipocytes and *agouti*-transgenic mice has demonstrated that an increase in ($[Ca^{2+}]_i$) stimulates the expression of and activity of fatty acid synthase (FAS), a key enzyme in *de novo* lipogenesis, and inhibits basal and agonist-stimulated lipolysis in both human and murine adipocytes (Xue & Zemel, 2000; Shi *et al.* 2001a). Furthermore Zemel's group demonstrated in 3T3 L1 adipocytes using mitochondrial uncouplers; that, mitochondrial uncoupling increases ($[Ca^{2+}]_i$) and down-regulates lipolysis via a calcium-dependent mechanism. Alternatively, mitochondrial uncoupling up-regulates FAS expression via a calcium-dependent mechanism, and suppression of FAS activity via a Ca^{2+}-independent mechanism (Sun & Zemel, 2003).

In the aP2-*agouti* mouse study by Sun and Zemel (2004), obesity was induced by a high-fat/high-sucrose diet followed by energy restriction on a high-calcium diet. The animals were then provided *ad libitum* basal (low calcium) diets, or high (cereal) calcium, high (milk) calcium, high (yoghurt) calcium or a cereal control diet. The animals on the low-calcium diet regained all the weight whereas the high-calcium diet animals reduced fat gain by 55%. All the high-calcium diets stimulated adipose tissue uncoupling protein (UCP2) and skeletal muscle UCP3 expression ($P<0.001$). Interestingly, only the high dairy diets showed a marked (>10-fold, $P<0.001$), increase in skeletal muscle peroxisome proliferator-activator receptor-α expression (PPAR-α) (a transcriptional factor that regulates cellular lipid metabolism). These mice also exhibited some trend towards an increase in core temperature (an indirect metabolic index associated with thermogenesis) in the high-calcium fed mice. This increase was demonstrated without a difference in food intake during the refeeding and is suggestive of a shift in energy metabolism from energy storage to thermogenesis.

Shi *et al.* (2001b) fed aP2-*agouti* transgenic mice a medium-dairy or a high-dairy diet. They observed that the animals core temperature was raised by 0.48, 0.57 and $0.67^{o}C$, respectively ($P<0.05$) across the 3 groups. Further evidence of changes in energy metabolism was further demonstrated by the increase in PPAR-α, which was 10-fold higher in the mice refed with high dairy-calcium diets compared with the

low-calcium diets. Similarly mice refed the high-calcium diets exhibited significantly lower adipocyte PPAR-γ expression than those refed the low-calcium diets ($P<0.03$), suggesting that dietary calcium may also inhibit fat mass regain by suppressing adipogenesis (Sun & Zemel, 2004).

1.3.6 The Control of Food Intake

1.3.6.1 Hunger, Satiation and Satiety

Most people who maintain a stable body weight spontaneously adapt their energy intake to a large range of energy expenditures through accurate mechanisms of control of food intake. Appetite involves a complex sequence of interactions amongst the peripheral and central mechanisms. The gastrointestinal tract contains chemo-and mechanoreceptors that relay the information about its nutrient content to the brain mainly via the vagus nerve. Impairment of appetite or satiety may arise from peripheral or central mechanisms. The amount of energy ingested over 24 hours depends on two major variables: the size of individual meals and the frequency with which meals are ingested. These two variables are regulated by distinct mechanisms. Hunger can be defined as the sensation felt by an individual that drives them to search for, and ingest food. This sensation is elicited after a variable period following the absorption of the nutrients ingested with the previous meal. Although its mechanisms remain poorly understood, it has been observed that a light drop in plasma glucose concentration precedes the initiation of food intake in both rats and humans. After the ingestion of a certain amount of food, a suppression of hunger occurs that will lead to the termination of food intake. This process is referred to as satiation, and the mechanisms that underlie it are the major determinants of meal size. The time of satiation is followed by a period of variable duration that is characterized by the absence of hunger; this is referred to as satiety. Termination of the period of satiety coincides with resurgence of the feeling of hunger, leading to consumption of the next meal, thus resuming the cycle of food intake. The mechanisms that promote satiation are different from those that determine the duration of satiety. Thus meal size and meal frequency are controlled by different factors.

The overall process of food intake control is governed by complex mechanisms. The macronutrient composition, size, and caloric density, along with their organoleptic properties (sight, smell, taste and texture), play an important role in the determination of satiation. In has been shown that subjects that are voluntarily energy restricted or energy overfed to lose and gain weight, respectively; once they are resumed on an *ad libitum* diet, initial body weight is soon reinstated. Thus, it is clear that whilst nutrient composition is important to regulation food intake, chronic body weight and composition play a more influential role in the control of food intake. What is becoming more evident is that food intake is now becoming more linked to social activities, and not necessarily linked to the hunger response.

1.3.6.1.1 Effect of Nutrients on Food Intake

The influence of nutrients on subsequent food intake has been extensively studied by providing blinded meals with differing food composition or energy content and observing the changes in the subsequent food composition of the next meal. It has generally been observed that an acute deficit in energy intake is rapidly compensated in the subsequent meals. Although the results of numerous studies are not conclusive, it is the general opinion that if one of the major macronutrients is in deficit at one meal, there is not a compensatory increase in the intake of that macronutrient but rather a compensatory ingestion of an equivalent number of calories from a mixed meal (Blundell, 1995; Blundell *et al.* 1995; Blundell *et al.* 1996). In contrast, excessive intake of nutrients generally decreases subsequent food intake. There is a hierarchy regarding the ability of the various macronutrients to suppress subsequent food intake. It is well-documented that kJ for kJ, protein is more satiating than carbohydrate or fat (Robinson *et al.* 1988; Hill & Blundell, 1990; Blundell *et al.* 1995; Stubbs *et al.* 1996; Hellstrom & Naslund, 2001; Hu, 2005). Evidence also suggests a hierarchy in macronutrient oxidation rate during the postprandial state with the sequence protein > carbohydrate > fat (Flatt, 1988; Flatt, 1995b; Stubbs *et al.* 1995). This line of research has therefore lead to the suggestion that a high satiating efficiency of a macronutrient may be related to its oxidation rate. It has also been documented that different protein sources have a different satiating capacity, with whey protein shown to be more satiating than casein (Hall *et al.* 2003). This is

of particular interest with regards the dairy and weight loss story, due to milk and milk-containing products having a higher whey content (see discussion below).

1.3.6.1.1.1 Dairy intake and it's effect on Food Intake

It has been suggested that it is the whey protein content of milk and dairy products that may regulate food intake. It was first suggested by Mellinkoff (1956) through his *aminostatic concept*, that the digestion and absorption of proteins could influence their satiating properties. The digestion and absorption of whey and casein differ quite considerably. Whey protein rapidly enters the jejunum in the form of intact protein, whereas casein is slow to appear and is mainly in the form of degraded peptides. This difference between the two proteins is mainly due to the greater clotting of casein in the stomach, giving it larger exposure to the gastric peptic hydrolysis (Mahe *et al.* 1996). As a result the over-all gastric emptying time for casein is longer and there is a smaller postprandial increase in plasma amino acids compared with non-coagulating whey protein. Whey proteins therefore elicit a greater satiating power as this protein empties faster from the stomach and has a higher postprandial excursion of plasma amino acids.

The data of Hall *et al*. (2003) confirms some aspects. A whey preload led to greater AA levels compared to casein, though clear cut differences in gastric emptying (as judged by paracetamol absorption test) could not be confirmed. Importantly, buffet meal food intake was significantly suppressed following the whey pre-load (Hall *et al.* 2003).

Another possible effect of whey proteins on satiety is the postprandial release of the hormone cholecystokinin (CCK). An increase in CCK has been associated with a meal-induced satiety. This has been demonstrated by Schneeman *et al.* whereby 24 men and women who were given either a high dairy or non-dairy breakfast meal (providing the same energy value and similar polyunsaturated to saturated fat ratios), consumption of the high dairy meals resulted in an enhanced CCK response (2003). This was also demonstrated by Hall *et al.* in subjects fed isocaloric liquid breakfast meals, the high whey meal elicited a 60% greater postprandial plasma CCK increase

(2003). In this study it was also demonstrated that the whey pre-load also increased two other hormones known to contribute to satiety, namely glucose-dependent insulinotrophic polypeptides and glucagon-like peptide-1, greater than the casein pre-load. One other proteolysis product that is important to food intake is caseinomacropeptide (CMP). CMP is the first digestion product of casein to be released from the stomach (Ledoux *et al.* 1999). It has been suggested that CMP may act as an appetite suppressant, in rats (Pedersen *et al.* 2000), CMP has been demonstrated to stimulate pancreatic secretion (a marker of CCK release) and in humans it has been reported to a potent stimulant of CCK release; however, clinical studies are limited (Gustafson *et al.* 2001).

If dairy products are to have an effect on weight reduction through mechanisms of greater satiety it would be anticipated that less food be ingested following the consumption of a high dairy meal. In our laboratory we have demonstrated a significant reduction in food consumption of human subjects following a high dairy, high vitamin D breakfast meal (HCB) versus a low dairy, low vitamin D meal (LCB). The differences in consumption were -444 kJ consumed during an *ad libitum* buffet meal (immediately post-testing), this difference widened to -765 kJ at the evening meal and there was less food consumed 24 hours after the experiment on the HCB (7143 $\pm$ 431 kJ vs. 8484 $\pm$ 699, $P<0.02$) (Ping-Delfos *et al.* 2004). Findings by Barr *et al*. (2000) have also hinted at a compensatory decrease in energy intake following dairy intake. In their randomised, controlled open trial, 101 subjects were advised to increase their intake of skim or 1% milk intake by 3 serves per day and compared to subjects advised to maintain their usual intake. This protocol was followed for 12 weeks. The milk group gained 0.6 kg more than the control group ($P<0.01$) but interestingly, the predicted weight gain due to an increase in energy intake of 200 to 250 kcal/day (836–1046 kJ) over a 12-week period should have been of least 2.5 kg.

Besides the other factors discussed in this thesis, food intake may be an additional facet of the calcium/dairy story. We therefore wished to examine the effects of calcium (pharmaceutical) and dairy calcium on feelings of hunger/satiety and subsequent food intake, as part of our studies.

1.3.7 Change in Fat Absorption

Another proposed mechanism by which calcium may impact body weight is that increased dietary calcium seems to bind more fatty acids in the colon, thereby inhibiting fat absorption. Welberg *et al*. in a small 1994 study (1994), showed that calcium supplementation increased the percentage of excretion of total fat as related to fat intake. The calcium supplementation in this study was either 2 or 4 g. Denke *et al.* (1993) supplemented 13 men with approximately 2 g of calcium per day. The percentage of dietary fat excreted per day increased from 6% to 13% with calcium supplementation. The Nestle Company supplemented chocolate with 900 mg of calcium and tested the faecal fat content of 10 men in a double-blind crossover study (Shahkhalili *et al.* 2001). Calcium supplementation of chocolate increased faecal fat from 4.4 to 8.4 g per day ($P < .0001$). These studies show a small effect of calcium on fat absorption that probably contributes to the antiobesity effects but does not explain it entirely. The degree of faecal fat loss in these studies of high calcium supplementation is only approximately 3% of that induced by medications such as orlistat.

1.4 Evidence to date: The Role of Dietary Calcium in Weight Loss

1.4.1 Introduction

Since the early work by Zemel and the initial epidemiological study by McCarron (1984) there has been much interest in the area of dietary calcium and weight loss. There are now at least 18 review studies published (Zemel, 2001; Heaney *et al.* 2002; Zemel, 2002; Barr, 2003; Parikh & Yanovski, 2003; Teegarden & Zemel, 2003; Zemel, 2003a; Zemel, 2003b; Elwood *et al.* 2005a; Harkness & Bonny, 2005; Harris, 2005; Ilich, 2005; Sakhaee & Maalouf, 2005; St-Onge, 2005; Teegarden, 2005; Zemel, 2005b); only two of which are systematic reviews (Barr, 2003; Trowman *et al.* 2006). However, what many of these authors agree on is that there is a lack of well-controlled randomised controlled studies of dairy and/or calcium-containing products on weight loss. The next section will discuss the evidence to date from

animal studies through to cross-sectional and longitudinal data to randomised controlled trials (refer to Tables 1:4-1:9). Each study has been graded on the National Health and Medical Research Council levels of evidence (2000) (Table 1:2). In a review of the data, expert consensus statements and evidence from experimental studies with animals and/or cells may provide valuable information and are given a rating of level V.

Table 1:2 National Health & Medical Research Council - levels of evidence

NHMRC level of evidence	
I	**Evidence obtained from a systematic review of level II studies**
II	**Evidence obtained from at least one properly designed randomised controlled trial**
III-1	**Evidence obtained from well-designed, pseudo-randomised controlled trials**
III-2	**Evidence obtained from comparative studies with concurrent controls and allocation not randomised, cohort studies, case-control studies or interrupted time series with a control group**
III-3	**Evidence obtained from comparative studies without concurrent controls, a historical control study, two or more single arm studies, or an interrupted time series without a control group**
IV	**Evidence obtained from case series, either post-test or pre-test/post-test**
V	**Evidence provided by expert consensus statements, experimental animal and cell studies**

1.4.1.1 Animal Studies

The first animal studies to be conducted came from Metz and colleagues, and demonstrated a reduction in body fat mass in 2 strains of hypertensive rats with higher calcium intake (1988). Spontaneously hypertensive rats and Wistar Kyoto rats were randomised to 2%, 1% and 0.1% calcium ($CaCO_3$) intakes, they exhibited a significant reduction in body weight ($P<0.05$) between the high and low calcium diets (evidence level-NHMRC V-animal study).

Much of the knowledge to date regarding the role of dairy and/or calcium has come from Zemel's work with the aP2-*agouti* transgenic mouse. Agouti is an obesity gene expressed in both rodents and human adipocytes, these mice exhibit a normal pattern of leptin expression and activity similar to that found in humans and exhibit a human pattern (adipocyte-specific) of *agouti* expression. These mice are a useful model to use as they are not obese when consuming a standard A1N-93G diet, but develop mild-to-moderate obesity when fed high-sucrose and/or high-fat diets (Zemel *et al.* 2000). In a three-phase study obesity was induced in these *agouti*-mice with a high-fat/high-sucrose diet (phase I), energy was then restricted using a high-calcium (1.3%) diet for 6 weeks to induce fat loss (phase II). After this 6 week period the mice group (n=50) were randomised into 5 groups and given free access to either a) a low-calcium (0.4%) diet b) a high-calcium (1.3%) cereal, c) milk plus the high-calcium cereal (1.3% calcium), d) yoghurt plus the high-calcium cereal (1.3% calcium) or e) a cereal-control diet (0.4% calcium) for 6 weeks. Re-feeding of the low-calcium diet to the mice caused a regain of all the weight and fat, whereas the high-calcium diets reduced weight gain by 55% ($P<0.01$) (Sun & Zemel, 2004) (evidence level-NHMRC V-animal study). Interestingly, fat pad weight was significantly affected in that the low-calcium diets had a 487% increase in fat pad mass, further the dairy-based, high-calcium diets were more effective than the high-calcium cereal diets, as the high-dairy diets prevented 85% of the fat gain compared to preventing 55% of the fat regain in the high-calcium diets.

Shi *et al.* (2001b) have fed agouti-mice with low calcium (0.4%) or high calcium ($CaCO_3$, 1.2%), or medium or high dairy calcium (non-fat dry milk, 1.2% or 2.4% calcium) diets. Energy restriction on the low-calcium diet resulted in an 11% body weight loss ($P<0.001$), compared with the *ad libitum* control (evidence level-NHMRC V-animal study). However, much greater weight reductions of 19%, 25% and 29% were observed in the high-calcium, medium-dairy and high dairy groups, respectively ($P<0.01$, vs. basal energy-restricted group). Consistent with these findings, the energy-restriction caused only an 8% decrease in fat pad mass on the low-calcium diet (NS), compared to the basal diet *ad libitum* group. However, on the energy-restricted diet with the high-calcium intake a 42% fat pad mass decrease was observed ($P<0.001$), which was further reduced by 60% and 69% for the medium and high-dairy groups, respectively ($P<0.001$, versus the basal energy-restricted group).

Similar results were reported by Papakonstantinou (2003b), whereby male Wistar rats were fed a control versus a high calcium diet for 85 days. It is important to note that unlike the Agouti mouse used by Zemel's group, the Wistar rat does not have any genetic mutations that have the potential to modify energy balance, but it is susceptible to dietary obesity (Papakonstantinou *et al.* 2003a). Rats fed the high-calcium diet gained less weight than the control (310 $\pm$ 10 g versus 335 $\pm$ 12 g, P=0.04), and had 29% less body fat than the controls. This finding was; however, contributable to the greater faecal fat content and hence significantly less digestible dietary energy provided to the high-calcium diet fed mice (evidence level-NHMRC V-animal study).

Not all findings from animal studies are in agreement. Zhang and Tordoff (2004) (evidence level-NHMRC V-animal study), have demonstrated no effect of differing concentrations of calcium in normal weight and obese, Sprague-Dawley rats and C57BL/6J mice. These animals were fed a dietary mix with 0.2, 0.6 or 1.8% of calcium. Interestingly, with this group of animal models, they were already obese, rather than young animals that were growing and obesity was induced, as in the three previously mentioned studies. Zhang and Tordoff found in their studies that when the Sprague-Dawley rats were fed *ad libitum* high versus low calcium diets, they ate significantly less on the high calcium diet ($P<0.00001$), indicating a lack of palatability of the diet. It has been suggested by these authors that in the studies on animals supplemented with higher calcium diets (Shi *et al.* 2001b; Papakonstantinou *et al.* 2003b; Sun & Zemel, 2004), the animals are simply smaller due to eating less of the unpalatable diet and this is restricting their growth and hence body fat, rather than the high calcium diets causing less gains in body fat. It is also important to note that differences in the results observed between animal species can be due to the fact that the initial observations that led to the current hypothesis were made in an agouti mouse model. When ectopically expressed, the agouti gene increases intracellular calcium in proportion to its level of expression in that tissue. This primary event leads to varying degrees of insulin resistance and obesity. Consequently, lowering of intracellular calcium, through high calcium diets, reverses these effects. The human homologue of agouti is expressed in human adiposites, and Zemel's group have shown that recombinant agouti protein increased adipocyte intracellular Ca^{2+}($[Ca^{2+}]_i$) and thereby stimulated lipogenesis and inhibited lipolysis in a coordinated manner.

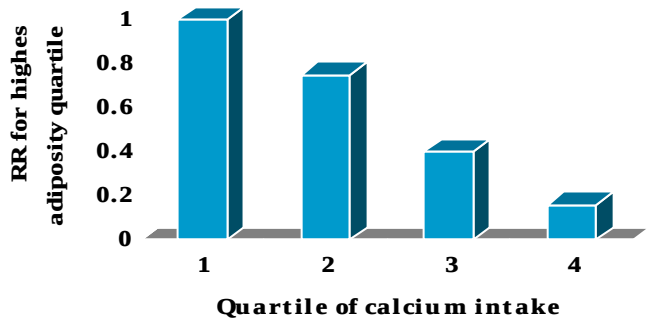
In other animal models of dietary obesity (C57BL/6J mice or Sprague-Dawley rats), dramatic changes in intracellular calcium are not the primary phenomenon leading to obesity. Hence the beneficial effects of dietary calcium may not be so clear cut.

1.4.1.2 Cross-sectional Observational Studies

(Refer to Table 1:4)

The first study to show a link between calcium intake and body weight was an analysis of the National Health and Nutrition Examination Survey I (NHANES-I) by McCarron *et al.* (1984) (evidence level-NHMRC IV). However, the lack of any plausible explanation for this finding merely relegated it to mild interest or a chance finding. Furthermore, Zemel *et al*. (2000) (evidence level-NHMRC IV) analysis of data from the NHANES III showed a strong inverse association for relative risk (RR) of obesity and calcium intake. These data showed that after controlling for energy intake, RR of high body adiposity decreased as calcium intake increased. The RR for women was 0.75. 0.40 and 0.16 for the second, third and fourth quartiles, respectively, with a similar inverse relationship shown in males (refer to Figure 1:8). Underreporting of dietary intake in overweight and obese subjects is a major confounder in dietary interventions (Black & Cole, 2001; Livingstone & Black, 2003). Hence in studies that employ reported dietary intakes, this confounding cannot always be fully controlled.

Figure 1:8 Relative Risk – highest adiposity quartile versus quartile of reported dietary calcium intake



(n=380, P<0.0009) (Zemel *et al.* 2000)

Since these two studies were published a range of cross-sectional studies from around the world have supported these findings.

Davies *et al.* (2000) (evidence level-NHMRC IV), re-analysed the results from 5 clinical studies looking at dietary calcium and bone mineral changes. The subjects' age was clustered towards the 3rd, 5th and 8th decade and results showed a significant negative association between calcium intake and weight for all age groups. However, it is pointed out by Davies, that weight/fat loss was not the end point in these studies. Jacqmain *et al.* (2003) (evidence level-NHMRC IV) analysing data from part II of the Québec Family Study (235 females and 235 males). After adjustment for age, daily energy intake, percentage dietary fat and protein, and markers of socio-economic status, there were negative correlations between daily calcium intake and percentage body fat, BMI, fat mass and waist circumference. Comparative trends were observed in men, but not after adjustment for the same covariates. Similarly, a negative inverse correlation was found between the intake of dietary calcium and abdominal obesity in 63 year old Swedish men (Rosell *et al.* 2004) (evidence level-NHMRC IV). This same relationship was found in black and white men and white women (but not in black women) (Loos *et al.* 2004) (evidence level-NHMRC IV). In a longitudinal study of milk intake and ishaemic heart disease and stroke in males from Wales, cross-sectional data at baseline indicated that men who drank at least one pint of milk (500 ml) had a BMI of 25.4 kg/m^2, whereas those who drank little or no milk had a BMI or 26.7 kg/m^2 ($P<0.001$) (Elwood *et al.* 2004) (evidence level-NHMRC IV). A similar relationship has also been demonstrated from an examination of the data from the National Nutrition Survey 1995 of Australia. Soares *et al.* (2004) showed that Australian men and women with higher calcium intakes, had lower BMIs as well as waist circumferences (data controlled for various confounders) (evidence level-NHMRC IV).

Three more recent studies have also demonstrated the link between dairy consumption and body composition. In the Tehran Lipid and Glucose Study (223 males, 239 females) a negative association was observed between dairy consumption and BMI (Mirmiran *et al.* 2005)(evidence level-NHMRC IV). This association was also demonstrated in Portugese men and pre-menopausal (Marques-Vidal *et al.* 2006) (evidence level-NHMRC IV) and pre-menopausal African-American women, who

described themselves as lactose-intolerant (and hence had a low calcium intake) had (Buchowski *et al.* 2002) (evidence level-NHMRC IV). Finally a number of studies that have addressed the link between calcium and/or dairy intake and risk of heart disease/stroke have cross-sectionally analysed the data at baseline and have concluded that a higher dairy intake (Shaper *et al.* 1991; Abbott *et al.* 1996; Bostick *et al.* 1999) or calcium intake (Van der Vijver *et al.* 1992; Iso *et al.* 1999) is associated with a reduced BMI (evidence level-NHMRC IV).

Only three reported studies have found no association between high and low tertiles of milk intake and BMI (Ness *et al.* 2001; Kamycheva *et al.* 2003; Venti *et al.* 2005) (evidence level-NHMRC IV).

Although a large number of cross-sectional studies from around the world are in agreement that there is an association between calcium and/or dairy in body composition, these studies do not indicate a causal link. In many of these studies dairy intake is often used as a surrogate measure for calcium intake; because dairy calcium comprises the primary source of dietary calcium in the US and Australian diet. It is also important to note that a low calcium intake may be associated with a less healthy lifestyle, diet or lower socio-economic status (Barger-Lux *et al.* 1992; Teegarden, 2005). It should also be noted that these types of studies use "reported dietary intake" and underreporting of dietary intake in overweight and obese subjects is a major confounder in these types of studies (Black & Cole, 2001; Livingstone & Black, 2003). It has also been suggested that dietary calcium may only have an effect under certain circumstances, such as during weight loss of obese subjects or in subjects with a vitamin D insufficiency. Likewise, it may be that there is a threshold effect for calcium, and epidemiological studies are not sensitive enough to pick up these effects.

1.4.1.3 Longitudinal Observational Studies

(Refer to Table 1:5)

Heaney *et al.* (2003) reanalysed data already published by Davies *et al.* (2000) from 2 cohorts of women studied from 1984 to 1985 and 1995 through to 1997. In total 564 women were enrolled in the studies examining calcium intake and bone density

(348 were studied once, 70 were studied for 8.5 years and 146 were studied for 22 years). The young women at the 25th percentile of calcium intake for the group had a 15% prevalence of being overweight, whereas when the women with the highest quartile for calcium intake (equivalent to the American Recommended Daily Allowance (RDA) had only a 4% prevalence of being overweight. In the analysis of women in midlife, women at the 25th percentile of intake of calcium gained weight on average of 0.42 kg per year; whereas the RDA of calcium was consumed there was a weight loss of 0.011 kg per year. Heaney goes on to conclude that although calcium intake only explains a small fraction of the variability in weight or weight gain, an increase of calcium intake across the population could reduce the prevalence of obesity by as much as 60-80%.

In the Amsterdam Growth and Health Longitudinal Study, subjects were tracked from age 13 years (1977) to age 36 (2000) (Boon *et al.* 2005b). The authors found a weak inverse relation of calcium intake to body composition. They also report no differences between the middle (800-1200 mg/day) and high (>1200 mg/day) groups of calcium intake, which may suggest that there is a threshold effect whereby greater than 800 mg / day has no additional benefits on body composition.

Two longitudinal studies in young adults (18-30 years) followed for 10 years (CARDIA Study) (Pereira *et al.* 2002) and a 2 year exercise study in young women (Lin *et al.* 2000) have shown similar results. In the CARDIA study at baseline, overweight individuals consumed dairy products less frequently than their normal weight counterparts (P=0.01). After a 10 year period the incidence of developing obesity in subjects who were overweight at baseline was 64.8% for those in the lowest quintile of dairy intake, compared with 45.1% for those in the highest quintile of dairy intake (P<0.001). When a BMI of 30 kg/m^2 and a WHR of 0.90 (men) and 0.85 (women) were evaluated separately, the odds of developing both conditions were lower (OR 0.81 for BMI and OR 0.89 for WHR) with each daily increment of total dairy intake over ten years in those who were overweight at baseline. In Lin *et al*. (2000) exercise study when calcium was adjusted for energy intake there was a negative relationship with the 2 year changes in total body weight and body fat and the young women with higher intakes of calcium gained less fat over the two years. Another study in young women who had followed a one-year dairy intervention (no

fat mass change at one-year); however, at 18 months of follow-up the high dairy group had maintained an elevated calcium intake compared with the control group. Using regression analysis the high calcium group also predicted a negative change in fat mass, controlled for BMI (Eagan *et al.* 2006).

Newby *et al.* (2004a) study looked at 459 men and women and assessed dietary intake (using 7-day food diaries) and changes in body composition were assessed over 25 months. Using a multivariate adjusted models in which highest and lowest quintiles for food pattern 1 (reduced fat dairy products, fruit and fibre) were compared; factor 1 was inversely associated with an annual change in BMI in women and inversely associated with waist circumference in both sexes. A sample of subjects from the Quebéc Family Study were measured twice ~6 years apart. Consumption of two food patterns (whole fruit intake and skimmed milk and partly skimmed milk) were negatively correlated with the changes of waist circumference and percentage body fat (Drapeau *et al.* 2004). Again using retrospective data it was demonstrated that middle-aged women (but not men) who habitually consumed greater than 500 mg of calcium per day, had less of a weight gain than non-calcium consumers over 10 years (Gonzalez *et al.* 2006).

More recently, Rajpathak and colleagues (2006) found no association between calcium, dairy intake or supplemental calcium and body weight changes over 12 years follow-up data from the Health Professional Study, which is a prospective cohort of men aged 40-75 years. Although the findings were that the men with the greatest increase in total dairy intake gained slightly more weight than did the men who decreased their consumption; it must be noted that this is likely contributable to the mens increased consumption of high-fat dairy foods.

All the above mentioned studies have been given a NHMRC level of evidence of IV. Although they are all prospective in nature, these studies are not intervention based, have no control group and merely determine predictions of weight / fat gain based on dietary calcium intake measured at certain time-series.

1.4.1.4 Randomised Clinical Trials

As the idea of calcium and in particular dairy calcium is a fairly new area of research, to date there have only been a limited number of well-designed randomised control trials. Some of the studies have been designed with weight loss as the primary outcome and hence the diet plans were hypocaloric, versus studies designed to address body composition changes whilst following eucaloric intakes, these two areas have therefore been divided into separate sections. Similarly, there has been interest in the effect of calcium supplements versus dairy; again these two topics will be covered separately.

1.4.1.4.1 Randomised Clinical Trials – adequate dairy intake versus inadequate dairy intake during energy restriction

(Refer to Table 1:6)

One of the first RCT that involved dairy foods and weight loss was a study designed to test a novel, but simple weight loss design versus a standard energy-restricted diet in obese outpatients. The outcome from this study was a greater weight loss on the milk (and unsweetened yoghurt) only diet (11.2 kg (SD 5.2) versus 2.6 kg (SD 4.1) on the standard diet. At the time this was an unexpected outcome; however, the authors contributed the result to the novelty and simplicity of the diet that the subjects had to follow (Summerbell *et al.* 1998) (evidence level-NHMRC II). More recently three clinical studies that were designed to specifically address the hypothesis of dairy calcium intake and body composition were undertaken by Zemel's group that have all shown a high dairy calcium diet has an enhanced effect on weight and fat loss particularly from the abdominal region, with a preservation of lean tissue mass (Zemel *et al.* 2004; 2005a; 2005b). In their 2004 study, Zemel *et al.* randomised 32 obese subjects to a standard calorie-deficient diet alone, a calorie-deficient diet supplemented with calcium or a calorie-deficient diet supplemented with dairy products. As was expected all subjects lost weight, the control group lost 6.4% of their body weight, the high-calcium group lost 26% more weight (8.6%), whilst the high dairy group lost 70% more weight (10.9% of body weight) ($P<0.01$). More interestingly greater fat was lost from the trunk region, this was 19% of total fat loss on the low calcium diet and this fraction was increased to 50.1% and 66.2% ($P<0.001$) on the high-calcium and high-dairy diets, respectively (Zemel *et al.* 2004)

(NHMRC – level of evidence III-I). In their second study, obese subjects were randomised to a control diet or a high-dairy (from yoghurt) diet on an energy-restricted 12-week weight loss study. As with the previous study, the high-dairy group lost significantly more fat (-4.43 kg versus -2.75 kg), whilst lean tissue was reduced by 31% on the yoghurt diet. Again trunk fat loss was augmented on the yoghurt versus the control diet and this was reflected by a greater reduction in waist circumference (Zemel *et al.* 2005a) (NHMRC – level of evidence III-I).

Zemel *et al*. third RCT was looking at weight loss and maintenance (discussed below) in African-American adults (2005b). Twenty-nine subjects were randomised to a low dairy (LD) (~500 mg calcium) versus a high dairy (HD) (~1300 mg of calcium) diet with calorie restriction of 500 kcal per day, the subjects followed this protocol for 6 months. Subjects achieved significant weight loss on both arms of the study; however, the HD diet achieved a 2-fold greater weight loss (LD -5.954 kg, HD -11.02 kg P=0.01), the loss of lean body mass was also markedly reduced with the HD diet (LD -1988 g, HD -148 g, P=0.001) (NHMRC – level of evidence III-I).

Not all data supports the findings of Zemel's group, a recent study by Harvey-Berino and colleagues (evidence level-NHMRC III-I), replicated the design of Zemel's weight loss studies. Fifty-four subjects were randomised onto a calorie restriction diet of 500 kcal/day and either following a low dairy or high dairy diet (2005). As would be expected from a weight-loss trial both groups lost a significant amount of weight or body fat at the end of the 6-month period; however, there was no significant difference between the dietary groups for weight (P=0.56) or body fat (P=0.37). Similarly, a study by Thompson *et al.* (2005) (evidence level-NHMRC III-I) comparing a moderate (not low) dairy calcium diet (800 mg Ca/d) with a high dairy calcium diet (~1400 mg Ca/d) and found no difference between the groups for weight or body fat. A lack of significant change in weight or body fat loss was also found in Bowen *et al.* study (2005) (evidence level-NHMRC III-I). It is important to note that this study; however, was manipulating the protein content of the diet, as well as calcium. The diets provided either a high-dairy protein, high-calcium diet (2400 mg Ca/d) versus a high mixed protein, moderate calcium (500 mg Ca/d) for 12 weeks of weight loss followed by 4 weeks of weight maintenance.

1.4.1.4.2 Randomised Controlled Trial – Calcium supplementation & weight loss

To date, there are limited RCT that have attempted to address the question of calcium supplementation and its effect on weight and fat loss in humans. Most of the studies found with weight loss end-points, were studies designed with bone mass or fracture rate incidence in mind.

1.4.1.4.2.1 Supplementation with energy restriction

In brief, four studies have included a supplemental protocol with restriction of calorie intake (Ricci *et al.* 1998; Jensen *et al.* 2001; Shapses *et al.* 2004; Major *et al.* 2007), whilst one study supplemented the subjects with calcium whilst they consumed an isocaloric diet (Reid *et al.* 2002).

Shapses *et al.* (2004) pooled the results from 3 separate, randomised, placebo-controlled trials, with subjects receiving 1 g of supplemental calcium (subjects were 100 pre- and post-menopausal women), followed for 25 weeks. Results showed no differences between body weight or fat between the supplemented versus the placebo group. Similarly, no differences in weight loss between groups following weight loss for 3 months (strict liquid formula diet) (Jensen *et al.* 2001), or in the supplemented group (provided with dietary counselling for calorie restriction) when measured at the end of 6-months (Ricci *et al.* 1998). In a more recent study conducted in 63 overweight/obese subjects, a 700 kcal/d energy-restricted diet was followed, and supplemented with 1.2 g/day of elemental calcium (plus 400 IU vitamin D) or a placebo, there was no difference between the groups for weight, fat mass or waist circumference (Major *et al.* 2007). Barr's (2003) review paper found 12 studies that provided information on calcium supplementation and weight loss end-points, a further 5 studies were included that weight loss end points could be obtained from the study authors. There was a strong conclusion that in 16 of these studies, the calcium supplemented group did not elicit greater weight loss than the placebo group. The one study that did find a reduction in weight (or lesser weight gain) in the supplemented group, was in a long-term (>4 yr) study, without energy restriction. The only other study that found greater reductions in weight, fat mass and trunkal fat, was Zemel's group, with one of his weight loss arms being given a supplement of 800 mg of calcium (total intake ~1300 mg) (2004).

1.4.1.4.2.2 Supplementation without energy restriction

In Reid *et al.* study (2002), subjects were provided with a 1 g/day calcium supplement or placebo, without changes to usual dietary intake. Subjects were assessed at 30-months; the results indicated no difference in weight lost or changes in fat mass.

In summary, only two of the above mentioned studies were able to demonstrate a relationship between a higher supplemental calcium intake and greater weight or body fat loss. However, it must be noted that all of studies, except Major *et al.* (2007) and Zemel *et al.* (2004), were designed with bone measures as end-points and therefore were possibly not powered to pick up differences in anthropometrical measurements. Similarly, in all but the two previously mentioned studies, the subjects did not follow strict dietary protocols for calorie restriction, but were rather provided with behaviour modification and general instructions on moderately reducing food intake. This throws open the question of compliance in such studies, especially when the length of the studies are considered.

1.4.1.4.3 Randomised Control Trial – weight maintenance

(Refer to Table 1:8)

More recently Zemel has suggested that the benefits of dairy on body composition can be achieved without energy-restriction. In his original trial, investigating the antihypertensive effect of calcium in obese African-Americans, it was observed that the group provided with 2 cups of yoghurt per day (increasing calcium from ~400 to 1000 mg /d) for a year resulted in a 4.9 kg reduction in body fat (Zemel *et al.* 2000). In his latest study (Zemel *et al.* 2005b), 34 obese African-Americans were maintained on a low calcium/low dairy diet (LD) or a high-dairy diet (HD) with no change in energy intake. Body weight remained stable for both groups; however, the high-dairy group had significant reductions in body fat (LD -0.169, HD -2.158, $P<0.01$), and trunk fat (LD -0.357, HD -1.026, $P<0.01$) and an increase in lean mass, whereas there were no significant changes in the low-dairy group (LD 0.283, HD 1.08, $P<0.04$).

The above findings were not replicated in Gunther *et al.* study (2005a) (evidence level-NH&MRC III-I). They studied 155 normal-weight young women, randomly assigned to one of 3 groups (1. control: maintain dietary intake; 2. medium-dairy: increase dairy intake to achieve 1100 mg calcium and isocaloric intake; 3. high-dairy group: substitute dairy products to achieve 1300 mg calcium and an isocaloric diet). The authors of this study did not find any weight loss differences between the three dietary groups at the end of the one-year period.

In an interesting review paper by Barr (2003) a MEDLINE search (1966-2001) (Table 1:9) located nine articles examining dairy supplementation and body weight or composition. In seven of these studies, no significant differences in the change in body weight or composition were detected between treatment and control groups. However, two studies conducted in older adults detected a significantly greater weight gain in the dairy treatment group. However, in interpreting these results, it is important to add that these studies did not instruct the participants to reduce their energy intake to compensate for the increase in energy from a high dairy intake. In fact one of the studies reported that although there was a greater weight gain on the dairy treatment group, the weight gain was not as much as would be expected from simply adding milk to the usual diet (Barr *et al.* 2000).

1.4.1.5 Pitfalls of current RCT Studies

What is clear from the studies previously mentioned is that there is a lack of consistency of study endpoints. Only, one group of researchers have been able to draw clear and significant conclusions for a role for calcium and dairy calcium in weight/fat loss (Zemel *et al.* 2004; 2005a; 2005b), or dairy alone (Zemel *et al.* 2005a). Other studies, which have looked at only the role of dairy calcium on weight loss, have failed to show any significant difference between the treatment groups (Bowen *et al.* 2005; Harvey-Berino *et al.* 2005; Thompson *et al.* 2005). Reasons for these discrepancies may include the following:

1. Lack of a true placebo-controlled experimental arm

The role of dairy and its effect on weight loss had become a very popular news item with the media (West Australian, Jan 22, 2003; Today Tonight, February 2003),

especially during the recruitment phase of our studies. The design of a true placebo-controlled study is required to eliminate bias form subjects not on the "dairy-rich" diet. Subjects who realise they are allocated to the "control" diet, can reduce their compliance and adherence to the study protocol.

2. Does calcium have an effect without vitamin D?

This was the first Australian study to test Zemel's hypothesis, and hence the use of a typical Australian diet meant the reliance on calcium-rich products that are not vitamin D enriched, as in the United States.

3. Does amount of fibre in the diet matter?

We also provided diets closer to the RDI for fibre; there were two reasons for this: (a) to ensure the diets were closer to usual/recommended intakes and (b) It has also been reported that higher intakes of calcium can lead to constipation (Arnaud & Sanchez, 1990; Soffer, 1999), to avoid this we ensured that the diets meet fibre recommendations ~28 g / day. Zemel's *et al*. studies gave only 8-12 g / day of fibre; however, they make no comment on symptoms reported by the subjects.

4. Energy deficit based on empirical kJ reduction or on body size considerations?

A fixed energy deficit (500 kcal or more) to all subjects means that, depending on the study design (see below), some would loose more and some less weight as it would tax different proportions of energy requirement. Our study was the first to utilise indirect calorimetry methods to determine BMR and hence fix the energy requirements of the subjects at the same plane of nutrition. This is not only a more accurate method, compared to using energy equations, but allows for greater control based on body size.

5. Study design consideration

Most studies have utilised an open parallel design which necessitates greater control of potential confounders between groups. No reported study has indeed made a good attempt to examine their data after controlling for confounders. Our study bypassed

these limitations by employing a with-in subject, cross-over designed study. Further our analysis will account for period, sequence and treatment effects.

1.4.1.6 Studies conducted in Children

The majority of dairy-weight loss studies have been conducted with adults; however, several longitudinal and cross-sectional studies are now emerging that have found an association between dairy in promoting a healthy weight or preventing an unhealthy weight gain among children and adolescents. As the focus of this thesis was utilising the adult population, only a brief overview of the studies carried out in children will be discussed.

A study in 8 year old children by Skinner *et al.* (2003) used statistical modelling to demonstrate that children could reduce their body fat by 0.4% simply by drinking an extra 200 ml glass of skim milk or 200 g of low-fat yoghurt each day. Similarly, Carruth and Skinner (2001) looked at the longitudinal food consumption of preschoolers aged 24-60 months and related the findings to body composition at 70 months. They concluded that higher intakes of calcium and servings of dairy were associated with lower body fat levels. Similar results have been found in a recent study of children aged 6 followed yearly until 13 years of age. These results concluded that children in the lowest sex-specific tertile of dairy intake at preschool (<1.25 serves per day for girls and <1.70 serves per day for boys) had significantly greater gains in body fat during childhood (as measured by sum of 4 skinfold measurements). In addition, by the time the cohort reached early adolescence, those in the lowest tertile of dairy intake had higher BMIs by ~2 units and an extra 25 mm of subcutaneous fat (Moore *et al.* 2006). Cross-sectional data by Novotny *et al.* (2004) indicates that total calcium and dairy-calcium intake were negatively associated with both iliac skinfold thickness and weight, and these associations were greatest in girls of Asian ancestry than Caucasian girls. A complex relationship was found in a cross-sectional and longitudinal study of hypercholesterolemic (HC) and normo-cholesterolemic (NC) children (Dixon *et al.* 2005). After adjustment for age, sex, energy intake and percentage energy from fat, calcium intake was inversely associated with BMI, sum of skinfolds, and trunk skinfolds at baseline and over 1 year in 7-10 year old non-HC children. An inverse relation was also found between

dairy food intake and measures of obesity at baseline in these children; however, calcium and dairy food intake was not associated with measures of obesity in HC children or in the 4- to 6-year old non-HC children. A large cohort (884) of Italian children (mean age 7.5 $\pm$ 2.1 years), had their height and weight measured along with lifestyle and dietary habits assessed, as part of a survey on childhood obesity. The frequency of consumption of milk was inversely and significantly associated (t=-2.964, P=0.003) with age-and sex-specific BMI z-scores when controlled for sex, age, physical activity, birth weight and parental overweight and education (Barba *et al.* 2005). Likewise, in cross-sectional data of 1701 children, a significant association was found between obesity and low intakes of dairy products (Olivares *et al.* 2004).

Several studies have now emerged that have found no association between dairy consumption and body weight/BMI. One was a prospective cohort study of 1,345 children (from low income families) aged 2 to 5 years (Newby *et al.* 2004b). Secondly, a longitudinal study in adolescents that found no association between dairy or calcium consumption and BMI-z score or percentage body fat (Phillips *et al.* 2003). Likewise, in cross-sectional data from an eating-behaviour study of 78 Pima Indian children (aged 10.4 $\pm$ 0.3 years), height, weight and DEXA measurements were taken and food intake measured by 24-hour recall, with assistance from parents. There were no significant associations between calcium intake and body weight (r=0.04, P=0.73), body fat (r=0.12, P=0.42) or body mass index (r=0.04, P=0.77) (Venti *et al.* 2005).

To date, only three randomised controlled trials have been conducted in children. It is important to note that whereas the adult studies have been interested in weight loss, in this group of subjects who are still growing (and hence gaining weight), the subjects have been supplemented with additional calcium and the difference in weight gain noted. The first study by Chan *et al.* (1995) divided 48 pubertal girls (age 11 years) into two groups of either consuming their usual dietary intake or supplemented with dairy foods to achieve ~1200 mg calcium per day. There were no differences between the total energy or fat intake between the groups and no differences between the groups for body weight or body fat levels; however, it is important to note that the main study outcome was bone mineral content and density rather than body composition changes. Similarly, in a 24-week study by Lappe's

group (2004), there were no differences in weight gain in pubertal (mean age 9 years), although the girls in the treatment group had consumed nearly twice as much dietary calcium. The increase in calcium came primarily from dairy foods, which had resulted in a ~150 calorie (628 kJ) greater consumption per day; however, they did not have greater increases in body weight, BMI or fat mass compared to girls consuming their usual dietary intake providing ~900 mg calcium per day. In a study by Lorenzen's group (2006) 110 girls (mean age 13.3 years) were assigned to two groups, either a medium or low calcium intake, based on their habitual calcium intake. The girls were then randomised to receiving 500 mg calcium carbonate or a placebo for one year. These researchers found that the girls habitual calcium intake was inversely associated with body fat (r=-0.242, P=0.011). However, calcium supplementation had no effect on height, body weight, or percentage body fat.

Only one study, that the author is aware of, reported a negative response of dairy and or calcium intake in children. Berkey *et al*. (2005) followed a cohort of 12,829 children aged 9 to 14 years. Annual weights and heights were recorded and food frequency questionnaires were completed to determine the past years food intake. The authors concluded that the children who drank more milk gained more weight. It is important to note, that the weight gain was probably due to excess calorie intake by the group who drank more milk. Interestingly, dietary calcium, skim and 1 % milk were associated with weight gain, but dairy fat was not.

1.4.1.6.1 Summary – Dietary Calcium's role in Weight Loss

Some animal studies (NHMRC – level of evidence V) have demonstrated a role for dairy / calcium and weight loss (Zemel *et al.* 2000; Sun & Zemel, 2004); however, these findings have not always been consistent (Zhang & Tordoff, 2004). Epidemiological studies (McCarron, 1983; Davies *et al.* 2000; Heaney, 2003; Jacqmain *et al.* 2003; Parikh & Yanovski, 2003; Soares *et al.* 2004; Azadbakht *et al.* 2005) support a clear role for calcium, and in particular, dairy foods in the regulation of weight. However, these types of studies do not indicate a causal link and it has been suggested that low calcium intake may also be associated with less healthy lifestyles, diet or other social status indicators (Barger-Lux *et al.* 1992; Teegarden, 2005). These types of observational studies in this review were allocated a NHMRC

level of evidence of IV. RCT are expected to provide a high level of evidence for a cause-effect link; however, due to the novelty of this topic the research to date is very limited. Only two reviews of RCT, one non-systematic (Barr, 2003) and the other a systematic review (Trowman *et al.* 2006) have concluded that there was no association between calcium supplementation and a reduction in body weight. However, with both these reviews, the data was drawn from studies that did not begin with weight regulation/ loss as an endpoint. To date, there are only five RCT designed specifically to relate dietary calcium/dairy to weight loss. Three of these studies, all from the same laboratory, have found a positive role of dairy calcium in augmenting weight loss (Zemel *et al.* 2004; Zemel, 2005a; Zemel, 2005b). Due to the limitation of blinding such studies (parallel design) the level of evidence (NHMRC) given to these studies was III-I. Two more recent RCT have replicated the design of Zemel's weight loss studies and found no difference between the groups for weight or body fat (Harvey-Berino *et al.* 2005; Thompson *et al.* 2005). Both these studies were given an NHMRC level of evidence of III-I. It is clear from the evidence to date, that there is a paucity of well-designed RCT, with NHMRC level of evidence II designed studies. The RCT's discussed above are parallel in design, which can lead to many confounding errors (see section 1.4.1.5). In an attempt to avoid such pitfalls our weight loss study was designed as a cross-over design in which the subjects were truly "blinded" to the dietary intake.

1.4.2 Dairy and Weight: other bioactive compounds

Along with the calcium component of dairy products there is growing evidence that there may be other bioactive compounds, which could be responsible for improvements to health outcomes. Milk contains approximately 5% lactose, 3.2% protein, 4% lipid and 0.7% mineral salts. It is these components that gives milk and milk products their nutritional value (Shah, 2000).

1.4.2.1 Whey

Whey proteins comprise approximately 20% of total milk proteins. β-Lactoglobulin represents half the total protein in the whey of cows' milk. Whey has been recognised as a rich source of bioactive compounds (Shah, 2000); and it has been

suggested by Zemel that they may act independently, as well as, synergistically with the calcium in milk to attenuate lipogenesis and accelerate lipolysis (Zemel, 2003b).

One of these health benefits seems to stem from the insulinotrophic effects that milk may elicit. The potential health aspects of the insulinotrophic effects of milk remain unclear. Hyperinsulinemia, mediated from hyperglycemia seems to be a risk factor for diseases that are collectively called the Metabolic Syndrome. It has been shown that experimentally induced hyperinsulinemia under normoglycemic conditions decreased insulin sensitivity in healthy subjects. Recent research seems to suggest that milk proteins, in particular the whey fraction have a stimulating effect on insulin secretion in healthy subjects (Nilsson *et al.* 2004). The mechanism for this stimulatory effect is unclear, but it has been suggested that specific insulinogenic amino acids or other bioactive peptides that are originally present in the whey or formed during digestion could promote these effects. Research by Nilsson's group has indicated that the incretin hormones seem to be involved, in particular glucose-dependent insulinotrophic polypeptide (GIP), which has been shown to significantly increase in plasma following the ingestion of a whey-based meal. Furthermore, glucagon-like peptide (GLP-1) is known to have insulinotrophic properties during normal plasma glucose concentrations (Vilsboll *et al.* 2003). A recent study has shown that the same elevated insulin response with a consequential reduction in the postprandial blood glucose area under the curve was observed in diabetic subjects when administered whey protein at a breakfast and lunch meal based on high glycemic foods (Frid *et al.* 2005).

As discussed previously in section 1.3.6.1.1.1, whey protein has a greater effect on food intake due to its faster absorption rate and hormonal response, compared to casein. Whey consumption leads to higher plasma concentrations of factors known to contribute to satiety including amino acids, glucose-dependent insulinotrophic polypeptides, GLP-1 and CCK. Whey proteins also contain significant angiotensin-converting enzyme (ACE) inhibitory peptides (see section 1.4.2.5).

1.4.2.2 Branched chain amino acids (BCAA)

The BCAA's leucine, isoleucine and valine, but especially leucine plays a specific metabolic role in the regulation of energy metabolism and muscle protein synthesis.

In skeletal muscle, leucine stimulates protein synthesis and inhibits protein catabolism. Leucine stimulates protein synthesis through the mammalian target of rapamycin (mTOR) pathways, 70-kDa ribosomal protein S6kinase activity and enhances eukaryotic initiation factor (elF)4E-binding protein phosphorylation and the association of elF4E with elF4G. Accordingly the three BCAA's are relatively abundant in the food supply, with dairy products being a particular rich source (Layman, 2003).

Layman (2003) proposes that leucine has a metabolic role in maintaining glucose homeostasis and stimulating protein synthesis, particularly when subjects are energy restricted with exercise. Layman's study had subjects following a high protein (1.5 g/kg body weight) and 10 g leucine/kg for 16 weeks with a 5-day/ week exercise protocol, versus a low protein diet and the same exercise. The high-protein diets elicited a greater weight loss, primarily from body fat; in addition to this at week 4, 10 and 16, the subjects on the high-protein protocol had higher lean body mass. These findings are consistent with other studies in that leucine appears to have a protein sparing effect under energy-restriction, weight-loss diets (Skov *et al.* 1999; Layman *et al.* 2003). However, the mechanism for this finding is yet to be established.

1.4.2.3 Vitamin D

Without adequate vitamin D, the small intestine absorbs no more than 10-15% of dietary calcium. In a person with vitamin D sufficiency, the small intestine absorbs, on average 30% of dietary calcium during growth, lactation and pregnancy, with the efficiency increasing to 80% (Holick, 2004). According to Bell *et al.* (1985) obesity is often associated with vitamin D deficiency. It has been shown that whether the vitamin D is ingested in the diet or obtained from sunlight, it becomes stored in the large body fat stores in obese subjects and is not bioavailable (Wortsman *et al.* 2000). In the US there is mandatory fortification of milk and butter/margarine products with vitamin D; whereas only a limited number of milk products are presently vitamin D fortified in Australia.

1.4.2.4 Conjugated Linoleic Acid (CLA)

Conjugated Linoleic Acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional and stereoisomers of conjugated dienoic octadecadienate. The predominate isomer found in foods is the *cis*-9, *trans*-11-CLA isomer, and this is found mostly in ruminant animals, and in particular dairy products (Belury *et al.* 2003). Park *et al.* were the first to report that incorporating 0.5% CLA in the diets of mice reduces body fat by ~60% (1997). These findings have since been supported by other mice studies (Park *et al.* 1997; West *et al.* 1998; Terpstra *et al.* 2002). The body-fat lowering effect of CLA has been reported in other experimental animals, such as pigs, rats, hamsters and chickens, but the effect has been less striking in mice (Terpstra, 2004). A review by Terpstra (2004), of eight human studies reported that none of the studies found a significant reduction in body weight, and only 2 studies showed a significant, but small body-fat lowering effect. Similarly, a RCT testing two CLA isomers on weight loss in 81 middle-aged men, failed to find a significant change in body composition (Malpuech-Brugére *et al.* 2004). Likewise, in Larsen *et al.* (2006), a study of 101 men who were assigned to the ingestion of 3.4 g/d of CLA or placebo of olive oil, with a modest calorie restricted diet, (following a lead-in of >8% weight loss for 1 year), the supplementation of the CLA failed to prevent weight or fat mass regain. Wahle *et al.* states that studies in humans are sparse and contradictory. They report a number of reasons for these differences in results; namely, lower dosages of CLA (g/kg bodyweight) are used in humans studies compared to the animal studies, and also growing animals may be more responsive than the mature human. It has been suggested that the CLA could be activating brown tissue in young animals. Brown adipose tissue is lost in older animals and humans (Wahle *et al.* 2004). It has also been reported that the CLA isomer that is involved in the body fat-lowering effect is the *trans*-10, *cis*-12 isomer, whereas > 90% of the total CLA intake from food in humans is accounted for by the *cis*-9, *trans*-11 isomer. It is also stated that CLA is only found in high fat containing dairy products and as all of the RCT studies that have found a positive effect of dairy on changes in body composition have used low-fat/non-fat milks and yoghurts (Zemel *et al.* 2004; 2005a; 2005b); it is unlikely to be the CLA content of these foods that is the significant contributor to the weight loss observed.

1.4.2.5 Angiotensin-Converting Enzyme (ACE) Inhibitory activity

The rennin-angiotensin-aldosterone system is a target for blood pressure control. Cleavage of angiotensinogen by rennin produces angiotensin I which is subsequently hydrolysed by angiotensin-I-converting enzyme (ACE), to angiotensin II, which is a potent vasoconstrictor. Milk proteins, both casein and whey, are a rich source of ACE inhibitory peptides (FitzGerald *et al.* 2004). The milk proteins contain ACE inhibitory peptides encrypted within their primary structures, these peptides can be released by enzymatic hydrolysis either during gastrointestinal digestion or during food processing. The ACE inhibitory activity is generally more associated with a antihypertensive effect; however, it has been reported that adipocytes have an autocrine/paracrine rennin-angiotensin system (RAS), and it has been suggested that adipocyte lipogenesis is regulated, in part by angiotensin II (Engeli *et al.* 2003; Zemel, 2003b; Abuissa *et al.* 2005). Animal studies support this theory that the inhibition of RAS mildly attenuates obesity in rodents (Reckelhoff & Fortepiani, 2004); however, limited human clinical studies support this (Zorad *et al.* 1995; Barton *et al.* 2003).

1.5 Dietary Calcium and its Other Health Benefits

The intake of dietary calcium has long been associated with the prevention of osteoporosis and there are a myriad of studies from all around the world to demonstrate this (Nordin, 1997; Reid, 1998; Heaney, 2000; Cosman, 2005; Nieves, 2005). However, dairy is now becoming more widely investigated for its links with improvements in other areas of health, namely lowering blood pressure, and improvements in the blood lipid parameters, along with reducing the risk of developing diabetes.

1.5.1.1 Hypertension and the role of dairy calcium

Hypertension is similarly a serious public health issue as it greatly increases the incidence from stroke and coronary heart disease. Recent Australian data quotes the prevalence of hypertension in Australia to be ~29% (Briganti *et al.* 2003). There is a direct positive relationship between overweight and hypertension, and it has been

estimated that the control of obesity may eliminate 48% of the hypertension in caucasians (El-Atat *et al.* 2003). Evidence that dietary calcium can have an impact on blood pressure first emerged with the publication of McCarron's paper in the early 1980s (McCarron *et al.* 1984). McCarron & Reusser (1999), in their paper "Finding consensus in the Dietary Calcium-Blood Pressure Debate" state that there is evidence from meta-analyses of 23 observational studies and 42 randomised controlled trials that identify statistically significant reductions in hypertension risk through an adequate calcium intake. Findings from these meta-analyses seem to demonstrate considerable diversity in the blood pressure responses. It seems that some of this diversity in results could be attributable to a possible "threshold effect".

1.5.1.2 Cardiovascular Disease

Cardiovascular disease risk factors along with risk factors for the Metabolic Syndrome and Type II diabetes are closely tied in with obesity, this section will be given considerable expansion.

Dairy consumption and in particular milk, have long being considered an important factor in coronary heart disease, because of the contribution it makes to the dietary intake of saturated fats. This belief that milk is a factor in heart disease along with weight gain, appears to have led to a decline in milk consumption in many countries (Elwood *et al.* 2005a), including Australia (Dairy Australia, 2005). For a brief overview of some of the available studies refer to Table 1:11.

A cross-sectional study of 470 men and women taking part in the Québec Family Study, found that daily calcium intake was negatively correlated with plasma low density lipoprotein (LDL) cholesterol, total cholesterol, and total:HDL cholesterol in both sexes, and after adjustment for variations in body fat mass and waist circumference ($P<0.05$) (Jacqmain *et al.* 2003). Similarly, in a study of 291 healthy men (age 62-64 years), with a range of insulin sensitivities, the subjects were divided into tertiles of percentage contribution of small dense-LDL (sd-LDL). It was found that individual fatty acids, typically found in milk products, were associated with a more favourable LDL profile (i.e. fewer sdLDL particles) (Sjogren *et al.* 2004). However, this was not the case in a cross-sectional study of 12,610 Japanese men

(age 30-69 years), the cohort was divided into dairy consumers (Yes) versus non-dairy consumers (No), based on a simply question "Do you drink a glass of cow's milk (180-200 ml) everyday". Multivariate models correcting for five confounding factors, demonstrated higher serum triglyceride levels ($P<0.001$) and LDL-cholesterol ($P<0.001$) in the dairy-consuming group (Nagaya *et al.* 1996).

In several further cross-sectional studies no difference or at least an insignificant change has been reported in blood lipid levels (Abbott *et al.* 1996; Barr *et al.* 2000; Ness *et al.* 2001; Elwood *et al.* 2004).

An early randomised controlled cross-over study (Steinmetz *et al.* 1994) showed that after a 6-week consumption of either a background diet, following the recommendations from the American Heart Association, with either skim milk or whole milk, that total cholesterol was lowered in both diets, but with a ~7% greater reduction on the skim milk versus the whole milk diet. Likewise, mean LDL-C were ~11% lower on the skim milk versus the whole milk diet. A randomised controlled trial in 13 healthy men with moderate hypercholesterolemia, who had randomly been administered a low calcium diet (410 mg / day) versus a high calcium (2200 mg / day) demonstrated a greater dietary saturated fat excretion per day (6 to 13%) with the calcium fortification. The high calcium diet significantly reduced total cholesterol by 6%, LDL cholesterol by 11% and apolipoprotein A1 by 7% ($P<0.05$) when compared with the low Ca diet (Denke *et al.* 1993). A review article by St Onge *et al.* (2000) seems to confirm overwhelmingly, that milk and dairy products have a cholesterol lowering effect.

A number of RCT looking at the effects of a high-dairy diet on weight loss have failed to show significant changes in blood lipids between the control and high-dairy diet. Two of Zemel *et al.* studies, one with a -500 kcal/day deficit diet and using yoghurt as the dairy source (2005a) and secondly, a 24-week weight loss trial, standard low dairy/calcium diet, a high dairy and a high calcium diet (Zemel *et al.* 2004), reported a significantly greater weight loss in the high dairy/calcium group; however, neither of these studies found any differences in blood lipids, between the diet groups. Similarly, in a weight maintenance trial in African-American subjects (Zemel *et al.* 2005b), where a significantly greater body fat reduction achieved in the

high dairy group, was 5.4%, P<0.01, no difference in blood lipids (total cholesterol, HDL-C or LDL-C) were reported.

Two further RCT that manipulated the calcium content of the diets, but did not report greater weight losses between the dietary interventions, also did not see differences in the blood lipid levels (Bowen *et al.* 2005; Thompson *et al.* 2005). Similarly, in a randomised-double-blind trial in 193 male and female subjects, given a calcium supplement of 1 or 2 g of calcium per day for 4 months, no significant differences between the high calcium versus the placebo groups for total cholesterol or high-density lipoprotein-cholesterol (Bostick *et al.* 2000). Barr *et al*. performed an open trail of 204 healthy subjects given advice to increase their milk intake to 3 cups per day, versus maintaining their usual diet for 12 weeks, no difference was found between the groups for total cholesterol, LDL-C, or the ratio of total cholesterol to HDL-C. However, triglyceride levels increased by 8%, but still within the normal range for the milk group, and remained stable for the control group (Barr *et al.* 2000).

The results from the research to date are mixed, but in summary it seems that no negative conclusion can be drawn between only milk intake and an increased risk from coronary heart disease. A recent review paper highlighted the results from 10 cohort studies and 2 case-control studies. The studies had been selected from a search on MEDLINE (using the key words: milk, dairy and dietary calcium and a range of words appropriate to vascular disease). The results from the 10 prospective studies were pooled, addressing the relative odds for ischaemic heart disease in the subjects with the highest milk intakes, compared to those with the lowest intakes, 0.87 (0.74-1.03) and for stroke the pooled estimate is 0.83 (0.77-0.90). A pooled estimate of the odds for a vascular event in the 10 prospective studies, either heart disease of stroke, is 0.84 (0.78-0.90). The results were reassessed using energy intake as a confounding factor; the results still gave similar estimates, namely 0.85 (0.70-1.03) (Elwood *et al.* 2004).

In a recently published paper from data using a sub-population of the original Caerphilly Cohort study (commenced 1979-1983); subjects were asked to weigh and record their food intake for 7 days, to assess total milk intake. The results indicated

that the relative odds of an event in the men whose milk consumption was the median or higher, relative to those with lower intakes of milk, were 0.52 (0.27-0.99) for an ischaemic stroke and 0.88 (0.56-1.40) for an ischaemic heart event. These results are in line with the previous pooled results from Elwood *et al*. (2005b).

In a Swedish case-control study to address whether intake of milk-fat influences cardiovascular risk, it was found that the intake of milk-fat was not negatively associated with cardiovascular disease and that the consumption of milk-fat did not increase the risk of a first acute myocardial infarction (Warensjö *et al.* 2004). Similar results were found in two other case-control studies, Gramenzi *et al.* (1990) in a retrospective case-control study, 287 women with acute myocardial infarction and 649 control women (with other acute disorders) who were admitted to hospital during 1983-89, were questioned about the frequency and amount of various foods consumed prior to the onset of symptoms of infarction. The odds ratio for the risk of myocardial infarction in the third of women with the highest consumption of milk, compared with the third with the lowest consumption, was 0.9. (1990). Similarly, in Tavani *et al*. (2002) data from a case-control of non-fatal acute myocardial infarctions (AMI) conducted in Milan, Italy, versus a control group who were admitted to hospital for acute conditions unrelated to known or likely AMI risk factors. Information on the diet was based on a validated food frequency questionnaire to determine prior consumption of milk and other dairy products. The odds ratio for infarction in patients who drank seven or more cups of milk per week, compared to patients who drunk no milk, as 0.78 (0.58-1.08) after adjustment for numerous possible confounding factors.

It is clear from these studies that dairy consumption, and in particular milk, does not cause an increase in blood lipid levels. And there does not appear to be a positive association between milk consumption and coronary heart disease risk.

1.5.1.3 Metabolic Syndrome & Type 2 Diabetes

If dairy calcium can be shown to improve weight or fat loss, an additional benefit may be seen by a concomitant reduction in insulin resistance. Insulin resistance is associated with several metabolic disorders, such as, impaired glucose tolerance, type

2 diabetes mellitus, central obesity, hypertension and dyslipidemia, which together constitute the metabolic syndrome or insulin resistant syndrome (Reaven, 1988; Reaven, 2002). The Metabolic Syndrome can be diagnosed according to guidelines established by the World Health Organisation (WHO) in 1998-99, or more recently published diagnostic criteria by the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) in 2001 (Table 1:3) (Alberti & Zimmet, 1998; Scheen & Luyckx, 2003). The central role of insulin resistance in the metabolic syndrome was established in the Bruneck Study, which evaluated the prevalence of insulin resistance in 888 subjects between the ages of 40-79 years (Bonora *et al.* 1998). The homeostasis model assessment method (HOMA), which takes fasting insulin and glucose levels into account, was used to estimate the level of insulin resistance. Insulin resistance was most prevalent in subjects with hypertriglyceridemia (TG > 2 mmol/L) (84.2%), low HDL cholesterol levels (HDL cholesterol < 1 mmol/L) (88.1%) and non-insulin dependent diabetes mellitus (NIDDM) (83.9%), and to a lesser degree hypercholesterolemia.

Table 1:3 Diagnostic criteria for the metabolic syndrome according to the WHO and NCEP ATP III guidelines.

Abbreviations: HOMA, homeostatic model assessment; BMI, body mass index; IGT, impaired glucose tolerance; WHO, World Health Organisation; NCEP ATP III, National Cholesterol Education Program Adult Treatment Panel III.

Risk Factor	WHO Defining level	NCEP ATP III Defining level
Waist-to-hip ratio	>0.9	-
Waist circumference (cm)	-	Men ≥102 Women ≥88
HDL cholesterol (mmol/L)	<0.9	Men <1.03 Women <1.29
Triglycerides (mmol/L)	≥1.7	≥1.69
Blood pressure (mmHg)	>160/90	>130/85
Fasting glucose (mmol/L)	-	>6.1
HOMA	>2	-
BMI (kg/m^2)	>30	-
Number of risk factors required for diagnosis	≥2 plus IGT and/or insulin resistance	≥3

A cross-sectional study of 2537 women and 2439 men assessed the habitual intake of several major food products, including dairy, by a food frequency questionnaire. They concluded that in men there was an inverse association with the presence of the metabolic syndrome (Mennen *et al.* 2000). More recently, a cross sectional study in 827 Tehranian adults, looked at several measures of the metabolic syndrome and concluded that those subjects in the highest quartile of dairy consumption had lower odds of having enlarged waist circumferences (p for trend <0.001), hypertension (P for trend <0.02) and the metabolic syndrome (p for trend <0.02) (Azadbakht *et al.* 2005). A large observational study over ten years in overweight adults, identified a link between dairy consumption and a reduced risk (72% risk reduction) for developing insulin resistance (Pereira *et al.* 2002).

The latest results from the ongoing Health Professionals Follow-Up Study have added some interesting findings on the relationship between dairy consumption and the risk of developing type 2 diabetes in men (Choi *et al.* 2005). This study involved longitudinal monitoring of the dietary intake and health status in 41,254 male health professionals for 12 years, after an initial dietary assessment using a semi-quantitative food frequency questionnaire. They concluded that there is a clear link between consuming more dairy foods (which included milk, yoghurt, cheese, ice cream and cream), and having a lower risk of developing type 2 diabetes over the following 12 years. After adjusting for 14 confounding variables, it was found that those in the two highest quintiles of dairy consumption were 23-25% less likely to develop the disease than those in the lowest quartile (P=0.003). The research suggested that for each single serve increase in dairy consumption per day, the risk of developing diabetes was reduced by 9%. The authors conclude that the protective effect of dairy is likely to be due to the lowering of risk factors from type 2 diabetes, such as body weight, hypertension and abnormal glucose homeostasis.

Of the several RCT that have been specifically designed to address the issue of dairy calcium intake and weight/fat loss, only two of the studies by Zemel's group indicated improvements in glucose function. Zemel *et al*. study (2004), giving 3 groups a -500 kcal deficit diet with either a low dairy (LD), high dairy (HD) or high calcium (HC) intake. Glucose tolerance was not significantly different at baseline; however, the HD group exhibited a significantly improved glucose tolerance at 24

weeks and a 27% reduced area under the postprandial curve for glucose following a standard glucose load ($P<0.01$). Fasting plasma insulin levels also showed a 44% decrease in the HD group ($P<0.01$). Neither the LD nor the HC group exhibited any difference with regards to glucose or insulin levels. In Zemel's study of African-American obese subjects either following a weight reduction or weight maintenance diet, along with either a LD or HD diet, the weight loss arm of the study the subjects from both groups exhibited decreases in circulating insulin levels; however, the HD group had significantly greater reductions than the control ($P<0.05$). Similarly, on the weight maintenance study, the HD group had significantly greater reductions in circulating insulin levels ($P<0.05$) (Zemel *et al.* 2005b). Not surprisingly, it would appear that these results were obtained due to a greater weight or fat loss from the HD groups in Zemel's studies, as Thompson *et al*. (2005) study comparing a moderate dairy calcium intake (~800 mg/day) versus a high dairy calcium intake (~1200 mg/day), they found no significant weight difference between the groups and although there were significant decreases in glucose and insulin levels, there was no treatment effect ($P=0.44$, $P=0.89$, for glucose and insulin, respectively). Likewise Bowen *et al.* reported a similar weight loss for subjects following a high protein (dairy) diet versus a moderate protein (low dairy) diet, fasting insulin levels decreased by the end of a 12 week (weight loss), and 4 week (weight maintenance), but was independent of treatment group. There was also no difference in an oral glucose tolerance test between dietary groups (Bowen *et al.* 2005). However, in Barr and colleagues study of a RCT–open trial of subjects instructed to increase their milk intake or maintain their usual intake, a small, but significant weight gain was reported in the milk intake group. Interestingly, the authors report that this modest weight gain of 0.6 kg was significantly less than expected for the additional energy intake. They reported a reduction of insulin in both groups, irrespective of treatment, a stable haemoglobin A_{1c}; however, there was a significantly ($P<0.01$) difference between groups for changes in glucose, the milk group had a less than 2% increase in glucose, whilst the control group had a reduction in glucose, but by less than 1% (Barr *et al.* 2000).

1.5.1.4 Bone Health

The Weight Loss industry is now a billion dollar business, with a wide array of diet programs and weight-loss centres to chose from (Weight Watchers, Jenny Craig Weight Loss Centres, Cohen's Lifestyle Clinics, SureSlim Wellness Clinics, to name a few). Along with these weight loss centres is a range of popular diets, often given much attention and hence promotion through the media, namely, The Atkin's Diet, South Beach Diet, SugarBusters, Scarsdale diet, the list goes on. The problem with many of these diets is that their, energy-restriction is often extreme and leads to nutrient deficiencies (Dwyer *et al.* 2005). In fact many of these diets advocate the use of nutritional supplements to ensure adequate nutrient intake. It is common amongst dieters to limit or avoid calcium rich foods, particularly the dairy products, as these are perceived as high fat foods (Radak, 2004; Dwyer *et al.* 2005). Recent guidelines for obesity treatment state that "*During weight loss, attention should be given to maintaining an adequate intake of vitamins and minerals; maintenance of the recommended calcium intakes of 1,000 to 1,500 mg/day is especially important for women who may be at risk of osteoporosis*" (National Institutes of Health, 1998). Studies have indicated that weight loss is associated with elevated calcium requirements; it would seem that even normal intakes of calcium during energy restriction can result in an inadequate calcium absorption which can ultimately compromise calcium balance and bone mass (Cifuentes *et al.* 2004). Several studies have indicated that the provision of at least 1 g/day of calcium (through a high calcium diet or calcium supplement) is likely to reduce loss of bone mineral and alter markers of bone resorption (Bowen *et al.* 2004; Radak, 2004). Some have suggested that a higher amount may be required to offset the weight loss associated bone changes (Riedt *et al.* 2005). Therefore, with or without, conclusive evidence that a high dietary calcium diet aids weight loss; it seems prudent and above all ethical to ensure that at least 1 g of calcium per day is achieved.

1.6 Concluding remarks

1.6.1 Summary

Australia like the rest of the developed world is in the grip of an epidemic of obesity. To date the role of macronutrients and weight management have been widely studied; however, the role of micronutrients have not until recently been investigated. A large number of cross-sectional and longitudinal studies from around the world are in agreement that there is an association between calcium and/or dairy in body composition; however, these studies can not be used to elicit a causal link. It is important to note that dairy intake is often a surrogate measure for calcium intake; because dairy calcium comprises the primary source of dairy intake in the US and Australian diets. In addition, a low calcium intake may be associated with a less healthy lifestyle, diet or social status.

As the concept of calcium, and in particular dairy calcium is a fairly new area of research, to date there have only been a limited number of well-designed randomised control trials. We would conclude from these studies that there is a lack of consistency of study endpoints. Only, one group of researchers have been able to draw consistent and significant conclusions of a role for calcium and dairy calcium in weight / fat loss (Zemel *et al.* 2004; 2005a; 2005b), or dairy alone (Zemel *et al.* 2005a; 2005b). Other studies, which have looked at only the role of dairy calcium on weight loss, have failed to show any significant difference between the treatment groups (Harvey-Berino *et al.* 2005; Thompson *et al.* 2005). We propose that there are some flaws in the methodology of these studies which may explain an inconsistency in results. These are lack of a true placebo-controlled experimental arm, the addition of vitamin D as well as calcium as the treatment, methodology used for determining energy deficiency (empirical versus measured) and the lack of control of confounders in the parallel-designed studies.

Therefore, by addressing some of the limitations currently present in the literature, our research adds invaluable knowledge towards the presently inconclusive link between dietary calcium and its role in weight and fat loss in the overweight/obese adult population.

Table 1:4 Cross-Sectional data from observational studies investigating the effects of dairy and /or calcium intake on body weight and adiposity

NH&MRC levels of evidence were only give to studies relating to main thesis topic of weight or fat change. Source: (National Health and Medical Research Council, 2000).

NHMRC level	Authors	Details of study	Results / Conclusion
IV	McCarron et al. 1984	NHANES I data 13,671 adults 18-74 years US data	Dietary calcium was negatively associated with BMI; r = -0.59, P<0.001
IV	Shaper et al. 1991	Prospective study of 7,735 men UK data	The proportion of men with a BMI $\geq$28.0 kg/m^2 was 27% in the quintile with the lowest milk intake and 16% in the quintile with the highest intake at baseline.
IV	Van der Vijver et al. 1992	Prospective study from 1953-1954 data of general health examination of 1,340 men and 1,265 women Calcium intake was assessed by 1-week food frequency recall Dutch data	Baseline BMI in men was 1.2 kg/m^2 greater in those in the lowest quintile of dietary calcium intakes ($\leq$585mg/d), compared with those is the highest quintile of calcium intake (>1245 mg/d). Similar patterns were obtained with corresponding figures of 2.0 kg/m^2 (low $\leq$ 445 mg/d versus > 850 mg/d).
IV	Abbott et al. 1996	Prospective study Honolulu Heart Program 3150 men (55 – 68 years) Food intake assessed by 24-hour recall	Baseline BMI was 0.8 kg/m^2 (25% of SD) greater in those in the lowest quintile of milk intakes compared with those in the highest quintile of milk intake.

NHMRC level	Authors	Details of study	Results / Conclusion
IV	Bostick et al. 1999	Prospective study 34,486 postmenopausal women (55 – 69 years) US data	Baseline BMI was 1.0 kg/m^2 greater in those in the lowest quintile of daily calcium intake (<696 mg/d) compared with those in the highest quintile of intake (>1,425 mg/d).
IV	Iso et al. 1999	Prospective study from 1980 data from Nurses' Health Study (age 34-59 years) 85,764 women US data	At baseline, 0.3% (9% of SD) more women had a BMI≥29 kg/m^2 in the lowest quintile of milk intakes, compared with those in the highest of dietary calcium intake (dietary intake included calcium from multivitamin supplements but not specifically calcium supplements).
IV	Zemel et al. 2000	Re-examination of NHANES III data (US data) (1988-1994) Body composition was assessed using anthropometric and bioelectrical impedance. Odds ratios for %BF and corresponding 95% CI were calculated.	The odds ratio of being in the highest quartile of body fat was markedly reduced from 1.00 (1^{st}) to 0.75, 0.40, 0.16 (2^{nd}, 3^{rd}, 4^{th}) in adult women. Males represented an inverse relationship between calcium and dairy intakes and body fat. These data demonstrate a reduction in the odds of being in the highest quartile of adiposity with increases in the intake of calcium and dairy product intakes.

NHMRC level	Authors	Details of study	Results / Conclusion
IV	Davies et al. 2000	Re-evaluation of 5 clinical studies (primary outcomes were skeletal end points) All subjects were women clustered around 3 main age groups:- 3rd, 5th and 8th decade (n=780) 4 studies were observational (2 cross sectional – BMI was regressed against entry level calcium and 2 longitudinal – change in weight over time was regressed against calcium intake, 1 double blind RCT – calcium supplementation)	Significant negative associations between calcium intake and weight were found for all three age groups, and the odds ratio for being overweight (BMI > 26 was 2.25 for the young women in the lower half of calcium intake (P<0.02). Relative to placebo, the calcium-treated subjects in the controlled trial had a greater weight loss, across 4 yr. Estimates of the relationship indicate that a 1000 mg calcium intake difference is associated with an 8 kg difference in mean body weight & that calcium intake explains ~3% of the variance in body weight.
IV	Ness et al. 2001	Prospective study 5,765 men (35-64 years) UK data	No difference – baseline BMI was 0.3 kg/m^2 greater in the highest tertile of milk intake, compared with the lowest tertile of milk intake (P=0.50)
IV	Kamycheva et al. 2002	Cross sectional study 9252 (males) and 9662 (females) 1994-95 completed food-frequency and lifestyle factor questionnaires Norwegian data	Ca intake and BMI had no correlation, but after correcting for the other variables with age, there was a +ve relation in men (P<0.001). No association was found in women. Dairy products supply ~75% of total calcium intake in Norway.

NHMRC level	Authors	Details of study	Results / Conclusion
IV	Buchowski et al. 2002	50 pre-menopausal African-America women US data	Lactose intolerant women (had a low dairy intake) had a higher BMI than lactose tolerant women (P<0.001). Calcium intake was negatively with BMI (R^2 = 0.470)
IV	Jacqmain, 2003	Cross-sectional study Quebec Family study (age 20- 65 years) 235 males, 235 females Canadian data Subjects divided into 3 group based on calcium intake 1) <600 mg, 2) 600-1000 mg, 3) >1000 mg	After adjusting for age, daily energy intake, % dietary fat, dietary protein and markers of socioeconomic status, there were –ve correlations between daily calcium intake and % BF (r = -0.19, P<0.01), BMI (r = -0.14, P=0.05), fat mass (r = -0.17, P=<0.05) and waist circumference (r = -0.15, P<0.05). Comparative trends were observed in men, but not after adjusting for the same covariates. Dairy supplied 62% and 60 % of daily calcium intake for women and men respectively.
IV	Loos et al. 2004	HERITAGE Family study 362 men (109 black, 253 white) 462 women (201 black, 261 white) Groups divided into tertiles of energy-adjusted Ca intake US data	At baseline, calcium intake was inversely associated with measures of adiposity in black men and white women. Black men in the top tertile of energy adjusted calcium intake had a BMI 3.3 kg/m2 lower than those in the lowest group (P=0.01). In white women there was a –ve association between energy adjusted calcium intake and BMI (P<0.02) and white men in the highest tertile of energy adjusted calcium intake had lower body fat than those in the lowest tertile (P=0.04). No significant associations were found with black women.

NHMRC level	Authors	Details of study	Results / Conclusion
IV	Rosell et al. 2004	Cross-sectional study 301, healthy males (age 63 years) with different degrees of fasting-insulin concentrations Sagittal abdominal obesity (SAD), dietary intake from 7 day food records, fatty acid composition in serum phospholipids and adipose tissue were measured. Under-reporters (UR) and non under-reporters (non-UR) were identified Swedish data	The intake of dairy fat (g/100 g fat) was inversely correlated with SAD (this was only observed in UR). The intake of calcium was inversely related in UR and non-UR. The intake of dairy fat was inversely correlated in the UR group. Calcium intake (g/10 MJ) was negatively correlated with abdominal obesity ($r = -0.22$, $P<0.001$).
IV	Elwood et al. 2004	Observational data from Caerphilly Cohort Study (1979-1983) 2512 men (45 – 59 years) Food intake assessed by semi-quantitative food frequency questionnaire UK data	At baseline, the BMI of men who drank at least 1 pint (500 ml) per day was 25.4 kg/m^2, whereas those who drank little or no milk had a BMI of 26.7 $kg.m^2$ ($P<0.001$).

NHMRC level	Authors	Details of study	Results / Conclusion
IV	Soares et al. 2004 (unpublished report)	Cross sectional data from the 1995/6 National Nutrition Survey Australian data	After controlling for age, energy intake, fat and protein intake, reporting bias and socio-economic status, both men and women in the top tertile of dietary calcium intake had significant lower BMI and waist circumference than those in the lower groups.
IV	Marques-Vidal et al. 2005	Cross sectional data 17,771 men and 19,742 women (>18 years) Portugal data	In men, milk intake was inversely related to BMI (r = -0.10, P<0.001), whereas the relationship in women was weaker (r = -0.06, P<0.001). In both genders the relevance of milk consumers increased with decreasing BMI – in men 62,68,71% in obese, overweight and normal weight men (P<0.001); in women the corresponding numbers were 71,72 and 76% (P<0.001). No relationship was found in women > 55 years.
IV	Azadbakht, 2005	Population-based cross-sectional 357 men, 470 women (age 18-74 years) Usual dietary intake was defined by 168-item semiquantitative FFQ Quartile cut-offs 1-4: <1, 1-<1.8, 1.8-<2.7 and >2.7 servings of dairy per day Iranian data	Subjects in highest quartile for dairy consumption had lower odds of having a larger waist circumference (1, 0.89, 0.74, 0.63 P<0.001).

NHMRC level	Authors	Details of study	Results / Conclusion
IV	Mirmiran et al. 2005	Cross sectional study 223 males, 239 females (age >16 years) randomly selected from the Tehran Lipid & Glucose Study. Dietary data collected from means of a FFQ for 1 y and 2 x 24-h dietary recall. Iranian data	After adjusting for confounding variables (age, energy intake, carbohydrate intake, fat intake, protein intake, dietary fibre intake and physical activity levels) men and women in the top quartile for dairy consumption had lower chances of being overweight (OR 0.78, 95%CI 0.43-0.92 for men and OR 0.89, 95%CI 0.53-0.95 for women) or obese (OR 0.73 95%CI 0.40-0.83 for men and OR 0.69 95%CI 0.34-0.80 for women) $P<0.05$.
IV	Venti et al. 2005	Cross-sectional study 35 men, 30 females (age 33$\pm$8 years) participating in a study of eating behaviour Height and weight measured, body composition (by DEXA) Food intake assessed using the Block 1998 Food questionnaire US (Pima Indian) data	There was no significant associations between calcium intake and body weight ($r=0.05$, $P=0.71$), body fat ($r=0.16$, $P=0.19$) and body mass index ($r=0.01$, $P=0.97$). One explanation for the lack of association may be that the high-fat, high-energy diet consumed by the subjects, overwhelmed the anti-obesogenic effect of calcium.

NHMRC level	Authors	Details of study	Results / Conclusion
IV	Murakami et al. 2006	Cross sectional study 1905 female dietetic students (age 18-20 years) Dietary intake was assessed over a 1-mo period, using diet history questionnaire and BMI (assessed by self-reported weight and height). Japanese data	Intakes of calcium and dairy products were not significantly associated with BMI (adjusted means in the lowest and highest quartiles were 20.7 and 20.8 for calcium, P for trend = 0.48 and 20.6 and 20.6 for dairy products, p for trend = 0.81). these results were also observed after excluding 481 energy under and over-reporters for calcium (20.4 and 20.5, respectively, P for trend =0.73) and dairy products (20.3 and 20.4, respectively, P for trend =0.73).

Table 1:5 Longitudinal data from observational studies investigating the effects of dairy and/or calcium intake on body weight and adiposity

NH&MRC	Authors	Details of study	Results / Conclusion
IV	Lin et al. 2000	Two year prospective exercise study 54 (normal weight females, age 18 – 31 years) US data	Total calcium/kcal –vely predicted changes in body wt. (R^2 = 0.19) and body fat (R^2 = 0.27).There was an interaction of calcium and energy intake in predicting changes in body wt, such that at the lower energy intakes, calcium intake (not adjusted for energy) predicted changes in body weight. In subjects with higher energy intakes, calcium had no impact on changes in body weight or body fat.
IV	Pereira et al. 2002	CARDIA Study 10 year prospective study of 3,157 young adults (age 18-30 years) Food intake measured by diet history interview US data	At baseline, overweight individuals consumed dairy products less frequently than their normal weight subjects (P<0.01). The 10-year cumulative incidence of developing obesity in subjects who were overweight at baseline was 64.8% for those in the lowest quintile of dairy intake, compared with 45.1% for those in the highest quintile of dairy intake (P<0.001). The odds ratio for adults who were overweight to become obese 10 years later were 0.84 (95% CI 0.73-0.97) for regular-fat dairy, 0.84 (0.70-1.02) for reduced-fat dairy and 0.82 (0.72-0.93) for all dairy products, per 1 daily eating occasion.

	When BMI of 30 kg/m^2 and a WHR of 0.90 for men and 0.85 for women were evaluated separately, the odds of developing both were lower (OR 0.81 for BMI and OR 0.89 for WHR) with each daily increment of total dairy over ten years in those who were overweight at baseline.

NH&MRC	Authors	Details of study	Results / Conclusion
IV	Heaney, 2003	Re-evaluation of Davies et al. 2000 data, focusing on distribution of values around the regression lines relating Ca intake to BMI. 564 women (348 were studied once, 70 studied 8.5 years and 146 were studied for 22 years) US data	At the 25$^{th\%ile}$ of Ca intakes, 15% of young women were overweight, this fell to 4% at Ca intakes in the range of current recommended values. Obesity prevalence also fell within this cohort from 1.4 to 0.2% across the same differences in Ca intake. At midlife women in the 25$^{th\%ile}$ of intake gained 0.42 kg/y, this gain dropped to –0.011 kg/y at recommended Ca intake levels. Although Ca intake explains only a small fraction of the variability in weight or wt gain, shifting the mean of the distribution downwards by increasing calcium intake can be estimated to reduce the prevalence of overweight and obesity by 60-80%.
IV	Newby et al. 2003	Baltimore Longitudinal Study of Aging – a prospective study 219 women and 240 men were followed for 25 $\pm$ 7 months Food intake was assessed by 7-day dietary records. US data	Six food patterns were derived from 40 food groups, Factor 1(reduced fat dairy products, fruit and fibre). Factor 1 was inversely associated with annual change in BMI (β = -0.51; 95% CI -0.82—0.20, P<0.05) in women and inversely associated with annual change in waist circumference (β = -1.06 cm; 95% CI -1.88 — 0.24 cm, P<0.05) in both sexes.

NH&MRC	Authors	Details of study	Results / Conclusion
IV	Drapeau et al. 2004	A sample of 248 volunteers from the Quebec Family Study were measured twice (1, 1989-1994 and 2, 1995-2000) 3-day food diaries were completed at visit 1 and food-based questionnaires indicating changes in the consumption of 10 food categories. Canadian data	Increases in the consumption of whole fruit, skimmed and partly skimmed milk were the two food patterns that negatively correlated with the changes in each body weight-related indicator; change in waist circumference (β = -0.23, $\pm$ 0.09 P = 0.02); change in percentage body fat (β = -0.14, $\pm$ 0.06 P = 0.02).
IV	Boon et al. 2005	Amsterdam Growth and Health Longitudinal Study followed men (296) and women (333) from age 13 (1977) until age 36 (2000) Dutch data	For men, in the age-adjusted model, a 1000 mg per day higher dietary calcium intake was related to a 0.21 cm lower sum of four skin folds (P=0.04). For women, the highest dietary calcium intake group (>1200 mg/d) had a significantly lower sum of four skinfolds than the group consuming less than 800 mg/d (β = -0.28; 95% CI -0.56—0.01 P=0.04) when adjusted for age. These effects disappeared after adjustment for dietary energy, fibre intake, and habitual physical activity. No difference were observed between the middle calcium intake (800-1200 mg/d) and high calcium intake (>1200 mg/d), suggesting a threshold of ~800 mg/d above which calcium intake has no additional beneficial effect on body composition.

NH&MRC	Authors	Details of study	Results / Conclusion
IV	Rajpathak et al. 2006	Data from the Health Professionals Follow-Up Study Prospective data from men aged 40-75 years in 1986. Data on lifestyle factors and diet updated biennially, with self-administered questionnaires. Participants reported their weight in 1986 and 1998. Multi-variant linear regression analysis used to examine how baseline calcium intake (n=23 504) and change in calcium intake (n=19 615), were associated with weight change. Similar associations were determined with dairy foods.	When adjustments were made for confounders, baseline or change in intake for calcium, dairy or supplemental calcium and changes in weight. The men with the largest increase in dairy intake gained slightly more weight than did the men who decreased intake the most (3.14 compared to 2.57 kg , P for trend = 0.001). This association was primarily due to an increase in high-fat dairy intake. Low-fat dairy intake was not significantly associated with weight change.
IV	Eagan et al. 2006	In a previous study 154 young women were randomised to a control (<800 mg Ca/d), medium dairy (1000 mg Ca/d) or high dairy (1300-1400 mg Ca/d) for a year trial. 51 women recalled 6 months post-study and dietary intake assessed and body	HD group maintained elevated Ca intake. Mean Ca intake predicted a negative change in fat mass (P=0.04).

		composition	
IV	Gonzalez et al. 2006	Retrospective data in 10,591 men & women aged >45 yr. Assessment of the relationship between calcium intake & weight gain over 10 years.	Women with a Ca supplement dose of > 500 mg had a 1.8 kg less weight gain than non-users (P=0.001).

Table 1:6 Randomised Controlled Studies investigating the effects of dairy with energy restriction on body weight and body fat

NH&MRC	Authors	Details of study	Results / Conclusion
II	Summerbell et al. 1998	3 arm randomised trial 16 week intervention 1) 3.4 MJ (control), 2) isocaloric milk only, 3) milk plus designated food only 45 patients (outpatient clinic) BMI > 27	Wt loss for each group was 1) 1.7 kg (95% CI -0.3-3.7), 2) 9.4 kg (5.9-12.9), 3) 7.0 kg (2.7-11.3). Dietary treatment can achieve as much wt loss in obese outpatients as has been reported with any drug treatment. But compliance is poor unless the diet is novel and simple.
III-I	Zemel et al. 2004	Parallel design 32 obese subjects (BMI 30 – 39.9 kg/m^2) (27 men, 5 women, age 49 $\pm$ 6 years) 24 week intervention 3 groups 1. low dairy (400-500 mg Ca) + placebo (n=10) 2. high-calcium diet (supplemented with 800 mg Ca) (n=11) or 3. high-dairy group (1200-1300 mg dietary calcium + placebo) (n=11). Energy-deficient –500 kcal/d	Weight loss 1. 6.60 $\pm$ 2.58 kg, 2. 8.58 $\pm$ 1.60 kg, 3. 11.07 $\pm$ 1.63 kg (P<0.01). Fat loss 1. 4.81 $\pm$ 1.22 kg, 2. 5.61 $\pm$ 0.98 kg, 3. 7.16 $\pm$ 1.22 kg (P<0.01). Trunk fat change 1. 1.38 $\pm$ 0.60, 2. 2.94 $\pm$ 0.73, 3. 3.74 $\pm$ 0.64 kg (P<0.01).

NH&MRC	Authors	Details of study	Results / Conclusion
III-I	Zemel et al. 2005a	Parallel design 34 obese subjects Two groups, both energy deficit (-500 kcal/d) 1. 400-500 mg Ca/d (n=16, 14 women, 2 men, age 42 ± 6) 2. 3 serves yoghurt 1100 mg Ca/d (n=18, 13 women, 5 men, age 39 ± 10) 12 week intervention	Weight loss was greater on the yogurt diet (1. 4.99 ± 0.5 kg, 2. 6.63 ± 0.6 kg, P<0.01) Fat loss greater by 61% on 2. (1. -2.75 ± 0.73 kg, 2. -4.43 ± 0.47, P<0.005). Less reduction in LBM on 2. (1. -1963 g, 2. 1361g, P<0.05). Greater waist circumference loss on yogurt diet (1. -0.58 ± 1.04 cm, 2. -3.99 ± 0.48, P=0.001)
III-I	Zemel et al. 2005b	Parallel design 29 African-American Two groups, both energy deficit (-500 kcal/d) 1. 400-500 mg Ca/d (n=12, 11 women, 1 man, age 42 ± 2.7) 2. 3 serves dairy 1000 mg Ca/d (n=17, 14 women, 3 men, age 42 ± 2.9) 24 week intervention	Weight loss was greater on high dairy diet (1. -5.954, 2. -11.02 kg, P<0.01). Fat loss was also greater on the high dairy diet (1. -3.97, 2. -9.08 kg, P<0.01) and trunk fat (1. -0.849, 2. -4.177 kg, P<0.01). Less reduction in LBM on high dairy diet (1. -1988, 2. -148 g, P<0.001).

NH&MRC	Authors	Details of study	Results / Conclusion
III-I	Harvey-Berino et al. 2005	Parallel design 54 overweight & obese (44 completers) Energy-deficit -500 kcal/d (calculated from 7-day food record. 1. Low dairy - 1 serve dairy (26 women, 2 men, age 45 ± 6.5 years) 2. High dairy - 3-4 serves of dairy (23 women, 2 men, age 45 ± 7.0 years) Encouraged to expend 1000 calories/wk in PA 1 year intervention	No difference in weight lost between groups (1. -9.6 ± 6.5, 2. -10.8 ± 5.9 kg, P=0.56) at 1 year and fat loss at 6 months (1. -9.0 ± 3.8 kg, 2. -10.1 ± 3.6 kg, P=0.37).
III-1	Bowen et al. 2005	Parallel design 50 overweight & obese (30 women, 20 men) 1. high dairy protein/high calcium (DP 2400 mg/d) (age men 49 ± 3.2, women 47 ± 2.4 years) 2. high mixed protein /moderate calcium (MP 500 mg/d) (age men 49 ± 4.2, women 46 ± 2.6 years	Weight loss or fat loss was not different between treatments. Weight change (men 1. -9.4 ± 1.3 kg, 2. -12.0 ± 1.5 kg, females 1. -9.4 ± 1.0 kg, 2. -7.8 ± 0.6 kg). Fat mass (men 1. -7.5 + 1.0 kg, 2. -9.6 + 1.1 kg, women 1. -9.2 + 0.9 2. -7.1 + 0.5).

NH&MRC	Authors	Details of study	Results / Conclusion
III-I	Thompson et al. 2005	Parallel study 72 completers, obese subjects Randomised into 1 of 3 groups, all with -500 kcal/d energy restriction 1. Control: 30% fat, 20% protein, 50% carbohydrate, provided average of 932 mg calcium & 16.2 g fibre (n=26) 2. as 1. with addition of 4 serves of dairy (2 serves from milk) (n=22) 3. as 2. additional fibre (from wholegrain, fruit, veg) and GI foods less than 100 encouraged (n=24) 48 week intervention	None of the 3 dietary groups achieved significantly greater weight loss. Weight loss (1. 10.0 $\pm$ 6.8 kg, 2. 11.8 $\pm$ 6.1 kg, 3. 10.6 $\pm$ 6.8 kg) and fat loss (1. 7.5 $\pm$ 6.6 kg, 2. 9.0 $\pm$ 6.0 kg, 3. 8.5 $\pm$ 7.8 kg).

* Weight loss arm of study

Table 1:7 Randomised Controlled Studies investigating the effects of calcium supplements with energy restriction on body weight and body fat

This table does not include studies that were reviewed in Barr, 2003 (obtained from a Medline search 1966-2001). The majority of those studies did not set out to establish the hypothesis that a greater intake of calcium would elicit a greater weight / fat loss. In a number of the studies the results had to be obtained by personal communication).

NH& MRC	Authors	Details of study	Results / Conclusion
III-I	Ricci et al. 1998	31postmenopausal women (BMI 28-42 kg/m^2), subjects had <800mg/d Ca. 6 month intervention Randomised to 1 g Ca citrate or placebo, along with calorie restriction (behaviour modification).	Both groups had similar weight loss (-9.0 (Ca) vs.-8.8 (placebo) kg), BMI (-3.3 (Ca) vs. -3.3 (placebo) kg/m^2), and fat mass (-7.3 (Ca) vs. -7.3 (placebo) kg).
III-I	Jensen et al. 2001	62 overweight & obese women, 6 month intervention (3 month 4.2 MJ supplemental diet, 3 months advised to eat normal diet with calorie reduction) in additional randomised to Ca supplement group (1 g) or placebo.	Both groups lost similar amounts of weight (5.6% (Ca supp) versus 5.5% (placebo). Ca supplementation should be encouraged during weight loss to prevent bone loss.

NH&MRC	Authors	Details of study	Results / Conclusion
III-I	Zemel et al. 2004	Parallel design 32 obese subjects (BMI 30 – 39.9 kg/m^2) (27 men, 5 women, age 49 $\pm$ 6 years) 24 week intervention 3 groups 1. low dairy (400-500 mg Ca) + placebo (n=10) 2. high-calcium diet (supplemented with 800 mg Ca) overall 1200-1300 mg/d (n=11) or 3. high-dairy group (1200-1300 mg dietary calcium + placebo) (n=11). Energy-deficient –500 kcal/d	Weight loss 1. 6.60 $\pm$ 2.58 kg, 2. 8.58 $\pm$ 1.60 kg, 3. 11.07 $\pm$ 1.63 kg (P<0.01). Fat loss 1. 4.81 $\pm$ 1.22 kg, 2. 5.61 $\pm$ 0.98 kg, 3. 7.16 + 1.22 kg (P<0.01). Trunk fat change 1. 1.38 $\pm$ 0.60, 2. 2.94 $\pm$ 0.73, 3. 3.74 $\pm$ 0.64 kg (P<0.01).
III-I	Shapses et al. 2004	Data combined from 3 x 25-week RCT of 1000 mg Ca supplementation 100 pre- and post-menopausal women (initial research with bone mineral change outcomes). Energy requirements based on Harris Benedict equation, added activity factor and subtracting 2100 kJ	No difference in weight change between calcium and placebo group (placebo –6.2 $\pm$ 0.7 kg, calcium –7.0 $\pm$ 0.7 kg). The mean change in body weight and fat were not significant different between calcium and placebo treatments in any of the studies.
III-I	Major et al. 2007	RCT- parallel design	No treatment effect on weight or body fat levels

	63 overweight/obese women, daily Ca intake <800 mg. Energy-restricted diet (700 kcal/d) 1. Elemental Ca 1200 mg + 400 IU vit D 2. placebo supplement	

Table 1:8 Randomised Controlled Studies investigating the effects of dairy with energy balance on body weight and body fat

NH&MRC	Authors	Details of study	Results / Conclusion
†	Zemel et al. 2000	11 African-American men (age – not reported) Consumed 2 cups of yoghurt daily 1 year intervention	Weight change – not reported. High dairy group lost 4.9 kg body fat (no control group).
III-I	Gunther et al. 2005	135 healthy weight females (age 18-30 years). Followed isocaloric intakes and divided into 1 of 3 groups. 1) control (n=22) 2) medium-dairy to achieve 1100-1200 mg Ca (n=23) 3) high-dairy to achieve 1300-1400 mg Ca (n=22) 1 year intervention	No significant change in body weight or fat mass between the 1), 2) or 3) (BW: 0.8 $\pm$ 2.8, 0.7 $\pm$ 3.0, 1.5 $\pm$ 4.1 kg, P=0.45; FM: -0.5 $\pm$ 2.5, 0.3 $\pm$ 2.7, 0.5 $\pm$ 3.5 kg, P=0.26)
III-I	Zemel et al. 2005b **	Parallel design 34 African-American (11 men, 23 women) Two groups, weight maintenance 1. low dairy - 500 mg Ca/d 2. high dairy - 3 serves dairy 1200 mg Ca/d 24 week intervention	There was no significant change in body weight (1. 0.2 $\pm$ 0.5, 2. 0.4 $\pm$ 0.6 kg). However, significant decrease in body fat in the high dairy group (1. -0.169, 2. -2.158 kg, P<0.01), trunk fat (1. -0.357, 2. -1.026 kg, P<0.01), with an increase in lean body mass in the high dairy group (1. 0.283, 2. 1.08 g, P<0.04).

† The details of this study are not reported, NH&MRC levels of evidence – unable to determine ** Weight maintenance arm of study

Table 1:9 Randomised Controlled Studies where energy intake was not controlled but subjects were given additional dairy

([1-6] adapted from Barr, SI, 2003)

Authors	Details of study	Results / Conclusion
Baran et al. 1989[1]	37 premenopausal women (age 30-42 years) 3 year randomised trial of increased dietary calcium (+610 mg/d) vs. usual diet Women in dairy supplement group were asked to increase their calcium intake (500-600 mg/d) by substituting low-fat high-calcium foods and eliminating unnecessary calories.	The control group gained 3.4 kg, the dairy group gained 4.2 kg (significance not reported)
Prince et al. 1995[2]	168 postmenopausal women (mean age 63 years) 2 year randomised trial of 1. placebo 2. milk powder (1 g Ca/d) and two other interventions. Due to the extra kilojoules from the milk powder subjects were asked to reduce their energy intake by 1200 kJ by avoiding high fat foods.	No change in BMI in the dairy group, not reported in the control group.
Storm et al. 1998[3]	60 postmenopausal women (age > 65 years) 2 year randomised trial of addition of 250 ml/d milk or placebo (or 1 other intervention)	No significant change differences in change in weight or body composition among treatment groups.

NH&MRC	Authors	Details of study
Barr et al. 2000[4]	200 (129 women and 71 men) (mean age 65 years) Randomised to ~2 cups/d low fat milk or usual diet. Milk group instructed to add 3, 240 ml servings of skim or 1% milk to their diet and to follow their usual diet. 12 week intervention	Men gained 1.0 kg, women 0.4 kg (control), and men gained 1.6 kg, women 1.9 kg (dairy). Overall the dairy group gained 0.6 kg more weight than the control (P<0.01). However the weight gain was less than predicted from the addition of the dairy calories (~2.5 kg may have been expected).
Lau et al. 2001[5]	185 Chinese women (age 55-59 years), > 5 yr postmenopausal. 2 year intervention of addition of 50 g/d high-calcium, low fat milk powder. No mention of substitution or addition – milk powder provided 175 kcal/d.	There was a significant gain in weight in the dairy group (control -0.26, dairy +0.52 kg, P<0.001). Fat change (control -0.14, dairy +0.42 kg) and lean change (control +0.2, dairy +0.3 kg).
Cleghorn et al. 2001[6]	115 Australian women < 5 year postmenopausal 2 year open-crossover 3 litres/week of calcium fortified milk (1 year on milk, 1 year off). Women were asked to drink an additional 3 litres of milk without changing their usual diet.	There was a 0.06 kg difference between the on/off added dairy products (95% CI: -0.71 to 0.83 kg, NS).

Table 1:10 Fat oxidation studies in Humans

NH&MRC	Authors	Details of study	Results / Conclusion
IV	Melanson et al. 2003	35 (21M, 14F) non-obese, healthy adults (age 31 + 6 y, weight 71.2 ± 12.3 kg, BMI 23.7 ± 2.9 kgm^2, fat% 21.4 ± 5.4 %). Cross sectional data was collected on 24 h energy expenditure, macronutrient oxidation using whole room indirect calorimetry, habitual Ca intake (4 day food records); acute ca intake estimated from measured food intake during 24 h stay in a room calorimeter.	Acute Ca intake was positively correlated with fat oxidation over 24 hr (r = 0.38, P=0.03), during sleep (r = 0.36, P=0.04) and during light physical activity (r = 0.32, P=0.07). Acute Ca intake was inversely correlated with 24-h RQ (r = -0.36, P=0.04) and RQ during sleep (r = -0.31, P=0.07). After the adjustment for fat mass, fat free mass, energy balance, acute fat intake, & habitual fat intake, acute Ca intake explained ~10% of the variance in 24-h fat oxidation. In backwards stepwise models, total Ca intake was a stronger predictor of 24 h oxidation than dairy Ca intake.
†	Melanson et al. 2004 (abstract)	18 subjects (10M, 8F), age 33 + 8 yr, BMI 27.6 + 3.2 kgm2, %fat 31.4 + 11.7%) consumed a low dairy (500 mg) or high dairy (1400 mg), isocaloric diet for 7 d. Each dietary condition was performed twice with a 1-3 week wash-out. RMR was measured on day 5 or 6 of the diet.	RMR did not differ between diets (1676 ± 214 kcal/d HD, 1650 ± 212 kcal/d LD, P=0.75). Similarly, there was no difference between RQ (0.78 ± 0.05 HD, 0.78 ± 0.03 LD, P=0.96). Conclusion – modifying dietary calcium intake between 500 and 1400 mg/d, has no effect on resting energy expenditure and substrate oxidation.

NH&MRC	Authors	Details of study	Results / Conclusion
II	Boon et al. 2005	12 healthy men (28 ± 2 yr, BMI 25.2 ± 0.06) given isocaloric diets (high calcium/high diary H/H, high calcium/low dairy H/L, low calcium/low dairy L/L) in a cross-over design, for 7 d. At the end of 7d measured 24-h energy expenditure, substrate metabolism, fat biopsies to determine mRNA expression in genes involved in lipolysis & lipogenesis.	24-h energy expenditure was 11.8 ± 0.3, 11.6 ± 0.3, and 11.7 ± 0.3 MJ/24 h in the H/H, H/L, and L/L conditions. Fat oxidation did not differ significantly, 108 ± 7, 105 ± 9, 100 ± 6 g/24 h (H/H, H/L, and L/L, respectively). $1,25(OH)_2D_3$ changed from 175 ± 16, 138 ± 15, 181 ± 23 to 159 ± 19, 164 ± 13 and 198 ± 19 pmol/L in the H/H, H/L, and L/L condition, this was significantly different between the H/H and the L/L conditions (P=0.05). There was no difference in the gene expression for any of the measures taken.
II	Gunther et al. 2005	Normal weight women (age 18-30 y) were assigned to a low (<800 mg/d, n=10) or high (1000-1400 mg/d, n=9) dietary calcium group of 1 y. Whole body fat oxidation was assessed after the subjects consumed 2 isocaloric liquid meals containing 100 or 500 mg Ca at baseline and 1 y. Fasting PTH was also measured at baseline and 1 y.	The mean 1 y change in fat oxidation was higher in the HC than the LC group after the consumption of a low calcium meal (0.10 + 0.05 versus 0.005 + 0.04 g/min, P<0.001) and a high calcium meal (0.06 + 0.05 versus 0.03 + 0.04 g/min, P<0.05). 1 y change in serum log PTH was negatively associated with the 1y changing in postprandial fat oxidation after a high calcium meal (partial r=-0.46, P<0.04) when controlled for the 1 y change in total body fat mass. Conclusion: the chronic consumption of high diary, calcium diet increases whole body fat oxidation from a meal, and increases in fasting serum PTH relate to decreases in postprandial whole body fat oxidation.

NH&MRC	Authors	Details of study	Results / Conclusion
II	Jacobsen et al. 2005	10 subjects followed an isocaloric diet for 1 week either: low calcium/normal protein ~500 mg/d (LCNP), high calcium/ normal protein~1800 mg/d (HCNP) or high calcium/high protein~1800 mg/d (HCHP). Diets were provided in a randomised, crossover design.	The calcium intake had no effect on 24-h energy expenditure or fat oxidation. Faecal fat excretion increased 2.5 fold during the HCNP diet compared with the LCNP and HCHP diets (14.2, 6.0 and 5.9 g/d, respectively, $P<0.05$). The HCNP diet also increased faecal energy excretion as compared with the LCNP and HCHP diets (1045, 684, 668 kJ/d, respectively, $P<0.05$). There were no effects on blood cholesterol, free fatty acids, triglycerides, insulin, leptin, or thyroid hormones.
II	Melanson et al. 2005	19 subjects (10 M, 9 F), consumed a low dairy (~500 mg/d), LD or high dairy (~1400 mg/d) energy balanced diet for 1 week. On the 7^{th} day, subjects were studied in a room calorimeter under one of four conditions (in a cross over design). Each dietary condition was performed under energy balance and energy restricted conditions. The energy restriction was only given to the subject within the 24 hr testing period and was achieved by energy restriction and exercise.	Under energy balance conditions, there was no effect of dietary treatment on RQ or 24 hour macronutrient oxidation. Under energy deficit conditions, 24 hr fat oxidation was significantly increased on the HD diet (HD 136 $\pm$ 13 g/d, LD 106 $\pm$ 7 g/d, $P=0.02$). Conclusion: consumption of a HD, energy restricted diet increased 24 hr fat oxidation. It is hypothesised that these effects are due to an increased fat oxidation during exercise.

NH&MRC	Authors	Details of study	Results / Conclusion
II	Melanson et al. 2006 (abstract)	10 subjects (4 F, 6 M), consumed a low dairy calcium (LDC) ~500mg/d or high dairy calcium (HDC) ~1400mg/d diet for 7 days. On the 7th day, pre-exercise (30 mins), exercise (50% max capacity, 60 mins) and post-exercise (120 mins) measures of palmitate & glycerol kinetics & substrate metabolism were made 4.5 hours following consumption of a standard breakfast (crossover design).	Fasting 1,25-vit D trended lower on the HDC diet (P=0.06). RQ did not differ significantly before, during or after exercise. There were no differences in circulating glucose, insulin, FFA concentration or glycerol/palmitate kinetics at any time point. This study does not support the hypothesis that lipolysis and FOX are increased short-term after the consumption of a HDC diet, despite a decrease in 1,25-vit D.

✝ The details of this study are not reported, NH&MRC levels of evidence – unable to determine

Table 1:11 Studies of dietary calcium / dairy in relation to cardiovascular risk factors and vascular outcomes

Abbreviations: IHD - Ischemic Heart Disease, CHD - Coronary Heart Disease, FA - Fatty Acids, sd - small dense, AHA - American Heart Association

Authors	Details of study	Results / Conclusion
Steinmetz et al. 1994	Randomised controlled study 8 healthy males (age 20-36 years) Subjects were given 236 ml of either skim or whole milk within a background diet according to the AHA recommendations. Plasma lipid were analysed at baseline, week 3 and 6.	After 6 weeks total cholesterol mean was 4.47 (skim) and 4.80 (whole) mmol/L (P<0.001) (~7% lower in skim than whole milk diet). Mean LDL-C were 2.64 (skim) and 2.96 (whole) mmol/L (P<0.001)(~11% lower in skim than whole milk diet). Mean apolipoprotein B decreased with skim milk and increased with whole milk.
Nagaya et al. 1996	Cross-sectional data 12,610 Japanese men (age 30-69 years) Cohort divided into dairy consumers (Yes) versus non-dairy consumers (No) based on a simply question of "Do you drink a glass of cow's milk (180-200 ml) everyday".	Regardless of age the "Yes" group had higher levels of serum TC, HDL-C and LDL-C than the "No" group (P<0.001); except for the age group 50-54. Multivariate models correcting for five confounding factors (age, body mass index, habitual exercise, smoking and drinking habits), demonstrated higher serum triglyceride levels (+1.5%, 5.280 vs. 5.201 mmol/L, P<0.001) and LDL-cholesterol (+2.0%, 3.382 vs. 3.316 mmol/L, P<0.001).

Authors	Details of study	Results / Conclusion
Bostick et al. 1999	Prospective cohort 34,486 postmenopausal women Age 55-69 years (without a history of IHD) Completed semi-quantitative food frequency questionnaire (1986) quartiles for calcium intake: 1 <696 mg, 2 696-1051 mg, 3 1052-1425 mg, 4 >1425 mg) 8 year follow-up US data	Relative Risks for highest versus the lowest quartiles of total calcium 0.67 (95% CI: 0.47-0.94,P for trend 0.09) and 0.94 (95% CI:0.66-1.35, P for trend 0.68) for milk products
Shaper et al. 1991	Prospective cohort 7735 men Age 40-59 years (1981) Questioned about milk intake and milk taken on cereal (divided into none milk drinkers, < ½ pint, <1 pint and > 1 pint) 9.5 year follow up UK data	Men with lower milk intake had higher rate of heart attack than high milk drinkers at baseline. There were big differences between the groups, and when background characteristics were adjusted for no significant association could be seen between the groups.

Authors	Details of study	Results / Conclusion
Mann et al. 1997	10802 male & female vegetarians Aged 16–79 years 13 year follow up UK data	The ratio of deaths from all causes in subjects who drank > ½ pint of milk per day, compared to < ½ pint, was 0.87 (0.68-1.13). Only 63 (16%) of deaths has been certified as due to ischaemic heart disease and the ratio in these was 1.50 (0.81-2.78) for the subjects in the highest milk consumption group, relative to the low-consumption group.
Elwood et al. 2004	Caerphilly Prospective study (set up in 1979-1983) 2512 men Age 45-59 years Semiquantitative food frequency questionnaire used to assess usual intake Group followed for 20-24 years and incidence of IHD and stroke events identified UK data	The hazard ratio in men with a milk consumption of 1 pint or more (0.57 L), relative to men who stated they consumed no milk, is 0.71 (0.40-1.26) for IHD and 0.66 (0.24-1.81) for ischaemic stroke.
Iso et al. 1999	Nurses Health Study (commenced 1976, followed up 1994) 85 764 women (no evidence of CHD) Aged 34-59 years Completed dietary questionnaires, calcium intake was assessed, US data	Women in the highest quartile of calcium intake had an adjusted relative risk of ishaemic stroke of 0.69 (95%CI, 0.50-0.95, P for trend 0.03) compared with those in the lowest quartile (this result was not restricted to milk, but observed for yoghurt, hard cheese and ice cream.

Authors	Details of study	Results / Conclusion
Ness et al. 2001	5765 men (commenced 1970), 25 year follow up Aged 35-64 years Completed health and lifestyle questionnaires which asked about daily milk consumption UK (Scotland) data	Men who drank more than 1 1/3 pints of milk per day had an adjusted risk of death from CHD, relative to men who drank < 1/3 pint
Kinjo et al. 1999	223170 men and women Asked about no. of times drank milk in a week 25 year follow up Japanese data	There were 3084 deaths due to cerebral embolism and thrombosis. Risk ratios, adjusted for confounding (sex, age, area, smoking, alcohol and occupation), were inversely associated with milk consumption. The relative risk in subjects who drank milk more than four times a week compared with those who drank milk less than once a week was 0.85 (0.77-0.92).
Abbott et al. 1996	Baseline data from Honolulu Heart Program 3150 men (age 55-68 years) Reports baseline dietary calcium intake on stroke risk in 22 years follow up US data (Japanese ancestry)	Men who were non-drinkers of milk experienced stroke at twice the rate ($P<0.05$) of men who consumed $\geq$2 cups of milk / day. While the rate of stroke decreased with increasing milk intake, the decline in stroke risk with increased consumption was modest for those who consumed less than 2 cups. Intake of dietary calcium was also associated with a reduced risk of stroke ($P<0.01$), although its association was confounded with milk consumption.
Snowdon et al. 1984	8724 men , 15048 women (age 40-65 years) Half of subjects were lacto-vegetarian; milk intake divided into 2 glasses or more per day. US data	In subjects with an intake of milk >2glasses the relative risk of coronary death in the following 20 years, compared with those that drank no milk, was 0.94 men and 1.11 in women (no confidence intervals available).

Authors	Details of study	Results / Conclusion
Vijver et al. 1992	Data obtained from the General Health examination in 1953-1954, 1340 men and 1265 women (40-65 years) Calcium intake was assessed at baseline by a 1-week food frequency recall, compared subjects in top fifth of total calcium intake vs. bottom fifth Cardiovascular deaths identified over the following 28 years	Adjusted odds ratios for cardiovascular disease in the fifth of subjects with the highest calcium intakes, compared with the lowest, were 0.77 (0.53-1.11) and 0.91(0.55-1.50) for males and females, respectively.
Barr et al. 2000	Randomised, controlled open trail 204 healthy men & women Age 55-85 years Advised to increase skim or 1% milk intake by 3 cups / day (n=101) or to maintain usual diet (n=103) for 12 weeks after a 4-week baseline period.	Total and low-density lipoprotein cholesterol levels and the ratio of TC to HDL-c were unchanged. Triglyceride levels increased within the normal range in the milk group (P=0.002).
Elwood et al. 2005a	Caerphilly Cohort, population sample of men age 45-59 years, 665 men completed 7 day food intake and milk consumption was determined. UK (Wales) data	The relative odds of an event in the men whose milk consumption was median or higher, relative to those with lower intakes of milk, were 0.52 (0.27-0.99) for an ishaemic stroke and 0.88 (0.56-1.40) for an ishaemic heart disease event.

Authors	Details of study	Results / Conclusion
Jacqmain *et al.* 2003	Cross-sectional data from phase 2 of Québec Family Study Adults age 20-65 years (235 men, 235 women) Subjects divided into 3 groups based on daily calcium intake A. <600 mg, B. 600-1000 mg, C. >1000 mg	Daily calcium intake was negatively correlated with plasma LDL cholesterol, total cholesterol, and total:HDL cholesterol in women and men after adjustment for variations in body fat mass and waist circumference ($P<0.05$).
Sjogren *et al.* 2004	Cross-sectional data 291 healthy men, age 62-64 Individuals completed 7-d dietary records, and fasting plasma insulin, lipid and lipoprotein concentrations as well as serum and adipose tissue FA composition were determined. Subjects were divided into tertiles of percentage distribution of sdLDL.	Small dense LDL was positively related to plasma triacylglycerol and fasting insulin concentrations ($P<0.001$) and inversely related to HDL cholesterol ($P<0.0001$). No strong relations were found between sdLDL and the reported intake of SFA, monounsaturated fatty acids or PUFA. Individual FAs typically found in milk products were associated with a more favourable LDL profile. This was shown for 4:0-10:0 and 14:0 in the diet ($P<0.05$), 15:0 and 17:0 in serum phospholipids (both $P<0.05$), and 15:0 in serum nonesterified FA ($P<0.01$).
Denke *et al.* 1993	Randomised controlled trial, single-blind 13 healthy men with moderate hypercholesterolemia Comparison of a low-calcium diet (34% fat, 13% SFA, 410 mg calcium) versus a fortified diet (2200 mg calcium / day – calcium citrate malate).	The percentage of dietary saturated fat excreted per day increased from 6 to 13% with calcium fortification. The high Ca diet significantly reduced total cholesterol 6% (5.99 to 5.66 mmol/L), LDL cholesterol 11% (4.13 to 3.67 mmol/L), and apolipoprotein A1 7% ($P<0.05$) when compared with the low Ca diet.

Authors	Details of study	Results / Conclusion
Bostick et al. 2000	Randomised, double-blind, placebo controlled trial Outpatient clinic 193 (men & women, age 30-74 year) Subjects supplemented with 1 or 2 g elemental calcium vs. placebo over a 4-month period (cholesterol outcomes) US data	Because of no apparent difference between the 1 and 2 g supplemented group these data were pooled and a comparison made with the placebo group. Mean cholesterol level dropped 0.07 mmol/L (1.3%) (P=0.43) more and the mean HDL-C level dropped 0.01 mmol/L (1.1%) (P=0.71) less in the calcium group than in the placebo group. Among participants without a history of hypercholesterolemia, the mean cholesterol level dropped 0.18 mmol/L (3.3%) (P=0.01). and the HDL-C level dropped 0.02 (1.5%) (P=0.61) more in the calcium group than in the placebo group.
Barr et al. 2000	Randomised, controlled open trial 240 healthy men & women (age 55-85 years) Who consumed < 1 ½ serves dairy per day – from 6 US academic health centres. Provided with advice to increase skim milk or 1% milk by 3 cups per day (n=101) or maintain usual diet (n=103) for 12 weeks after a 4 week baseline period.	Total and LDL-C and the ratio of TC to HDL-C were unchanged. Triglycerides increased within normal range in the milk group (P=0.002).

Authors	Details of study	Results / Conclusion
Bowen et al. 2005	Parallel design 50 overweight & obese (30 women, 20 men) 1. high dairy protein/high calcium (DP 2400 mg/d) (age men 49 $\pm$ 3.2, women 47 $\pm$ 2.4 years) 2. high mixed protein /moderate calcium (MP 500 mg/d) (age men 49 $\pm$ 4.2, women 46 $\pm$ 2.6 years Subjects all subjects given 5.5 MJ/d (inc. to 7 MJ/d if active) 12 weeks energy restriction - ER (4 week energy balance - EB)	Fasting plasma levels of lipids changed throughout the study, with no differences between dietary groups or gender. Overall, TC decreased by 0.62 $\pm$ 0.11 mmol/L during ER, and increased by 0.21 $\pm$ 0.10 mmol/L during EB, resulting in a net reduction in TC (-0.41 $\pm$ 0.07 mmol/L; P<0.001). LDL-C decreased 0.60 $\pm$ 0.10 mol/L after 12 weeks of ER (P<0.001), at week 16, LDLD-C remained 0.36 $\pm$ 0.10 mmol/L below baseline (P<0.001). Fasting triglycerides decreased by 0.35 $\pm$ 0.06 mmol/L during ER, by week 16, there was a 0.24 + 0.06 mmol/L decrease in triglyceride concentration compared to baseline (P<0.001). Fasting HDL-C remained unchanged during the ER and EB phases.
Thompson et al. 2005	Refer to table 1.6	Each group showed a significant increase in HDL-C and significant reductions in total cholesterol, LDL-C and triglycerides - but there were no differences between the groups (P=0.71, TC; P=0.24, HDL-C; P=0.77, LDL-C; P=0.40, triglycerides)
Zemel et al. 2004	Refer to table 1.6	No significant treatment effect on TC, HDL-C or LDL-C (no values reported)
Zemel et al. 2005a	Refer to table 1.6	No significant treatment effect on circulating lipids (no values reported)
Zemel et al. 2005b	Refer to table 1.6	No significant treatment effect on circulating lipids (weight maintenance) Not reported on the weight loss (phase 2) study
Major et al. 2007	RCT- parallel design	After 15 wk intervention > decrease LDL (P=0.05)and LDL:HDL (P<0.01)

63 overweight/obese women, daily Ca intake <800 mg. Energy-restricted diet (700 kcal/d) for 15 wk 1. Elemental Ca 1200 mg + 400 IU vit D 2. placebo only	in treatment group independent of changes in wt and fat loss.

Chapter 2: General Materials & Methods

2.1 Materials

- **Bio-Rad** 550 microplate reader
- **Blood pressure** machine: Datex-Engstrom Cardiocap™ II and Model T8, Omron, Japan
- **Calcium fortified milk powder**: NatraCal™, Murray Goulburn Co-Operative Ltd. Brunswick, VIC, Australia
- **Calcium tablets**: Citrical, Mission Pharmacal Australasia, Sydney, Australia
- **Cholesterol** and triglyceride enzymatic colorimetric kit: TRACE Scientific Ltd, Melbourne, VIC, Australia
- **Dual energy X-ray absorptometry (DEXA)**: Hologic QDR-4500A, version 8.6, Bedford, MA, USA
- **Dietary Scales**: Philips electronic scales, VIC, Australia
- **Digital Scale**: Tanita System 502, Tokyo, Japan
- **Glucose** (finger prick – acute study only): ACCU-CHEK Active blood glucose strips, Roche Diagnostics, NSW, Australia
- **Glucose** colorimetric assay kit (chronic study): Randox Laboratories Ltd. Ardmore, Co.Antrim, UK
- **Glycerol** kit: Randox Laboratories Ltd. Ardmore, Co.Antrim, UK
- **Indirect calorimetry**: Deltatrac II metabolic monitor, Datex Ohmeda, Finland
- **Insulin** ELISA kits: DAKO Diagnostic, Cambridgeshire, UK
- **Medi**-swab alcohol swabs: Smith and Nephew, Mount Waverley, VIC, Australia
- **Microplates** 96-well: NUNC, Naperville, USA
- **Milk products**: Murray Goulburn Co-Operative Ltd. Brunswick, VIC, Australia
- **Non-esterified fatty acid** kit: Wako Pure Chemical Industries, Ltd. Osaka, Japan
- **Polyjoule**: Nutricia, Mount Wellington, New Zealand

- **Skinfold Calipers**: Harpenden brand, Diagnostic Instruments Pty. Ltd. Victoria Park, West Australia
- **Syringes:** Terumo, Elkton, USA
- **Tape measure**: Rosscraft Pty. Ltd. Vancouver, Canada
- **Urinary calcium**: Atomic Absorption Spectrometer, Avanta Σ, GBC Scientific Equipment Pty Ltd. VIC, Australia
- **Urine containers**: 4 litre stock bottles, Viscount Plastics, Canning Vale, West Australia
- **Vacutainer adaptors**: Becton Dickinson, Franklin Lakes, USA
- **Vacutainer EDTA** collection tubes: Greiner bio-one, Kremunster, Austria
- **Vacutainer SST (sera)** collection tubes: Greiner bio-one, Kremunster, Austria
- **Winged-infusion** sets: Terumo, Tokyo, Japan

2.2 Methods

2.2.1 Subjects and experimental protocol

The investigation into the effect of dairy calcium consumption on weight and fat loss was conducted in the acute and chronic setting. The details concerning the specific characteristics of the subjects recruited and the experimental protocol of the respective studies will be described in subsequent chapters. This chapter will cover specific methods which were used for both the acute and chronic studies.

2.2.1.1 Measurements of Body Composition

2.2.1.1.1 Body weight and height

Body weight was measured after an overnight fast on each occasion, immediately after voiding, with subjects wearing underwear and a light surgical gown, on a digital balance (Tanita System 502, Tokyo, Japan) and recorded to the nearest 100 g. Standing height was measured using a stadiometer fixed to the wall and recorded to the nearest 0.1 cm

2.2.1.1.2 Waist and hip circumferences

Waist and hip circumferences were measured as described by Norton and Olds (2000). The waist measurement is taken at the level of the narrowest point between the lower costal (rib) border and the iliac crest. If there is no obvious narrowing then the measurement was taken at the mid-point between these two landmarks. Each measurement was taken at the end of a normal expiration with the arms relaxed at the sides. The waist was measured twice and a mean value recorded for each subject. Waist circumference measurements were only taken by the chief investigator. Unpublished repeatability measurements of 25 overweight/obese clinical patients gave a CV% of 1.1. The hip measurement was taken at the level of the greatest posterior protuberance of the buttocks. The measurer stood by the side of the subject and measured with the tape in a horizontal plane.

2.2.1.1.3 Dual Energy X-ray absorptometry

Body composition was determined by Dual energy X-ray absorptometry (DEXA) (Hologic QDR-4500A, version 8.6, Bedford, MA, USA) and were performed at an accredited DEXA centre off-site but close to Curtin University Bentley Campus. The scan mode used was Array. Daily scanning of the anthropometric spine phantom were conducted (using the Hologic protocol). In addition a daily reading is checked using Shewart Multi-rule Charts and Cusum plots (Lu *et al.* 1996). In addition a weekly step phantom is analysed to enable standard body composition measurements to be compared. An abdominal region of interest (ROI) was delineated as previously described by Ley *et al.* (1992), in brief, it is between the upper part of the body of T12 and the iliac crest and its sides lateral to any trunk soft tissue. The software system used was the Hologic Dos Version. CVs for the measurement of a total body scan were >4%.

2.2.2 Measurement of blood pressure

In all studies subjects were measured under standardised conditions, this included, an overnight fast (of at least 12 hours), and having rested in a supine position for at least 1 hour.

2.2.2.1 Acute Study

During the acute studies the Blood Pressure machine (Datex-Engstrom Cardiocap™ II) was used. The subjects were lying in a supine position, as for the measurement of energy expenditure. The blood pressure measurement was taken during the last 5 minutes of measurement, to ensure that subjects were relaxed during measurements. Three blood pressure measurements were taken, with a minute rest in between and the mean of the three measurements recorded for each subject.

2.2.2.2 Chronic Study

During the weight loss study systolic and diastolic blood pressure were measured at baseline, week 6 and at the end of each diet period, using the Omron blood pressure machine (Model T8, Omron, Japan). Three blood pressure measurements were taken, with a minute rest in between and the mean of the three measurements recorded for each subject.

2.2.3 Using Indirect Calorimetry

Since Basal Metabolic Rate (BMR) constitutes up to 70% for the total energy output, is reasonably stable and easily measurable, it is appropriate that the energy needs of man are based on measures of BMR as an approximate for the total energy output (Soares *et al.* 1989). Resting Metabolic Rate (RMR) is usually used as synonym to BMR, but is conceptually different. BMR measurements need to be conducted immediately after the subject wakes up, but are not feasible when overnight facilities are not available. However, Soares *et al.* have shown that these two methods yield similar results, when proper rest periods are observed (1989).

There are several ways of measuring energy expenditure. The accurate, but invasive way is the whole body calorimetry or direct calorimetry. This method involves the subject being placed in a thermically isolated chamber, where the heat that he/she is dissipating is accurately collected and precisely measured (Ferranini, 1988). In the present studies RMR was measured by indirect calorimetry using a Deltatrac II metabolic monitor (Datex Ohmeda, Finland); an open-circuit ventilated canopy

measurement system. Indirect calorimetry is a non-invasive method, by which the type and rate of substrate utilisation and energy metabolism are estimated in vivo starting from gas exchange measurements (measurements of oxygen consumption and carbon dioxide production). The background behind the indirect calorimetry is that the main goal of nutrient metabolism is to produce energy, involving the extraction of the chemical energy of a substrate (being carbohydrate, protein and fat) via complete oxidation to carbon dioxide and water. The heat produced by these biological combustions is used to maintain body temperature. The concept mentioned above is based on the assumption that all the oxygen is used to oxidise degradable fuels, and that all the carbon dioxide evolved by this means is recovered, therefore making it possible to calculate the total amount of energy produced (Ferranini, 1988). Energy produced means the conversion of the chemical free-energy from nutrients into energy of adenosine triphosphate (ATP) plus some energy loss during the oxidation process. O_2 and CO_2 concentrations were measured by means of a paramagnetic O_2 analyser and infrared CO_2 analyser. Humidity measurements are also performed for correction to standard temperature and pressure (STPD). The flow meters were set at 46.4 L/min to account for the size of an adult subject, and the gas analysers are manually calibrated before each use by a calibration gas mixture (see below). The measurements of VO_2 and VCO_2 are used to calculate Respiratory Quotient (RQ) using the equation VCO_2/VO_2 and Resting Energy Expenditure using the following equation by Ferranini (1988).

Energy expenditure (kJ/hr): $(3.91 \times VO_2/1000) + (1.1 \times VCO_2) - (3.34 \times N) \times 60 \times 4.184$

Where N is the total nitrogen excreted in the urine.

Figure 2:1 Overview of Patient Connections in Canopy Mode

1. Mixing chamber inlet, 2. Canopy hose, 3. Canopy, 4. Flow generator inlet, 5. Sampling line, 6. & 7. Canopy outlet fittings, 8. Plastic seal

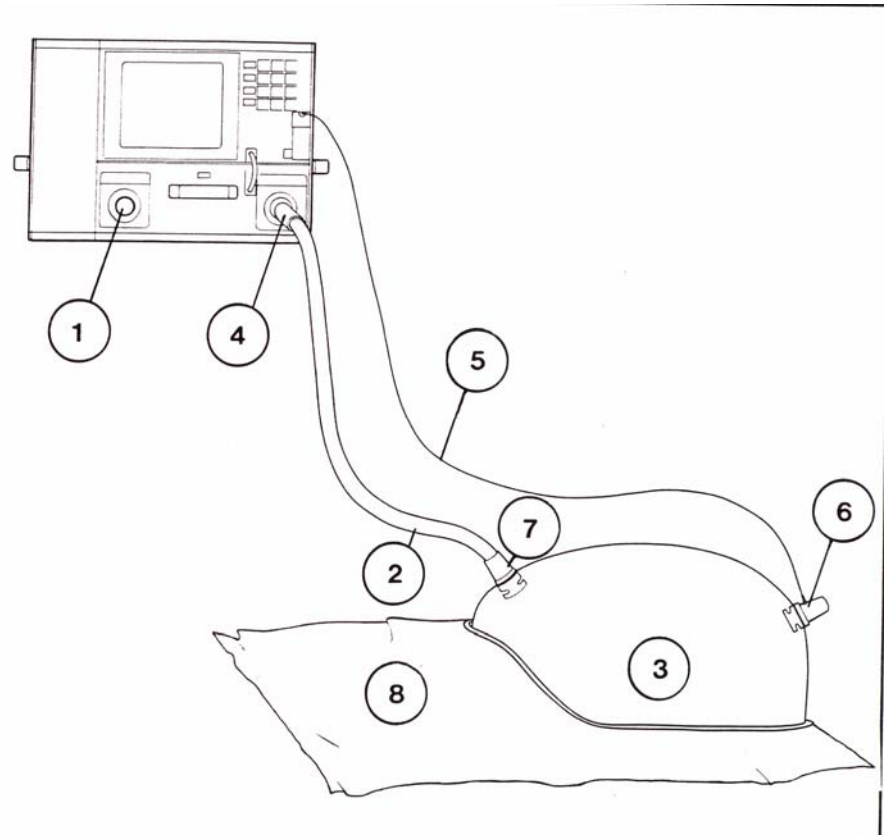
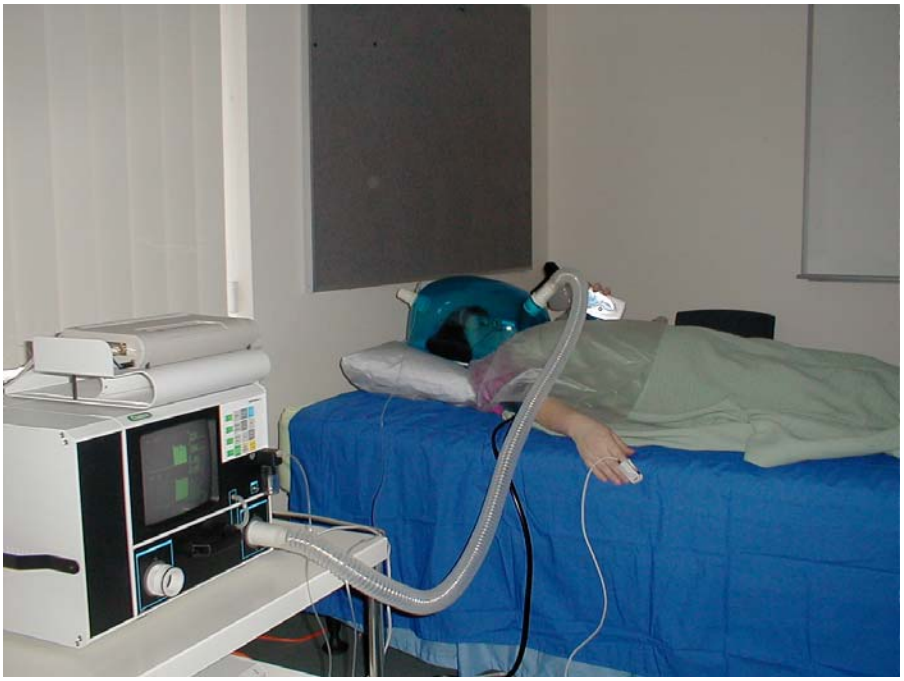


Figure 2:1 demonstrates the direction of the air flow when the Deltatrac II is used for indirect calorimetry measurements. Atmospheric air enters the canopy at 6. as the person breathes in, and the exhaled air exits through tube 2. A sample of air is taken through tube 1, as well as a sample of exhaled air and they are analysed separately by the Deltatrac II.

Figure 2:2 Subject during testing phase



2.2.4 Calibration of Deltatrac II

The Deltatrac II was allowed to warm-up for 30 minutes each morning of testing, followed by a calibration protocol. The calibration protocol allows for the automatic calibration of the gas supply. The Datex calibration gas contains 95% oxygen and 5% carbon dioxide, in the automatic mode the monitor compares the measured gas concentration with calibration gas values stored in the monitor memory, and adjusts the measurement accordingly. The adjustment value will not allow a greater than 3% variance, and will require re-calibrating if this arises. Performance of the instrument was also checked at regular intervals during the study period by monitoring the ratio of carbon dioxide produced to oxygen consumed, during a 30-minute ethanol burn. During all PhD experiments the mean (SD) ratio for ethanol burns was 0.664 (0.007) with a CV of 1.5 %.

2.2.4.1 Measurement of Resting Metabolic Rate

The measurement was conducted under standardised conditions, as in previous studies (Piers *et al.* 2002a) with subjects lying (a) at complete physical rest; (b) in a thermally neutral environment; (c) 12-14 hours after their last meal and a minimum

of 8 hours of sleep; (d) awake and emotionally undisturbed; and (e) without disease and fever. The within-subject coefficient of variation (CV) in RMR was 3.3%.

2.2.5 Diet Induced thermogenesis

Diet induced thermogenesis (DIT) was measured as described previously (Piers *et al.* 2002a). In brief, twenty minutes into each postprandial hour, subjects returned to the supine position and rested for 10 minutes. The canopy was then placed over their head and measurements made for the last 30 minutes of the hour. The first 5 minutes was not included in subsequent data analysis. In between measurements subjects were allowed to sit up in bed and listen to music, or read. Some elected to sit at a table close to the bed, where they did craft work or read a book. An intermittent measurement protocol provides accurate postprandial data relative to that obtained by continuous minute-to-minute measurement over the postprandial period (Piers *et al.* 1992). Results were expressed in absolute values (kJ/h) and as a percentage of the energy in the breakfast meal.

Whole-body substrate oxidation rates were calculated at rest (fasting), and for every hour up to 5 hours (6 hours – acute studies) postprandially, using measures of oxygen consumption (VO_2), carbon dioxide production (VCO_2) and urinary nitrogen excretion in that postprandial phase. The equations of (Ferrannini, 1988) were used to calculate energy expenditure and substrate oxidation rates (see next page).

2.2.5.1 Calculation for Fat, Carbohydrate & Protein Oxidation

Figure 2:3 Equations for the calculation of protein, fat and carbohydrate oxidation

Protein oxidation (g/hr) =

$$6.25 \times N$$

Fat oxidation (g/hr) =

$$1.67 \times (VO_2 - VCO_2)/1000 - (1.92 \times N) \times 60$$

Carbohydrate oxidation (g/hr) =

$$(4.55 \times VCO_2/1000) - (3.21 \times VO_2/1000) - (2.87 \times N) \times 60$$

Where N is the total nitrogen excreted in urine (g/hr), VO2 is the oxygen consumption (L/hr) and VCO2 is the carbon dioxide produced (L/hr) (Ferranini, 1988).

Indirect calorimetry measurements are very delicate, and consequently there are a number of conditions that need to be fulfilled in order to obtain accurate results.

1. Reduction of Physical Activity at least 36 hours prior to the experiment: In a review by (Granata & Brandon, 2002) they showed that strenuous physical activity has been demonstrated to increase resting energy expenditure for as long as 24 to 48 hours. Also physical activity increases fat oxidation and decreases glucose metabolism rates for up to 24 hours. To achieve the most accurate indirect calorimetry results, it is recommended that subjects refrain from strenuous physical activity 36 hours before the test day.

2. Controlling emotional state: Glucose metabolism has been demonstrated to be elevated during stressful situations by the increased secretion of the counter-regulatory hormones (to insulin), cortisol, catecholamines and glucagon. Elevation of these hormones results in the elevation of endogenous glucose production which is secondary to accelerated hepatic gluconeogenesis (Weissman, 1999). The increased glucose production, together with the peripheral tissue resistance to insulin, results in

reduced glucose utilisation and triggers hyperglycaemia. In this situation insulin levels are within normal or mildly elevated range, but not high enough to counteract the hyperglycaemia. In addition to the hyperglycaemia, fat oxidation is also increased and glucose oxidation is decreased. The fatty acids released via lipolysis undergo β-oxidation, which in a stressed person is the main ATP-production pathway. Hence it is essential a subject is well-rested prior to the test day, and is familiar with the equipment used for the testing to prevent any apprehension. The emotional state of a subject can affect his/her BMR by varying the levels of circulating epinephrine and norepinephrine levels. Anxiety and tension in a subject has been shown to elevate BMR as an increase in epinephrine secretion results. To this purpose all subjects underwent an initial visit, when they were put under the calorimetry hood and allowed to rest for at least 5 minutes to experience the test day process. Subjects who felt at all apprehensive during this process were eliminated from the study.

3. Restlessness or hypo/hyper ventilation may affect the volume of gases exchanged: Although intra-individual variation has been demonstrated to be insignificant (Shetty & Soares, 1988). It is essential that each test day is meticulously controlled, and that subjects stay relaxed during the testing period.

4. Menstrual cycle: It has been found that the thermic effect of a test meal increases significantly after ovulation (Piers, 1994). Although the change demonstrated was small, it is recommended that females subjects are tested after their menstruation and before ovulation, this is usually determined as between day 5 and 10 after commencement of menstruation. This was not a necessary consideration during the acute studies, as only women 2 years post-menopause were recruited. However, in the chronic weight loss study, two subjects were pre-menopausal and were tested during day 5 and 10 of their cycles.

5. Previous Dietary Intake: In a review by Granata and Brandon (2002) it was demonstrated that the preceding dietary intake influences the amount of lipogenesis and the magnitude of thermogenesis after a high-carbohydrate meal. In addition, research demonstrates that overfeeding increases resting energy expenditure (REE) and the thermic effect of food (TEF). Therefore in all studies that included a fasted testing, the subjects were provided with a dinner pack of known energy and nutrient value

6. Environmental temperature: Body temperature is very tightly controlled within the body. To this end, any changes in the environmental temperature will trigger

temperature control mechanisms so that the body is maintained at a constant temperature. For example, the metabolic rate will be elevated if the room temperature drops and the body's heat conserving mechanisms, such as shivering are activated. The metabolic testing room was therefore maintained at a comfortable 22-23^{o}C, and subjects were regularly asked if there were comfortable.

Indirect calorimetry necessitates the measurement of urinary non-protein nitrogen excretion, as it is the predominant (>90%) mechanism of nitrogen removal in normal subjects (Ferranini, 1988). Urinary nitrogen excretion is the most feasible and practical method to use; the Kjeldahl method was utilised for this purpose.

2.2.5.2 Measurement of nitrogen excretion

The Kjeldahl method was employed to determine the nitrogen content of the urine samples. The Kjeldahl method used during these experiments has been adapted from Egan *et al.* (1981). In brief, the Kjeldahl method involves digesting the sample with an acid mixture until the carbon and hydrogen are oxidised and the protein nitrogen is converted into ammonium sulphate. The addition of a catalyst reduces the digestion time by raising the digestion temperature. The addition of hydrogen peroxide accelerates digestion and decreases foaming. When the solution is made alkaline the ammonium is converted to free ammonia. The ammonia is distilled from the solution and the condensate collected in a boric acid solution. This solution (which contains an indicator) is directly titrated with a standard acid. The percentage nitrogen can then be calculated in the sample (see equation below). The CV_{intra}% and CV_{inter}% were 1.2 and 1.9, respectively.

Figure 2:4 Equation to calculation nitrogen content of urine sample

(sample titre mL – blank titre mL) x M HCL x 14.1 x 100 / mg sample

2.2.6 Blood collection

Venous blood samples were taken from subjects lying in a supine position and rested for at least 30 minutes. Blood was drawn into specimen tubes (vacutainers)

containing EDTA (plasma) and SST (sera), an additional tube was collected anaerobically by drawing blood into a vacutainer containing a gel separator. The SST vacutainer tubes were left to stand for 30 minutes at room temperature and then centrifuged at 3000 x g for 10 min, EDTA tubes were spun 5 minutes after collection. Serum and plasma was extracted and the samples stored at -80ºC for later analysis. The anaerobically-collected tubes (for the determination of ionised calcium) were sent directly to an accredited laboratory and assayed within 12 hours.

2.2.6.1 Measurement of blood lipids

Serum triglycerides (TG) and total cholesterol were measured by enzymatic colorimetric kits (TRACE Scientific LTD, Melbourne, Australia). Serum HDL cholesterol was determined after precipitation of apo-B-containing lipoproteins with phosphotungstic acid and $MgCl_2$ (diluted 1:2 with precipitant). HDL cholesterol contained in the supernatant was determined by enzymatic colorimetry (TRACE Scientific LTD, Melbourne, Australia). Samples were analysed in triplicate according to the manufacturer's instructions. In brief, serum samples for the determination of triglycerides and total cholesterol were diluted 1:100 with the manufacturer's reagent, and serum/plasma samples for the determination of HDL cholesterol were diluted 1:10 with the manufacturer's reagent. The diluted samples and standards were mixed briefly (using microplate reader) then incubated at 37^{o}C for 15 minutes. Samples were remixed and left to stand at room temperature for 7 minutes. The absorbance of samples was determined at a wavelength of 490nm using a Bio-Rad 550 microplate reader and compared against the absorbance of the standards. Serum LDL cholesterol was determined by using a modified version of the Friedewald equation (Bairaktari *et al.* 2000). The mean intra- and inter-assay coefficients of variance for total cholesterol, LDL- and HDL-cholesterol were less than 2%.

LDL cholesterol = [total cholesterol]-[HDL cholesterol] – (0.46 x [TG]).

The enzymatic determination of cholesterol is based on the following reactions:

$$\text{Cholesterol Esters} \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol + Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{hydroxybenzoic acid} + \text{4-amioantipyrine} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye} + 4H_2O$$

The enzymatic determination of triglyceride is based on the following reactions

$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Free Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetone phosphate} + 2H_2O_2$$

$$H_2O_3 + \text{4-aminoantipyrine} + \text{3,5–dichloro–2–hydroxybenzene sulphonate} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine Dye (red)} + 2\,H_2O$$

2.2.6.2 Measurement of blood glucose

2.2.6.2.1 Acute Studies

Blood glucose was measured using the Accu-Chek Active glucose strips (Roche Diagnostic, Australia). Whole venous blood was applied to the strips and reacts with glucose dehydrogenase and NAD^+ to produce a small electrical current which is directly proportional to the amount of glucose present in the blood sample.

2.2.6.2.2 Chronic Study

Serum glucose was measured according to manufacturers' directions (Randox Laboratories Ltd. Ardmore, Co.Antrim, UK). In brief, glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide produced, reacts catalysed by peroxidase, with phenol and 4-aminophenazone to

form a red-violet colour (using quinoneimine dye as indictor). The intensity of the colour is directly proportional to the glucose concentration and is measured at a wavelength of 505 nm.

The enzymatic determination of glucose is based on the following reactions:

Glucose + 0_2 + H_20 ⟶ gluconic acid + H_20_2

2 H_20_2 + 4-aminophenazone + phenol ⟶ quinoneimine + $4H_20$

2.2.6.3 Measurement of insulin and insulin resistance

Serum insulin was measured by enzyme-linked immunosorbent assay (ELISA) based on two monoclonal antibodies (Dako Diagnostic, UK) according to the manufacturer's instructions. Briefly, calibrators (containing biosynthetic insulin in a protein matrix) as well as serum samples are incubated with an enzyme-labelled antibody for 1 hour in a microplate well coated with a specific anti-insulin antibody. Simultaneous incubation of the samples and antibody forms a complex, and subsequent washing in an organic buffered solution removes unbound enzyme-labelled antibodies. The bound conjugate is detected by reaction with the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding 1mol/L phosphoric acid to give a colorimetric endpoint that is read spectrophotometrically at a wavelength of 450nm. Construction of a calibration curve allows for the determination of insulin concentration in the samples. The mean intra- and inter-assay coefficients of variance for insulin are less than 5%.

The homeostasis model assessment (HOMA score) was used as a surrogate estimate of the state of insulin sensitivity by multiplying fasting insulin concentration (mIU/L) by fasting glucose concentration (mM) and dividing by 22.5 (Matthews *et al.* 1985). The HOMA score has been shown to be a reliable surrogate marker for in vivo insulin sensitivity in humans (Bonora *et al.* 2000). Bonora *et al.* reported a strong positive correlation between euglycaemic, hyperinsulinaemic clamp-measured total glucose disposal (gold standard of insulin sensitivity) and HOMA-estimated insulin sensitivity (r = 0.82, P 0.0001) in 115 subjects with varying degrees of glucose tolerance and insulin sensitivity.

2.2.6.4 Measurement of glycerol

Serum glycerol was measured using the colorimetric method (Randox Laboratories Ltd. Ardmore, Co.Antrim, UK) according to manufacturer's instructions. In brief, the direct colorimetric procedure for the measurement of glycerol is described utilising a quinoneimine chromagen system in the presence of glycerol kinase, peroxidise and glycerol phosphate oxidase.

The enzymatic determination of glycerol is based on the following reactions:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + 0_2 \xrightarrow{\text{Glycerol phosphate oxidase}} H_20_2 + \text{Dihydroxyacetone-phosphate}$$

$$2H_20_2 + \text{3,5-dichloro-2-hydroxybenzene sulphonic acid} + \text{4-aminophenazone} \xrightarrow{\text{Peroxidase}} \text{n-(4-antipyryl)-3-chloro-5-sulphonate-p-benzoquinoneimine}$$

The mean intra- and inter-assay coefficients of variance for glycerol are less than 2%.

2.2.6.5 Measurement of Non-Esterified Fatty Acids (NEFA)

Serum NEFA was measured using an enzymatic method (Wako Pure Chemical Industries, Ltd. Osaka, Japan), according to manufacturer's instructions. In brief, this method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase. The acyl-CoA thus produced is oxidised by added acyl-CoA oxidase with the generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase permits the oxidative condensation of 30methyl-N-ethyl-N-(β-hydro-xyethyl)-aniline with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at a wavelength of 550 nm. The mean intra- and inter-assay coefficients of variance for NEFA are less than 2%.

The following measurements were made at PathWest Laboratory, Perth WA.

2.2.6.6 Measurement of Intact Parathyroid Hormone (iPTH)

Plasma iPTH was measured using the Chemiluminescence Immunoassay method (Nichols Institute Diagnostics, San Clemente, USA), as per the manufacturer's instructions. In brief, this method utilises two goat polyclonal antibodies to human iPTH. One of the polyclonal antibodies is coupled to biotin, while the other polyclonal antibody is labelled with acridinium ester for detection. The iPTH is sandwiched between these antibodies. After an initial incubation period with the acridinium labeled antibody, streptavidin coated magnetic particles and the capture antibody are added to the reaction mixture, a second incubation follows. Free labelled antibody is separated from the labeled antibody bound to the magnetic particles by aspiration of the reaction mixture and subsequent washing. The wells containing the washed magnetic particles are transported into the system luminometer, which automatically injects trigger 1 and trigger 2, initiating the chemiluminescence reaction. The light is quantitated by the luminometer and expressed as relative light units. The amount of bound labelled antibody is directly proportional to the concentration of iPTH in the sample. The mean intra- and inter-assay coefficients of variance for iPTH are 4.2 % (concentration level range 7.58-69.7 pmol/L) and 6.3 % (concentration level range 5.69-40.8 pmol/L), respectively.

2.2.6.7 Measurement of Haemoglobin A1c

Serum HbA1c was measured using the Ion Exchange High Performance Liquid Chromatography method (BioRad Variant Haemoglobin testing system, 270-2101, Munchen, Germany), as per manufacturer's instructions. In brief, glucose binds to HbA in a two stage process, the first stage is fast but reversible and the product is an aldimine or Schiff base. The second stage is a slow, irreversible conversion of this product to the keroamine, HbA1c. Prepared haemolysate is injected onto the cation ion exchange analytical cartridge strongly retaining all haemoglobin fractions. A gradual increase in the ionic strength of a two buffer gradient system then displaces the strongly retaining haemoglobin fractions, which elute off at characteristic retention times. A dual-wavelength filter photometer monitors and records the

absorbance (415 and 690 nm) for each heamoglobin fraction. The mean intra- and inter-assay coefficients of variance for HbA1c are within the range of 1.6-2.9 % (concentration levels 5.5-9.8 mmol/L).

2.2.6.8 Measurement of Triiodothyronine (3,5,3'-L-triiodothyronine, T3) – free T3

Triiodothyronine is a hormone synthesised and secreted from the thyroid gland. T_3 and $_4$ are secreted into the circulation in response to thyroid stimulating hormone and play an important part in regulating metabolism. In the circulation, 99.7% of T_3 is reversibly bound to transport proteins, primarily thyroxine-binding globulin (TBG) and to a lesser extent albumin and prealbumin. The remaining T_3 does not bind to transport proteins, but is free in the circulation. This unbound fraction of the total T_3 concentration is free triiodothyronine (FT_3). Serum FT_3 was measured using the ADVIA® Centaur™ System (Bayer Corporation, Diagnostics Division, Tarrytown, New York, USA), as per manufactures instructions. The ADVIA Centaur FT_3 assay is a competitive immunoassay using direct chemiluminescent technology. FT_3 in the sample competes with a T_3 analog, which is covalently coupled to paramagnetic particles in the Solid Phase for a limited amount of a combination of acridinium ester-labeled monoclonal mouse anti-T_3 antibodies in the Lite Reagent. An inverse relationship exists between the amount of FT_3 present in the patient sample and the amount of relative light units detected by the system. The mean intra- and inter-assay coefficients of variance for FT_3 are less than 5%.

2.2.6.9 Measurement of Paracetamol (Acetaminophen)

Serum paracetamol was measured using the method as per manufacturer's instructions (Cambridge Life Sciences plc, Cambridgeshire, UK). The principle of this assay is based on the use of an enzyme specific for the amide body of acylated aromatic amines. It cleaves the paracetamol molecule, yielding p-aminophenol, which reacts specifically with o-cresol in ammoniacal copper solution to produce a blue colour. The mean intra- and inter-assay coefficients of variance for Paracetamol are less than 1.4% .

2.2.6.10 Measurement of 25-Hydroxyvitamin D

Serum 25-Hydroxyvitamin D was measured as per manufacturers instructions (DiaSorin Inc. Minnesota, USA). In brief, as calciferol enters the circulation it is metabolized to several forms, the major one being 25-hydroxycalciferol. The first step in the metabolism of vitamin D, 25-hydroxylation, occurs mainly in the liver. Only a small amount of 25-OH-D is metabolized in the kidney to other dihydroxyvitamin D metabolites in man. Since 25-OH-D is the predominant circulating form of vitamin D in the normal population, it is considered the most reliable index of vitamin D status. This assay consists of a two-step procedure. The first procedure involves a rapid extraction of 25-OH-D and other metabolites from serum with acetonitrile. Following extraction, the treated sample is then assayed using radio-immuno-assay (RIA) method. The RIA method is based on an antibody, specific to 25-OH-D. The sample, antibody and tracer are incubated for 90 minutes at 20-25^{o}C. Following this phase separation is accomplished after a 20 minute incubation at 20-25^{o}C with a second antibody precipitating complex. A buffer is then added to this solution and centrifuged to reduce non-specific binding. The mean intra- and inter-assay coefficients of variance for 25-Hydroxyvitamin D are less than 5 %.

2.2.6.11 Measurement of Ionised calcium

Serum ionised calcium was measured using the calcium sensor method (Bayer Corporation, Diagnostics Division, Tarrytown, New York, USA). We used pH corrected ionised calcium results, as hydrogen ions compete with calcium for the calcium binding sites and hence a change in pH can have a direct effect on the calcium levels. The calcium value adjusted to pH of 7.40 reflects the true ionised calcium concentration of blood normalized to pH 7.40. Calcium is corrected according to the following equation.

$$\text{Adjusted } Ca^{2+} = Ca^{2+} \text{ measured} \times 10^{-0.178\,(\text{required pH} - \text{measured pH})}$$

The calcium sensor used is a half-cell that combines with the external reference sensor to form a complete electrochemical cell capable of measuring calcium levels

in a blood sample. The sensor contains a silver and silver chloride wire surrounded by an electrolyte solution that has a fixed concentration of calcium ions. A membrane, consisting of an ionophore imbedded in a polyvinyl chloride membrane, separates the electrolyte solution from the sample. The ionophore is a compound that is highly selective for calcium ions over other ions. When the sample comes in contact with the membrane of the measuring sensor, a membrane potential develops as calcium ions interact with the membrane. This membrane potential is compared to the constant potential of the external reference sensor. The final measured potential is proportional to the calcium ion concentration in the sample. The mean intra- and inter-assay coefficients of variance for ionised calcium are less than 2%.

2.2.6.12 Measurement of Urine Samples

Collection of urine was undertaken during metabolic test days, for the determination of nitrogen (protein oxidation) – see section 2.2.5.2. Twenty-four hour complete urine collections were made by each subject participating on the chronic weight loss study. Subjects were provided with a 4 litre container, which contained a weak hydrochloric acid solution to prevent the growth of bacteria. On the urine collection day, subjects were asked to empty their bladders on rising and note the time. The subjects then collected all urine for a total of 24 hours. On collection of the urine from the subjects, the volume was recorded, and 3 x 20 mL aliquots of urine decanted and frozen at -80^{o}C.

2.2.6.13 Measurement of Urinary Calcium

Urine samples were first defrosted thoroughly; secondly a 2 ml sample was digested using 0.5 ml of concentrated nitric acid (for one hour in a 100oC water bath). This was allowed to cool and 1 ml of potassium chloride (500 mg/L potassium chloride, KCl) was added as a releasing agent, and diluted to 10 ml. The digestion process is carried out before reading the absorbance to free any bound calcium (such as calcium bound with protein). Two samples for each urine collection were prepared and analysis was done in duplicate. The method of Atomic Absorption Spectrometer (AAS) (Avanta Σ, GBC Scientific Equipment Pty Ltd. Victoria, Australia) was used to determine the levels of calcium in each sample, as per the manufacturer's recommended methodology. Flame atomic absorption is a common technique for detecting metals in environmental samples. This technique is based on the fact that

ground state metals absorb light at specific wavelengths. Metal ions in a solution are converted to atomic state by means of a flame. Light of the appropriate wavelength is supplied and the amount of light absorbed is measured against a standard curve. The AAS was first standardised with 5 mg/L, 10 mg/L, 20 mg/L, 30 mg/L and 40 mg/L of potassium chloride and the absorbance of the urine samples read at 422.7 nm.

During sample testing the following parameters were kept constant.
Flame type: nitrous oxide / acetylene; Fuel flow: 4.94 l/min; Oxidant flow: 11.61 l/min; Burner angle: 43.6^{o}.

Chapter 3: The acute effect of dairy and non-dairy calcium on diet induced thermogenesis, substrate utilisation & appetite

3.1 Introduction

Obesity along with its co-morbidities of dyslipidemia, insulin resistance and cardiovascular disease, is now a world-wide problem. The prevalence of overweight/obesity in Australia increases with age and is greatest in the 55-64 year age group (Thorburn, 2005). Overall, it is more common for Australians to have a weight problem, with 48% men and 30% of women being overweight and a further 19% of men and 22% of women, obese (National Health and Medical Research Council, 1997). Interestingly, 50% of the adult Australian population are consuming calcium at well below the Recommended Dietary Intake (RDI) (Australian Bureau of Statistics, 1998). Vitamin D, a key regulator in calcium status, is also of concern. Recent Australian data states that 70% of the elderly and institutionalised Australian population have a frank or sub-optimal vitamin D status, while 23% of younger Australians seem to have a poor vitamin D status (Nowson & Margerison, 2002). The co-existence of poor calcium status and overweight/obesity presents a tantalizing paradigm for public health intervention.

The regulation of calcium homeostasis is important to a large number of physiological processes, from blood clotting to muscle contraction to enzyme secretion. Over the years the role of calcium in osteoporosis and hypertension has been well-established. However, until recently calcium's role in obesity and type-2 diabetes were unheard of. Zemel *et al.* (2000) work proposes that intracellular calcium holds the key to regulation of insulin sensitivity, lipid storage and synthesis. Interestingly Zemel's tenet arose from clinical trials on hypertension, where it was observed that increasing the intake of calcium from ~400 mg/day to 1000 mg/day resulted in significant weight loss. Zemel speculated that the increased calcitrophic hormones, parathyroid hormone (PTH) and vitamin D_3 secondary to a low calcium

intake, stimulated adipocyte calcium influx, which in turn stimulated fatty acid synthase activity and hence lipid storage (Zemel *et al.* 2000). If this is true, increasing calcium intake should lower calcitrophic hormones, reduce intracellular calcium and protect against fat gain.

A number of epidemiological studies have supported Zemel's original findings with a strong inverse correlation between calcium intake and adiposity (Parikh & Yanovski, 2003) in that persons with the lowest calcium intake tend to have the highest body weight. Similarly data from the National Health and Nutrition Examination Survey III (NHANES III) also showed a strong inverse association for relative risk (RR) of obesity and calcium intakes (Zemel *et al.* 2000). Similar relationships have also been demonstrated in the Australian population, based on an examination of the data from the National Nutrition Survey 1995. The results indicated that Australian men and women with higher calcium intakes, had lower body mass indices as well as waist circumferences, on controlling for various confounders (Soares *et al.* 2004). Retrospective analyses lend support to the thesis that calcium may influence the regulation of body weight. Davies *et al*. re-evaluated the results from 5 clinical studies (one intervention and four observational studies) looking at dietary calcium and bone mineral content (2000). Davies' results showed a significant negative association between calcium intake and weight for all age groups; however it is important to point out that with these early studies weight / fat loss was not the major end point. More recently three clinical studies have documented that a high dairy calcium diet accelerated fat loss, particularly from the abdominal region. These results were obtained under energy deficit conditions (Zemel *et al.* 2004; 2005a; 2005b), as well as during weight maintenance (Zemel *et al.* 2005b). Two studies by Thompson *et al*. (2005) and Harvey-Berino *et al.* (2005); however, did not replicate these outcomes. Both studies concluded that a high-calcium diet did not augment weight loss above that obtained from calorie restriction alone. Clearly, the issue is far from settled.

The influence of calcium on appetite is another factor that has been suggested as a mechanism for changes in body weight. Most people who maintain a stable body weight spontaneously adapt their energy intake over a large range of energy expenditure through accurate mechanisms of control of food intake. Appetite

involves a complex sequence of interactions amongst the peripheral and central mechanisms. There has been some suggestion that components in milk may reduce food intake (Schneeman *et al.* 2003; Anderson & Moore, 2004; Moran, 2004). In our laboratory we have demonstrated a significant reduction in food consumption of human subjects following a high dairy versus a low dairy breakfast meal fed acutely. There was a trend for a less energy intake following the high dairy diet, this difference in energy intake was significantly different 24 hours post-testing (Ping-Delfos *et al.* 2004). Findings by Barr *et al*. (2000) have also hinted at a compensatory decrease in energy intake following a 3 serves of milk a day intake versus maintaining their usual intake. The milk group gained 0.6 kg more than the control group ($P<0.01$) but interestingly, the predicted weight gain due to an increase in energy intake of 200 to 250 kcal/day (836–1046 kJ) over a 12-week period should have been of least 2.5 kg.

The present study examined the acute effects of increasing calcium intake on postprandial thermogenesis and substrate oxidation in overweight and obese subjects. It was based on the hypothesis that an increase in calcium intake would acutely stimulate fat oxidation and thermogenesis, and that calcium from dairy sources would be superior to elemental (non-dairy) calcium in these effects. Furthermore, we wished to examine the effect of different sources of calcium on feelings of hungry/satiety and subsequent food intake.

3.1.1 Methods

3.1.1.1 Study design

The study was a single blind, within-subject randomised comparison of the acute responses to three isocaloric mixed meals. The interval between trials ranged from 2 to 3 weeks, and subjects were instructed to maintain their habitual intake and activity patterns throughout the study.

3.1.1.2 Subjects

Subjects were recruited by advertisement in the local media. Subjects were initially screened by a telephone interview. Inclusion criteria included: (i) absence of clinical

signs or symptoms of chronic disease; (ii) history of weight stability (± 2 kg for the preceding 12 months); (iii) not on medication affecting metabolic rate or body composition; (iv) resting diastolic blood pressure <90 mm Hg (checked at introductory appointment); and in the women (v) at least 2 years postmenopausal; (iv) not on hormone replacement therapy. Suitable recruits (from the initial telephone screening) were sent a map of Curtin University of Technology and were booked in for an introductory appointment. The purpose of this first visit was to meet with the chief investigator, to show the subjects the testing room and explain the test day protocol and most importantly familiarise the subjects with indirect calorimetry measurements. This familiarisation was an important part of the study as it is critical to prevent any apprehension from the subjects on the test day. At this appointment subjects were also asked to complete a calcium food frequency questionnaire (Appendix A), they were also provided with an information sheet describing the study protocol (Appendix B) and asked to sign the written informed consent (Appendix B). The subjects were also provided with a checklist of instructions to follow before the experimental day (Appendix C).

Eight subjects, 6 men and 2 postmenopausal women, aged between 47 and 66 years and body mass index (BMI) within the range 27.6 – 36.1 kg/m^2 were recruited for the study (Table 3:1). The Human Research Ethics Committee of Curtin University of Technology (HR 245-2001) approved the protocol. All measurements were made in the clinical rooms of the Bentley Campus of Curtin University, Perth.

Table 3:1 Physical and metabolic characteristics of subjects.

Age (yr.)	53.7 ± 0.29
Gender	Males n=6 Females n=2
Weight (kg)	92.4 ± 0.87
Body mass index (kg/m^2)	32.5 ± 0.12
Fat mass (kg)	31.6 ± 0.35
Fat free mass (kg)	63.3 ± 0.90
Waist circumference (cm)	108.1 ± 0.47
Serum Vitamin D3 (nmol/L)	75.5 ± 0.95
Fasting TG (mM)	1.7 ± 0.003
Fasting TC (mM)	5.6 ± 0.003
Fasting LDL (mM)	3.4 ± 0.0075
Fasting HDL (mM)	0.7 ± 0.00073

Values are mean ± SD

3.1.1.3 Anthropometry and body composition measurements

Standing height was measured using a stadiometer fixed to the wall and recorded to the nearest 0.1 cm. Body weight was measured after an overnight fast on each occasion, immediately after voiding, with subjects wearing underwear and a light surgical gown, on a digital balance and recorded to the nearest 100 g. Waist and hip circumferences were measured as described by Norton and Olds (2000) (refer to Chapter 2). Body composition was determined by Dual energy X-ray absorptometry (DEXA) (Lunar DPX-L, Madison, WI) at a certified commercial Bone Densitometry Centre (refer to Chapter 2).

3.1.1.4 Measurement protocol

Subjects were requested to abstain from any strenuous exercise for 36 h prior to the measurement.

3.1.1.4.1 Pre-experimental day dinner meal

A frozen dinner meal was provided to each subject to be consumed the night before the test day. The meal chosen was Kan Tong Chicken Chow Mien, this meal was chosen for its easy of preparation and low calcium content (<50 mg). Each meal weighs 340g, and its composition per 100 g is as follows:

Energy 380 kJ
Protein 5.1 g
Fat 0.4 g
Carbohydrate 15.2 g
Sugars 0.8 g
Calcium < 50 mg
Sodium 280 mg
Potassium 95 mg

To further control calcium intake the night before the test day, each subject was provided with a 2 L bottle of deionised distilled water, for *ad libitum* consumption.

Subjects arrived at the laboratory after a 12 h overnight fast, and emptied their bladder (the time was recorded), the subjects were then weighed. They lay supine for a mandatory 30-min rest period, while the Deltatrac II metabolic monitor (Datex, Finland) was calibrated. At the end of this rest period, the canopy was placed over the head of the subject and they were asked to remain awake and motionless, as far as possible, for a 30 min resting metabolic rate (RMR) measurement. This protocol yields an RMR not very different from a basal metabolic rate measurement obtained immediately on waking, after an overnight stay in the laboratory (Soares *et al.* 1989).

After the RMR measurement, the plastic canopy was removed and a fasting blood sample (15 ml) was collected. Following the blood collection subjects made a fasting

collection of urine. They were given a breakfast meal, which they consumed within 10 minutes.

3.1.1.4.2 Test breakfast meal

The composition of the test breakfasts are provided in Table 2:2. The meals contained the following foods to meet the percentage macronutrients and calcium goals (Table 2:2): white bread (Goodman Fielder; Malaga, West Australia), butter (Bowland Dairies Pty Ltd; Rowville, Victoria, Australia), Adelphi ham and tomato (purchased from Coles Supermarket, Karawara, West Australia), muesli bar (Uncle Toby's; Wahgunyah, Victoria, Australia). The LD meal was supplemented by a low calcium, low vitamin D UHT milk and the HD meal was supplemented with a high calcium, high vitamin D milk (all milks were low in fat (1%) and provided by Murray Goulburn Co-Operative Co. Ltd; Brunswick, Victoria, Australia). The HC meal was supplemented with orange juice (Berri Ltd; Carlton, Victoria, Australia), and consumed with 2 tablets of Citracal (Mission Pharmacal Australia Pty. Ltd. Sydney, NSW, Australia). The solid component of all meals were of a similar weight (~400 g) and fluids consumed were of identical volumes (200 ml). The macronutrient content of the breakfast meals was determined using Foodworks analysis package 3.0 (Xyris software, Highgate Hill, Australia), the database uses data from AusNut, 1995, along with the manufacturer's product information. All subjects completed a palatability questionnaire that enquired about amount, taste, and overall acceptability of each meal. Each answer was scored on a 15 cm visual analogue scale anchored by the most negative to the most positive response for each question (Appendix D).

Table 3:2 Nutrient Composition of Breakfast Meals

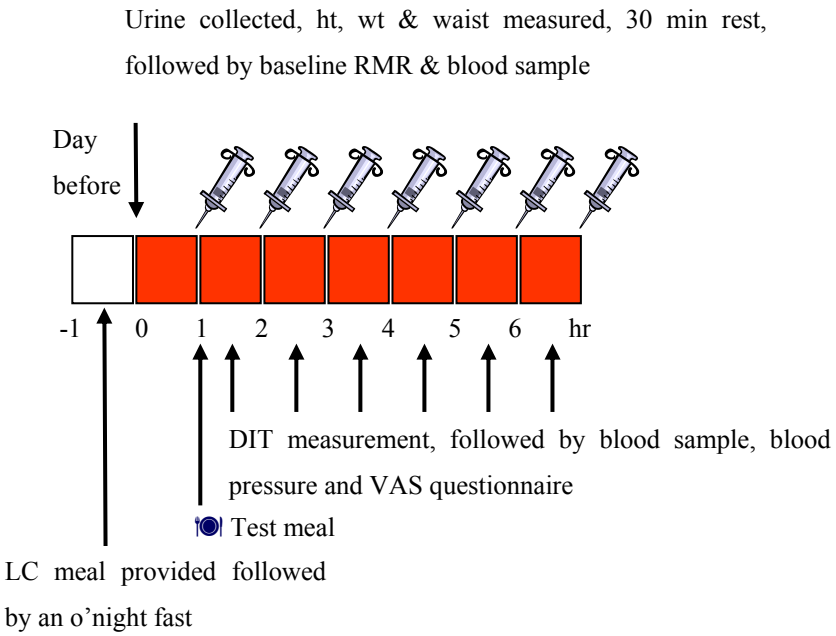
	Low dairy calcium (LD)	High dairy calcium (HD)	High non-dairy calcium (HC)
Energy content (kJ)	2391.0 ± 0.028	2372.8 ± 0.46	2386.9 ± 0.29
Protein (g)	20.7 ± 0.004	21.1 ± 0.004	20.7 ± 0.002
(% of total energy)	(14.7 ± 0.002)	(15.1 ± 0.001)	(14.7 ± 0.001)
Total fat (g)	22.0 ± 0.003	22.1 ± 0.001	21.6 ± 0.009
(% of total energy)	(34.0 ± 0.004)	(34.5 ± 0.005)	(33.4 ± 0.013)
Carbohydrate (g)	69.9 ± 0.00	68.3 ± 0.03	70.7 ± 0.016
(% of total energy)	(46.8 ± 0.005)	(46.0 ± 0.01)	(47.4 ± 0.006)
Fibre (g)	4.9 ± 0.00	4.7 ± 0.005	4.9 ± 0.002
Calcium (mg)	175.8 ± 0.04	531.7 ± 0.05	575 ± 0.00
Vitamin D (μg)*	1.7	4.8	1.9
Volume (mL)**	365	362	350
Weight (g)	401.1 ± 0.05	408.7 ± 0.04	408.1 ± 0.032
GI/GL	67/47	62/44	69/49

Data are mean ± SEM. * Vitamin D content was calculated using the NUTTAB database for one sample of each of the three test meals (Food Standards Australia & New Zealand, 2006) ** Volume was calculated by the analysis of one meal as a sample of each of the three test meals. GL – glycaemic index, GL – glycaemic load.

Figure 3:1 Example of high dairy/calcium breakfast meal

The palatability questionnaire was completed at this time. Twenty minutes into each postprandial hour, subjects returned to the supine position and rested for 10 min. The canopy was then placed over their head and measurements made for the last 30 min of the hour. The first 5 min was not included in subsequent data analysis. In between measurements subjects were allowed to sit up in bed and listen to music, or read. Some elected to sit at a table close to the bed, where they did craft work or read a book. An intermittent measurement protocol provides accurate postprandial data relative to that obtained by continuous minute-to-minute measurement over the postprandial period (Piers *et al.* 1992). De-ionised water was allowed *ad libitum* over the first postprandial visit, and the amount consumed was noted. These amounts were kept constant for subsequent visits. All subjects made two separate urine collections, hours 0-3 and 3-6 after the meal. The weight and duration of all collections were noted, urines were acidified and an aliquot was frozen at –80^{o}C. Total urinary nitrogen excretion was estimated by the Kjeldahl technique.

Figure 3:2 Overview of Study Protocol



3.1.1.4.3 Resting metabolic rate

Resting metabolic rate (RMR) was measured by indirect calorimetry using a Deltatrac II metabolic monitor (Datex Ohmeda, Finland); an open-circuit ventilated canopy measurement system. The measurement was conducted under standardised conditions, as per the protocol of (Piers *et al.* 2002) with subjects lying (a) at complete physical rest; (b) in a thermally neutral environment; (c) 12-14 hours after their last meal and a minimum of 8 hours of sleep; (d) awake and emotionally undisturbed; and (e) without disease and fever. The within-subject coefficient of variation (CV) in RMR was 3.3% in this study. The Deltatrac II was calibrated on the morning of each experimental day. Performance of the instrument was also checked at regular intervals during the study period by monitoring the ratio of carbon dioxide produced to oxygen consumed, during a 30 min ethanol burn. The mean (SD) ratio for 20 ethanol burns was 0.663 (0.008) with a CV of 1.2 %.

Figure 3:3 Indirect Calorimetry method

3.1.1.4.4 Diet induced thermogenesis and substrate oxidation rates

Diet induced thermogenesis (DIT) was measured as described previously (Piers *et al.* 1992), and expressed in absolute values (kJ/6h) and as a percentage of the energy in the breakfast meal. Whole-body substrate oxidation rates were calculated at rest (fasting), and for every hour up to 6h postprandial, using measures of oxygen consumption, carbon dioxide production and urinary nitrogen excretion in that postprandial phase. The equations of Ferranini (1988) were used to calculate energy expenditure and substrate oxidation rates.

3.1.1.4.5 Blood pressure measurement

The blood pressure measurement was taken during the last 5 minutes of RMR measurement, to ensure that subjects were relaxed during measurements. The blood pressure machine used was the Datex-Engstrom Cardiocap™ II. The subjects were lying in a supine position, as for the measurement of energy expenditure, with the arm cuff already attached to the arm. Three blood pressure measurements were

taken, with a minute rest in between and the mean of the three measurements recorded for each subject.

3.1.1.4.6 Temperature

Core body temperature was measured, at the tympanic membrane, through the ear canal in order to monitor change in temperature occurring as food is digested during the test day. This procedure was undertaken immediately after each blood draw, once the subject was in the seated position.

3.1.1.4.7 Blood assays

Venous blood samples were drawn at baseline and at the end of each 30 min RMR measurement. Blood for determination of insulin, total cholesterol (TC), low density lipoprotein (LDL) cholesterol (by calculation), high density lipoprotein (HDL) cholesterol and triaclyglycerol (TG), non-esterified free fatty acids (NEFA) and glycerol was left to stand for 30 minutes at room temperature and then centrifuged at 3000 x g for 10 min. Serum was extracted and the samples stored at -80ºC until later analysis. A finger prick blood sample was also taken and blood glucose was measured using Accu-Chek Active glucose strips (Roche Diagnostic, Australia). Serum insulin was measured by enzyme-linked immunosorbent assay (ELISA) based on two monoclonal antibodies (Dako Diagnostic, UK) according to manufacturer's instructions. Serum TG and TC were measured by enzymatic colorimetric kits (TRACE Scientific Ltd, Melbourne, Australia). Serum HDL cholesterol was determined after prior precipitation of apo B-containing lipoproteins with phosphotungstic acid and $MgCl_2$ by enzymatic colorimetry (TRACE Scientific Ltd, Melbourne, Australia). Serum LDL cholesterol was determined by using a modified version on the Friedewald equation: LDL cholesterol = [total cholesterol] – [HDL cholesterol] – (0.46 x [TG]) (Bairaktari *et al.* 2000). NEFA and Glycerol were determined by an enzymatic colorimetric method (WAKO NEFA C Kit, Novachem and RANDOX Ltd, United Kingdom, respectively). Individual CV_{intra}% were <2.1% (glucose), CV_{intra}% and CV_{inter}%, <5% (insulin), <2% (TC), <2% (HDL), <2% (TG) and <5% for both NEFA and glycerol.

In addition, an anaerobically collected serum sample was taken from each subject, this was sent to an accredited laboratory for the determination of ionised calcium.

Also an aliquot of plasma was kept and sent to the same accredited laboratory for the determination of intact parathyroid hormone.

3.1.1.4.8 Appetite, satiety and sensory ratings

To determine the subject's subjective feelings of hunger, appetite and satiety the Visual Analogue Scale (VAS) was utilised. The VAS questionnaire is a 10 cm scale anchored by the most negative to the most positive response for each question. The questions asked were: 1. How hungry do you feel?, 2. How full do you feel?, 3. How strong is your desire to eat?, 4. How much do you think you could eat now? and 5. Your preoccupation with thoughts of food? (Appendix E) (Stubbs *et al.* 2000). The subjects make a vertical mark or cross on the scale to determine their response. The VAS has the advantage of being easy, quick to use and very simple to interpret. The questionnaire shows good reproducibility under controlled conditions, provided they are used in a with-in subject design (as was the case in this study). The VAS questionnaire was administered to each subject following the temperature measurement.

3.1.1.4.9 Energy consumption after experimental period

Following the 6 hour testing period all subjects were offered a buffet meal before they returned home. The subjects were encouraged to eat the buffet *ad libitum,* an assistant investigator sat with each subject to chat and assist in creating a relaxed atmosphere. Once the subjects had finished eating they were provided with a food recording booklet (Appendix F), and provided with instructions on how to complete an accurate record of their intake for the following 24 hours. The booklet also includes photographs of standard portion serves, to assist with the accurate completion of the food diary.

Following consumption of the buffet and once the subject had left the test dining-hall all foods provided were re-weighed and actual food consumption was recorded. The foods provided to the subjects is demonstrated in table 3:3.

Table 3:3 List of Foods Provided to the Subjects and consumed ad libitum

Item	Brand name	Amount
Soup	Heinz Very Special Cream of Pumpkin Soup (98% fat free)	500 g
French stick – bread	Coles brand	150 g
Yoghurt	Yoplait – Pineapple & Mango (99% fat free)	2 x 200 g tub
Savoury biscuits	Arnott's Vita Weat crackers (original)	6 crackers
Cheese	Coon Tasty Dairy Farmers	3 slices – cut in half
Carrot sticks		80 g
Almonds	Sunbeam	100 g
Turkish apricots	SunBeam sun dried	150 g
Canned fruits	Goulbourn Valley Two fruits in light juice (pears & peaches)	2 x 140 g tub
Apple bars	Farmland oven baked bar (97% fat free)	2 bars
Milk chocolate	Cadbury dairy milk chocolate	6 small bars (~15.8 g without wrapper)
Orange juice	Just Juice 99.9% orange juice	900 ml
Milk	Brownes Hi-Lo Calcium rich	150 ml
Tea	Lipton's teabags	*
Coffee	Nescafe – Blend 43	*
Sugar	Farmland white sugar	100 g

Tea and coffee were served according to subject request (coffee powder was also weighed)

Figure 3:4 Layout of buffet meal provided to each subject post-testing

3.1.1.4.10 Calcium bioavailability

The bioavailability of the three test meals was determined using three standard methods; ionised calcium (pH corrected) and intact parathyroid hormone (both measured by a accredited laboratory in Perth). As the third method, urinary excretion of calcium was measured using the GBC Flame Atomic absorption Spectrophotometer (Avanta Σ, GBC Scientific Equipment Pty Ltd. Victoria, Australia) – refer to Chapter 2 for details of these methods.

3.1.1.4.11 Gastric emptying

When comparing the effect of different dietary intakes on energy metabolism and substrate utilisation it is essential to determine that the rate of gastric emptying is not a confounding factor. Varying rates of gastric emptying will obviously influence the rate of nutrient absorption and therefore energy expenditure resulting in differences in the diets due to intra-subject variations and not the direct affect of the nutrient intervention. The rate of gastric emptying influences physiological variables including glycaemia and insulin responses (Mourot *et al.* 1988), along with subjective feelings of hunger and satiety (Robinson *et al.* 1988). The paracetamol

marker method of gastric emptying is a simple, non-invasive technique, well accepted by subjects (Medhus *et al.* 1998; Willems *et al.* 2001). Studies have also examined the paracetamol marker method against the gold standard of gastric emptying, the scintigraphic technique, and concluded that the paracetamol method offers an accurate approximation of gastric emptying (Naslund *et al.* 2000; Willems *et al.* 2001). In a sub-set of subjects 1000 mg of Paracetamol™ (Glaxo Smith Kline, Erminton, NSW, Australia), this equated to 2 tablets, was provided and consumed with the test breakfast meal. A blood sample was taken every hour and the sample sent to an accredited laboratory in Perth for the determination of levels of paracetamol.

3.1.1.4.12 Glycaemic Index / Load of test meals

To ensure that interpretation of the results were not confounded by the glycaemic index (GI) or load (GL) of the meals, these values were determined using data from the International Tables of Glycaemic Index (Foster-Powell & Miller, 1995; Foster-Powell *et al.* 2002). Glycaemic Load was further calculated using the equation below (Foster-Powell & Miller, 1995; Foster-Powell *et al.* 2002).

GL = GI x carbohydrate content of food

3.1.1.5 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Change between fasting and fed states, was calculated by subtracting the fasting value x duration of measurement, from the total postprandial value over 6h. Since the intervals of measurement in the postprandial period were equal, this summary statistic was analogous to determining the incremental area under the curve (Mathews *et al.* 1990). Total postprandial fat and carbohydrate oxidation were also adjusted for their respective fasting values using the method of Ravussin & Bogardus (1989). A repeated measures ANOVA was used to determine statistical significance, which was set at the 5% level. Post-hoc test used the LSD procedure. Pearson's correlation coefficients for all anthropometric, body composition and metabolic variables were separately calculated for each meal. Data were analysed using the SPSS for Windows (Version 11, SPSS Inc. USA) statistical software package.

3.1.2 Results

The physical characteristics of the subjects are shown in Table 3:1.

3.1.2.1 Effect of breakfast meals

3.1.2.1.1 Energy expenditure & substrate oxidation

There was no significant difference in body weight or RMR between the three trials. However, basal FOR (fat oxidation rate) was significantly lower following the high calcium meals, and COR (carbohydrate oxidation rate) significantly higher following the HC meal, despite randomisation of the test meals (Table 3:4). DIT, in absolute terms (kJ/6h) or as percentage of energy in meal (%), was not different between test meals (Table 3:4). There was a significant rise in RQ following all meals, but the change in RQ was significantly (P=0.029) lower by 12.7% and 7.8% following the HC and HD meals, respectively (Table 3:5). Fat oxidation was significantly suppressed after each meal, but the suppression was significantly less by 38% and 44% following the HC and HD meals respectively. Reciprocally, the increase in carbohydrate oxidation (ΔCOR) was significantly (P=0.027) lower by 60% (HC) and 55.4% (HD) compared to the LD meal (Table 3:5). Sum of postprandial FOR adjusted for fasting values, was also significantly different between meals (P = 0.039), with the rank order LD< HC<HD (Table 3:5). The sum of postprandial COR adjusted for fasting values showed a trend to be lowest following HD (P=0.06, Table 3:5).

Table 3:4 Fasting measurements prior to the three test meals.

	Low dairy calcium (LD)	High dairy calcium (HD)	High non-dairy calcium (HC)	Significance by ANOVA for repeated measures
RMR (kJ/ hr)	298.5 ± 23.1	296.1 ± 22.4	292.9 ± 24.1	P=0.31
RQ	0.80 ± 0.02 [a]	0.85 ± 0.02 [b]	0.89 ± 0.02 [b]	P=0.009
Protein oxidation (g/hr)	3.0 ± 0.4	3.8 ± 0.7	3.33 ± 0.2	P=0.26
Fat oxidation (g/hr)	4.1 ± 0.5 [a]	2.9 ± 0.6 [b]	1.9 ± 0.7 [b]	P=0.003
Carbohydrate oxidation (g/hr)	5.3 ± 1.2 [a]	7.4 ± 1.1	10.2 ± 1.6 [b]	P=0.026
Serum glucose (mmol/L	5.25 + 0.2	5.13 + 0.14	5.09 + 0.98	P=0.22
Serum insulin (uIU/ml)	11.83 + 4.20	10.63 + 4.29	13.11 + 4.84	P=0.31
Serum NEFA (mmol/L)	0.65 + 0.076	0.615 + 0.092	0.627 + 0.076	P=0.54
Serum glycerol (μmol/L)	77.86 + 5.45	76.39 + 7.99	73.73 + 5.84	P=0.27

Values are mean ± SEM, n = 8. Values with unlike superscripts in the same row were significantly different on a post hoc LSD procedure (P<0.05).

Table 3:5 The influence of the source of calcium on postprandial thermogenesis and substrate oxidation.

	Low dairy calcium (LD)	High dairy calcium (HD)	High non-dairy calcium (HC)	Significance by ANOVA for repeated measures
Thermogenesis (kJ/6h)†	155.3 ± 26.3	165.7 ± 19.5	170.8 ± 32.4	P=0.59
DIT (%)	6.5 ± 1.1	7.0 ± 0.8	7.2 ± 1.4	P=0.58
Change in RQ†	0.32 ± 0.09 [a]	-0.024 ± 0.11 [b]	-0.039± 0.08	P=0.029
Change in protein oxidation (g/6hr)†	-11.5 ± 2.4	-16.7 ± 4.4	-13.7 ± 0.7	P=0.31
Change in carbohydrate oxidation (g/6hr) †	34.1 ± 7.6 [a]	15.2 ± 7.1 [b]	13.6 ± 7.5 [b]	P=0.032
Adjusted sum of carbohydrate oxidation (g/6hr)‡	68.0 ± 5.1	59.8 ± 7.5	72.5 ± 4.8	P=0.061
Change in fat oxidation (g/6hr)†	-6.5 ± 2.2 [a]	3.3 ± 2.5 [b]	2.9 ± 2.3 [b]	P=0.005
Adjusted sum of fat oxidation (g/6hr) ‡	14.1 ± 1.8 [a]	20.9 ± 2.4 [b]	18.2 ± 1.7	P=0.039
Change in glucose (mmol/L)	2.04 ± 1.25	1.73 ± 1.02	1.65 ± 0.50	P=0.76
Change in insulin (uIU/ml) †	181.93 ± 50.18	193.09 ± 40.16	188.18 ± 31.90	P=0.73
Change in NEFA (mmol/L) †	-1.50 ± 0.26 [a]	-0.94 ± 0.27 [b]	-1.22 ± 0.32	P=0.035
Change in glycerol (μmol/L) †	-48.29 ± 27.0	2.31 ± 26.78	0.99 ± 25.62	P=0.31

Values are mean ± SEM, n = 8. †Calculated as sum of postprandial values over 6 hr–(fasting value per hr x 6). ‡Calculated as sum of postprandial values over 6h adjusted for fasting baseline. Values with unlike superscripts in the same row were significantly different on a post hoc LSD procedure (P<0.05).

3.1.2.1.2 Substrates & hormones

There were no significant differences between the three meals for basal or postprandial glucose or insulin concentrations (Table 3:5). Following all meals postprandial NEFA levels were suppressed, with the HD meal significantly less suppressed over the 6h postprandial period relative to the LD (LD –1.50 ± 0.26, HC –1.22 ± 0.32 and HD –0.94 ± 0.27 mmol/L, P=0.035). Serum glycerol was less suppressed after the HD meal by almost 70% but results did not attain statistical significance (LD -48.30 ± 27.0, HC 1.0 ± 25.62 and HD 2.3 ± 26.78 μmol/L, P=0.31). Pearson's correlations did not detect any significant relationships between variables.

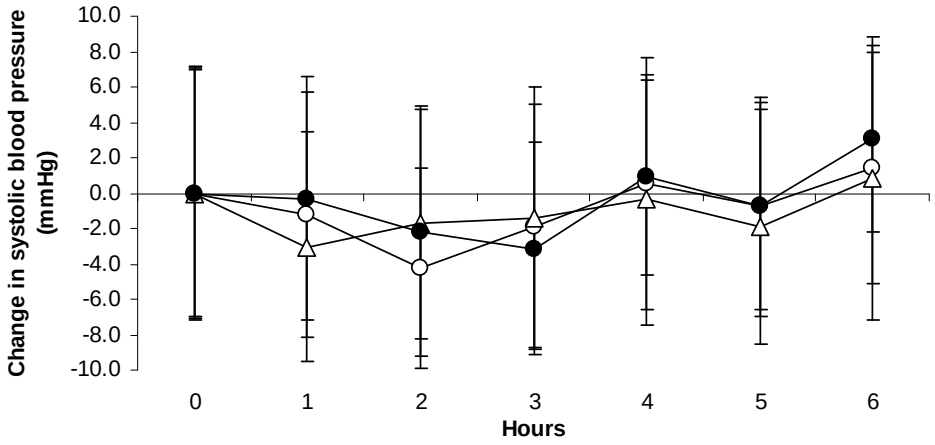
3.1.2.1.3 Blood Pressure

3.1.2.1.3.1 Systolic Blood Pressure

Blood pressure was measured five minutes at the end of each indirect calorimetry measurement to ensure that the subjects were relaxed during the testing period. There was no significant difference in the change in systolic blood pressure from baseline over the 6 hours of testing between each of the breakfast meals (RM-AVOVA P=0.58, LD -2.9 ± 11.1, HD -13.3 ± 12.0, HC -4.3 ± 13.0 mmHg).

Figure 3:5 Systolic blood pressure, measured each hour following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Values are means ± SEM shown by vertical bars.

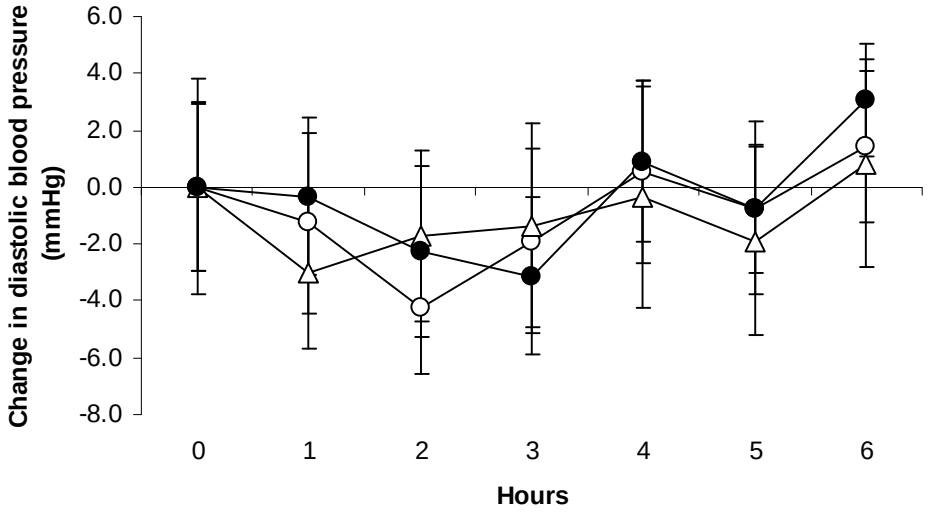


3.1.2.1.3.2 Diastolic Blood Pressure

The measurement of diastolic blood pressure also remainder consistent between the three breakfast meals, with no differences in the change from baseline (RM-ANOVA, P=0.45, LD -2.1 ± 4.3, HD -13.3 ± 7.7, HC -11.3 ± 8.4 mmHg).

Figure 3:6 Diastolic blood pressure, measured each hour following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Values are means ± SEM shown by vertical bars.

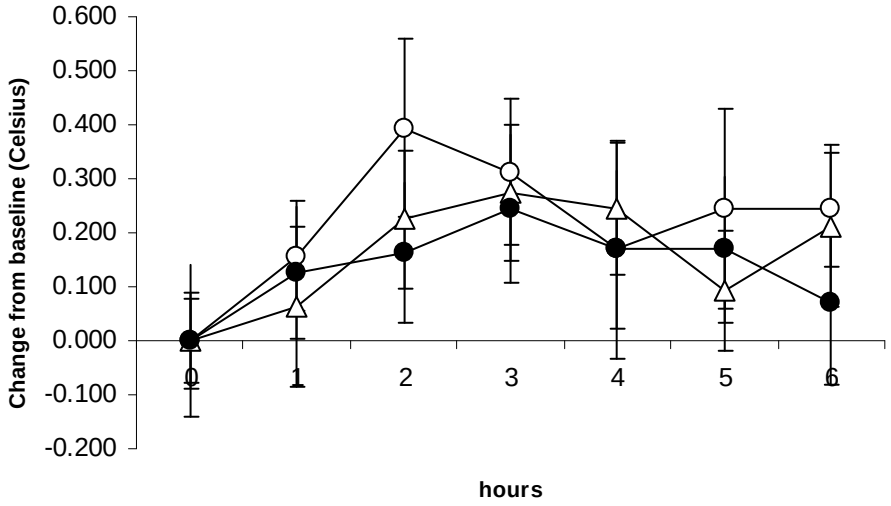


3.1.2.1.4 Temperature

Core body temperature was measured at the tympanic membrane, in order to monitor the change in temperature occurring over time following the consumption of a test breakfast test meal. There was no significantly different between the breakfast meals (LD 1.52 ± 0.52, HD 1.11 ± 0.45, HC 0.94 ± 0.46 ^{o}C, RM-ANOVA P=0.44).

Figure 3:7 Change in temperature measured each hour following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Values are means ± SEM shown by vertical bars.



3.1.2.1.5 Sensory evaluation of the test meals

Sensory evaluation of the test meals showed no significant difference in the scores for 'amount' (LD 9.0 ± 1.0 cm, HD 7.0 ± 0.8 cm, HC 7.3 ± 1.1 cm, RM-ANOVA, P=0.19), 'taste' (LD 12.8 ± 0.9 cm, HD 11.8 ± 1.2 cm, HC 12.7 ± 0.9, RM-ANOVA, P=0.35) and 'overall acceptability' of each meal (LD 12.9 ± 0.9 cm, HD 13.2 ± 0.4 cm, HC 14.1 ± 0.2 cm, RM-ANOVA, P=0.22).

3.1.2.1.6 Hunger/fullness ratings

Subjects were asked to fill in visual analogue scale questionnaires at baseline and after each hour following the consumption of the breakfast meal. The purpose of

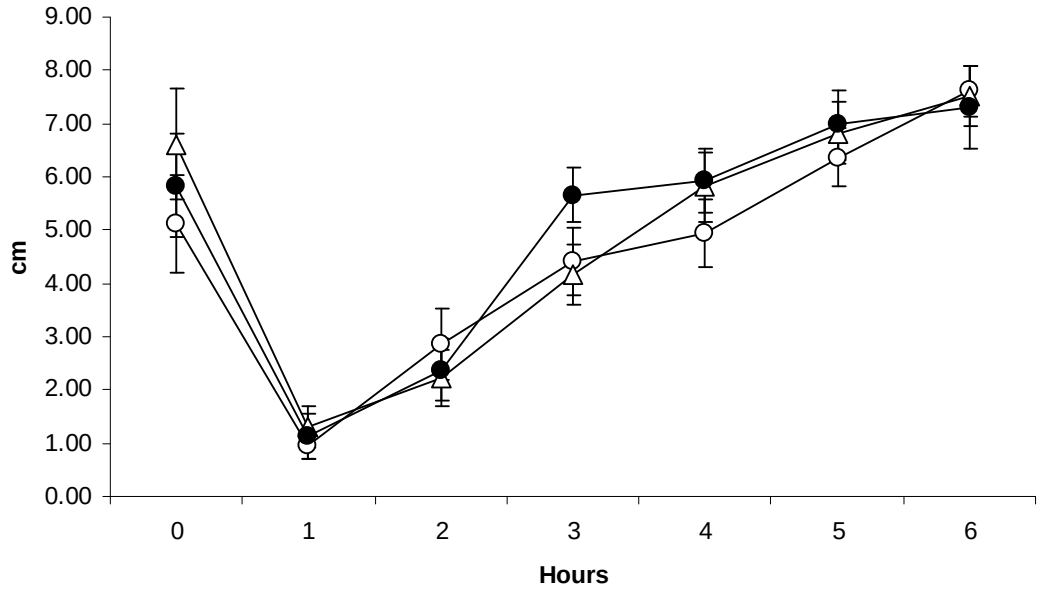
these questionnaires is to determine the subject's subjective feelings of hunger and satiety.

3.1.2.1.6.1 Question 1 – How hungry do you feel?

Question 1 asked, How hungry do you feel? The y-axis indicates 0, not hungry at all to 10, as hungry as I've ever felt. Analysis by repeated-measures ANOVA demonstrates that there was no significant difference between the three diets consumed on the subjects feelings of hunger/satiety as a change from baseline (P=0.17, LD -3.6 $\pm$ 5.6. HD -11.8 $\pm$ 5.3. HC -5.6 $\pm$ 5.2 cm). Similarly there was no significant difference between the diets consumed and subjective feelings of hunger or satiety at time point 0 (before the diet was consumed), hour 3 or hour 6 (P=0.22, P=0.06 and P=0.57, respectively).

Figure 3:8 Responses to Question 1 – How hungry do you feel? following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Bars represent SEM. 0= not hungry at all, 10=as hungry as I've ever felt.

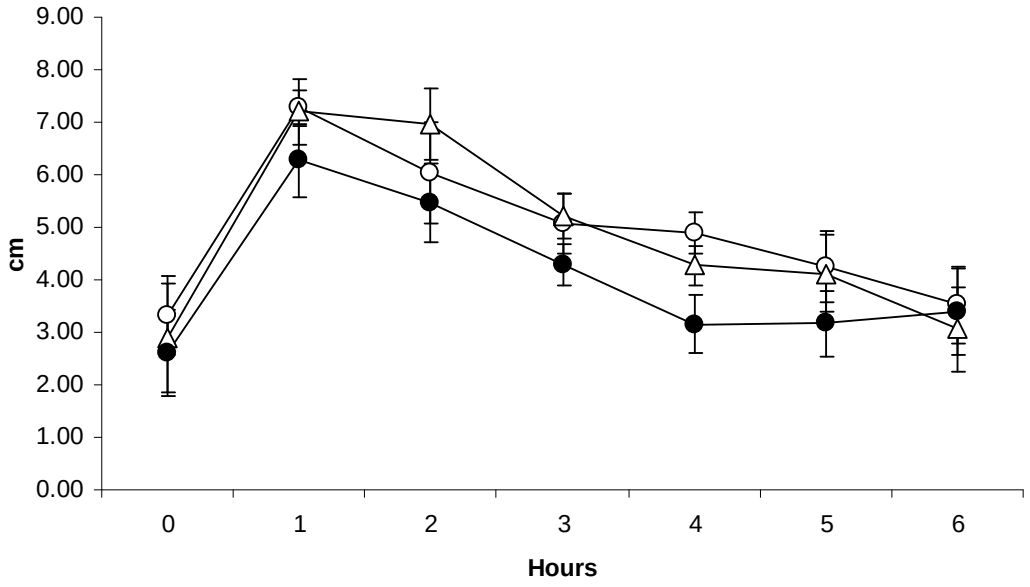


3.1.2.1.6.2 Question 2 - How full do you feel?

Question 2 asked, How full do you feel? The y-axis indicates 0, not at all full to 10, as full as I've ever felt. Analysis by repeated-measures ANOVA demonstrates that there was no significant difference between the three diets consumed on the subjects feelings of hunger/satiety as a change from baseline (P=0.53, LD 3.9 ± 3.7, HD 6.3 ± 5.9, HC 3.8 ± 4.6 cm).

Figure 3:9 Responses to Question 2 – How full do you feel? following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Bars represent SEM. 0= not hungry at all, 10=as hungry as I've ever felt.



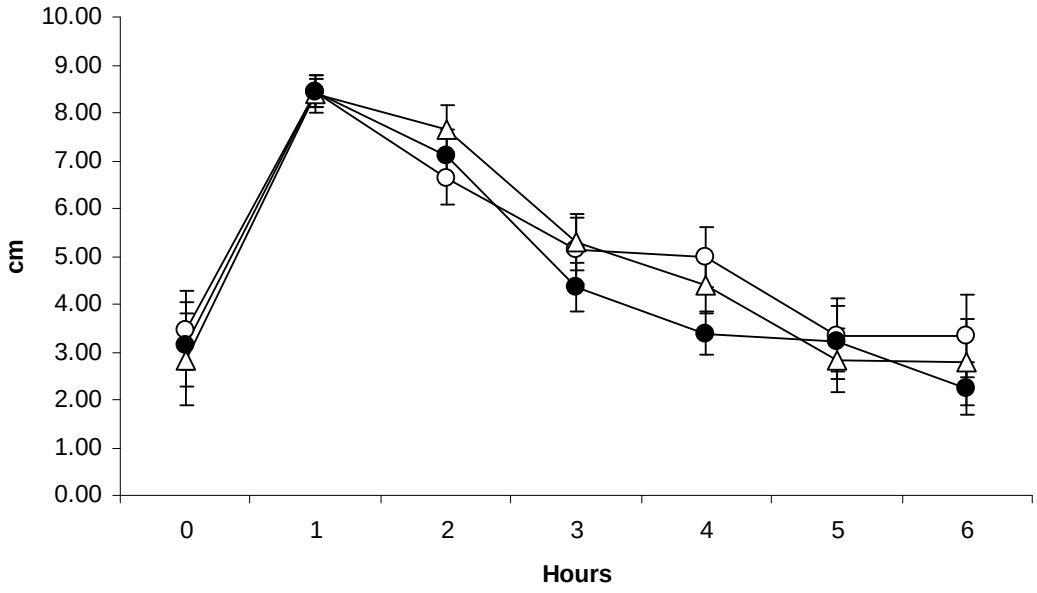
3.1.2.1.6.3 Question 3 – How strong is your desire to eat?

Question 3 asked, How strong is your desire to eat? With this question the response options were weight from 0, very strong desire to eat to 10, very weak desire to eat. It is important to have a question within this type of design, to ensure subjects are reading the questions before answering. Analysis by repeated-measures ANOVA demonstrates that there was no significant difference between the three diets consumed on the subjects feelings of hunger/satiety as a change from baseline (P=0.37, LD 11.2 ± 5.1, HD 14.4 ± 5.1, HC 9.8 ± 5.9 cm). At the end of the test day

there was no significantly greater desire to eat by the subjects after consuming any of the diets (RM-ANOVA, P=0.25, LD 3.3 ± 0.9, HD 2.8 ± 0.9, 2.2 ± 0.6 cm).

Figure 3:10 Responses to Question 3 – How strong is your desire to eat? following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Bars represent SEM. 0= very strong, 10=very weak.

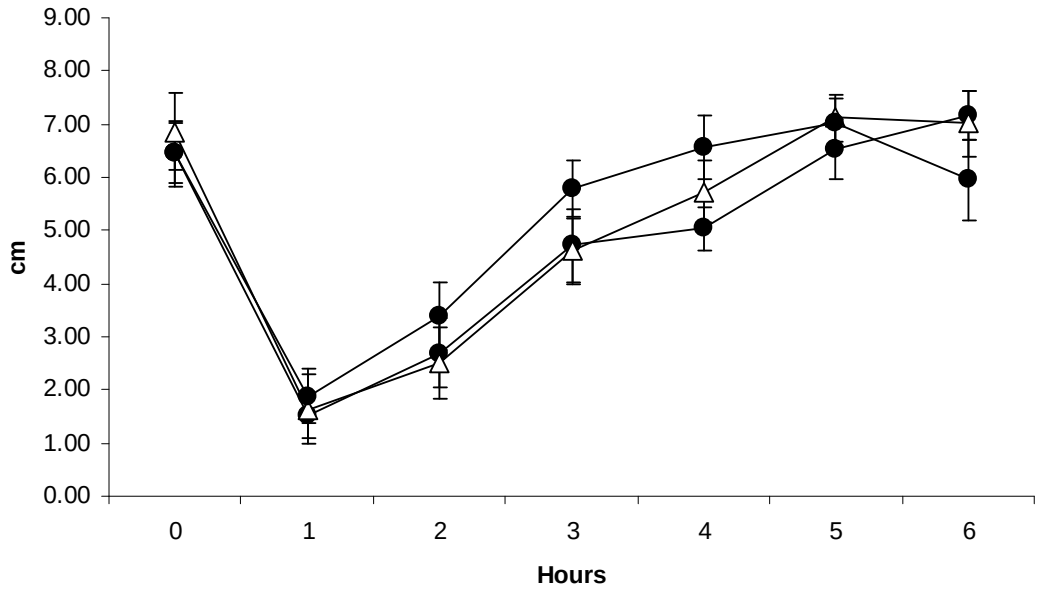


3.1.2.1.6.4 Question 4 – How much do you think you could eat now?

Question 4 addressed the question, How much do you think you could eat now? With this question the response options were weighted from 0, nothing at all to 10, a large amount. Analysis by repeated-measures ANOVA demonstrates that there was no significant difference between the three diets consumed on the subjects feelings of hunger/satiety as a change from baseline (P=0.38, LD -11.0 ± 3.4, HD -12.6 ± 3.4, HC -8.1 ± 3.6 cm). At the end of the test day there was no significantly greater desire to eat by the subjects after consuming any of the diets (RM-ANOVA, P=0.14, LD 7.2 ± 0.5, HD 7.0 ± 0.6, 6.0 ± 0.8 cm).

Figure 3:11 Responses to Question 4 – How much do you think you could eat now? following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Bars represent SEM. 0= Nothing at all, 10=A large amount.

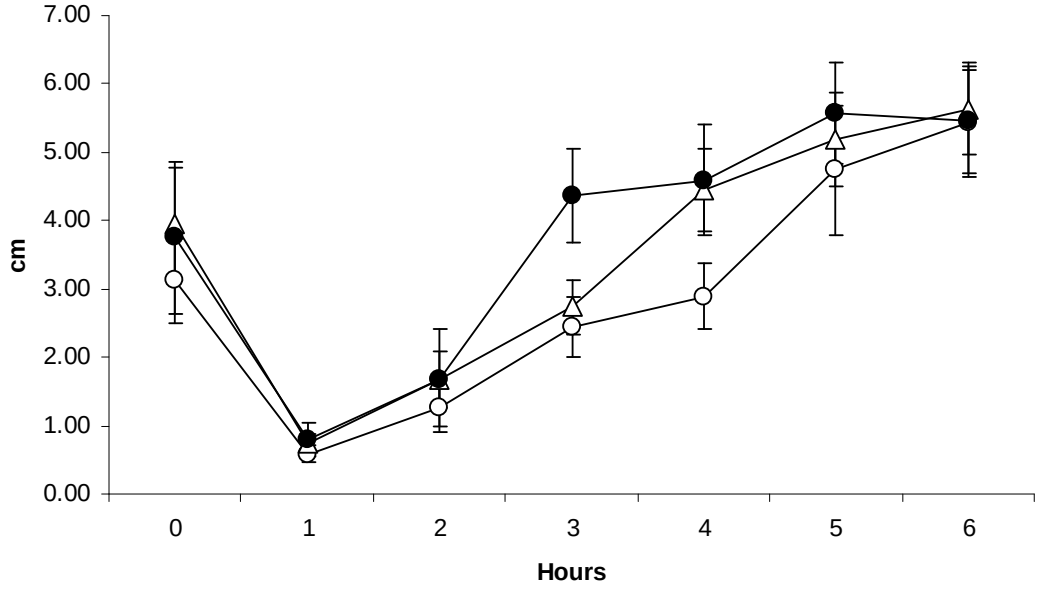


3.1.2.1.6.5 Question 5 – Preoccupation with thoughts of food

Question 5 addressed the question, Your preoccupation with thoughts of food? With this question the response options were weighted from 0, No thoughts of food to 10, very difficult to concentrate on other things. Analysis by repeated-measures ANOVA demonstrates that there was no significant difference between the three diets consumed on the subjects subjective response to this question, as a change from baseline (P=0.66, LD -1.3 $\pm$ 3.3, HD –3.3 $\pm$ 5.0, HC -0.04 $\pm$ 5.1 cm). At time point 3 (3 hours post-prandially) there is a distinct separation between the diets and the subjects when fed the high calcium (HC) diet reported that they found it harder to concentration on this diet (RM-ANOVA, P=0.02, LD 2.5 $\pm$ 0.4, HD 2.7 $\pm$ 0.4, HC 4.4 $\pm$ 0.7 cm). However, by the end of the test day this difference had dissipated and the subjects reported no difference in there preoccupation with food between the 3 diets (RM-ANOVA, P=0.54, LD 5.4 $\pm$ 0.8, HD 5.6 $\pm$ 0.7, HC 5.5 $\pm$ 0.8 cm).

Figure 3:12 Responses to Question 5 – Preoccupation with thoughts of food? following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Bars represent SEM. 0= No thoughts of food, 10=Very difficult to concentrate on other things.



3.1.2.1.7 Food consumption after test period

3.1.2.1.7.1 At buffet meal

The buffet meal provided was an *ad libitum* meal; however, the actual amount of food consumed was measured once the subject had left the premises. There was no significant difference between either of the test meals on total energy (Table 3:6) protein, fat or carbohydrate consumed (Table 3:6).

Table 3:6 Nutrient intake following the consumption of a buffet meal (n=8)

Meal	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
LD	3625.3 ± 512.6	26.3 ± 3.4	30.0 ± 4.5	116.9 ±18.8
HD	4251.2 ± 504.4	30.2 ± 2.6	31.2 ± 2.3	147.8 ± 21.9
HC	3564.6 ± 236.2	26.1 ± 1.7	25.7 ± 2.0	124.0 ± 12.0
RM-ANOVA (P value)	0.10	0.96	0.14	0.11

3.1.2.1.7.2 Food intake at the end of the test day

Subjects were provided with detailed instructions to complete a food record diary. All foods and drinks consumed had to be detailed in the diary and this was confirmed with a telephone conversation by the chief investigator to ensure the completeness of the diary. The time periods examined were up to the end of the test day and then total consumption 24 hours following the buffet meal. There were no significant differences between the three test meals on total energy intake, protein and carbohydrate intake; however, a trend for a reduced consumption of fat in the HC versus the LD diet was observed.

Table 3:7 Nutrient intake of the buffet meal and spontaneous food intake recorded at home, up until midnight (n=8)

Meal	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
LD	7706.0 ± 1245.2	75.7 ± 15.2	74.3 ± 14.8 *	197.8 ± 34.7
HD	7934.7 ± 1473.7	82.8 ± 23.5	60.6 ± 14.4	221.2 ± 35.4
HC	6810.6 ± 865.2	64.2 ± 7.5	50.8 ± 8.0 *	194.2 ± 25.3
RM-ANOVA (P value)	0.31	0.33	0.051	0.39

3.1.2.1.7.2.1 Time lag between the end of the buffet meal and next meal intake

Subjects were asked to record the time at which they consumed their next meal on returning home from the study (post-buffet meal). The time at which they had finished eating had also been recorded by the chief researcher and hence the time lag for each meal was calculated. We observed a significant difference between the three breakfast meals, in the order of HD>HC>LD (RM-ANOVA, P=0.03, LD 170.9 ± 30.4, HD 238.0 ± 14.5, HC 212.8 ± 11.7 minutes). These differences were not significant by a LSD post-hoc test.

3.1.2.1.7.3 Food intake 24 hours post-buffet

Total food intake recorded for a 24 hour period following the consumption of the buffet meal was not significantly different for total energy consumed, protein, fat or carbohydrate intake.

Table 3:8 Nutrient intake 24 hours after the buffet meal (n=8)

Meal	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)
LD	11119.2 ± 1554.8	112.3 ± 18.0	101.6 ± 18.1	298.4 ±39.7
HD	11976.2 ± 1357.1	125.1 ± 22.6	99.0 ± 11.2	327.2 ± 41.1
HC	12001.1 ± 1140.5	118.6 ± 11.5	99.5 ± 12.6	331.1 ± 37.8
RM-ANOVA (P value)	0.39	0.55	0.89	0.31

3.1.2.1.8 Bioavailability of calcium

Three standard approaches were used to determine the bioavailability of calcium from the breakfast meals; serum ionised calcium, pH corrected (iCa), intact parathyroid hormone (iPTH) and urinary calcium excretion of calcium, expressed as mg calcium per g of nitrogen excreted (UC). All results were expressed as a percent change from baseline.

3.1.2.1.8.1 Ionised calcium

There was no significant difference between the three breakfast meals consumed and the percent change from baseline for pH corrected ionised calcium (LD 11.71 ± 3.31, HD 8.66 ± 9.40, HC 11.64 ± 9.64 %, RM-ANOVA, P=0.99).

3.1.2.1.8.2 Intact Parathyroid Hormone

There was a trend for the suppression of iPTH (expressed as percent change from baseline) following the consumption of the HC versus the LD breakfast meal; however, this did not reach significance (LD 10.40 ± 41.28, HD -28.50 ± 19.64, HC -107.00 ± 43.32, RM-ANOVA, P=0.08).

3.1.2.1.8.3 Urinary calcium

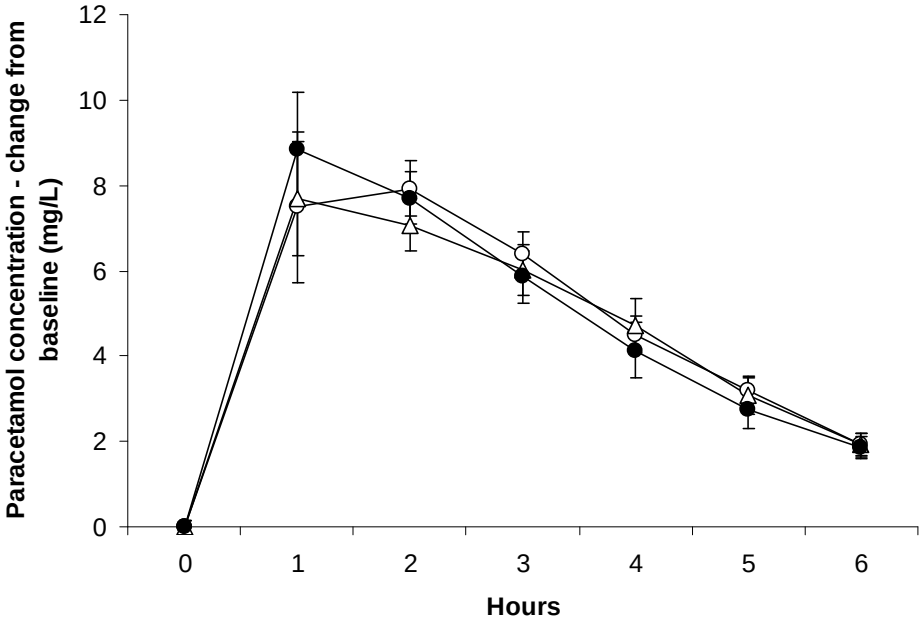
There was a trend for a greater urinary calcium excretion (expressed as percent change from baseline) following the consumption of the HC versus the LD breakfast meal; however, this did not reach significance (LD 93.51 ± 47.75, HD 278.12 ± 148.76, HC 303.49 ± 90.65, RM-ANOVA, P=0.08).

3.1.2.1.9 Gastric emptying

There was no significant difference in delta postprandial paracetamol concentration (LD 31.4 ± 2.7, HD 30.5 ± 1.9, HC 31.2 ± 2.3 mg/L, P=0.60), time taken to reach maximum concentration (LD 105 ± 18.8, HD 105 ± 22.0, HC 75 ± 9.8 minutes, P=0.23) nor the maximum paracetamol concentration (LD 14.3 ± 0.9, HD 13.6 ± 0.8, HC 14.8 ± 0.9 mg/L, P=0.36) reached between any of the test meals.

Figure 3:13 Gastric emptying following the breakfast meals responses in eight subjects, following breakfast meals low in dairy & calcium (○), high in dairy and calcium (△) and no dairy but high in calcium (●).

Bars represent SEM.



3.1.2.1.10 Glycaemic Index/Load

There was no significant difference between each of the test meals for GI (LD 67, HD 62, HC 69) or GL (LD 47, HD 44, HC 49).

3.1.3 Discussion

The regulation of fat balance is critical to energy balance and the equilibrium of fat stores in the body (Flatt, 1995). Fat balance is determined by the body's ability to match fat oxidation to fat intake. Any dietary factor that can stimulate fat oxidation over a prolonged period would hence reduce fat stores, provided there was no compensatory increase in fat intake. We pursued the idea that calcium and other ingredients in dairy, may help alter body composition if they could either, stimulate whole body fat oxidation, increase postprandial energy expenditure or accelerate mobilization of fat stores (lipolysis). For this purpose the control test meal was low in dairy (and calcium) and vitamin D (LD). The second meal (HD) was high in dairy and hence calcium with additional vitamin D. Vitamin D was added to enhance the absorption of calcium (Holick, 2004) and to mimic the US practice of fortifying all dairy products with vitamin D (Newmark *et al.* 2004). The third experimental meal (HC) was a high calcium meal using calcium citrate (pharmacological). There is evidence to indicate that calcium is better absorbed as calcium citrate in both fasting and mixed meal situations (Sakhaee *et al.* 1999). In addition this meal was served with orange juice to maximise the absorption of the calcium citrate tablets (Mehansho *et al.* 1989; Heaney *et al.* 2005).

There were no differences in the taste preference and overall acceptability of each meal as determined by sensory assessment questionnaires. Palatability of a test meal is known to affect insulin responses and ultimately substrate utilization (Sawaya *et al.* 2001). The amount and volume of each meal was also similar (Table 3:2). It was also important to determine that our results were not affected by differencing rates of gastric emptying; to this end the paracetamol absorption test was used. We demonstrated no difference between either of the breakfast test meals for post-prandial paracetamol concentration, time taken to reach maximum concentration nor maximum paracetamol concentration reached. Hence, we can confidently conclude

that neither palatability nor gastric emptying of the test meals confounded the outcomes.

We observed that subjects acutely fed a high dairy or high calcium meal exhibited a lesser suppression of FOR over a 6 hr postprandial period, when compared to a low dairy calcium meal. These findings have been confirmed by our group using a sequential meal design (Soares *et al.* 2004). Our results also compare favourably with evidence from the literature. In a cross-sectional study, Melanson *et al.* (2003) showed a positive correlation (r=0.38, P=0.03) between acute calcium intake and fat oxidation using whole-body room calorimetry. Moreover, in a prospective study of energy restriction, these authors showed a greater 24 hour fat oxidation following high calcium intakes (Melanson *et al.* 2005). One long-term study has also demonstrated a greater fat oxidation rate in subjects who followed a high calcium diet for a year (Gunther *et al.* 2005). In contrast, Jacobsen *et al.* (2005) manipulated calcium and protein intakes over 2 week periods and did not find any change in 24h energy expenditure nor fat oxidation between diets.

In this study circulating levels of NEFA were less suppressed following both high calcium meals, which paralleled the changes in fat oxidation. The similar glycaemic and insulinemic responses following all three meals (Table 3:5) would suggest that the relatively elevated NEFA levels did not result from a reduced insulin action. Frayn (1998) and Coppack *et al.* (1994) have argued that following a mixed meal, chylomicron triglyceride is preferentially acted upon by adipose tissue lipoprotein lipase. While re-esterification into TG does occur, much of this NEFA fails to be 'trapped' within adipose tissue and finds itself back in circulation.

A role for the sympathetic nervous system (SNS) in postprandial events cannot be discounted. SNS activity contributes to meal induced thermogenesis, and is a potent stimulator of adipose tissue lipolysis (Coppack *et al.* 1994). Although not statistically significant, the postprandial changes in serum glycerol were similar between the two high calcium meals, and around 70% higher relative to the low calcium meal. Evidence of a significantly higher glycerol level following high calcium intakes, has also been obtained under hypocaloric conditions (Zemel *et al.* 2005a; Zemel *et al.* 2005b), as well as during weight maintenance (Zemel *et al.* 2005a; Zemel *et al.* 2005b). While we acknowledge that measurements of circulating glycerol are not the

best quantitative index of adipose tissue lipolysis, the consistency of effects across studies merits further investigation. We anticipated a higher DIT between meals, but could not demonstrate such an effect. Hence higher intakes of calcium acutely modulate the type of fuel being utilized in the postprandial state, without a change in energy production. The calcium content of the meals tested here (530-575 mg), would in all probability exceed what is habitually consumed at individuals meals. Whether this would result in an attenuation of the effect on fat oxidation remains a possibility, but requires dose response studies for confirmation. Secondly, an increased frequency of calcium consumption would increase net amount absorbed for the day. Hence both factors are important when extrapolating results from acute meal based studies to dietary prescription for body weight regulation.

With a greater increase in fat oxidation demonstrated with the high calcium diets it would be expected that an increase in energy expenditure would be demonstrated. Although there was a greater increase in energy expenditure and diet induced thermogenesis with both high calcium containing diets, these did not reach significance. This is likely due to the reciprocal decrease in carbohydrate oxidation as the fat oxidation increased (was less suppressed). It must be noted that other acute studies examining the role of dairy/calcium in energy expenditure although have demonstrated a greater fat oxidation have failed to show the enhanced energy expenditure (Melanson *et al.* 2003; Jacobsen *et al.* 2005; Melanson *et al.* 2005). We also endeavoured to capture the change in thermogenesis through the measurement of auditory temperature to demonstrate a change in temperature induced by the diet; however no significant differences were observed between the three diets provided.

It has been suggested that dairy foods have a satiating effect due to their effect on the hormone, cholecystokinin (CCK). An increase in CCK has been associated with a meal-induced satiety (Schneeman *et al.* 2003). We were unable to measure CCK, but used VAS questionnaires to determine the subject's feelings of hunger and satiety before, during and following the testing period. The VAS questionnaire is a very simple and easy method to use to determine subjects feelings of hunger and satiety, and has been validated for use especially in a with-in study design, such as this study (Flint *et al.* 2000). We were unable to demonstrate any differences in feelings of hunger or fullness between the 3 test meals provided. Of all 5 of the

questions asked to determine the subject's subjective feelings of satiety, the results were consistent across all 3 diets. In other research from our laboratory, we similarly did not find any difference between subjects subjective feelings of satiety following a high versus a low dairy breakfast, followed by a low calcium meal provided at lunchtime (Ping-Delfos *et al.* 2004). One recently published study by Tsuchiya *et al.* (2006) has demonstrated a greater fullness and reduced hunger following the consumption of a yoghurt and a liquid yoghurt drink compared to a fruit drink and dairy beverage; however, it seems more likely that the greater satiety following these meals could have been due to the thicker consistency of the yoghurt test meals (Mattes & Rothacker, 2001) and a significantly higher protein content of the yoghurt based meals could have contributed to the effect (Hu, 2005; Orr & Davy, 2005). In this study the protein content as well as the viscosity of the breakfast meals were consistent across the 3 test meals.

We also calculated the subject's food intake following the end of the test period and 24 hour post-testing. We were unable to demonstrate any significant differences between either of the test breakfast meals. The food consumed after the buffet meal and up until midnight, demonstrated a trend for a greater fat intake on the LD diet versus the HC meal (P=0.051); however, no differences emerged for total energy intake nor intake 24 hours post-testing. In our laboratory we have demonstrated a significant reduction in food consumption of human subjects following a high dairy, (high vitamin D) breakfast meal versus a low dairy, (low vitamin D meal), the differences in consumption only reached significance once the food intake had been observed for 24 hours, with less food consumed following the high dairy meal (7143 $\pm$ 431 kJ vs. 8484 $\pm$ 699, P<0.02) (Ping-Delfos *et al.* 2004). The difference between these results and the present study may be due to the slightly difference in study design, with Ping-Delfos *et al*. study the subjects were given a high versus a low dairy breakfast meal, followed by a low calcium lunch time meal. This present study was only interested in the responses following the acute intake of a breakfast meal. Interestingly, although Tsuchiya *et al*. (2006) study demonstrated a significantly greater satiety with the yoghurt meals, the subjects did not reduce their energy consumption at the next meal. It has been argued by some that there is a lack of consistency between appetite rating scales and the following energy intake, in both normal weight (Mattes, 1990) and overweight subjects (Doucet *et al.* 2003).

If dairy / calcium is to be beneficial in the long-term management of weight control, a reduction in the time taken to eat the next meal or snack maybe important. We measured time to consumption of the next meal following the consumption of the buffet meal and demonstrated a trend (P=0.07) for a longer time lag before the next meal is consumed between the HD and LD meals (~ 60 minutes). This is very similar to our other findings (Ping-Delfos *et al.* 2004) with a greater time lag of 77 minutes between the high and low dairy breakfast meal.

In any study involving the manipulation of a nutrient it is important to be reassured that the results are not biased by poor absorbance of that nutrient. We examined 3 markers of calcium bioavailability; serum ionised calcium, intact parathyroid hormone suppression and urinary calcium excretion. It has been suggested by some authors that serum ionised calcium is not a good measure of calcium bioavailability as this nutrient is tightly regulated with the body and small changes over time will not elicit a good understand of the absorption of calcium (Heaney, 2001). However, our data for parathyroid hormone suppression and urinary calcium excretion, although not significant, showed a trend for a rank order of bioavailability across these two methods with LD<HD<HC. The observations that FOR following HD were similar to HC, despite poorer bioavailability of calcium, may indicate the presence of other bioactive components in dairy that influence fat oxidation (Zemel, 2002; Parikh & Yanovski, 2003; Zemel, 2003).

3.1.3.1 Conclusion

To the best of our knowledge, this is the first prospective study that shows a greater postprandial fat oxidation rate following high calcium meals, both dairy and non-dairy. The lesser postprandial suppression of NEFA would drive the increase in the fat oxidation, and the trend for a greater lipolysis would serve to maintain the relatively higher NEFA levels following such meals. Calcium rich meals did not alter subjective feelings of hunger or satiety, nor immediate food intake (buffet) following such meals. However, the inter-meal interval was prolonged relative to a low calcium diet, with some indication of a decrease in fat intake. Overall, 24 hour food intake was similar across meals. Such data provide a mechanistic framework

for understanding how the intake of calcium rich foods may influence human energy balance. Confirmation of these putative effects through well designed dietary interventions are required to confirm calcium's role in the regulation of body composition.

Chapter 4: The Potential Role of Dietary Calcium in Obesity – a randomised controlled trial

4.1 Introduction

In our acute study we have demonstrated a greater postprandial fat oxidation rate following the consumption of a single high calcium, high vitamin D, from both dairy and non-dairy meals. To date there are limited prospective randomised clinical studies designed to elucidate the effect of calcium and dairy on weight loss and body composition changes in humans. Importantly the studies that do exist are not consistent in their findings, making the outcomes of this PhD research particularly pertinent.

To date, there have only been six randomised clinical trials designed to specifically relate calcium from dairy sources to weight loss. These studies had been discussed thoroughly in chapter 1 (Literature Review) and a review table has been added at the end of this chapter for reference. In brief, 3 studies have found a positive role of dairy calcium in aiding weight loss (Zemel *et al.* 2004b; 2005a; 2005b). However, two studies that had similar study designs to Zemels group failed to find significant body composition differences between treatment groups (Harvey-Berino *et al.* 2005; Thompson *et al.* 2005). A lack of significant difference between treatment groups was also concluded from Bowen *et al.* (2005); however, this study manipulated the protein content as well as calcium and was therefore not included in the tables.

Clearly, there are several important issues that needed to be resolved. Firstly, the question arose whether calcium did in fact have a weight reducing effect per se, and secondly, whether other components in dairy (particularly protein) would have an added benefit. All previous studies had employed a double or multi-stranded parallel design, which in essence necessitated a between-subject comparison. The key facets that determine the success of such a design, is how well the subjects are matched at the start, the relative compliance to dietary prescription and the absence of unforseen

confounders arising during the study. It was therefore important to proceed with a weight loss study that asked a simple question, using a good design with proper attention to study execution.

4.1.1 Study design

We employed a single-blind, randomised crossover design comparing the effects of a high calcium diet (milk mineral powder) ~1200-1300 mg/d against a low calcium (~550-700 mg/d) hypocaloric mixed meal diet. The study involved 2 x 12 weeks of weight loss with a 12-week wash-out period (timed to coincide with the Christmas season (Figure 4:1). Postprandial measurements were made in the 10th week of each diet period using a low calcium, high fat test meal (refer to chapter 5).

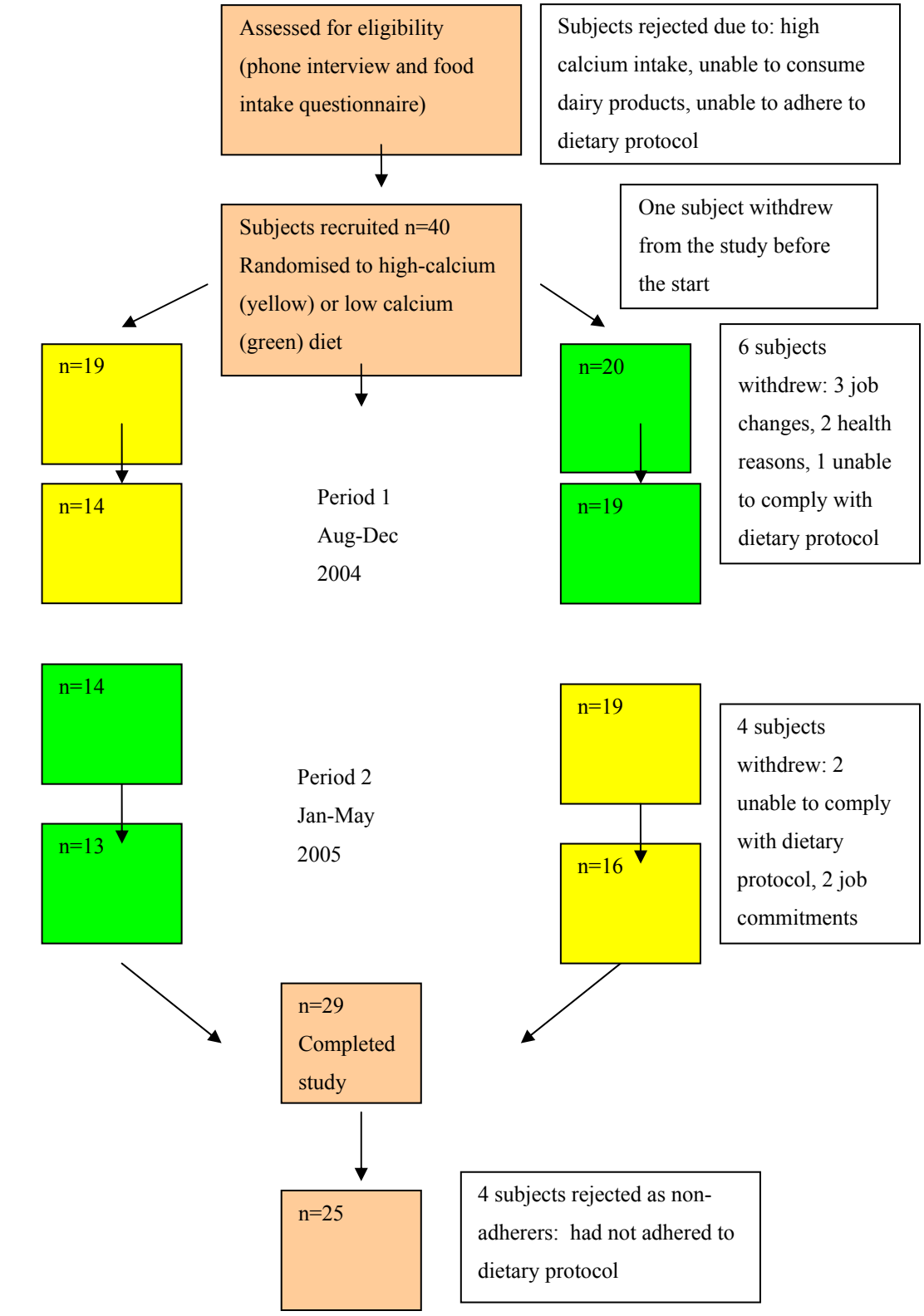


Figure 4:1 Study design

(HC = high calcium (dark shading), LC = low calcium (light shading)

4.1.2 Subjects

Subjects were recruited from the local media (ABC radio and Curtin University FM) and all subjects resided in Perth, WA. Subjects were screened initially through a telephone interview, which included a brief overview of the study, questions about previous weight loss, expectations on the study and a subjective assessment of subject suitability. A nutritional questionnaire was also undertaken, which was adapted by Professor Caryl Nowson (personal communication) using data from Angus & Eisman (1988; 1989). The questionnaire asks simple frequency (weekly or daily) consumption of some common calcium containing foods. Following this telephone screening, suitable subjects were sent a letter detailing the study protocol and time requirements during the study, along with a consent form (Appendix G). Once the signed consent form had been received by the researcher, subjects were asked to attend a medical screening appointment at the Health Centre, Curtin University. Ninety-seven subjects responded to our call, and 40 subjects were selected based on the following inclusion criteria; i) absence of clinical signs or symptoms of chronic disease; ii) history of weigh stability ($\pm$ 2 kg for the preceding 12 months); iii) not on medication affecting metabolic rate, body composition or calcium metabolism; iv) measured BMI > 28 kg/m^2 v) able to consume dairy products vi) current calcium consumption < 800 mg/day (due to paucity of suitable subjects a few subjects did not meet this criteria) vii) vitamin D status >50 nmol/L indicating vitamin D replete (4 subjects did not meet this criteria) . Subjects were assessed for medical suitability to participate in a weight loss study and a blood sample was taken to determine baseline thyroid function.

From an initial cohort of 40 subjects, 29 completed both arms of the study (reasons for drop-out included time commitment, commencement of a new job, diagnosed with breast cancer, and difficulty following the prescribed diet). Of the 29 subjects, a further 4 subjects were omitted from the analysis as they were deemed; non-adherers with the intervention (see comments below).

4.1.3 Standardised diets

The aim of the intervention was to increase the intake of dairy calcium while on a hypocaloric diet. The intake of calcium on the low calcium diet was ~500-600 mg/d, whilst the high calcium diet was 1200-1300 mg/d. Each subject had an individualised diet based on their measured resting metabolic rate (RMR) x 1.5 (activity factor). This was calculated at the start of each arm of the study. Dietary energy was then prescribed at 70% of that value with protein, fat (including saturated to unsaturated fat) and carbohydrate held constant and composed of 20, 25 and 55% of the diet, respectively (Table 4:1). The subjects were provided with dietary modules (5 options for breakfast, 5 options for lunch and 8 options for dinner) each breakfast, lunch and dinner module was matched for energy, protein, fat, carbohydrate, calcium and fibre content. The diets were based on typical eating patterns of Australians, as well as the Australian Dietary Guidelines (Nutbeam *et al.* 1993) (Table 4:1). Ongoing dietary instruction and support was provided by the principle researcher. The majority of food amounts were provided in standard metric cup sizes, to aid compliance. Calibrated digital scales were used to measure all other items of food. The subjects ingested their own food for the duration of the study. However, intervention foods were provided as 100 g UHT milk cartons (Murray Goulburn Co-Operative Co. Ltd; Brunswick, Victoria, Australia) and 1 litre containers of frozen yoghurt (daily consumption of 2 x 50 g portions); either mixed with Polyjoule for the low calcium diet or with NatraCal for the high calcium milk powder [NatraCal™ is a natural milk powder manufactured from skim milk and natural milk calcium extracted from fresh whey]. The intervention foods (milk and yoghurt) were matched in energy, protein, fat, carbohydrate content.

Since bioavailability of calcium decreases exponentially with increasing calcium intake (Heaney *et al.* 1990), all subjects were instructed to consume their dairy products in 3 equally divided doses over the day. The subjects completed daily dietary sheets, whereby they noted the module number they had chosen at each meal time and marked a box with M (milk) or Y (yoghurt) to confirm they had consumed the 3 dairy serves allocated and additional space was provided for the purpose of note taking with regards to deviation from the prescribed diet (Appendix H). The

food recording sheets were discussed with the chief researcher at each fortnightly visit. In addition, each subject was contacted by phone in the week between their appointment and asked general compliance questions, given a reminder about the next visit and any specific protocol instructions for the coming week (for example, fasting or physical activity diary to be completed). In this manner each subject received weekly contact with the principle researcher and dietitian. During week 6 of the study subjects were asked to complete a 3-day dietary intake record using household measures and/or scales.

Table 4:1 Comparison of the Study Diets in Part 1 & 2 to the Australian Dietary Goals.

Macronutrient	Study Diet		Australian
	Part 1	Part 2	Dietary Goals
Carbohydrate (% of total kJ)	55 ± 0.75	55 ± 0.98	> 50
Protein (% of total kJ)	20 ± 0.41	20 ± 0.46	15 – 20
Fat (% of total kJ)	25 ± 0.73	25 ± 0.73	15 – 30
Fibre (g)	29 ± 1.11	29 ± 0.93	30

Values provided as means (± SD).

4.1.4 Physical Activity assessment

Subjects were asked to maintain a complete 3-day physical activity diary, including 2 week days and 1 weekend day, before commencement of the study. This physical activity diary was then repeated for 3-days during week 4 and 8. The diary consisted of one-hour blocks from 07.00 hours until 23.00 hours. It was assumed that subjects slept between 23.00 and 07.00 hours; however, they were asked to indicate the number of minutes of sleep versus general activity. The activity diary listed a number of activities e.g. walking, sport & leisure, housework, gardening and office activities. Subjects were asked to indicate the number of minutes spent in each activity over

each hour (Appendix I). MET (Metabolic Equivalent) values for each activity were assessed from the American College of Sports Medicine manual (Bouchard *et al.* 1983; American College of Sports Medicine, 2001). A MET score is equivalent to 3.5ml $O_2.kg^{-1}.min^{-1}$. For the purpose of comparing baseline activity with activity during the intervention, each subject's data was calculated as an absolute MET hour score. Mean MET hour scores were calculated for the 3-day records at baseline and compared to mean values for each 3-day recording period for week 4 and 8 combined (= 6 days).

4.1.5 Anthropometry and Body Composition

Standing height was measured using a wall-fixed stadiometer and recorded to the nearest 1 mm. Body weight was measured immediately after voiding and after an overnight fast (baseline and then at fortnightly meetings with the chief researcher). Subjects wore underwear only and changed into a light surgical gown. The measurement was taken on a digital scale recorded to the nearest 100 g. Waist and hip circumferences were measured as described by Norton and Olds (2000). Body composition was determined by Dual energy X-ray absorptometry (DEXA) (Hologic QDR-4500A) at a certified commercial Bone Densitometry Centre. Daily scanning of the anthropometric spine phantom were conducted (using the Hologic protocol). In addition a daily reading is checked using Shewart Multi-rule Charts and Cusum plots (Lu *et al.* 1996). In addition a weekly step phantom is analysed to enable standard body composition measurements to be compared. An abdominal region of interest (ROI) was delineated as previously described by Ley *et al.* (1992). In brief, the area is bound by the upper part of the body of T12 and the iliac crest and its sides lateral to any trunk soft tissue.

4.1.6 Blood Pressure measurements

Systolic and diastolic blood pressure were measured at baseline, week 6 and at the end of each diet period under standardised conditions (after an overnight fast, in a

post-absorptive state and having rested in a supine position for at least 1 hour). The Omron blood pressure machine (Model T8, Omron Corporation, Japan) was used for all measurements. This blood pressure machine has previously been cross-validated by the Bland-Altman approach in a group of 25 subjects, and showed intra-class correlation coefficients of 0.94, 0.89 and 0.981 and no difference between machines for SBP, DBP or HR respectively. Intra-individual variations in BP had a coefficient of variation of <1.5% (Sivakumar & Soares, 2006).

4.1.7 Measurement of biochemical indicators

Fasting venous blood samples were drawn at baseline and at the end of each dietary period (week 12). Blood samples were used for the determination of insulin, glucose, total cholesterol (TC), high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol (by calculation), and triaclyglycerol (TG), as determined by the chief researcher (for details of methodology refer to chapter 2). Blood samples were collected in plasma and serum tubes, the former allowed to stand on ice for 30 minutes and the latter at room temperature, all tubes were then centrifuged at 3000xg for 10 minutes. Serum and plasma were extracted and the samples stored at -80ºC for later analysis. Additional samples were collected for the determination of 25, dihydroxy-vitamin D, ionised calcium, intact parathyroid hormone and free 3,5,3' triiodothyronine, these were sent to a certified laboratory in Perth (refer to methods section, chapter 2 for details of assay methods).

4.1.8 Measurement of urinary nitrogen excretion

On twenty-four complete urine collections was undertaken by each subject at the start intervention and during week 6 (refer to section 2.2.5.2 for details of methodology).

4.1.9 Dietary Assessment Questionnaire

At the end of each dietary intervention period (12 weeks), subjects were given a questionnaire to determine their assessment of the diet quality. For each question they were asked to make a mark (l or X) on a 150 mm line which extended between two extreme ranges. The length of the mark was then measured with a ruler. The questions were: 1. How would you rate the diet provided? 2. How would you rate the taste of the milk provided by the researcher? 3. How would you rate the taste of the yoghurt provided by the researcher?

4.1.10 Postprandial measurements (week 10)

During week 10 of the study postprandial measurements were taken over a 5 hour period following the consumption of a low calcium, high fat test meal, to determine differences in substrate oxidation rates following the high calcium versus the low calcium dietary intervention (refer to chapter 5 for details of this study).

4.1.11 Power calculations

The power calculations were based on a cross-over design. Conservatively a 2kg difference in body fat loss was expected between the two diets. For a 100 kg person, the precision of body fat estimates from DEXA, is at worst 3 kg. For an α error of 5%, 24 subjects are required to give a power of 80%. This sample size is sufficient to uncover differences in biochemical parameters as well. However, a total of 40 subjects were recruited for the intervention study, to allow for a 20% drop out, plus a safety margin to allow for other confounders.

4.1.12 Statistical analysis

Data were analysed using the STATA Statistical Software (Release 8.0, College Station, TX, USA) package. All results are presented as mean values and standard

errors of the mean (SEM), unless otherwise stated and all data was assessed for normality. All variables not conforming to normality were log transformed. The outcome variables of interest were body weight, fat mass, lean mass (total and abdominal region of interest), waist and hip measurements. The change in a variable of interest associated with a particular diet and the difference between the changes in a variable of interest between the two diet periods, in the same subject, were obtained as follows.

Change in variable = measure after diet – measure before diet
and
Difference in change = change on the high calcium diet – change on the low calcium diet

The difference between the changes in the variables of interest, or the differences in variables at the end of each diet period, were analysed using ANOVA for the 2 x 2 crossover study design. This is similar to repeated measures, except that the treatment factor (effects of the two diets in question) was assigned across the time periods. Each subject received both treatments in a particular sequence (sequence in which the diets were administered: HC diet then LC diet = 1, LC diet then HC diet = 2). Within each subject, a period effect was also assessed. A significant sequence effect, was ignored, as recommended by (Senn, 1994), when assessing TREATMENT effects using an ANOVA for a 2 x 2 cross-over study.

A paired t-test was used to compare paired data within individuals. Regression analysis with robust variance estimates was used to test for any treatment, sequence and period effects, after adjusting for confounding factors (STATA Reference Manual: Version 8.0, 2003; STATA Corporation).

As measurements regarding weight often result in large variability, correlations were run between weight loss and a number of relevant confounders (Appendix J). Variables of interest where then used as confounders with a regression model with robust clusters.

To determine changes over time a repeated measures ANOVA was used to determine statistical significance, which was set at the 5% level. Post-hoc test used the LSD procedure (unless otherwise stated). Data analysed this way used the SPSS for Windows (Version 11, SPSS Inc. USA) statistical software package.

4.1.13 Ethics

The Human Research Ethics Committee of Curtin University of Technology (HR 245-2001) approved the protocol and conformed to the Helsinki Declaration. All measurements were made in the clinical rooms of the Bentley Campus of Curtin University, Perth.

4.2 Results

4.2.1 Subject Characteristics

Table 4:2 Clinical characteristics of subjects at baseline

	Part 1.			Part 2.		
± SD	HC n=14 (♂2)	LC n=19 (♂4)	Independent t-test (P value)	LC n=13 (♂2)	HC n=16 (♂4)	Independent t-test (P value)
Age (yr)	53.3 ± 5.3	54.1 ± 4.8	0.67	53.3 ± 5.5	54.1 ± 4.4	0.67
Weight (kg)	88.26 ± 15.85	90.37 ± 13.24	0.69	83.72 ± 16.07	83.54 ± 11.24	0.97
Height (m)	1.65 ± 0.08	1.65 ± 0.10	0.82	1.64 ± 0.09	1.67 ± 0.09	0.52
BMI (kg/m^2)	32.42 ± 3.54	33.21 ± 5.38	0.61	30.77 ± 3.72	30.14 ± 3.89	0.66
% total body fat	39.65 ± 6.44	38.14 ± 7.01	0.53	37.22 ± 6.73	35.19 ± 6.93	0.43
FFM	52.38 ± 12.60	54.55 ± 10.17	0.59	52.10 ± 12.70	53.52 ± 9.23	0.73
TC (mM)	5.75 ± 1.01	5.79 ± 0.99	0.78	5.65 ± 0.85	5.82 ± 1.11	0.65
Ca intake (mg) (baseline)*	558.2 ± 203.70	609.7 ± 213.55	0.49	NA	NA	NA
Baseline energy intake (kJ)	9185 ± 123	8923 ± 143	0.75	8247 ± 89	8483 ± 98	0.67

HC – high calcium, LC – low calcium, NA – not applicable. Values are means ± standard deviations).
Abbreviations: BMI - body mass index, Ca - calcium. TC – total cholesterol, Gluc. – Glucose.
* Ca intake was assessed from the mean value obtained from a 3-day food diary (Appendix F).

Table 4:2 outlines the clinical characteristics of the subjects comparing the completers in part 1 (n=33), between dietary treatments and the completers on part 2 (n=29) of the study, measured at baseline or during the screening process. There

were no significant group differences in age, weight, height, body mass index (BMI), total body fat (measured by DEXA), nor baseline calcium intake, as measured by a validated nutritional questionnaire.

4.2.2 Dietary Assessment Questionnaire

The subjects did not rate the diets differently when following the high calcium versus the low calcium diet, which indicates that they were well-blinded to the diets provided. Question 1. overall rating of the diet provided (HC 85 $\pm$ 13, LC 83 $\pm$ 12, ANOVA, P=0.75). Question 2. How would you rate the taste of the milk provided (HC 75 $\pm$ 8, LC 63 $\pm$ 9, ANOVA, P=0.64) and Question 3. How would your rate the taste of the yoghurt (HC 89 $\pm$ 10, LC 85 $\pm$ 9, ANOVA, P=0.78).

4.2.3 Monitoring compliance on the diets

4.2.3.1 Objective methods of compliance

4.2.3.1.1 Dietary Intake

As previously mentioned subjects were prescribed an individualised diet, based on their measured RMR and an activity factor of 1.5. Energy intake was then prescribed at 70% of this value to achieve weight loss. This prescribed value was compared with mean values from 3-day food diaries, completed during week 6 of the study. There were no significant differences between treatment groups or period effects, for recorded versus actual (prescribed) dietary intake, indicating good compliance across both groups (Table 4:3). There were no significant differences between groups for calcium intake prescribed versus actual calcium intake consumed (as measured by a 3-day food diary) (Table 4:4), indicating good compliance across the groups.

Table 4:3 Comparison of energy intake between the dietary treatments and periods 1 & 2

Treatment	Recorded (kJ)	Diet provided (kJ)	Difference (kJ)	Paired t-test (P value)
High calcium	5962.7 ± 176.3	6003.3 ± 167.4	40.6 ± 66.5	0.55
Low calcium	5965.3 ± 208.8	6158.9 ± 207.2	193.6 ± 103.8	0.07
Period effect				
Part 1	6080.6 ± 184.8	6251.1 ± 189.8	170.5 ± 105.9	0.12
Part 2	5847.4 ± 190.1	5911.1 ± 181.8	63.72 ± 65.1	0.34

Values are means ± SEM, n=25

Table 4:4 Comparison of calcium intake between the dietary treatments and periods 1 & 2

Treatment	Recorded (kJ)	Diet provided (kJ)	Difference (kJ)	Paired t-test (P value)
High calcium	1340.7 ± 17.3	1341.0 ± 14.6	1.7 ± 2.7	0.96
Low calcium	640.4 ± 14.6	634.8 ± 12.1	5.6 ± 2.5	0.57
Part 1				
HC	1367.1 ± 21.6	1359.4 ± 18.8	-7.7 ± 2.8	0.47
LC	659.5 ± 16.1	651.4 ± 12.6	-8.4 ± 3.5	0.58
Part 2				
HC	1317.6 ± 11.1	1324.9 ± 9.0	7.3 ± 2.2	0.29
LC	612.6 ± 10.3	610.6 ± 9.8	-2.0 ± 0.5	0.86

Values are mean ± SEM, n=25

4.2.3.1.2 Physical activity

Each subject completed 3-days x 24-hour activity charts prior to commencing the study. These 3-day activity charts were repeated at week 4 and 8 (mean value compared with baseline) to ensure subjects did not increase or decrease their activity

during the intervention. There were no significant between-group differences nor period effects for subjects reported activity (MET hours) at baseline versus mean values for week 4 & 8.

Table 4:5 Comparison of energy expended before the intervention versus during the intervention (between the treatments and periods).

Treatment	Baseline (MET hours)	Mean week 4 & 8 (2 x 3 days of recording)	Difference	Paired t-test P value
High calcium	84.30 ± 2.40	86.48 ± 1.72	-2.18 ± 1.72	0.22
Low calcium	88.14 ± 1.75	86.97 ± 1.89	1.17 ± 1.61	0.47
Period effect				
Part 1	88.28 ± 2.42	88.25 ± 1.96	0.036 ± 2.02	0.99
Part 2	84.16 ± 1.71	85.20 ± 1.59	-1.04 ± 1.30	0.43

Measured as MET hours. Values are mean ± SEM, n=25

4.2.3.1.3 Weight loss

Body weight was monitored at baseline and on each visit by the chief researcher. Results in Figure 4:2, 4:3 and 4:4 indicate a significant drop in weight on every visit between 0-12 weeks, as determined by repeated measured ANOVA with a Bonferroni adjustment for multiple comparisons (P=0.0005). This was consistent on each diet and during each period on the RCT.

Figure 4:2 Change in weight (kg) between dietary groups during period 1

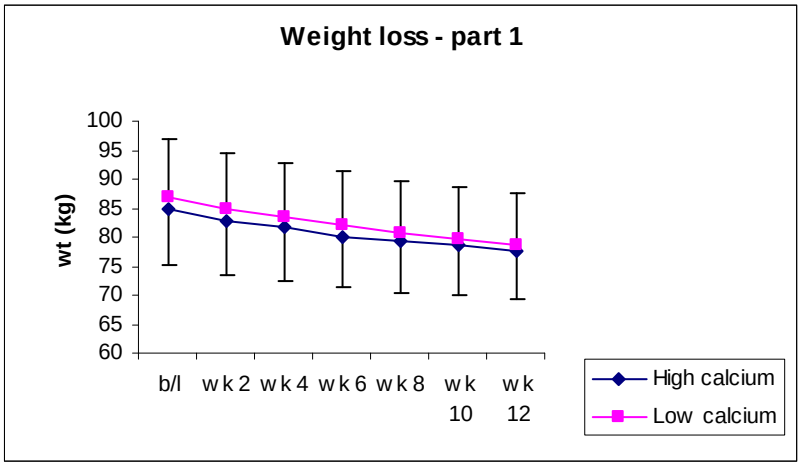


Figure 4:3 Change in weight (kg) between dietary groups during period 2

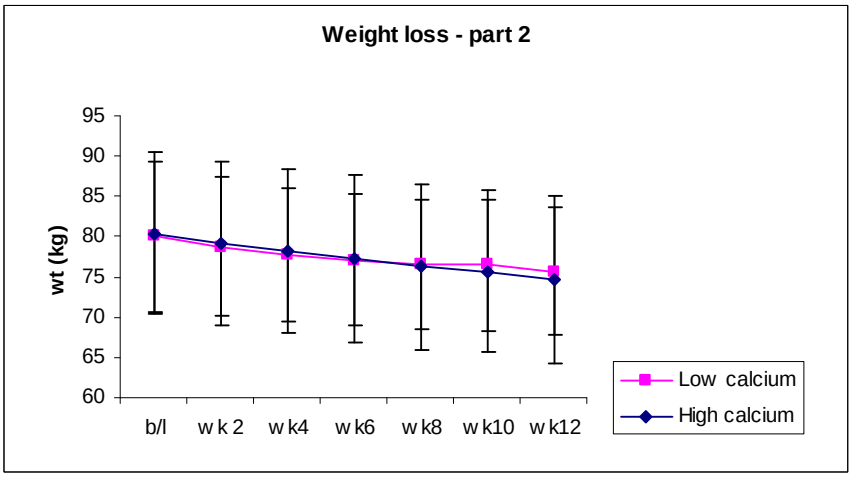
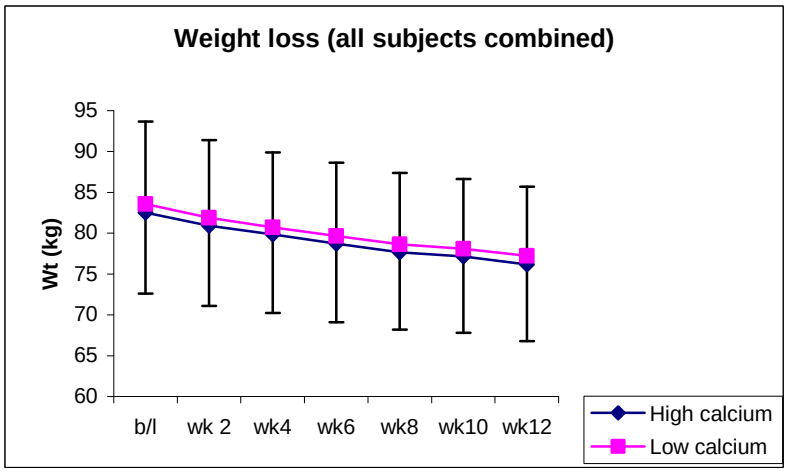


Figure 4:4 Change in weight (kg) between dietary groups, all subjects combined.



4.2.3.1.4 Nitrogen excretion versus protein intake

To determine dietary compliance amongst our cohort, we correlated urinary nitrogen excretion versus dietary protein intake (expressed as nitrogen). This method has been used widely as a marker of dietary compliance (Bingham, 2003).

Within each dietary treatment urinary nitrogen excretion correlated positively with dietary nitrogen intake (Table 4:6). Similarly, significant positive correlations were demonstrated for each dietary treatment during period 1 of the study; however, no correlations were observed during period 2.

Table 4:6 Urinary nitrogen excretion (g) correlated against dietary protein (expressed as nitrogen) intake from the diet

	g nitrogen in urine	g nitrogen from diet	Correlation (R)	P value
High calcium (n=25)	10.38 ± 0.47	11.94 ± 0.53	0.55	0.004
Low calcium (n=25)	9.83 ± 0.48	11.18 ± 0.44	0.44	0.03
Period 1. High calcium (n=12)	11.31 ± 0.82	12.28 ± 0.78	0.64	0.02
Low calcium (n=13)	9.45 ± 0.61	11.52 ± 0.69	0.61	0.03
Period 2. Low calcium (n=12)	10.23 ± 0.76	10.80 ± 0.53	0.34	0.27
High calcium (n=13)	9.53 ± 0.38	11.64 ± 0.74	0.47	0.11

Values are mean ± SEM

4.2.3.1.5 Calcium metabolism

4.2.3.1.5.1 Intact parathyroid hormone (iPTH)

Intact parathyroid hormone was measured at baseline and at the end of the study (week 12). The change from baseline was used to determine if iPTH had been suppressed in the high dairy group with less suppression in the low dairy group. This was not the case in this study and there was no difference in iPTH when combined across both arms. Refer to Table 4.7.

Table 4:7 With-in diet and treatment effect of parathyroid hormone (μmol/L), following a high versus a low calcium diet.

HC			LC			Within diet	Treatment effect
B'line	**End**	**Diff.**	**B'line**	**End**	**Diff.**		
3.46 ± 0.14	3.34 ± 0.18	-0.12 ± 0.18				0.52	
			3.45 ± 0.20	3.21 ± 0.23	-0.23 ± 0.18	0.20	0.83

Values are mean ± SEM, n=25

4.2.3.1.5.2 Urinary calcium excretion

Twenty-four urinary calcium excretion has been widely used as a measure of dietary calcium intake, with the explanation that a greater intake of calcium will cause a greater increase in urinary calcium excretion due to the down regulation of intestinal calcium absorption (Bronner, 2003). However, there are suggestions that 24-hour urinary calcium may not be the most accurate indicator of calcium intake (Toren & Norman, 2005; Weaver, 2006). Data from this study showed no consistent patterns or statistically significant correlations between urinary calcium levels and calcium intake.

Table 4:8 Comparison of correlations between 24-hour urinary calcium excretion (mg) and dietary calcium intake (mg)

	mg Ca / 24 hour collection	mg Ca in diet	Correlation (R)	P Value
High calcium (n=25)	153.70 ± 14.14	1332.28 ± 14.27	0.12	0.58
Low calcium (n=25)	146.74 ± 13.12	626.28 ± 11.06	-0.18	0.40
Period 1– High calcium (n=12)	154.0 ± 17.22	1352 ± 27.37	0.48	0.112
Low calcium (n=13)	111.9 ± 11.33	647 ± 15.97	0.023	0.942
Period 2 – Low calcium (n=12)	184.5 ± 19.54	604 ± 12.97	0.10	0.755
High calcium (n=13)	153.4 ± 22.75	1314 ± 10.06	-0.47	0.111

Values are mean ± SEM

Table 4:9 Changes in urinary calcium (mg) from baseline to end of study

HC			LC			Paired t-test
Baseline	**End**	**Diff.**	**Baseline**	**End**	**Diff.**	**P value**
154.81 ± 13.10	153.70 ± 14.14	1.11 ± 14.56				0.94
			151.03 ± 11.8	146.74 ± 13.12	4.29 ± 11.05	0.70

Values are mean ± SEM, n=25

4.2.3.1.5.3 Vitamin D status

The vitamin D status of subjects is important as it plays a major role in the absorption of calcium. This study was designed to minimise changes in vitamin D status by conducting the study during the spring and summer seasons. Serum 25-hydroxyvitamin D_3, (Nowson & Margerison, 2002) was used as a measure of vitamin D status and measured at baseline and at week 12 on each dietary treatment. We found no differences between the change from baseline between the 2 treatments (P=0.30).

Table 4:10 With-in diet and treatment effect on vitamin D (nmol/L), following a high (HC) versus a low calcium (LC) diet.

HC			LC			Within diet effect	Treatm't effect
B'line	**End**	**Diff.**	**B'line**	**End**	**Diff.**		
70.91 ± 5.28	70.34 ± 4.10	-0.57 ± 2.44				0.82	
			66.58 ± 4.15	69.55 ± 4.41	2.96 ± 2.65	0.28	0.30

Values are mean ± SEM

4.2.3.1.5.4 Free 3,5,3'-triiodothyronine T3

Thyroid function was assessed, as measured by free triiodothyronine (fT3), at baseline and week 12 of each period of the study. As would be expected with any weight loss, fT3 declines (Douyon & Schteingart, 2002). Our study shows a very similar (non-significant) decrease in fT3, comparing the treatment groups (Table 4:11).

Table 4:11 Changes in Free 3,5,3'-triiodothyronine T3, following an RCT of hypocaloric high calcium (HC) and low calcium (LC) diets.

HC			LC			Within diet effect (P value)	Treatment effect
Baseline	**End**	**Diff.**	**Baseline**	**End**	**Diff.**		
4.54 ± 0.09	4.46 ± 0.08	0.088± 0.06				0.12	
			4.70 ± 0.08	4.60 ± 0.08	-0.10 ± 0.09	0.27	0.93

Values are mean ± SEM

4.2.3.2 Subjective measures of compliance

4.2.3.2.1 Completion of daily food diaries

All subjects had to complete a proforma outlining the food intake module they had chosen for breakfast, lunch and dinner and any deviations from the prescribed module had to be noted. The subjects also entered an M (milk) and Y (yoghurt) to indicate when they had consumed these products (Appendix H). The chief researcher also checked the appropriate number of returned, empty yoghurt containers and milk containers at each fortnightly visit. Results from this method of assessment indicated excellent compliance (98.6 ± 0.4 %) to the intake of the intervention products.

4.2.3.2.2 Qualitative assessment of non-adherers

Detailed notes were made of conversations with all subjects at face-to-face interviews (fortnightly) and over the phone (alternate week with appointment). Subjects were asked further questions, if comments were made regarding their compliance over the proceeding week. Subjects were also asked to complete a questionnaire (on completion of the study) asking if they had done anything differently throughout the study during period 1 versus period 2.

4.2.4 Changes in pre-and post- measurements

Table 4:12 Changes in anthropometry and body composition measurements between periods on each diet.

Measurement	Period	Treatment HC	Treatment LC	Period	Treatment HC	Treatment LC	Sig. P value Period effect (paired *t* test)
Weight (kg)	1	-7.03 ± 0.43	-8.18 ± 0.42	2	-5.74 ± 0.48	-4.4 ± 0.38	0.00005
Waist (cm)	1	-5.83 ± 0.64	-6.18 ± 0.83	2	-7.35 ± 0.67	-4.67 ± 0.42	0.98
Hip (cm)	1	-4.96 ± 0.53	-6.22 ± 0.40	2	-4.62 ± 0.47	-4.00 ± 0.30	0.02
Total body fat (%)	1	-1.91 ± 0.18	-2.62 ± 0.27	2	-1.69 ± 0.32	-0.9 ± 0.14	0.00005
Total FM (kg)	1	-3.88 ± 0.20	-4.69 ± 0.26	2	-2.98 ± 0.35	-2.30 ± 0.17	0.00005
Total FFM (kg)	1	-2.27 ± 0.27	-2.13 ± 0.21	2	-2.32 ± 0.28	-1.96 ± 0.24	0.51
Trunk fat (kg)	1	-2.11± 0.23	-3.43 ± 0.38	2	-2.35 ± 0.47	-1.24 ± 0.22	0.02
ROI fat (kg)	1	-2.15 ± 0.29	-3.81 ± 0.57	2	-3.48 ± 0.56	-1.79 ± 0.28	0.39

Values are mean ± SEM, n=25, Abbreviations: FM fat mass, FFM fat free mass, ROI region of interest

Table 4:13 Whole Body Composition measurements to demonstrate within diet effects (change from baseline) and treatment effect

	HC			LC			Within diet effect (P value)	Treatment effect (P value) ANOVA
	Baseline	**End**	**Diff.**	**Baseline**	**End**	**Diff.**	**P value**	**P value**
Weight (kg)	82.53 ± 1.98	76.17 ± 1.88	-6.36 ± 0.47				0.0005	
				83.58±2.01	77.22 ± 1.70	-6.36 ± 0.55	0.0005	0.78
%Wt change			-7.70 ± 0.50					
						-7.47 ± 0.57	0.0005	0.78
TBF %	37.90 ± 1.36	36.12 ± 1.46	-1.80 ± 0.26				0.0005	
				38.29 ± 1.26	36.50 ± 1.33	-1.79 ± 0.27	0.0005	0.85
Total FM(kg)	30.83 ± 1.40	27.42 ± 1.45	-3.41 ± 0.30				0.0005	
				31.50 ± 1.33	27.95 ± 1.26	-3.54 ± 0.33	0.0005	0.79
Total FFM (kg)	50.36 ± 1.52	48.07 ± 1.34	-2.29 ± 0.27				0.0005	
				50.70 ± 1.51	48.55 ± 1.38	-2.15 ± 0.23	0.0005	0.51

Values are mean ± SEM, n=25, Abbreviations: FFM fat free mass, FM fat mass, ROI region of interest, Diff. Difference (between baseline and end of study), TBF % Total Body Fat %.

Table 4:14 Regional Body Composition measurements to demonstrate within diet effects (change from baseline) and treatment effect

Measurement	HC			LC			Within diet effect	Treatment effect ANOVA 2x2
	Baseline	End	Diff.	Baseline	End	Diff.	P value	P value
Waist (cm)	96.66 ± 2.09	90.04 ± 1.97	-6.62 ± 0.69	97.83 ± 2.22	92.43 ± 1.99	-5.39 ± 0.68	0.0005 0.0005	0.052
Hip (cm)	111.62 ± 1.63	106.84 ± 1.53	-4.78 ± 0.51	112.36 ±1.45	106.3 ± 1.38	-6.06 ± 0.83	0.0005 0.0005	0.53
Trunk fat %	36.72 ± 1.31	34.48 ± 1.48	-2.23 ± 0.37	37.45 ± 1.29	35.07 ± 1.36	-2.38 ± 0.38	0.0005 0.0005	0.78
Trunk FFM (kg)	24.60 ± 0.71	23.42 ± 0.62	-1.18 ± 0.15	24.71 ± 0.71	23.65 ± 0.64	-1.06 ± 0.12	0.0005 0.0005	0.78
Trunk FM (kg)	14.36 ± 0.68	12.53 ± 0.73	-1.83 ± 0.17	14.84 ± 0.65	12.84 ± 0.61	-2.00 ± 0.20	0.0005 0.0005	0.42
ROI fat %	34.91 ± 1.15	32.07 ± 1.38	-2.84 ± 0.46	35.73 ± 1.14	32.89 ± 1.20	-2.84 ± 0.49	0.0005 0.0005	0.97
ROI FFM (kg)	6.20 ± 0.16	5.84 ± 0.18	-0.36 ± 0.13	6.26 ± 0.19	5.93 ± 0.16	-0.33 ± 0.12	0.01 0.01	0.80
ROI FM (kg)	3.40 ± 0.20	2.87 ± 0.21	-0.53 ± 0.08	3.55 ± 0.21	3.06 ± 0.22	-0.49 ± 0.12	0.0005 0.0005	0.67

Values are mean + SEM, n=25, except waist and hip n=23. Abbreviations: FFM fat free mass, FM fat mass, ROI region of interest, Diff. Difference (between baseline and end of study).

Table 4:15 Body Composition measurements to demonstrate the treatment effect (includes all completers, n=29)

Measurement (change from baseline)	Treatment		Sig. P value (ANOVA for 2x2 crossover design)	
	HC	LC	Treatment effect	Period effect
Weight (kg)	-5.9 ± 0.50	-6.36 ± 0.53	0.86	0.0005
% weight	-7.10 ± 0.55	-7.09 ± 0.56	0.95	0.0005
Waist (cm)	-6.42 ± 0.59	-5.56 ± 0.60	0.14	0.49
% total body fat	-1.57 ± 0.23	-1.63 ± 0.25	0.72	0.0001

Values are mean ± SEM

Table 4:16 Intention-to-Treat analysis: Body Composition measurements to demonstrate the treatment and period effect

Measurement (change from baseline)	Treatment		Sig. P value (ANOVA for 2x2 crossover design)	
	HC	LC	Treatment effect	Period effect
Weight (kg) n=40	-5.08 + 0.46	-5.57 + 0.48	0.71	0.0005
Waist (cm) n=35	-6.07 + 0.64	-5.12 + 0.56	0.14	0.48
Hip (cm) n=35	-4.43 + 0.50	-4.44 + 0.43	0.76	0.001
Total body fat (%) n=35	-1.56 + 0.21	-1.57 + 0.23	0.51	0.0005
Total FFM (kg) n=35	-2.11 + 0.22	-2.12 + 0.21	0.86	0.13
Total FM (kg) n=35	-3.09 + 0.27	-3.25 + 0.30	0.66	0.0005

Table 4:17 Changes in weight (kg) between dietary treatments, on including only those with baseline calcium intake <600mg/day (n=19)

			Sig. P value (ANOVA for 2x2 crossover design)	
HC	**LC**	**Difference**	**Treatment effect**	**Period effect**
-6.48 ± 0.45	-5.58 ± 0.44	-0.9 ± 0.009	0.27	0.0012

Figure 4:5 Correlation of resting metabolic rate (RMR) and change in total fat mass (kg), in subjects on the high calcium (o) and low calcium diets (□).

HC r=-0.08, P=0.71, LC r=-0.59, P=0.002

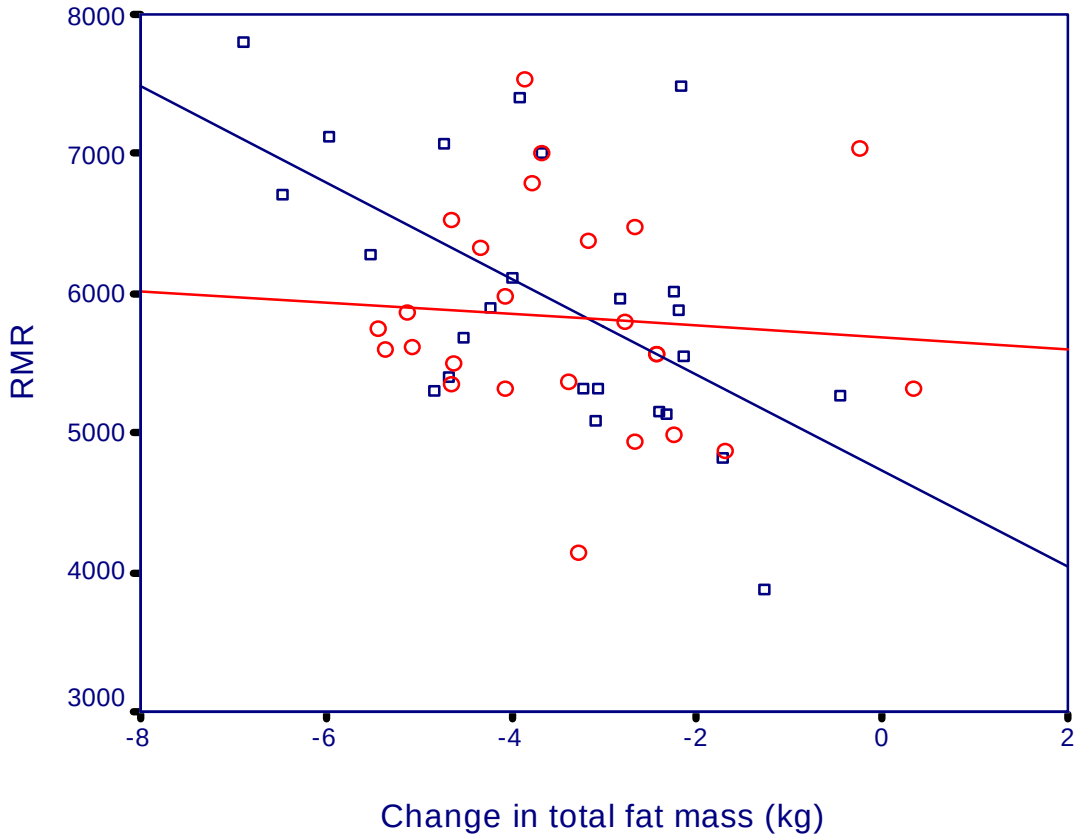


Table 4:18 Partial correlation coefficients between RMR and change in endpoint variables controlling for fat free mass and fat mass at the start of the study

	ΔWeight	ΔWaist	ΔHip	ΔTotal fat mass	ΔTotal fat free mass	ΔTotal fat mass
Diet:HC						
R	0.0355	-0.3142	0.2709	-0.1995	0.0866	-0.1311
P value	0.87	0.14	0.21	0.36	0.69	0.55
Diet:LC						
R	-0.5746	-0.2147	-0.3757	-0.4448	-0.0928	-0.5441
P value	0.006	0.35	0.093	0.043	0.69	0.011

Δ = change in variable (12 week – 0 week); HC = high calcium, LC = low calcium

Figure 4:6 Reduction in waist circumference (cm) between dietary treatments.

There was a significant with-in diet effect between baseline measurements and week 12 on the high and the low calcium diets (T-test, P=0.0005) and a significant treatment effect with the high calcium treatment eliciting a greater reduction in waist circumference (RM-ANOVA for 2x2 crossover design, P=0.052)

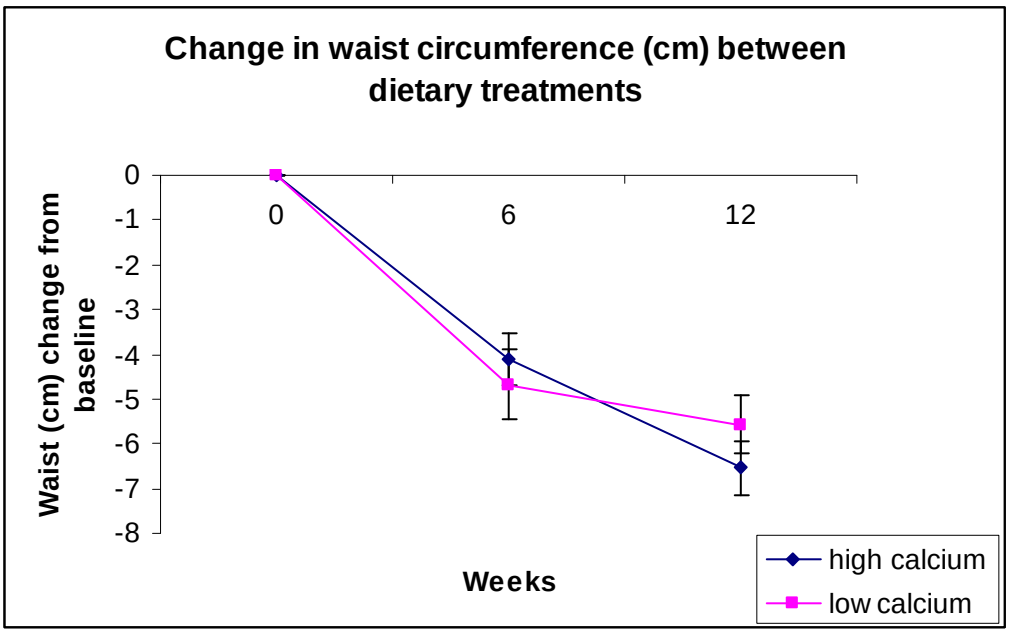
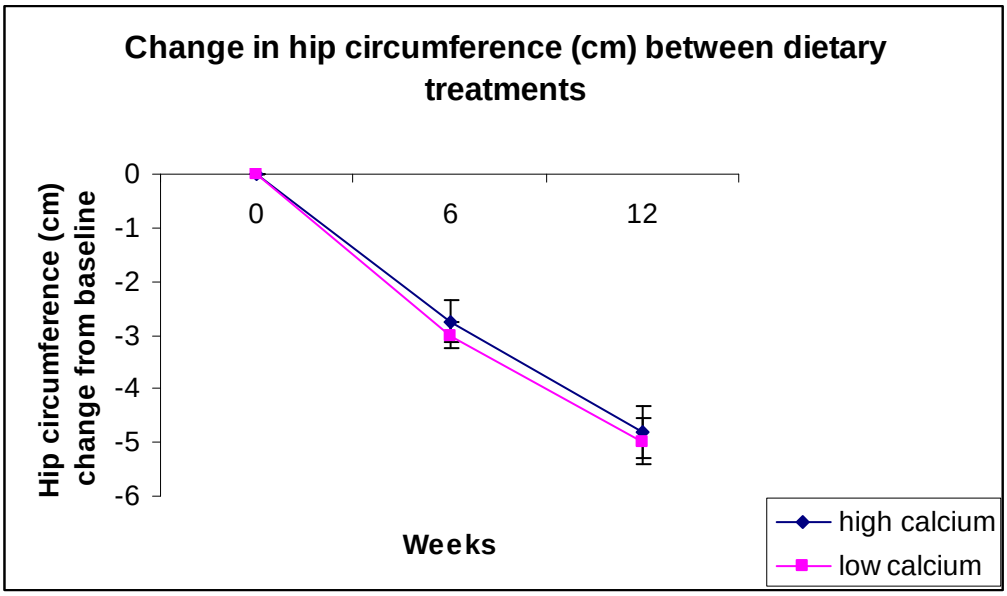


Figure 4:7 Reduction in hip circumference (cm) between dietary treatments.

There was a significant with-in diet effect between baseline measurements and week 12 on the high and the low calcium diets (T-test, P=0.0005). There was no significant difference between dietary treatments and reduction in hip circumference.



4.2.4.1 Body composition changes

As can be seen in Table 4:13 and 4:14 the high dairy calcium diet did not result in greater weight or body fat loss as measured by weight, total body fat %, total fat mass, trunk fat nor by an abdominal region of interest (fat % or absolute FFM). Similarly, there was no significant difference between treatment groups on total fat free mass. A greater reduction in waist circumference of 1.23 cm was observed when subjects had consumed the HC diet; this however was just short of significance (P=0.052). This observation was consistent in both of the crossover trial (Table 4:14 & Figure 4:6).

To investigate why we may not have achieved differences in body composition measurements between treatment groups various correlations were run with possible confounders of interest (refer to Appendix J). The most reoccurring relationship was between resting metabolic rate (RMR) at the start of the study and various body composition measurements. Correlations of RMR and change in total fat mass (Figure 4:5) demonstrated a significant negative relationship within subjects on the

LD diet (P=0.002), but not when the subjects consumed the HC diet. Furthermore, this relationship persisted when controlled for fat free mass and fat mass at the start of the study. On the low calcium diet RMR correlated negatively with weight loss (P=0.006), total fat percentage lost (P=0.043) and total fat mass lost (P=0.011). However, these variables were not correlated with RMR on the high calcium diets (Table 4:18). Table 4:13 & 4:14 also shows the with-in diet effect of the various body composition measurements. All body composition measurements (weight, waist circumference, total body fat %, total fat mass & fat free mass, trunk fat mass and an abdominal region of interest, showed significantly changes from baseline to the end of the study across both dietary treatments.

Table 4:12 shows body composition measurements for the 2 study periods (1 & 2). There is a significant period effect for weight loss, hip circumference, total body fat %, total fat mass and truck fat, with period 2, showing a lesser change. This may indicate less compliance or study fatigue of the subjects during this period.

The analysis was also completed on all subjects who had completed both arms of the study (n=29), this reanalysis did not change the previously reported results (Table 4:15). Similarly an intention-to-treat analysis did not alter the results with no change between treatment groups for weight, waist and hip circumference, total body fat percentage, total fat free mass and total fat mass (Table 4:16).

4.2.4.2 Fasting glucose, insulin levels and HOMA score

Fasting blood glucose levels were not altered by consumption of a high calcium diet versus a low calcium diet (treatment effect). However, both diets resulted in significant reductions in fasting glucose levels (Table 4:19 & 4:20). Fasting insulin levels and HOMA score were not significantly different between the dietary treatments. As with the fasting glucose levels, there were significant with-in diet effects for both high and low calcium diets. There were no significant differences for Hb_{a1c} changes from baseline, either a treatment effect or with-in diet effect.

4.2.4.3 Fasting Lipid levels

4.2.4.3.1 Fasting total cholesterol

There was no significant treatment effect on fasting cholesterol levels; however, both diets individually resulted in a small decrease in fasting cholesterol levels (HC -0.17 ± 0.60, LC -0.41 ± 0.81 mM, P=0.07) (Table 4:19).

4.2.4.3.2 Fasting LDL-cholesterol levels

There was a significant treatment effect on fasting LDL-cholesterol levels, with both dietary treatments eliciting a small increase in LDL-C (HC 0.38 ± 0.65, LC 0.03 ± 0.76 mM, P= 0.04), there was a greater increase within the HC group. Fasting LDL-cholesterol levels were slightly higher on the low calcium diet at the start of the study; however, this did not reach significance (HC 3.24 ± 1.03, LC 3.52 ± 0.89. P=0.07) (Table 4:19).

4.2.4.3.3 Fasting HDL-cholesterol levels

There was no significant treatment effect on fasting HDL-cholesterol levels; however, both dietary treatments resulted in a small decrease from baseline (Table 4:19).

4.2.4.3.4 Fasting triglycerides levels

No treatment effect was demonstrated on either diet; however, there was a with-in diet effect with the HC and LC diets both showing significant reductions in fasting triglyceride levels (Table 4:19).

4.2.4.4 Blood Pressure

Systolic and diastolic blood pressure were significantly reduced following both dietary interventions (with-in diet effect). However, no treatment effect was demonstrated (Table 4:19).

Table 4:19 Changes in fasting levels of glucose, insulin, HOMA score and Hba1c, total cholesterol, HDL and LDL-cholesterol, triglycerides and blood pressure (treatment and period effect)

	HC			LC			ANOVA 2x2 crossover design (P value)	
	Change from baseline	Period 1	Period 2	Change from baseline	Period 1	Period 2	Treat' effect	Period effect
Insulin (uIU/ml)	-1.33 ± 0.63	-3.04 ± 0.64	0.25 ± 0.41	-1.65 ± 0.92	-2.48 ± 1.23	-0.75 ± 0.36	0.84	0.03
Gluc.(mmol/L)	-0.54 ± 0.12	-0.45 ± 0.12	-0.63 ± 0.13	-0.68 + 0.12	-0.63 ± 0.12	-0.74 ± 0.13	0.39	0.37
HOMA score	-1.33 ± 0.63	-3.04 + 0.64	0.26 + 0.41	-2.32 ± 0.63	-3.88 ± 0.64	-0.75 ± 0.36	0.19	0.0002
Hb_{a1c}	-0.0083 ± 0.05	-0.05 ± 0.05	0.033 ± 0.05	0.029 ± 0.06	-0.03 ± 0.07	0.092 ± 0.034	0.60	0.16
TC (mM)	-0.17 ± 0.12	0.11 ± 0.13	-0.42 ± 0.09	-0.41 ± 0.16	-0.083 ± 0.14	-0.76 ± 0.16	0.07	0.0003

	HC			LC			ANOV 2 x 2 crossover design (P value)	
	Change from baseline	**Period 1**	**Period 2**	**Change from baseline**	**Period 1**	**Period 2**	**Treat' effect**	**Period effect**
HDL-C (mM)	-0.15 ± 0.07			-0.17 ± 0.07			0.78	
		-0.09 ± 0.02	-0.20 ± 0.10		-0.071 ± 0.05	-0.27 ± 0.08		0.10
LDL-C (mM)	0.38 ± 0.13			0.03 ± 0.76			0.04	
		0.62 ± 0.09	0.16 ± 0.15		0.41 ± 0.14	-0.38 ± 0.12		0.001
Trigs. (mM)	-0.74 ± 0.13			-0.69 ± 0.12			0.63	
Systolic BP (mmHg)	-5.86 ± 1.65			-7.1 ± 1.51			0.57	
		-7.08 ± 1.66	-4.73 ± 1.67		-9.15 ± 1.62	-4.88 ± 1.31		0.10
Diastolic BP (mmHg)	-1.86 ± 1.10			-2.86 ± 0.99			0.45	
		-1.63 ± 0.92	-2.08 ± 1.28		-4.88 ± 1.02	-0.67 ± 0.77		0.14

Values are mean ± SEM, P-values for with-in diet effects and treatment effects.

Table 4:20 Changes in fasting levels of glucose, insulin, HOMA score and Hba1c, total cholesterol, HDL and LDL-cholesterol, triglycerides and blood pressure (with-in diet effect)

	HC			LC			
	Baseline	**End**	**Change**	**Baseline**	**End**	**Change**	**With-in diet effect (P value)**
Insulin (uIU/ml)	8.51 ± 1.38	7.18 ± 1.13	-1.33 ± 0.63				0.04
				8.42 ± 1.14	6.10 ± 0.77	-2.32 ± 0.62	0.0013
Gluc(mmol/L)	5.62 ± 0.12	5.07 ± 0.10	-0.54 ± 0.12				0.0002
				5.79 ± 0.13	5.11 ± 0.08	-0.68 ± 0.12	0.0005
HOMA score	2.12 ± 0.33	1.61 ± 0.25	-0.51 ± 0.17				0.005
				2.12 ± 0.27	1.31 ± 0.17	0.81 ± 0.17	0.0005
Hb_{a1c}	5.33 ± 0.06	5.33 ± 0.04	-0.0083 ± 0.05				0.87
				5.37 ± 0.06	5.39 ± 0.06	0.029 ± 0.06	0.66
TC (mM)	5.78 ± 0.22	5.62 ± 0.21	-0.17 ± 0.12				0.18
				5.97 ± 0.19	5.57 ± 0.18	-0.41 ± 0.16	0.02
HDL-C (mM)	1.60 ± 0.07	1.45 ± 0.07	-0.15 ± 0.07				0.05
				1.61 ± 0.08	1.45 ± 0.08	-0.17 ± 0.07	0.02

	HC			LC			
	Baseline	**End**	**Change**	**Baseline**	**End**	**Change**	**With-in diet effect (P value)**
LDL-C (mM)	3.24 ± 0.21	3.61 ± 0.17	0.38 ± 0.13				0.008
				3.52 ± 0.18	3.55 ± 0.17	0.03 ± 0.15	0.84
Trigs. (mM)	1.94 ± 0.18	1.20 ± 0.12	-0.74 ± 0.13				0.00005
				1.92 ± 0.18	1.23 ± 0.13	-0.69 ± 0.12	0.00005
Systolic BP (mmHg)	120.18 ± 2.42	114.32 ± 1.96	-5.86 ± 1.65				0.002
				121.94 ± 2.80	114.84 ± 2.12	-7.1 ± 1.51	0.0001
Diastolic BP (mmHg)	72.9 ± 1.42	71.04 ± 1.34	-1.86 ± 1.10				0.10
				73.2 ± 1.17	70.3 ± 1.14	-2.86 ± 0.99	0.008

Values are mean ± SEM, P-values for with-in diet effects (n=25, except 1 missing value for HOMA and Insulin)

4.3 Discussion

4.3.1 Body Composition

Most prospective studies in this area have employed an RCT with a double or multi-stranded parallel design. In this design, it is imperative that all volunteers are similar in body composition and in metabolic function at the start. This is not always reported and hence the end result is open to interpretation. Confounding variables may therefore mask or amplify the expected endpoint. Our study chose to circumvent these issues by having each subject acting as their own control. We believe this is the first attempt to conduct a randomised cross-over weight loss trial examining the effect of dietary calcium. In addition, we measured subject compliance through a variety of ways; dietetic interviews, bias of reported energy intake, correlation between urinary nitrogen excretion and protein intake as well as intervention food counts. Overall, we were confident that 25 of the 29, who completed the program, could be classified as adherers.

In chapter 3 we demonstrated, for the first time, that the acute consumption of a high-calcium meal, both dairy and pharmaceutical, elicited a greater postprandial fat oxidation. Based on these findings and the results from some randomised-controlled studies (Zemel *et al.* 2004b; 2005a; 2005b), we anticipated that the chronic consumption of a high calcium meal, when combined with a hypocaloric diet, would result in subjects losing a greater amount of weight and/or body fat. The results of this study demonstrate that while subjects lost weight on both diets in both periods, neither weight nor fat loss was greater when subjects consumed the higher calcium diet. The lack of differences in weight and fat loss is similar to outcomes obtained by Thompson *et al.* (2005) and Harvey-Berino *et al.* (2005) who examined the effect of dairy rather than calcium per se. Additionally in a RCT of overweight/obese subjects following an energy-restricted diet with a supplement of 1200 mg of calcium plus vitamin D, no differences in weight or fat loss were observed (Major *et al.* 2007). This study does however, demonstrate a trend towards a greater reduction in waist circumference on the high calcium diet, this was just short of significance (P=0.052).

Based on a reanalysis of data from a nationally representative sample, it was predicted that Australians with higher calcium intakes could have lower BMI and waist circumferences (Soares *et al.* 2004). Therefore some confirmation is obtained from this study. In all three of Zemel's studies a greater waist circumference along with trunkal fat was reported (Zemel *et al.* 2004b; 2005a; 2005b).

Our finding of a trend for a greater reduction in waist circumference in subjects consuming a high calcium diet is of particular important to health, as abdominal obesity has been linked with the metabolic syndrome along with an increased risk from cardiovascular disease. In a recent meta-regression analysis study it was demonstrated that a 1 cm increase in waist circumference increased the relative risk of a cardiovascular disease event by 2% (95% CI: 1-3%)(de Koning *et al.* 2007). Visceral fat has been classified as being highly metabolic, with a high lipolytic rate (Wajchenberg, 2000). It has been suggested that the improvements in health outcomes with a small amount of weight loss, is due to the reduction in visceral fat mass and has been linked with the activation of the sympathetic nervous system (Rupp & Maisch, 2003). It has been suggested by Zemel (2004) that there may be a role for autocrine production of cortisol by adipose tissue. Human adipose tissue expresses significant 11-β-hydro-xysteroid dehydrogenase-1 (11-β-HSD-1), which can generate active cortisol from cortisone. It has been demonstrated that visceral adipose tissue (greatest in the waist region) has greater 11-β-HSD-1 expression than does subcutaneous adipose tissue (Rask *et al.* 2001). In the mice model over-expression of 11-β-HSD-1 has lead to central obesity (Masuzaki *et al.* 2001), whilst 11-β-HSD-1 knockout mice exhibit protection from features of the metabolic syndrome (Kotelevstev *et al.* 1997). In prelimary *in vitro* studies calcium agonists (such as calcitriol), exert substantial stimulation of cortisol production in human adipocytes (Morris & Zemel, 2005). This could have implications for our study, indicating that the possible reduction in waist circumference seen in our subjects consuming the high calcium diet could be attributable to a reduction in cortisol production by visceral adipocytes.

RMR was negatively correlated to weight loss, total fat loss and total fat free mass loss but only when the subjects had consumed the low calcium diet. It has long been established that a low RMR can predict long-term body weight gain (Ravussin *et al.* 1988; Astrup *et al.* 1999; Buscemi *et al.* 2005). In this study we demonstrated that subjects with a greater RMR at the start of the study tended towards a greater fat loss. In fact RMR contributed to ~32% of the variability of weight loss. Similar observations were made in a meta-analysis study demonstrating a 4% greater number of subjects with a higher RMR in a control versus a formerly obese group, this however, did not reach significance (Astrup *et al.* 1999). Similarly, a high baseline RMR has been suggested as predictor for successful weight maintenance following a period of weight loss (Vogels *et al.* 2005). However, the fact that the relationship of a higher RMR with a greater fat loss was only observed on the low calcium diet (consistent in both periods) would indicate that the ingestion of calcium abolishes this relationship. This is a novel finding indicating that higher calcium seems to 'equate' the amount of fat lost by taking away the effect of initial body size/composition.

4.3.1.1 Comparison of studies: weight & fat loss

Of interest between all these studies is the magnitude of weight change between the two/three treatment groups. In our study, when grouping the total weight loss over period 1 and 2 (24 weeks) and comparing the dietary treatments, the HC group achieved a 12.8 kg versus 12.6 kg loss (LC group). A similar magnitude of weight loss was seen in Harvey-Berino's study (2005), with their high dairy-calcium group losing 10 kg versus 9.3 kg for the low dairy-calcium group at 6 months. It is important to add that a greater weight loss would be expected on our study, as a greater calorie deficit was given -676 kcal/d versus -500 kcal/d. In the case of Zemel and colleagues' two studies that ran for a period of 6 months; the weight loss achieved was -6.6 kg, -8.6 kg and -11.1 kg, following the low dairy, high calcium and high dairy-calcium, respectively (Zemel *et al.* 2004b). Interestingly, in a recent multi-site trial, that replicated the protocols from the Zemel studies, except the study ran for 12 weeks and included subjects with a BMI >25. Although the dairy group achieved a two-fold greater weight loss, than the low calcium group, there was no

difference with the high calcium group (Teegarden, 2006). Similarly, in the low versus high dairy study, the weight loss was -6 (LD) versus -11 kg (HD) (Zemel *et al.* 2005a). So the weight loss achieved following the high dairy-calcium diets is very similar in all the studies that followed an identical protocol of -500 kcal/d energy deficit, or proportional to the calorie-deficit introduced (this thesis) (Zemel *et al.* 2004a; Zemel *et al.* 2004b; Harvey-Berino *et al.* 2005; Zemel *et al.* 2005a; Teegarden, 2006). It therefore appears that the ability to demonstrate differences between treatment protocols, seems to rest with the amount of weight lost on the 'low' or control arm of the design.

With regard to changes in body fat, participants in our study both lost a significant amount of body fat; however, there was no treatment effect (P=0.51). Our subjects following the high calcium diet (periods 1 & 2) lost 6.9 kg on the high-calcium and 7.0 kg on the low-calcium diet, these figures are comparable with the fat loss (7.2 kg) by the high-dairy group on Zemel's study (2004b). As with the weight loss our low-calcium group did not achieve the lower fat loss that Zemel's group reports (-4.8 kg). In Zemel *et al*. study in African-American subjects a greater fat loss of 9.1 kg was achieved, which may also reflect the fact they were heavier at baseline, with average body weights of 101.5 kg and mean body fat content of 52.8 kg (Zemel *et al.* 2005b), compared to our study body weights 89.3 kg and 33.7 kg of body fat. We do not believe we lacked the power to detect differences with the final sample size achieved. As is clear from our analyses, we can detect a difference of ~1.3 kg with ease (Table 4:12, period effect=0.00005).

4.3.1.2 Potential reasons for diverse effects

Recently it has been suggested that African-Americans have a resistance to the bone resorptive action of parathyroid hormone, which results in better urinary conservation of calcium along with a more efficient intestinal calcium absorption. However, long term this adaptation appears to put African–Americans at greater risk from several chronic diseases, including cardiovascular disease, obesity and insulin resistance syndrome. This may also explain the greater response of this group to a high calcium diet and greater weight loss in Zemel's study (Heaney, 2006). Whether

this 'ethnic' effect in weight loss with calcium is a feature of other population groups remains to be determined.

It has been suggested by some authors that there could be a threshold effect for the effects of calcium, and that calcium enhances weight and fat loss when individuals are consuming a low calcium intake (<500 mg Ca/d) (Heaney *et al.* 2002; Parikh & Yanovski, 2003; Sakhaee & Maalouf, 2005). In our study it was in fact difficult to recruit enough subjects, who habitually consumed a very low calcium intake; our group consumed a mean of 587 mg Ca/d (range 139-980 mg Ca/d). It is the authors view that some of the subjects may have over-estimated their calcium intake, in the believe that they had more chance of being selected for the study, as radio advertising had *sold* the story of a link between dairy intake and weight loss. In our study the low calcium arm of the study was provided at 634.8 (± 60.5) mg per day versus 1341 (± 72.9) mg for the high calcium diet. The low calcium arm of the study is somewhat higher than that provided in Zemel's studies and due to the explanation of a threshold effect could explain the lack of a weight loss result achieved in our study. However, Harvey-Berino et al. (2005) also did not report a weight loss difference between the high and low calcium intake at a similar calcium intake as Zemel's studies. A sub-analysis of the data was conducted with the high calcium consumers (> 600 mg/day) removed from the data set, there was still no difference in weight loss between the dietary treatments (P=0.27) (Table 4:17).

4.3.2 Could other dietary components mask calcium's effect?

One important difference between the dietary recommendations in our study is that the diets provide adequate fibre intake to meet nutrient guidelines for fibre recommendations ~28g/day, compared with Zemel *et al.* studies which gave only 8-12g/day of fibre. We chose this level of fibre intake for two reasons, 1. to ensure diets were close to recommended intakes and 2. to reduce the risk of constipation, often associated with higher calcium intakes (Arnaud & Sanchez, 1990; Soffer, 1999; Prince *et al.* 2006). Although our diets did meet fibre recommendations, a number of female subjects reported symptoms of constipation during the high calcium arm of

the study, this was remedied by ensuring adequate fluid intake and exchanging prunes within the fruit allowance. There is no mention of such adverse events in Zemel's studies, which are low in fibre intake, whilst following a high calcium intake. Fibre was once considered to reduce the bioavailability of calcium in whole-wheat bread; however, recent research suggests that purified fibres have little effect on calcium bioavailability and the fibres in low oxalate vegetables do not reduce calcium bioavailability relative to milk (Weaver & Heaney, 2006). It has been suggested that it is more likely the phytate content of fibre, rather than the fibre *per se* that can elicit a negative calcium balance. The diets provided for our study were not particularly high in phytates, regular breakfast cereals were recommended and the Health Department message of 2 fruits and 5 vegetables each day. In addition the calcium (milk and milk powder combined into yoghurt) was consumed in 3 doses distributed over the day.

Another difference between the diets provided to the subjects in this study is the addition of vitamin D to dairy products in the United States (Holick *et al.* 1992; Heaney, 2000). For example with the addition of 3 serves of dairy per day in Zemel's studies an intake of ~300 IU of vitamin D would have been consumed, compared to our non-fortified diet of ~50 IU per day (Food Standards Australia & New Zealand, 2006). Vitamin D is required for the optimal absorption of calcium and a study providing a high vitamin D intake in combination with a high calcium diet was shown to suppress PTH greater than the high calcium only diet (Pfeifer *et al.* 2001). This may explain the reason why Zemel's studies have achieved a weight change difference between the high and low calcium treatments. However, this does not explain why Harvey-Berino's group did not exhibit the same weight losses considering the protocols were very similar and presuming the high dairy diets would also be vitamin D-fortified. More recently in Major *et al*. (2007) a RCT with subjects undertaking a 15 week energy-restricted diet supplemented with 1200 mg of elemental calcium and 400 IU of vitamin D, no greater weight or fat loss was observed with the treatment compared to placebo.

4.3.3 Glucose metabolism

We reported a reduction in fasting glucose levels, fasting insulin and HOMA score on both arms of the study, as might be expected when weight loss is achieved. However, there was no significant treatment effect, as in a high calcium diet did not elicit a greater reduction in these measures compared with a low calcium intake. This demonstrates improvements in insulin resistance that have been experienced by both dietary interventions, and is the result of weight loss achieved by the subjects. This is in agreement with two other studies Thompson *et al*. (2005) study comparing a moderate dairy calcium intake (~800 mg/day) versus a high dairy calcium intake (~1200 mg/day), they found no significant weight difference between the groups and although there were significant decreases in glucose and insulin levels, there was no treatment effect (P=0.44, P=0.89, for glucose and insulin, respectively). Likewise Bowen *et al.* reported a similar weight loss for subjects following a high protein (dairy) diet versus a moderate protein (low dairy) diet, fasting insulin levels decreased by the end of a 12 week (weight loss), and 4 week (weight maintenance), but was independent of treatment group. There was also no difference in an oral glucose tolerance test between dietary groups (2005).

Of the several RCT that have been specifically designed to address the issue of dairy calcium intake and weight/fat loss, only two of the studies by Zemel's group indicated improvements in glucose function. Zemel *et al*. study (2004b), giving 3 groups a -500 kcal deficit diet with either a low dairy (LD), high calcium (HC) or a high dairy (HD) intake. Glucose tolerance was not significantly different at baseline; however, the HD group exhibited a significantly improved glucose tolerance at 24 weeks and a 27% reduced area under the curve for glucose ($P<0.01$). Fasting plasma insulin levels also showed a 44% decrease in the HD group ($P<0.01$). Neither the LD nor the HC group exhibited any difference with regards to glucose or insulin levels. In Zemel's study of African-American obese subjects, either following a weight reduction or weight maintenance diet, along with either a LD or HD diet, the weight loss arm of the study the subjects from both groups exhibited decreases in circulating insulin levels; however, the HD group had significantly greater reductions than the control ($P<0.05$). Similarly, on the weight maintenance study, the HD group had

significantly greater reductions in circulating insulin levels (P<0.05) (Zemel *et al.* 2005b). Not surprisingly, it would appear that these results were obtained due to a greater weight or fat loss from the HD groups in Zemel's studies.

4.3.4 Lipid metabolism

We demonstrated no treatment effect on fasting total cholesterol or HDL-cholesterol from the start of the study to week 12. We demonstrated a treatment effect on change in fasting LDL-cholesterol levels, with the high calcium diet eliciting a greater increase than the low calcium diet (P=0.04). One explanation could be the slightly higher (but non-significant) fasting LDL-cholesterol level with in the subjects on the low-calcium diet (HC 3.24 $\pm$ 1.03, LC 3.52 $\pm$ 0.89 mM, P=0.07). However, the final LDL-cholesterol levels were still with in normal ranges (HC 3.61 $\pm$ 0.83, LC 3.55 $\pm$ 0.84). We also demonstrated a small decrease in fasting triglyceride levels with both dietary treatments, but there was no treatment effect. These findings are consistent with recent research, based on omega-3 fatty acids, suggesting that modest decreases in triglyceride are frequently accompanied by increases in the levels of LDL cholesterol (Balk *et al.* 2006). The reasoning behind this phenomenon has been suggested as a decrease in the hepatic production of triglyceride rich particles occurs (VLDL, the lipoprotein responsible for transporting triglycerides for subsequent delipidation by lipoprotein and hepatic lipases by peripheral tissues and the liver, respectively) along with an increase in fractional clearance rates. In addition to this it has been suggested that (omega-3 fatty acids) increase the conversion rate of VLDL to LDL, similar to fibrate drugs (Despres *et al.* 2004). It is suggested that this could be occurring in this study.

Two RCT that have found significant weight loss changes between a diet high in dairy/calcium versus a control diet failed to show significant changes in blood lipids between the dietary groups (Zemel *et al.* 2004b; 2005a). Two further RCT that manipulated the calcium content of the diets, but did not report greater weight losses between a high versus a low dairy/calcium intake, were also unable to demonstrate a positive change in blood lipid levels (Bowen *et al.* 2005; Thompson *et al.* 2005).

Similarly, in a RCT in 193 male and female subjects, given a calcium supplement of 1 or 2 g of calcium per day for 4 months, no significant differences between the high calcium versus the placebo groups for total cholesterol or high-density lipoprotein-cholesterol were observed (Bostick *et al.* 2000).

4.3.5 Blood Pressure measurements

Both systolic and diastolic blood pressure exhibited a significant reduction from the start of the study; however, we did not demonstrate a treatment effect. A reduction in blood pressure would be expected with weight loss and a meta-analysis of short-term studies has indicated an expected reduction in blood pressure of 1:1, to weight loss (kg) (Neter *et al.* 2003). Our study was consistent with this finding with regards to systolic blood pressure reduction (HC weight loss 12.8 kg, SBP reduction 11.8 mmHg; LC 12.6 versus 14.0 mmHg); however, a slightly lower diastolic blood pressure reduction was shown (HC 3.7 mmHg; LC 5.6 mmHg).

Evidence that dietary calcium can have an impact on blood pressure first emerged with the publication of McCarron's paper in the early 1980s (McCarron *et al.* 1984). McCarron & Reusser (1999), in their paper "Finding consensus in the Dietary Calcium-Blood Pressure Debate" state that there is evidence from meta-analyses of 23 observational studies and 42 randomised controlled trials that identify statistically significant reductions in hypertension risk through an adequate calcium intake. Findings from these meta-analyses seem to demonstrate considerable diversity in the blood pressure responses. The variations in the results of blood pressure lowering seem also to be due to the intake of additional nutrients. In fact, in studies that used dietary sources of calcium as compared to calcium supplements, a two-fold greater effect was demonstrated on blood pressure (McCarron & Reusser, 1999). As our study used a milk-calcium powder as the high-calcium source, rather than dairy foods, this may explain why we were unable to demonstrate a significantly greater blood pressure reduction between the two diets. Zemel *et al*. initial weight loss study with subjects either on a low calcium, high calcium or high dairy diet demonstrated a

significant reduction in systolic blood pressure on only the high dairy group; however, no difference was observed with diastolic blood pressure (2004b).

It has also been suggested that calcium's effect on blood pressure could be attributable to a "threshold effect". From McCarron's paper it suggests that the threshold could be 600-700 mg calcium per day. As our low calcium diets were close to this cut off threshold, the additional calcium received by the high-calcium consumers may explain why we did not elicit a greater blood pressure response with in this group of subjects. It has also been suggested that subjects within a normal range of blood pressure may not experience greater blood pressure reductions with the addition of more dietary calcium (Barr *et al.* 2000). Our subjects were within normal blood pressure ranges (<130/85) at the start of the study.

4.3.6 Positive aspects of this study

Positives of this study is that this was the first Australian study to test the hypothesis proposed by Zemel *et al*. (2000) that a diet high in calcium would elicit a greater weight loss in adults. For our study we used a typical Australian diet, this meant the reliance on calcium-rich products that are not fortified with vitamin D, as is the case in the United States (as discussed above).

Our study was the only one (out of the studies designed to address weight loss on a high versus low calcium intake) to utilise Indirect Calorimetry methods to determine the energy requirements of the subjects. This is a more accurate method, compared to using energy equations and allows for individualised weight loss programs. In contrast other studies have used fixed energy deficits for all subjects, which would introduce a variable weight loss depending on subject's energy intake at the start. In a parallel design, if subjects are not matched at the start then a difference in weight loss would easily be assigned to a treatment effect.

Our study was also unique in that we used a cross-over design and controlled for a period, sequence and treatment effect. This type of study allowed for blinding of

subjects to which diet they were on. We were also able to demonstrate that the subjects had been well blinded, as our questions at the end of each arm of the study indicated that they rated the milk and yoghurt provided, to the same level of satisfaction. Similar studies where a parallel design was used, the subjects were either consuming dairy products or not or the report did not mention any attempt to blinding the diets (Zemel *et al.* 2004b; Thompson *et al.* 2005; Zemel *et al.* 2005a; Zemel *et al.* 2005b). Only the study by Harvey-Berino *et al.* which also found no difference between treatment groups, mentioned that the participants were blinded to the purpose of the study and the nature of the diets being studies (2005). Subjects who realise they are allocated to the "control" diet, can reduce their compliance and adherence to the study protocol.

Another strength of this study was that one investigator was responsible for all aspects of the study, which involved the recruitment of the subjects and the ongoing dietary appointments, which gave the researcher a strong rapport with all the subjects. Hence adherence to the diet and study protocol was left in the hands of an experienced dietitian. It is for this reason that we could be confident to include a group of participants as non-adherers and exclude them from the data analysis. Interestingly, only one other study (Thompson *et al.* 2005) mentions non-adherers, and this group were treated with additional appointments with the researchers, which in itself may have caused bias amongst the participants.

Finally, we have used a number of objective, as well as subjective measures to determine compliance. We charted weight loss at each time point (every 2 weeks) over the duration of the study and demonstrated a significant weight loss at each time point and across both dietary treatments. Most studies fail to mention this aspect of their study. Harvey–Berino *et al*. (2005) in their year long weight loss study briefly mentions that at 6 months the subjects on the low dairy-calcium diet have lost 9.3 kg, whereas the high-dairy consumers lost 10 kg; however, at the end of the study (another 6 months), the subjects had only lost a further 0.6 kg and 0.8 kg, respectively. Likewise, in Thompson *et al.* (2005) study, also one year in duration, states that their subjects achieved greater weight loss in the first 24 weeks of the

study; however, a graph clearly displays a plateau effect after the half-way mark. It must be suggested that this is likely due to study fatigue or a reduction in compliance to the study protocol, in addition as weight loss is achieved and metabolic rate reduces, over time a reduction in energy intake is also going to be required. Either way it is an important point to consider in study design as lack of weight loss is an often cited reason for subject drop out (Dalle Grave *et al.* 2005). It appears that not only is it difficult to get subjects to remain on weight loss studies, there have been reports of attrition rates ranging from 10-80% depending on the clinical setting and experimental design (Grossi *et al.* 2006). Considering each participant in this study underwent two weight loss periods, a loss of 27.5% (includes non-adheres) – 37.5% (excluding non-adheres) of the original sample is not unreasonable.

4.3.7 Limitations of this study

It was the intention of this study to eliminate previous biases of other parallel designed studies, such as initial baseline differences between the groups, non-blinding of the control versus the test diet. A crossover design does however have its own limitations, the main one is that of study fatigue possibly leading to poor compliance. Whilst we aimed to limit this by a long washout period (during the Christmas holiday period), our participants lost more weight during the first arm of the study.

During this study 24 hour urinary collections were used to determine nitrogen and hence protein intake. In the first period of the study there was a strong correlation between the recorded dietary protein intake and 24-hour urinary protein excretion; however, no correlation was reported between these two measures during the second period of the study. This could indicate poor compliance to the dietary intervention during this period; however, it could also be suggested that there was a poor compliance to complete 24-hour urine collections. The lack of completeness of urine collection seems more likely. Anecdotal feedback by all subjects indicated that ‘urine collections’ was the “worst” / “most difficult” task of being a subject on this study.

Future studies would certainly require the verification of completeness of urinary collections, as recommended by (Bingham, 2003).

DEXA had not been commissioned at Curtin University at the start of this study, and so we had no choice but to utilize the services of a clinical/commercial facility close to our campus. While the centre is accredited, we are unable to guarantee the control over each measurement protocol/scan analysis. It would have been more appropriate for all outcome measures to be undertaken by one investigator.

Finally, the question of a threshold effect of calcium on body composition needs to be considered. Two changes that would shed more light on a similar designed study would include, 1. Recruitment of subjects with habitually low calcium consumption and 2. Provision a really low calcium diet that has less than 500 mg calcium per day.

4.4 Summary

Based on a crossover design, we could not demonstrate an added benefit of calcium intake on weight loss or fat mass loss. However, a high calcium intake modified fat distribution by demonstrating a trend towards a greater reduction in waist circumference. It is very common for dieters, in particular women to eliminate dairy foods from their diets, believing them to be high fat foods. However, our study shows that by substituting milk power (providing ~1300 mg Ca/d) into a regular eating pattern can elicit weight loss over a 12 week period. Although this eating pattern did not cause a greater weight loss than a low calcium diet, it still demonstrates that dairy containing foods do not have to be eliminated from the diet to assist with weight loss, providing the energy intake is hypocaloric. Because calcium intake is associated with many other significant health benefits, notably the prevention of osteoporosis and hypertension (Nordin, 1997; McCarron & Reusser, 1999; Heaney, 2000; Sakhaee & Maalouf, 2005), it is suggested that public health messages should still include the promotion of dairy products for optimal health, with out the causing the fear of weight gain. However, our study suggests that a

high-calcium diet does not improve weight or fat loss beyond that achieved by a regular hypocaloric diet.

Chapter 5: Chronic effects of Dietary Calcium on postprandial energy metabolism

5.1 Introduction

Zemel's hypothesis states that an increase in calcium intake should lower the calcitrophic hormones, parathyroid (PTH) and vitamin D_3, reduce intracellular calcium and protect against fat gain (Zemel *et al.* 2000). Furthermore, high-calcium diets protect against fat gain by creating a balance of lipolysis over lipogenesis in adipocytes. This increase in lipolysis may in turn promote an increase in fat oxidation. Zemel's hypothesis is based on animal studies demonstrating a greater liver carnitine palmitoyltransferase (CPT) activity, demonstrating a greater liver fat oxidation rate, in animals fed a high dairy and/or high calcium intake (Sun & Zemel, 2004). In addition Zemel's group have also demonstrated increase in lipolysis (in the animal model), as measured by incubation of perirenal adipose tissue and the ensuing glycerol release into the culture medium (Sun & Zemel, 2004). In our acute study we demonstrated a greater fat oxidation rate following the consumption of a high dairy and a high calcium breakfast meal (chapter 3). We also demonstrated a 70% higher (relative to a low calcium intake) higher serum glycerol level (although his did not reach significance). Evidence of a significantly higher glycerol level following high calcium intakes, has also been obtained under hypocaloric weight loss conditions in humans (Zemel, 2005a; Zemel, 2005b). A greater 24 hour fat oxidation following higher calcium intake has also been demonstrated under energy restricted conditions (Melanson *et al.* 2005).

Our weight loss study (chapter 4), was hence based on the expectation that higher calcium intakes would result in greater weight/fat loss. It was important to investigate whether feeding a high calcium diet would have similar postprandial effects as was seen acutely with high calcium. To enable us to determine chronic effects of calcium, we utilized a low calcium standard meal on both arms of the study. As has been demonstrated in other (non-calcium) studies, if the test meal provided mimics the background diet given to the subject, an acute on chronic effect

is determined (Weintraub *et al.* 1988). The postprandial testing was conducted during week 10 (rather than at the end of the study at week 12), to ensure that metabolic measurements were taken during a dynamic period of weight loss rather than during a possible plateau.

The largest component of energy expenditure is, for most people, the basal metabolic rate (BMR), the BMR is closely related to the amount of non-fat tissue (or fat free mass) (Frayn, 2003). As weight loss pursues, a persons fat free mass also decreases which is one reason why a decrease in energy expenditure would be expected on a weight restricted diet. In addition to a reduction in energy expenditure, a reduction in fat oxidation has been demonstrated (Weyer *et al.* 2000). The further decline in EE can be explained by a decrease in the thermic effect of food (due to a lower energy intake), along with a reduced cost of physical activity, due to the lower body weight (Ravussin *et al.* 1985). As this study design was based on giving the subjects hypocaloric diets, both a reduction in energy expenditure and fat oxidation were expected; however, we wished to demonstrate an attenuation this lowering of energy expenditure and fat oxidation rates when subjects were fed the high calcium diet.

Many of the metabolic changes in obesity have been shown to be linked with insulin resistance. Along with an increased susceptibility to the development of type-2 diabetes; insulin resistance also has effects on lipid metabolism, in particular the metabolic picture of obesity is for a tendency to have elevated LDL-cholesterol concentration, a depression of HDL-cholesterol and an elevation of plasma triaclyglycerol concentration.

5.1.1 Study design

The study employed a single-blind, randomised crossover design comparing the effects of a high calcium diet (milk mineral powder) ~1200-1300 mg/d against a low calcium (~550-700 mg/d) hypocaloric mixed meal diet. The study involved 2 x 12 weeks of weight loss with a 12-week wash-out period (timed to coincide with the Christmas season (refer to Chapter 4). Postprandial measurements were made in the 10th week of each diet period using a low calcium, high fat test meal.

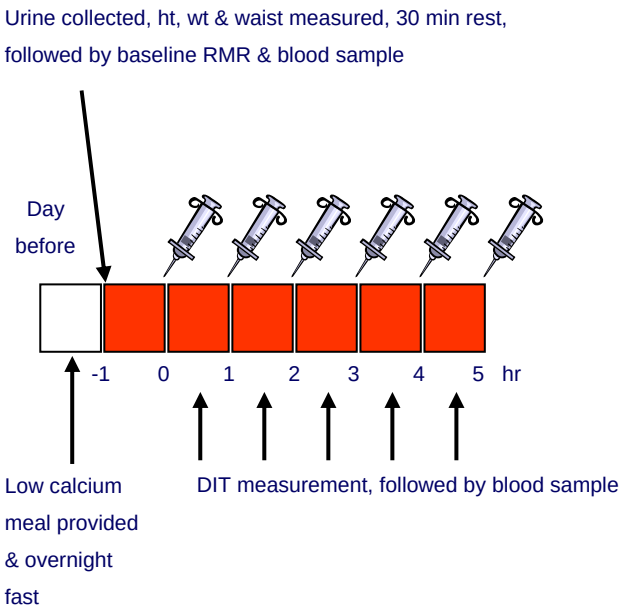
5.1.2 Subjects

Refer to chapter 4 for subject details.

5.1.3 Postprandial measurements (week 10)

Postprandial measurements were taken over a 5 hour period, following the consumption of a low calcium, high fat test meal, to determine differences in substrate oxidation rates following the high calcium versus the low calcium diets.

Figure 5:1 Postprandial measurements



5.1.3.1 Postprandial Study Design

Refer to section 2.2.4.1 for details of postprandial study design. The postprandial testing period was 5 hours in duration and hence all subjects made two separate urine collections, baseline and 0-5 hours after the meal.

5.1.3.2 Standard meal used for Postprandial Study

The low calcium, high fat breakfast meal provided consisted of 45 g cornflakes (Kellogg™, Pagewood, NSW, Australia), 20 g sultanas (Sunbeam sultanas, Sunbeam

Foods, Irymple, VIC, Australia), 35 g cream (Pura Pure cream, National Foods Ltd, VIC, Australia), 70 g full-cream milk (Brown's Dairy, Balcatta, WA, Australia) and made up to 145g with de-ionised water. This meal provided 1680 kJ of energy; 7% as protein, 35% as fat and 58% as carbohydrate. The fibre content of the meal was 2.0 g. The macronutrient content of the breakfast meals was determined using the nutrient analysis package FoodWorks (Version 3.0, 2003, Xyris software, Brisbane, Australia), incorporating nutrient tables for use in Australia (AUSNUT, Canberra, 2000) (Table 5:2).

5.1.3.3 Dietary assessment questionnaire

After the consumption of each breakfast meal, subjects were given a questionnaire to determine their assessment of the meal provided. For each question they were asked to make a mark (l or X) on a 150 mm line which extended between two extreme ranges. The length of the mark was then measured with a ruler. The questions were: 1. How would you rate the overall amount of the meal provided? 2. How would you rate the taste of the meal provided? 3. How would you rate the overall acceptability of the meal provided?

5.1.3.4 Blood samples

Fasting venous blood samples were drawn at baseline and at the end of each 30 minute RMR measurement. Blood samples for the determination of insulin, glucose and triaclyglycerol (TG) were left to stand for 30 minutes at room temperature or on ice for EDTA plasma tubes, and then centrifuged at 3000xg for 10 minutes. Methodology for assay measurements (refer to Chapter 2). A sample of plasma for the determination of intact PTH, and an anaerobically collected serum sample, for the determination of ionised calcium were couriered to an accredited pathology laboratory in Perth at the end of the test day (refer to Chapter 2 for methodology). As recommended by Heaney iPTH was expressed as percentage change from baseline (Heaney, 2003b). For consistency all blood assay results were reported in this manner.

5.1.4 Statistical analysis

All data are presented as mean values ± standard errors of the mean (unless otherwise stated). Change between fasting and fed states, was calculated by subtracting the fasting value x duration of measurement, from the total postprandial value over 5 hours. Since the intervals of measurement in the postprandial period were equal, this summary statistic was analogous to determining the incremental area under the curve (Mathews *et al.* 1990). The difference between the changes in the variables of interest, or the differences in variables at the end of each diet period, were analysed using ANOVA for the 2 x 2 crossover study design. This is similar to repeated measures, except that the treatment factor (effects of the two diets in question) was assigned across the time periods. Each subject received both treatments in a particular sequence (sequence in which the diets were administered: HC diet then LC diet = 1, LC diet then HC diet = 2). Within each subject, a period effect was also assessed. A significant sequence effect, was ignored, as recommended by (Senn, 1994), when assessing TREATMENT effects using an ANOVA for a 2 x 2 cross-over study. A paired t-test was used to compare paired data within individuals. Data were analysed using the STATA Statistical Software (Release 8.0, College Station, TX, USA) package.

Regression analysis with robust variance estimates was used to test for any treatment, sequence and period effects, after adjusting for confounding factors (STATA Reference Manual: Version 8.0, 2003; STATA Corporation).

5.1.5 Ethics

The Human Research Ethics Committee of Curtin University of Technology (HR 245-2001) approved the protocol and conformed to the Helsinki Declaration. All measurements were made in the clinical rooms of the Bentley Campus of Curtin University, Perth.

5.2 Results

5.2.1 Subject characteristics

Table 5:1 Physical characteristics of subjects

	Value
age (years)	54.04 ± 5.09 (38 - 65)
weight (kg)	85.86 ± 9.65 (65.9 - 104)
BMI (kg/m^2)	32.19 ± 3.44 (27.19 - 38.05)
waist (cm)	99.26 ± 10.22 (83.5 - 116)
% body fat	39.38 ± 6.18 (25.9 - 47.8)
fat free mass (kg)	50.96 ± 6.64 (34.98 - 68.05)

All values are mean ± SD, n=25. BMI – body mass index.

The average age of the subjects was 54.04 ± 5.09 years. The average BMI was 32.19 ± 3.44 kg/m^2.

5.2.2 Dietary intake

Table 5:2 Energy, macronutrient and calcium composition of breakfast meals provided to the subjects

	HC	LC	ANOVA 2x2 crossover design (P value)
Energy (kJ)	1678.27 ± 3.46	1675.94 ± 7.62	0.23
Protein (g)	6.68 ± 0.01	6.68 ± 0.01	0.24
Carbohydrate (g)	57.35 ± 0.20	57.32 ± 0.16	0.62
Fat (g)	15.99 ± 0.04	15.94 ± 0.18	0.21
Calcium (mg)	116.17 ± 0.19	116.14 ± 0.27	0.78

All values are mean ± SD. n=25. HC = high calcium diet, LC = low calcium diet.

The breakfast meals provided to the subjects were of the same energy, macronutrient and calcium composition between the time they were on the high calcium versus the low calcium dietary period.

5.2.2.1 Acceptability of the breakfast meals provided

The breakfast meals provided to the subjects were well accepted and there was no significant difference recorded for 1. Overall amount; HC (8.15 ± 1.91) versus LC

(9.00 ± 2.67) (ANOVA, P=0.17). 2. Overall taste; HC (11.99 ± 2.21) versus LC (11.82 ± 2.36) (P=0.77) and 3. Overall acceptability; HC (12.07 ± 2.52) versus LC 12.19 ± 2.26) (P=0.85).

5.2.3 Metabolic measurements

5.2.3.1 Energy expenditure and substrate oxidation (week 0 (fasting) to week 10 (fasting)

Table 5:3 With-in diet effects of changes in fasting substrate oxidation, Respiratory Quotient and Energy Expenditure between baseline and week 10

	HC initial	HC week 10	Diff.	LC initial	LC week 10	Diff.	Paired *t* test (P value)	ANOVA for treatment effect
RQ	0.83 ± 0.008	0.82 ± 0.008	-0.02 ± 0.01	0.83 ± 0.01	0.82 ± 0.012	-0.01 ± 0.014	0.08 0.51	0.68
FOR (g/hr)	2.32 ± 0.23	2.84 ± 0.22	0.51 ± 0.28	2.66 ± 0.24	2.69 ± 0.24	0.024 ± 0.29	0.08 0.94	0.28
COR (g/hr)	5.34 ± 0.39	4.51 ± 0.41	-0.83 ± 0.56	5.34 ± 0.45	4.61 ± 0.55	-0.72 ± 0.73	0.15 0.33	0.91
POR (g/hr)	3.44 ± 0.26	2.64 ± 0.19	-0.80 ± 0.28	3.02 ± 0.17	2.57 ± 0.16	-0.44 ± 0.18	0.008 0.02	0.24
EE (kJ/hr)	235.47 ± 6.32	224.32 ± 5.61	-11.15 ± 2.21	241.53 ± 7.90	223.53 ± 5.79	-18.00 ± 3.24	0.0005 0.0005	0.06

All values are mean ± SEM. FOR fat oxidation rate, COR carbohydrate oxidation rate, POR protein oxidation rate, EE energy expenditure, HC = high calcium, LC = low calcium.

There was a trend towards a greater decrease in respiratory quotient from baseline to week 10, when the subjects consumed the high calcium diet (P=0.08). A trend for a greater fasting fat oxidation rate between baseline to week 10 when subjects were

consuming the high calcium diet (P=0.08), but not when the subjects consumed the low calcium diet (with-in diet effect). There were significant decreases in fasting protein oxidation rates when both diets were consumed (HC, P=0.008, LC, P=0.02). No treatment effects were observed between any of these variables. Significant reductions in fasting energy expenditure were observed by subjects consuming both diets (HC, P=0.0005, LC, P=0.0005). A trend (P=0.06) was observed for a lesser reduction in fasting energy expenditure (from baseline to week 10) following the high calcium diet.

5.2.3.2 Fasting levels

Table 5:4 Fasting levels of fat, protein, carbohydrate oxidation rates, Respiratory Quotient and Energy Expenditure at week 10

Measurement	HC	LC	ANOVA 2x2 crossover design (P value)
FOR (g/h)	2.84 ± 0.22	2.69 ± 0.24	0.56
COR (g/h)	4.51 ± 0.41	4.61 ± 0.55	0.88
POR (g/h)	2.64 ± 0.19	2.57 ± 0.16	0.59
RQ	0.81 ± 0.008	0.82 ± 0.012	0.78
EE (kJ/h)	224.32 ± 5.61	223.53 ± 5.79	0.61

All values are mean ± SEM. FOR fat oxidation rate, COR carbohydrate oxidation rate, POR protein oxidation rate, RQ respiratory quotient, EE Energy Expenditure. HC = high calcium, LC = low calcium.

There were no differences between fasting levels during each dietary period for each of the metabolic measurements undertaken.

Table 5:5 The influence of diet composition (high versus low calcium) on postprandial thermogenesis and substrate oxidation following a standard meal

Measurement	Treatment		Period effect (ANOVA 2x2 crossover design) P value	Treatment effect (ANOVA 2x2 crossover design) P value
	HC	LC		
Change in RQ*	0.17 ± 0.034	0.15 ± 0.052	0.09	0.71
DIT (%)	4.72 ± 0.46	5.52 ± 0.61	0.46	0.21
Change in energy expenditure*	79.25 ± 7.63	92.43 ± 10.30	0.46	0.22
Change in fat oxidation (g/5h)*	-2.30 ± 0.77	-2.52 ± 1.25	0.21	0.91
Change in carbohydrate oxidation (g/5h)*	10.70 ± 1.66	9.87 ± 2.63	0.03	0.74

Values are mean ± SEM. n=25. HC, high calcium; LC, low calcium. DIT, diet induced thermogenesis.

*Calculated as sum of postprandial values over 5 h – (fasting value per h x 5).

Table 5:6 The influence of diet (high versus low calcium) on postprandial thermogenesis and substrate oxidation with all subjects included (n=27)

	HC	LC	Treatment effect (ANOVA 2x2 crossover design) P value
Change in fat oxidation rate (g/5h)	-2.19 ± 0.73	-2.77 ± 1.16	0.90
Change in carbohydrate (g/5h)	10.76 ± 1.55	10.33 ± 2.55	0.55
Change in energy expenditure (kJ/5h)	84.27 ± 7.91	93.58 ± 9.29	0.41

We observed no treatment effects for any of the postprandial measurements (Table 5:5). There was a significant period effect on change in carbohydrate oxidation, with period 1 having a greater oxidation rate compared to period 2 (14.04 ± 9.24, 6.54 ± 11.32 g/5h) (Table 5:5). We also observed a trend (P=0.06) for a lesser reduction in fasting energy expenditure when subjects consumed the high versus the low calcium diet (HC -11.15 ± 11.03, LC -18.00 ± 16.20 kJ/h) (Table 5:3). However, no difference was observed with RQ, between subjects consuming the high versus the low calcium diet (-0.019 ± 0.05, -0.009 ± 0.074, P=0.65). Fat and carbohydrate oxidation rates and energy expenditure were examined with 2 additional subjects who were discarded as non-adherers to the study protocol; this did not change our study outcomes (Table 5:6).

5.2.3.3 Changes in postprandial blood levels

Table 5:7 Fasting levels for triglycerides, glucose, insulin, parathyroid hormone and ionised calcium

Fasting levels	Treatment		Treatment effect (ANOVA 2x2 crossover design) P value
	HC	LC	
Triglycerides (mM)	1.66 ± 0.13	1.65 ± 0.13	0.88
Glucose (mmol/L)	5.70 ± 0.1	5.51 ± 0.12	0.44
Insulin (μIU/ml)	8.74 ± 1.13	9.55 ± 0.99	0.32
iPTH (pmol/L)	2.90 ± 0.1	3.28 ± 0.16	0.03
pH corrected ionised calcium (mmol/L)	1.19 ± 0.006	1.20 ± 0.008	0.96

Values are mean + SEM, n=25

There were no differences observed between fasting levels for triglycerides, glucose, insulin or pH corrected ionised calcium. However, iPTH demonstrated a significantly lower baseline level when the subjects commenced the high calcium diet. When the baseline iPTH was corrected for, no treatment effect on postprandial iPTH (% change from baseline) (P=0.82) was observed.

Table 5:8 The influence of dietary treatment on blood parameters over 5 hours of testing (% change from baseline)

Measurement (% change from baseline)	Treatment		Period effect (ANOVA 2x2 crossover design P value	Treatment effect (ANOVA 2x2 crossover design) P value
	HC	LC		
Triglycerides (mM)	54.98 ± 29.78	57.23 ± 16.26	0.053	0.94
Glucose (mmol/L)	28.70 ± 8.06	41.53 ± 9.97	0.38	0.29
Insulin (μIU/ml)	756.36 ± 97.12	754.45 ± 94.48	0.004	0.99
iPTH (pmol/L)	31.19 ± 12.95	12.64 ± 23.99	0.66	0.57
pH corrected ionised calcium (mmol/L)	-0.13 ± 1.59	0.38 ± 1.36	0.26	0.77

All values are mean ± SEM, n=23 (due to incomplete sampling from 2 subjects). Values are percentage change from baseline.

There was no treatment effect demonstrated for any of the blood parameters measured; glucose, insulin, triglycerides, intact parathyroid hormone nor ionised calcium, 5 hours postprandially following the consumption of a low calcium, high fat breakfast meal. We observed a period effect for percentage change in insulin, with period 1 eliciting a greater percentage change from baseline over the postprandial period, compared to period 2 (924.75 $\pm$ 427.14, 586.06 $\pm$ 446.21%). A similar observation was demonstrated in percentage change in triglycerides with period 1 (86.45 $\pm$ 106.76 %) eliciting a greater change compared to period 2 (25.76 $\pm$ 114.90 %). Both these variables were regressed using robust clusters to determine whether the greater changes in insulin and triglyceride were due to the subject's fat mass (kg) at the start of each period, this was shown to be the case for both change from baseline for insulin and triglycerides.

5.3 Discussion

An increased 24 hour RQ has been associated with high rates of weight gain (Zurlo *et al.* 1990). This inability to oxidise lipids has been implicated in the aetiology of obesity and type-2 diabetes (Kelley *et al.* 2001; Achten & Jeukendrup, 2004). It has been suggested that only relatively small differences in energy expenditure (70 cal/d or 293 kJ/d) and / or substrate oxidation could impact markedly on body weight over time (Ravussin *et al.* 1988). However, Weyer *et al.* (2000) demonstrated that responses of both 24 hour energy expenditure and fat oxidation can vary widely amongst individuals.

The study of postprandial metabolism within the design of the dietary intervention, was to understand the potential mechanisms underlying weight loss on low and high calcium intakes. Accordingly, subjects were measured during the dynamic phase of weight loss. As expected in subjects undergoing weight loss there was a significant reduction in RMR. This was true for both diets (Table 5:3). A reduction in RMR is an adaptive mechanism, aimed at lowering energy requirements so that further losses in FM and FFM are minimised. However, the RMR was less on the high calcium diet. As we did not observe any significant differences in FFM between diets (chapter 4), these data suggest that increasing calcium intake resulted in a trend (P=0.06) for the stimulation of resting energy metabolism. It was interesting that the

change in RQ (P=0.08) and FOR (P=0.08) on this diet favoured a greater fasting fat oxidation, relative to the low calcium arm of the study (Table 5:3); however, it is acknowledged that no treatment effect was observed. Overall, higher calcium intakes stimulated resting (fasting) energy expenditure even under hypocaloric conditions, and favoured the utilisation of fat as a fuel source. These data extend our previous observations of the acute effects of calcium during weight stability.

The postprandial responses to the same standard low calcium meal, were not different on the two dietary arms. There were no differences on DIT or substrate oxidation, suggesting that under conditions of calorie deficiency, calcium does not modulate postprandial metabolism. Alternatively, changes in fat oxidation and EE may have occurred earlier in the study such that by week 10, differences between diets were indistinguishable. Also DIT accounts for at most 10-15% of total energy expenditure, so that small differences in metabolism may be difficult to detect. In contrast resting metabolism accounts for 60% of total energy expenditure, making the effects of calcium more readily seen.

There were no detectable postprandial differences in other hormones and substrates between dietary arms (Table 5:8). We observed improvements in insulin sensitivity and triglyceride concentrations between period 1 and 2, but no significant treatment effects. A possible reason for the former is the greater fat mass lost during period 1 versus period 2 (chapter 4).

To the best of our knowledge, the present study is the first to investigate mechanisms for weight/fat loss following high calcium intakes. We found no effect of calcium on postprandial events when measured, towards the end of a weight loss program. In contrast, resting energy expenditure was less reduced on the high calcium arm and was accompanied by a relatively greater fat oxidation rate. These effects occurred during weight loss, a period that normally favours a reduction in RMR and an increase in fat oxidation. Calcium may hence modulate thermogenesis and substrate oxidation in man.

Chapter 6: Summary & Future Directions

6.1 Overview of Thesis

Australia like the rest of the developed world is in the grip of an epidemic of obesity. To date, the role of macronutrients and weight management have been widely studied; however, the role of micronutrients have not until recently been investigated. A large number of cross-sectional and longitudinal studies from around the world are in agreement that there is an association between calcium and/or dairy in body composition; however, these studies cannot be used to elicit a causal link. It is important to note that dairy intake is often a surrogate measure for calcium intake; because dairy calcium comprises the primary source of dairy intake in the United States and Australian diets. In addition, a low calcium intake may be associated with a less healthier lifestyle, diet or social status (Barger-Lux *et al.* 1992; Teegarden, 2005).

As the concept of calcium, and in particular dairy calcium is a fairly new area of research there have only been a limited number of well-designed randomised control trials. In chapter 1 these studies were graded according to the National Health and Medical Research Councils, levels of evidence I to V (2000). We demonstrated a lack of level I and II evidence based studies. We would also conclude from these studies that there is a lack of consistency of study endpoints. Only, one group of researchers have been able to draw consistent and significant conclusions of a role for calcium and dairy calcium in weight/fat loss (Zemel *et al.* 2004b; 2005a; 2005b), or dairy alone (Zemel *et al.* 2005a; 2005b). Other studies, which have looked at only the role of dairy calcium on weight loss, have failed to show any significant difference between the treatment groups (Harvey-Berino *et al.* 2005; Thompson *et al.* 2005). We propose that there are some flaws in the methodology of these studies which may explain an inconsistency in results. There is a lack of a true placebo-controlled experimental arm, the addition of vitamin D as well as calcium as the treatment, methodology used for determining energy deficiency (empirical versus measured) and the lack of control of confounders in the parallel-designed studies.

We therefore propose that the findings from this thesis have addressed some of the limitations currently present in the literature, and hence adds invaluable knowledge towards the presently inconclusive link between dietary calcium and its role in weight and fat loss in the overweight / obese adult population.

6.1.1 Summary of Findings

Overall, the background for this PhD research stemmed from inconsistent findings, lack of prospective RCT designed with weight loss in mind and a need to clarify potential mechanistic outcomes. As this was the first study to be conducted in Australia, we believed that the outcomes of this PhD research are particularly pertinent for the health and well-being of Australians.

This thesis focused on overweight/obese subjects with the primary aims being:-

(1) To study the acute (meal related) effects of dairy calcium and pharmaceutical calcium on postprandial thermogenesis, substrate oxidation and lipemia, along with hunger/satiety ratings and subsequent food intake.

(2) To determine the chronic (dietary) effects of dairy calcium on body composition and fat distribution following weight loss.

Our findings suggest that in an acute setting when subjects are fed a breakfast meal that is high in dairy calcium and high in calcium (pharmaceutical preparation) a greater fat oxidation is achieved over a 6 hour postprandial period (Chapter 3). This finding was accompanied by lesser suppression of NEFA, which may indicate a greater lipolysis in the postprandial period. We found no differences in the subject's feelings of hunger or satiety, nor immediate food intake (buffet), nor 24-hour food intake following the 3 test meals (Chapter 3). However, we demonstrated a trend for a greater inter-meal interval in the order of HD>HC>LD. There was also a reduction in the intake of fat between the HC and the LD meal, with food intake recorded immediately post-testing until mid-night.

In addition, when subjects were fed an energy-restricted diet, high in calcium, there was no greater weight loss or total body fat loss compared to a low calcium diet. However, a trend (P=0.052) for a greater reduction in waist circumference was observed when subjects consumed a high calcium diet (Chapter 4). Mechanistically, the greater postprandial fat oxidation seen after an acute meal, was not evident when a standard (low calcium) meal was tested on the high calcium diet. However fasting fat oxidation increased from start of the study to week 10 only on the high calcium arm of the crossover trial (Chapter 5). In the weight loss trial we have consistent effects of a good inverse correlation between weight loss and adjusted RMR indicating that a higher energy expenditure is associated with greater weight loss. However, this was true for the low calcium but not the high calcium arm of the trial (Chapter 4). This finding is of interest as it indicates that higher calcium seems to 'equate' the amount of fat lost by taking away the effect of initial body size/composition.

6.1.2 Implications of these findings

We believe we have developed a RCT that eliminates many of the pitfalls of present RCT research. In brief:-

1. Previous studies had utilised open parallel designs, which necessitates greater control of potential confounders between groups, no other study has made a good attempt to examine their data controlling for confounders. We chose to eliminate many of these issues by employing a with-in subject, cross-over designed study. Our analysis allowed us to account for a period, sequence and treatment effect.

2. Our study was the first RCT to utilise Indirect Calorimetry methods to determine BMR and hence fix the energy requirements of the subjects at the same level of intake. This is not only a more accurate method, but also allows for more control based on fat/fat free mass. This is particularly pertinent relevant to our findings discussed above (section 6.1.1).

3. Our study design being a true placebo-controlled design, allowed the elimination of bias from subjects not on the "dairy-rich" diet. Lack of blinding can result in subjects being less compliant to the study protocol.

4. This study was the first Australian study to address the issue of dietary calcium and body composition and hence the use of Australian foods meant only calcium-rich foods that were not vitamin D enriched, as in the US. We also opted for higher fibre intakes (compared to Zemel's studies - (Zemel, 2004; 2005a; 2005b) to a. be closer to Australian dietary recommendations and b. avoid constipation that can occur with high calcium intakes (Arnaud & Sanchez, 1990; Prince *et al.* 1995; Soffer, 1999; Prince *et al.* 2006).

We believe that the controlling of these above factors have lead to a tightly controlled weight loss study designed specifically to address the effect of a high versus a low calcium intake on overweight and obese subjects.

Our findings of a trend for a greater reduction in waist circumference tie in neatly with both our acute study findings of a greater fat oxidation in subjects given a high dairy and high calcium meal, along with a trend for an increase in fasting fat oxidation from the start of a weight loss study to measurements taken at week 10. The regulation of fat balance is critical to energy balance and the equilibrium of fat stores in the body (Flatt, 1995b). Fat balance is determined by the body's ability to match fat oxidation to fat intake; hence any dietary factor that can stimulate fat oxidation over a prolonged period would hence reduce fat stores, providing there was no compensatory increase in fat intake. These findings have been confirmed by our group using a sequential meal design (Soares *et al.* 2004b), along with population data (Soares *et al.* 2004a). Our results also compare favourably with cross-sectional evidence, (Melanson *et al.* 2003) and prospective data (Melanson *et al.* 2005). One long-term study has also demonstrated a greater fat oxidation rate in subjects who followed a high calcium diet for a year (Gunther *et al.* 2005b). In our acute study circulating levels of NEFA were less suppressed following both high calcium meals, which parallel the changes in fat oxidation. The findings of an increase in fat oxidation in our acute studies, supports Zemel's hypothesis of an increased lipolytic effect of calcium from cellular (Zemel *et al.* 2000; Shi *et al.* 2002) and animal

studies (Shi *et al.* 2001b; Sun & Zemel, 2004), and more recently human studies using the measurement of glycerol (Zemel *et al.* 2005a).

Visceral adipocytes are more lipolytic (Masuzaki *et al.* 2001; Rask *et al.* 2001) and the higher fasting FOX on the high calcium diet may explain the lower waist circumference we observed on the high calcium arm of the weight loss study. An attempt to verify this greater loss of visceral adiposity through a model of body composition from DEXA; however, did not prove fruitful. Additionally, we have observed that chronic increases in calcium intake may also modulate weight loss through changes to RMR. While there is sufficient evidence linking a low relative RMR to weight gain, a strong case for the opposite effect is seen only in some studies (Hansen *et al.* 2001). With a greater increase in fat oxidation demonstrated with the high calcium diets, it may be expected that this will elicit a greater increase in energy expenditure. Although there was a greater increase in energy expenditure and diet induced thermogenesis with both high calcium containing diets fed acutely, these did not reach significance (Chapter 3). This is likely due to the reciprocal decrease in carbohydrate oxidation as the fat oxidation increased (was less suppressed). As far as we are aware, our chronic study is the only one that has investigated mechanisms for weight/fat loss following high calcium intakes. We found no effect of calcium on postprandial events when measured, towards the end of a weight loss program. In contrast, resting energy expenditure was less reduced on the high calcium arm and was accompanied by a relatively greater fat oxidation rate. These effects occurred during weight loss, a period that normally favours a reduction in RMR and an increase in fat oxidation.

The intake of dietary calcium has long been associated with the prevention of osteoporosis and there are a vast array of studies and reviews linking a role for calcium and bone health (Nordin, 1997; Heaney, 2000; Cosman, 2005; Nieves, 2005). However, more recently studies have indicated that weight loss is associated with elevated calcium requirements; it has even been suggested that normal intake of calcium during energy restriction can result in inadequate calcium absorption; and compromising calcium balance and bone mass (Cifuentes *et al.* 2004). This is pertinent as it is common for dieters to restrict the intake of dairy products, believing these foods to be fattening (Radak, 2004; Dwyer *et al.* 2005). Results from our

weight loss study show that if subjects are educated to follow an energy restricted diet (as would be required to elicit weight loss), that high calcium containing foods, such as dairy products, are an acceptable alternative, as long as the intake is energy restricted and dairy snacks are replacing other snack foods. Several studies have indicated that the provision of at least 1 g / day of calcium is likely to reduce loss of bone mineral and alter makers of bone resorption (Bowen *et al.* 2004; Radak, 2004), whereas some studies have suggested a higher calcium intake may be required (Riedt *et al.* 2005). It would therefore seem prudent and ethical to ensure that at least 1 g of calcium per day is consumed, whilst undergoing energy restriction.

6.1.3 Future directions

Our acute data demonstrated a role for dairy calcium and pharmaceutical calcium given in a one off dose over the day. It could be said that 500 mg is a large dose to consume in one sitting and further studies need to be developed to elicit whether there is in fact a dose response for the intake of calcium. Some authors have suggested that there could be a threshold effect for the weight loss benefits of calcium, and that calcium only enhances body composition changes when individuals are consuming a low calcium intake (<500 mg Ca/d) (Heaney *et al.* 2002; Parikh & Yanovski, 2003; Sakhaee & Maalouf, 2005). In our weight loss study, due to the nature of our design (cross-over and single-blind) our low calcium group were given intakes of 550-700 mg of calcium per day. In Zemel's studies the LC diet was achieved by intakes of 500 mg (Zemel *et al.* 2004b). In our blinded-design this could only be achieved by specifically designed very low calcium dairy products. It must also be noted that in our weight loss study we found it difficult to recruit enough subjects, who habitually consumed a very low calcium intake (mean 587 mg, range 139-980 mg Ca/d). We tried to eliminate this possible confounder by running a sub-analysis, removing the high calcium consumers (>600 mg Ca/d) from the data set, this did not change our conclusion of no treatment effect on weight loss. Although a couple of studies have not found a difference in weight loss between a low-calcium (500-600 mg) and a high-calcium diet (Ricci *et al.* 1998; Bowen *et al.* 2005), this "threshold-effect" theory warrants further investigation.

One of the differences between Zemel's studies and our study is that dairy foods fortified with vitamin D were used, as is the practice in the US. Bell *et al*. (1985) have proposed that obesity is often associated with vitamin D deficiency, as this becomes stored in the large fat stores of overweight people and is not bioavailable (Wortsman *et al.* 2000). We endeavoured to eliminate any possible confounders of vitamin D sufficiency, but firstly ensuring our subjects were vitamin D replete, as measured by 25, dihydroxy vitamin D_3 at the start of the study. We also designed the crossover design to fall within the sunniest seasons within Australia (August to December and January to May). It is also worth mentioning that even though the issue of vitamin D is worth further investigation, it does not explain the lack of consistent results found in other US based studies (Harvey-Berino *et al.* 2005; Thompson *et al.* 2005).

As with the findings of our study and supported by several other studies (Zemel *et al.* 2004b; 2005a; 2005b) a greater reduction in waist circumference was observed. With recent evidence linking visceral adipocytes to a lesser cortisol production. Future studies need to investigate this further by the measurement of visceral fat through radiographic methods, such as computerised tomography or magnetic resonance imaging.

It is not only difficult to get subjects to remain on weight loss studies, attrition rates have been reported from 10–80% depending on the clinical setting and experimental design (Grossi *et al.* 2006); however, the other issue is compliance. Within our study we built in many measures, both subjective and objective to determine compliance and controlled for this by the elimination of non-adherers in the data set. Interesting, the RCT designed to address weight loss and dietary calcium, either make no correction for non-adherers (Zemel *et al.* 2004b; Harvey-Berino *et al.* 2005; Zemel *et al.* 2005a; Zemel *et al.* 2005b) or implement measures to enhance adherence, which may lead to bias (such as additional appointments with the dietitian) (Thompson *et al.* 2005). It is suggested that future studies should invest in doubly-labelled water techniques to ensure an independent measure of compliance.

There has been some suggestion that dietary calcium may have a greater role on adiposity, gender and ethnicity (Heaney, 2003a), it is therefore suggested that future studies are tightly controlled to ensure homogenous subject groups.

Future research into this area is worth considering the aspect of weight loss versus maintenance. When the body undergoes a period of energy deficit, it is under a considerable amount of stress. The role of calcium and its effect on weight loss have been described by some as small, in one review paper the magnitude of ~3% is suggested (Parikh & Yanovski, 2003). Therefore it is proposed that calcium's effects may be difficult to uncover in the weight loss situation. Mechanistically we were able to demonstrate a role for calcium in weight stable situation (acutely) but not chronically. Research has demonstrated a role for calcium in weight stable subjects (Zemel *et al.* 2005b), and this warrants further investigation.

Finally, calcium has been linked with eliciting a greater satiety, it has been suggested that this may be due to certain components in milk (Schneeman *et al.* 2003; Moran, 2004). In our laboratory we have demonstrated a significant reduction in food consumption of human subjects following a HD breakfast meal versus a LD meal (Ping-Delfos *et al.* 2004). In this thesis we utilised VAS questionnaires to determine the subjects' feelings of hunger and satiety before, during and after the test period; however, we observed no significant differences between the dietary treatments. We did; however, observe a trend (P=0.07) for a longer time lag before the next meal was consumed with the high dairy meal versus the low dairy meal. This aspect of satiety warrants further investigation within the area of dietary calcium and weight loss studies.

6.1.4 Conclusion

In conclusion this thesis was the first prospective study that demonstrates a greater postprandial fat oxidation rate following high calcium meals, both dairy and non-dairy. It is suggested that the lesser postprandial suppression of NEFA would drive the increase in the fat oxidation, and the trend for a greater lipolysis would serve to maintain the relatively higher NEFA levels following such meals. These acute studies did not demonstrate that calcium rich meals altered subjective feelings of

hunger or satiety, nor immediate food intake (*ad libitum* buffet). However, the inter-meal interval was prolonged relative to a low calcium diet, with some indication of a decrease in fat intake. Such data provide a mechanistic framework for understanding how the intake of calcium rich foods may influence human energy balance.

Furthermore, subjects fed high calcium, energy-restricted diets achieved the same weight and fat loss compared to subjects on a low calcium diet over a 12 week period. However, a trend for a greater reduction in waist circumference was achieved by the subjects when consuming the energy-restricted, high calcium diet and may have long-term health benefits for overweight/obese adults with the Metabolic Syndrome.

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Appendix A. Food Frequency Questionnaire

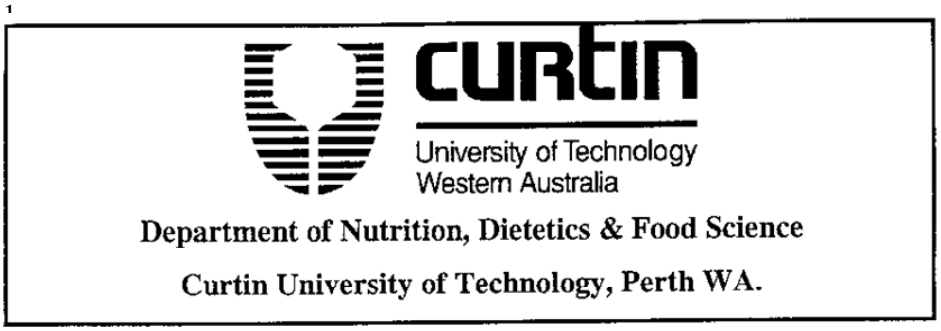
Food Type	Serving Amount	I Eat This Food: Every Week	I Eat This Food: Every Day	mg Ca / Serving	mg Ca/week (Investigator Use Only)	mg Ca/wk (Investigator Use Only)	mg Ca/day (Investigator Use Only)
Full cream milk, UHT, reconstituted full cream powder, soy milk (full cream or 'lite') and flavoured milk	1 Cup			290	0	0	0
Low fat modified milk (incl. Fitness, reconstituted skim powder, & Slim)	1 Cup			420	0	0	0
Reduced fat modified milk - Hi Lo, Hi Lo (UHT),	1 Cup			360	0	0	0
Yogurt, low fat, plain	200g carton			420	0	0	0
Yoghurt, flavoured low fat or regular, and plain regular	200g carton			340			
Hard cheese	30 g			220	0	0	0
Cheese slices	1 slice			190	0	0	0
Ricotta cheese	1 Tbs			50	0	0	0

Cottage cheese	1 Tbs			15	0	0	0
Cream cheese	1 Tbs			25	0	0	0
Ice cream and 'lite' ice cream	2 Scoops			120	0	0	0
Custard	1 Cup			260	0	0	0
Salmon canned	1/2 Cup			280	0	0	0
White fish	1 Fillet			20	0	0	0
Sardine, canned, drained	5 Whole			290	0	0	0
Cappuccino	1 Cup			190	0	0	0
Coffee, black	1 Cup			5	0	0	0
Soup, canned or packet	1 Cup			45	0	0	0
Cauliflower cheese	1 Cup			190	0	0	0
Cauliflower or broccoli, boiled	1 Piece			15	0	0	0
Carrot/pumpkin/peas/cabbage, boiled or steamed	1/2 Cup			20	0	0	0
Lettuce	2 Leaves			15	0	0	0
Tomato	1 Whole			10	0	0	0
Orange or grapefruit	1 Whole			40	0	0	0

Orange juice (100%, unsweetened)	1 Cup			20	0	0	0
Mandarin	1 Whole			15	0	0	0
Strawberry	1/2 Cup			10	0	0	0
Banana	1 Whole			10	0	0	0
Figs, dried	3 Pieces			70	0	0	0
Prunes, dried	3 Medium			10	0	0	0
Egg	1 Egg			20	0	0	0
Baked beans	1/2 Cup			60	0	0	0
Almonds	10 Nuts			40	0	0	0
Bread - wholemeal, mixed grain, white, fibre-increased white or wholemeal, rye, fruit loaf	1 Slice			15	0	0	0
Muesli, untoasted	1/2 Cup			65	0	0	0
Breakfast cereals (toasted muesli, rolled oats, all-bran)	1/2 Cup			20	0	0	0
Chocolate cake or a scone	1 Serving			40	0	0	0
Chocolate	4 Squares			50	0	0	0

Wine, red or white	1 Glass			30	0	0	0
Beer	375mls			5	0	0	0
		Total Calcium Intake/Day					

Appendix B. Study Design & Consent Form



Investigation into the effects of dairy calcium on fat & glucose utilization and blood fat patterns

Project Title: Dairy calcium and its effects on rates of whole-body nutrient utilization (HR 245/2001)

INFORMATION TO VOLUNTEERS

Thank you for agreeing to participate in the above-mentioned study. This document is to assist you to understand what is involved in this project. There is a consent form attached, which you will be asked to sign at the time of your visit. Please be sure you fully understand the nature of the study and any potential risks before signing.

BACKGROUND INFORMATION

Previous surveys have shown that many Australians are either overweight or obese, which can put them at greater risk of developing diabetes and heart disease. Both these conditions arise from the body's inability to properly handle glucose and fat that comes from the diet. There is evidence to suggest that calcium, particularly dairy calcium, has beneficial effects on the metabolism of fat and glucose. This study is the first step in determining whether dietary calcium can assist the body in handling both fat and glucose.

STUDY AIM

The aim of this study is to determine the amount of energy spent in digestion, absorption and utilization of fat and glucose when the diet is supplemented with calcium. This will enable us to calculate the amount of fat and carbohydrate (glucose) that is burnt by the body, and evaluate the beneficial effects of calcium.

STUDY PLAN/REQUIRMENTS

Visit 1 (1-2 hours only) will give you an opportunity to meet the investigators and have the study procedures and protocol fully explained to you. At this stage you will be asked to read and sign the 'Informed Consent' form. You will also complete a Food Frequency Questionnaire, which looks at your typical eating patterns.

Visit 2, 3 & 4 will be 7-hour visits to the clinical rooms of the School of Public Health, Curtin University. On each of these visits, you will be given the same type of breakfast

each meal you will also be asked to consume two (2) 500mg tablets of Paracetamol [Panadol]. This is a safe over the counter drug commonly used for relief of headache, muscular and joint pain. We use this drug to trace the absorption pattern of the meal.

At each of these visits you will be required to fast for 12-14 hours (the night before). On arrival, you will be asked to rest for half an hour before testing begins. We will place a large semi-transparent plastic hood over your head, through which you will be breathing room air whilst lying comfortably on a bed. This allows us to measure levels of oxygen and carbon dioxide. After 15 minutes, you will be given one of the special meals, the hood will be replaced and we will continue the testing for up to 6 hours following the meal. Measurements are made at hourly intervals and take approximately 30 minutes. In between measurements you can listen to music, read, chat, or just relax.

During the 7 hours of testing, we will be taking regular blood samples of 15 ml each. We will only be taking around 100 mL in total (approximately 6 tablespoons) of blood at each visit. To make the sampling more comfortable for you, a certified person (phlebotomist) will insert an indwelling catheter in your arm, similar to that of a hospital drip. We will also be collecting any urine passed during the study, for which you will be able to go to the toilet as per usual. The blood and urine samples will be used to measure such things as hormones, glucose, triglycerides etc. In the rare event of a medical emergency, fully qualified physicians & nursing staff are available.

Following the testing period it is likely that you will be quite hungry and we will provide you with a buffet meal. As this is still part of the study protocol it is important that you are able to stay for this part of the study period.

For the next 24-hours (following the test day) we require you to complete a food diary. You will be provided with a diary and instructions on how to complete the information accurately. We will provide you with a stamped addressed envelope so you can post the completed diary back to the researchers.

FURTHER INFORMATION

We assure you confidentiality of information collected as part of this study. Participation is purely voluntary and you may withdraw at any point in time.

If you have any further queries, please do not hesitate to contact:

Dr Mario Soares MBBS, Ph.D. R.Nutr OR **Ms Nikki Cummings** BSc, Grad Dip Diet
Telephone: (08)- 9266 3220 Telephone: (08) – 9266 2817

Department of Nutrition, Dietetics & Food Science, Curtin University.

This study HR 245/2001, has been approved by the Curtin University Human Research Ethics Committee. IF needed, verification of this approval can be obtained either in writing to the Curtin University Human Research Ethics Committee c/- Office of Research Development, Curtin University of Technology, GPO Box U1987, Perth 6845, or by telephoning 9266 2784.

Investigation into the effects of dairy calcium on fat & glucose utilization and blood fat patterns.

Project Title: Dairy calcium and its effects on rates of whole-body nutrient utilization (HR 245/2001)

CONSENT FORM

I,_____

Hereby consent to be a volunteer for the above-mentioned study. I understand that as part of the study, I will allow myself to be screened for suitability as a volunteer.

I have read the patient information sheet and have satisfied myself that this is a safe technique. I consent to ingesting a breakfast meal on three separate occasions. These meals may or may not be supplemented with calcium. With each breakfast meal I agree to swallow two 500 mg tablet of Panadol (Paracetamol) or equivalent. I consent to have my oxygen and carbon dioxide measured for the duration of 7 hours on each of these occasions. I also consent to having an indwelling catheter inserted into a vein in my arm/hand for the purposes of blood collection. I understand that a total of 100 ml of blood will be collected on each visit.

I fully understand all of the potential risks of these procedures as explained to me. I also understand that my participation is purely voluntary and that signing this form DOES NOT prevent me from withdrawing from the study at any time. I have been assured that confidentiality will be maintained at all stages throughout the study.

Signature:_____ Date:_____

Witness:_____ Date:_____

Appendix C. Information for Volunteers

Investigation into the effects of dairy calcium on fat & glucose utilization and blood fat patterns

INFORMATION TO VOLUNTEERS

Thank you for agreeing to participate in the above-mentioned study. To ensure the same study conditions occur before each of the three test days, please can you follow the protocol outlined below.

1. Do not undertake any strenuous exercise 24-hours before the test day.
2. Do not undertake any exercise (not even a light walk) the evening before the test-day.
3. The evening before the test-day you will be provided with a meal, please eat this no later than 7:30pm and only drink the water (deionised) provided to you.
4. After you have completed the meal, do not eat any other food (**do not consume coffee or tea** after this time). You must only consume the deionised water provded.
5. Please ensure you have a good nights rest (~ 8hours)
6. The morning of the test
    a. Do not consume any food and only drink the deionised water provide (do not drink any coffee or tea)
    b. Do not shower, but you can have a light-wash
    c. If possible have a friend or family member drive you to the centre at Curtin.
    d. If you drive yourself, you will be provided with a parking permit for the day.
    e. If are running late, please do not panic (I need you to be calm on the morning of testing).
    f. Wear light comfortable clothing
    g. It is suggested you bring a book, magazine or walk-man (if you bring a tape, I can provide you with a walk-man)

If you have any questions about this study protocol, please do not hesitate to contact Nikki Cummings 9266 2817 or 0412 705 355. Or leave a message on 9266 3220.

Appendix D. Sensory Evaluation of meal

Sensory Evaluation of meal(s)

Subject ID: _____ Date: _____

Time:_____ Study code: _____

Please mark a point on the line which best corresponds to your response to the question.

1. **How did you feel about the amount of the meal?**

Too small Too much

|_____|

2. **How did you feel about the taste of the meal?**

Very bad Very good

|_____|

3. **How did you feel about the overall acceptability of the meal?**

Un-acceptable Very acceptable

|_____|

Appendix E. Visual Analogue Scale

VAS Questionnaire

Subject ID: _____ Date: _____

Time:_____

Please mark a point on the line which best corresponds to your response to the question.

1. **How hungry do you feel?**

Not hungry at all ——— As hungry as I've ever felt

2. **How full do you feel?**

Not at all full ——— As full as I've ever felt

3. **How strong is your desire to eat?**

Very strong ——— Very weak

4. **How much do you think you could eat now?**

Nothing at all ——— A large amount

5. **Preoccupation with thoughts of food?**

No thoughts of food ——— Very difficult to concentrate on other things

Appendix F. Food Recording Booklet

Instructions for completing your 7-day food & drink diary

1. Please RECORD everything that you eat and drink for seven consecutive days.

Start a new page for each day. Record the date, the day and the time. Record each item as close to the time of eating/drinking as possible.

2. Give a FULL DESCRIPTION of your food.

We would like you to record:

- the *type* and *brand* of each item, which cut of meat is used and whether the fat has been trimmed, which brand of spread is used and which type of bread
- how the food has been prepared and which cooking method has been used (for example: boiling, frying, roasting or baking). If fat has been added, please state the brand and the amount.
- when 'take-aways' have been bought, please say where they have been bought from.

3. DESCRIBE the amount of food you eat.

We have listed on the foldout flaps some foods which are eaten regularly. Each of these food items is described as a 'medium portion'. There are pictures at the back of the diary which show what many of these 'medium portion sizes' look like.

- A serving of roast meat is described by the number of thin slices, for example a 'medium portion' is 3 thin slices
- Vegetables are described by the number of cups served, a 'medium portion' of cooked vegetables is 1/3 cup
- Potatoes are compared to the size of a hen's egg, a 'medium portion' is 2 hen's egg sized potatoes.

Following these instructions is an example page that shows you how to record food and drinks in your diary. Some foods (such as packets of crisps or chocolate bars) have a weight on the packet – just write this down.

The list of foods and the pictures of 'medium portion sizes' may not be the same amount that you eat, but they can be used to help you describe the amount you eat. We would like you to use the 'medium portion size' as a guide to describe smaller or larger portions in the following way:

- If your portion size is **similar** to that in the picture please record **medium (M)** in the *'amount eaten'* column.
- If your portion size is about **half** as much as that in the picture please record **small (S)** in the *'amount eaten'* column.
- If your portion is about **one and a half times** as much as that in the picture please record **large (L)** in the *'amount eaten'* column.
- If your portion is about **twice** as much as that in the picture please record **extra large (XL)** in the *'amount eaten'* column.

Here is an example:

You eat pasta (spaghetti) with a bolognaise sauce. *Picture 6e* shows a 'medium portion' of pasta; *picture 6d* shows a 'medium portion' of bolognaise sauce.

- If your portion size is **similar** to that in the picture, you record **medium (M)** in the *'amount eaten'* column.
- If your portion size is about **half** as much as the serving in the picture, you record **small (S)** in the *'amount eaten'* column.
- If your portion is about **one and a half times** as much as the serving in the picture, you record **large (L)** in the *'amount eaten'* column.
- If your portion is about **twice** as much as the serving in the picture, you record **extra large (XL)** in the *'amount eaten'* column.

The pictures and 'medium portion sizes' can also be used for foods not listed. For instance a medium serving of baked beans might be estimated by referring to *picture 7c* of peas. A medium serving of gateau cake can be estimated by referring to *picture 12e* of sponge cake: this is shown in the example overleaf.

If you describe your food by spoonfuls, please say what size of spoon you are using: for example, a tablespoon, a dessert spoon or a teaspoon.

4. RECORD each individual food item.

Please think of each meal in terms of individual food items and record each one separately. For example, a cheese and salad sandwich is bread, spread (eg margarine/ butter), cheese, lettuce, tomato etc (this is shown in the example overleaf).

Record all recipes: there is a *'notes'* section at the end of each page for this. Record the recipe and then tell us how much of the total is your portion (this is shown in the example overleaf).

5. EATING away from home.

When you eat away from home, it will not be possible to include recipes but we would like you to describe the food you eat in as much detail as possible.

6. Please REMEMBER:

- Record what you eat and drink as close as possible to the time that you consume it.
- At the end of the day think back – you may have forgotten to record a biscuit or a piece of cake eaten with a cup of tea; please write it all down.

Most important:

Please record everything that you eat and drink

Note: Main meals are pictured on a standard dinner plate which is 25cm in diameter (including the rim) and the side plate shown is 21cm in diameter (including the rim).

Date 22 October 1999

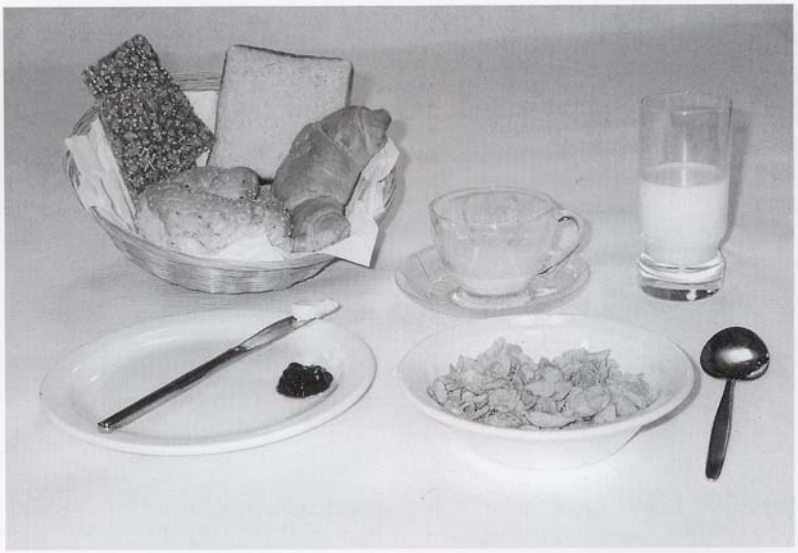
Day of week Friday

Example of how to record what you eat and drink

Time	Food and drink (include brand names, cooking method & place of purchase for foods eaten away from home)	Amount eaten (record as much detail as possible)
7.30 am	Cornflakes – Kellogg's	Picture 1a (M)
	Milk - Brownes Calcium Plus	Picture 1b (L)
	White sugar	1 teaspoon
	Toast, white - homemade	2 large thick slices pic 1d (L)
	Margarine - Flora Light	3 teaspoons
	Baked beans, tomato sauce - Heinz	Picture 7c (M)
	Tea with milk + sugar	1 mug
	Milk - Brownes Calcium Plus	Picture 1c (XL)
	Sugar	1 teaspoon
10.30am	Coffee with milk	1 cup
	skim milk - Brownes	picture 1c (M)
	Granita - Arnotts	1 biscuit
1.00pm	Bought from deli - 1 Cheese +	
	salad sandwich	
	white bread - unknown	2 standard slices - pic 4e (M)
	Butter - unknown	thickly spread - pict 4e (L)
	Cheese, grated - unknown	picture 6h (XL)
	Lettuce	Picture 7h (S)
	Tomato	3 medium slices
	Beetroot, canned	3 slices
	Apple - red delicious	large, picture 10i (L)
3.30 pm	Coffee with milk	1 cup
	skim milk - Brownes	picture 1c (M)
	Chocolate slice, from deli	2 x picture 12f (L)
6.00pm	Emu Export	1 x 375mL can
8.00pm	Spaghetti, white - Maggi	Large portion - pic 6e (L)
	Bolognaise sauce - lean mince	1/3 of recipe (see below)
	Parmesan Cheese - Kraft	(L) picture 6f
	Red wine, Shiraz	1/4 of 750mL bottle
	Salad	
	- carrots	(m) picture 7g
	- tomatoes	(S) picture 7e
	- lettuce	(S) picture 7h
	French salad dressing - Kraft free	1 tablespoon
8.30pm	Black Forest cake - from Miss Maudes	1 x picture 12e (M)

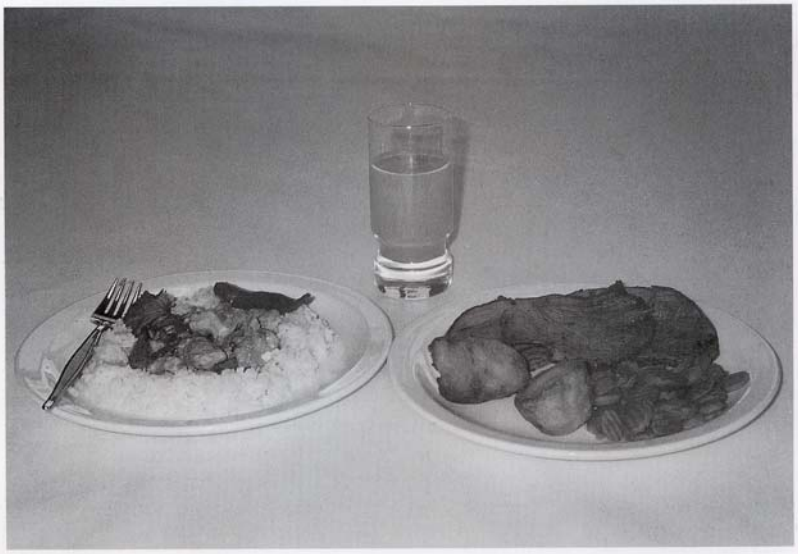
Notes/comment/recipes:

Bolognaise Sauce: 500g lean beef mince, 1 x 420g can whole tomatoes, 2 tablespoons Leggo tomato paste, 1 tablespoon canola oil, 1/2 cup dry white wine, oregano, thyme, basil, salt, pepper



1

a Cornflakes, *1 cup*
b Milk for cereal, *1/2 cup*
c Milk for tea/coffee, *25mL*
d Wholemeal bread, *1 slice*
e Bread roll, *1 medium*
f Croissant, *1 regular size*
g Ryvita, *2*
h Margarine/butter, *1 teaspoon*
i Jam, *1 heaped teaspoon*



2

a Chicken curry, *3/4 cup*
b Boiled rice, *3/4 cup*
c Roast beef, *3 thin slices*
d Roast potato, *2 halves*
e Carrots, *1/3 cup*
f Orange juice, *2/3 cup*

Appendix G. Information to volunteers – Chronic Study

INFORMATION TO VOLUNTEERS

Thank you for agreeing to participate in the above-mentioned study. This document is to assist you to understand what is involved in this project. For my study to be successful I need volunteers who are able to adhere to all parts of the study – please read this protocol carefully before you agree to take part in the study. There is a consent form attached, which you will be asked to sign at the time of your first visit. Please be sure you fully understand the nature of the study and any potential risks before signing.

BACKGROUND INFORMATION

Previous surveys have shown that many Australians are either overweight or obese, which can put them at greater risk of developing diabetes and heart disease. Both these conditions arise from the body's inability to properly handle glucose and fat that comes from the diet. There is evidence to suggest that calcium, particularly dairy calcium, has beneficial effects on the metabolism of fat and glucose and further to this may assist in weight loss.

STUDY AIM

The aim of this study is to determine if a hypocaloric diet supplemented with dairy calcium will induce a greater weight loss compared to a low calcium diet.

STUDY DESIGN

You will follow a prescribed diet for 12-weeks, followed by a second 12-week period (November 2004 – January 2005) whereby you will resume normal eating habits, with minimal intervention from the research team. The dietary intervention will then resume at the end of January / early February for a further 12-weeks. **Please be aware that you need to undertake both 12-week weight loss periods to participate in this study.**

STUDY PLAN/REQUIRMENTS

Visit 1 (1-2 hours only)

This visit will give you an opportunity to meet the investigators and have the study procedures and protocol fully explained to you. At this stage you will be asked to read and sign the 'Informed Consent' form. I will also take a blood sample at this stage and will test this for vitamin D status and thyroid function. You will also complete a food frequency questionnaire to determine your usual eating habits. At this visit I will also explain how to complete a detailed food diary and physical activity diary (which you will complete over the next week). I will provide you with a large coloured bottle, which will be used to collect a 24-hour urine sample (this will be repeated in week 6). I will also book you a medical appointment at the Health Centre (Curtin University) to ensure that there is no medical reason you may be ineligible to participate in the study.

Visit 2 (Sir Charles Gardiner Hospital)

You will be allocated a time to attend SCGH for a Dual Energy X-ray (DEXA) scan. DEXA is used to measure your body composition (amount of fat, bone and lean tissue). As the name denotes this technique utilizes a very small dose of X-rays, the dose of radiation is very small, in fact less than the amount of natural background radiation that we all receive each day. This test will be repeated in week 12 (following the dietary intervention).

Visit 3 (Curtin University, Building 400 room 101)
On this day I will be determining your resting metabolic rate (how much energy your body uses) using a method called Indirect Calorimetry. This machine is a very sensitive measure of energy usage and it is important that you attend each test day in the "same state". Therefore you will be provided with a meal that must be consumed the night before, you will then fast (not eat anything) until you arrive at Curtin early in the morning (time to be determined).

On arrival you will empty your bladder and then rest for 30 minutes. A large semi-transparent plastic hood will be placed over your head, through which you will be breathing room air whilst lying comfortably on a bed. This allows us to measure levels of oxygen and carbon dioxide. Following this testing period we will take a blood sample of 15 ml. The blood sample will be used to measure hormones, glucose, and blood fats. In the rare event of a medical emergency, fully qualified physicians & nursing staff are available close by. Please note that you will be required for ~2 hours for this test.

A week following this testing day you will commence the dietary intervention period. You will be provided with a detailed diet (listing types and amounts of foods). This diet is a hypocaloric diet (less energy than you require), and you will be provided with some dairy foods each week that you need to consume (as specified). **It is important that you only eat the types and amounts of foods as prescribed by the researcher**. During the dietary intervention period you will be requested to complete 2 food diaries and 2 activity charts. During the study it is important that you maintain your usual exercise habits (**please do not increase your physical activity**).

Visit 4,5,6,7 (week 2,4,6,8)
Each fortnight you will attend Curtin University in a fasted state (early in the morning) to have your weight measured. At this appointment you will also be asked about your progress on the diet (approximate appointment time ~ 1 hour). During your visit in week 6 I will also record your weight, hip/waist and blood pressure, and a fasting blood sample of 15ml will be taken. You will be provided with a cup of tea or coffee and a snack before you leave.

Visit 8 (week 10)
This visit will require you to attend Curtin University for a 7 hour period. The aim of this day is to determine your energy expenditure and substrate utilisation following the consumption of a test meal. You will be provided with a meal that you consume the night before and then yo will fast over-night. On arrival you will empty your bladder and then rest for 30 minutes. We will then measure your levels of oxygen and carbon dioxide consumption. A 15 ml blood sample will be taken at this time. You will then be given a test meal and the above procedure will be repeated every hour. A total of 90ml (~4 ½ tablespoons) of blood will be taken over the day. We will also be collecting any urine passed during the study, for which you will be able to go to the toilet as per usual.

Visit 9 (week 12)
Repeat of DEXA at SCGH, followed by final weight, waist, and hip and fasting blood sample taken at Curtin University.

Following this initial 12-week period you have a 12-week period (approx Nov 2004 – Jan 2005) whereby the dietary rules will be relaxed and there will be less contact with the researchers. The second dietary intervention (commencing end-January-February 2005) will follow the same protocol (as outlined above).

FURTHER INFORMATION

To be included in the study it is essential that you remain in the study for the two diet periods (12-week diet period followed by a 12-week period, and then a final 12-week dietary intervention period). This study is due to commence July/August 2004, with a 12-week break over the Christmas period. The second dietary period (12-weeks) commences at the end of January/February and will finish April /May 2005.

In summary

To participate in this study you need to be able to:

- ✓ Attend Curtin University for a medical examination, initial appointment with the researchers, initial resting metabolic rate test (2 hours), body fat test (Victoria Park)
- ✓ Be available to attend fortnightly meetings (~ 1 hour) with the researchers (at Curtin University)
- ✓ Attend Curtin University in week 10 for a 7-hour test day (blood collection will be required)
- ✓ Maintain your present activity levels (do not increase or decrease) during the study period
- ✓ Strictly follow the diet plan and consume dairy products provided by the researchers

As a participant on this study you will be provided with the following benefits:

- ☺ A nutritionally-balanced personal weight loss program that has been designed by an Accredited Practicing Dietitian (APD)
- ☺ Motivation ! If you have previously tried to lose weight without success, this program will provide you with ongoing support (fortnightly visits with two Accredited Practicing Dietitians, alternated with a fortnightly phone call)
- ☺ On completion of the study you will be provided with a complete medical & health profile
  - blood test results will include; total cholesterol, HDL and LDL, triglycerides, glucose, insulin, free fatty acids, apo B48.
  - additional your blood will be analysed for specific hormones associated with fat and calcium metabolism (vitamin D status, ionised calcium, parathyroid hormone, leptin, glucagon-like-peptide)
  - you will also receive your body composition results (weight, waist, hip) and body fat levels (including fat mass, and fat free-mass as provided by Dual Energy X-ray Absorptiometry).
- ☺ You will also receive a complete nutrition & metabolic-testing profile outlining energy expenditure, fat and carbohydrate oxidation (usage), and further advice on appropriate food choices – this information can be used to help you achieve further weight loss or weight maintenance once the study is complete.
- ☺ The opportunity to lose weight safely & effectively under the guidance of a medically qualified doctor and Accredited Practicing Dietitians.

** Unfortunately medical, health and nutritional profiles will not be available to subjects not completing the whole study.*

We assure you confidentiality of information collected as part of this study. Participation is purely voluntary and you may withdraw at any point in time.

If you have any further queries, please do not hesitate to contact:

Ms Nikki Cummings BSc, Grad Dip Diet RNutr
9266 2817 or 0412 705 355

Dr Mario Soares MBBS, PhD,
9266 3220

curtin
University of Technology
Western Australia
Department of Nutrition, Dietetics & Food Science
Curtin University of Technology, Perth WA.

Project Title: The Potential Role for Dairy Calcium & Weight Loss
CONSENT FORM

I,_____

Hereby consent to be a volunteer for the above-mentioned study. I understand that as part of the study, I will allow myself to be screened for suitability as a volunteer.

I have read the patient information sheet and have satisfied myself that this is a safe technique.

I consent to the following measures:

1. To attend for a medical examination, including an Electrocardiogram
2. I agree to have a blood sample taken a part of the screening process to check for vitamin D and thyroid function.
3. To have my oxygen and carbon dioxide measured using the Indirect Calorimetry method
4. To undergo blood collection on 4 separate visits. On visit 1,6 and 9 only 15ml of blood with be collected. During visit 7, 90 ml (4 ½ tablespoons) of blood will be taken.
5. To have a total body scan by DEXA; I understand that this procedure involves a very small amount of radiation. This test will be undertaken 12 weeks apart (on 4 occasions).
6. To collect a 24-hour urine sample on 4 separate days.

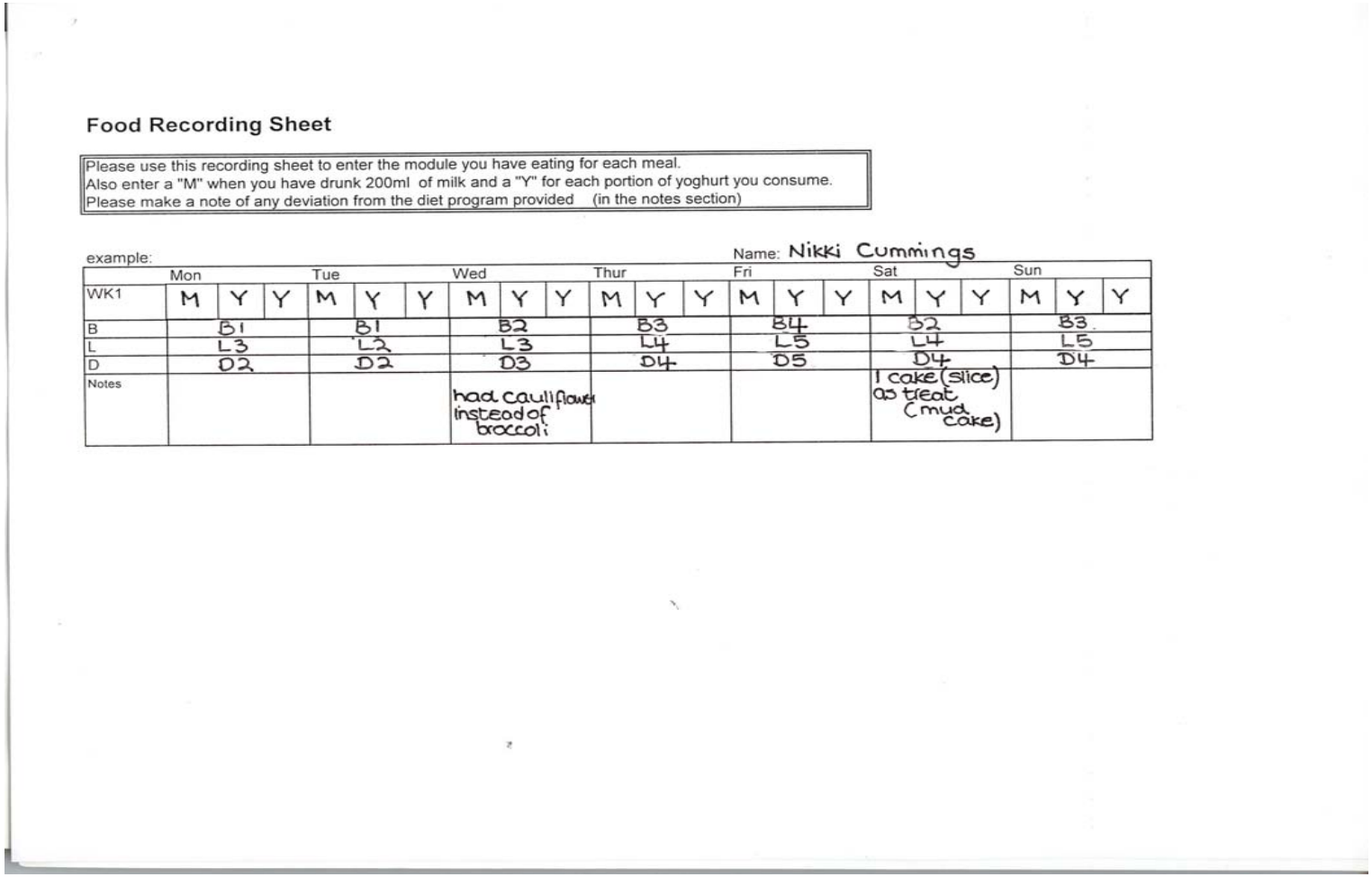
I consent to the following procedures:

1. I agree to complete a 3-day food diary and 3-day activity chart on 6 occasions.
2. I agree to follow the dietary protocol as administered by the chief researcher
3. I agree to consume the dairy products provided by the researchers.
4. I am able to attend all appointments as outlined in the protocol attached.

I fully understand all of the potential risks of these procedures as explained to me. I also understand that my participation is purely voluntary and that signing this form DOES NOT prevent me from withdrawing from the study at any time. I have been assured that confidentiality will be maintained at all stages throughout the study.

Signature:_____ Date:_____

Appendix H. Food intake recording sheets (sample and blank)



Food Recording Sheet

Please use this recording sheet to enter the module you have eating for each meal.
Also enter a "M" when you have drunk 200ml of milk and a "Y" for each portion of yoghurt you consume.
Please make a note of any deviation from the diet program provided (in the notes section)

example: Name: Nikki Cummings

	Mon			Tue			Wed			Thur			Fri			Sat			Sun		
WK1	M	Y	Y	M	Y	Y	M	Y	Y	M	Y	Y	M	Y	Y	M	Y	Y	M	Y	Y
B	B1			B1			B2			B3			B4			B2			B3		
L	L3			L2			L3			L4			L5			L4			L5		
D	D2			D2			D3			D4			D5			D4			D4		
Notes							had cauliflower instead of broccoli									1 cake (slice) as treat (mud cake)					

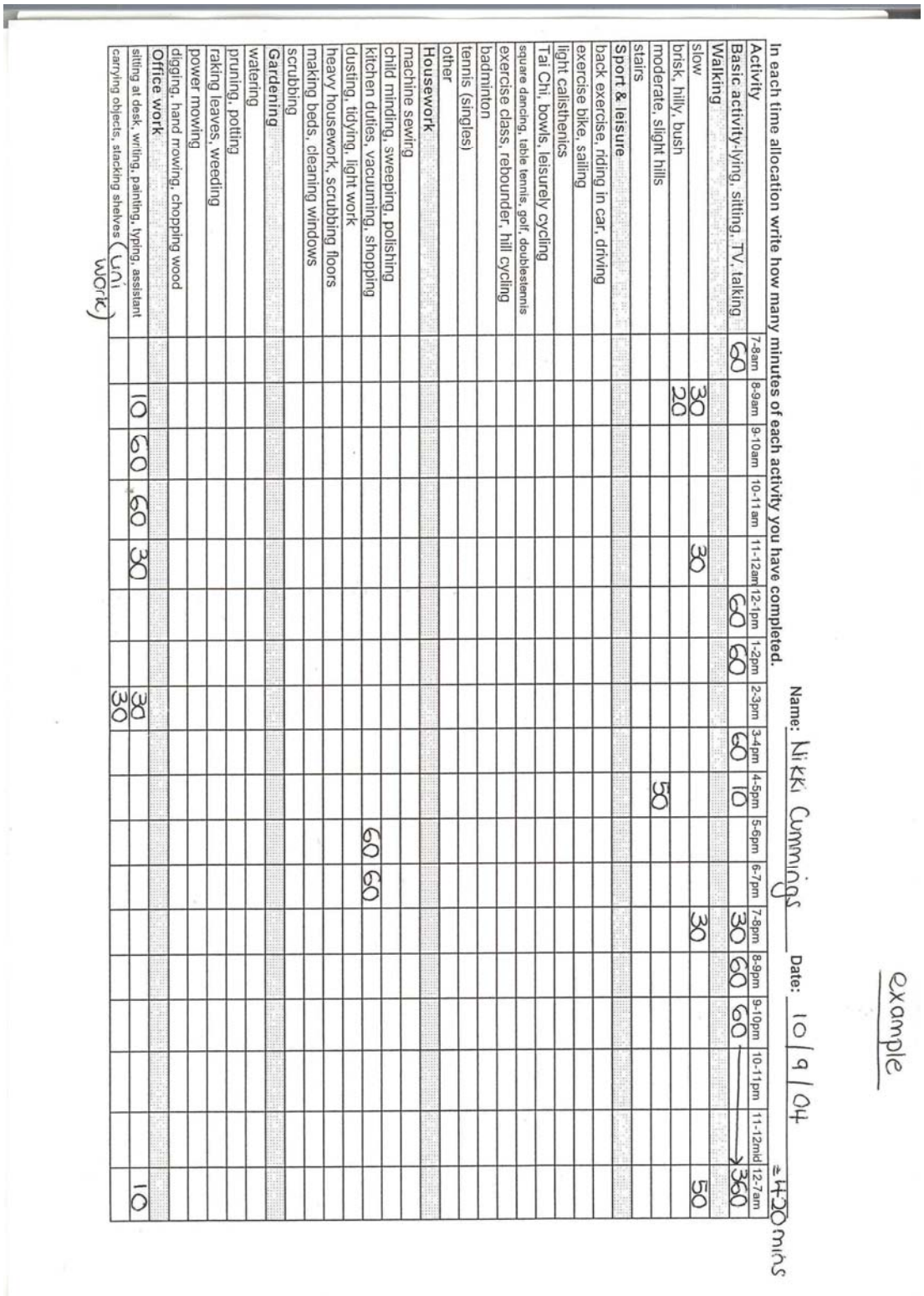
Name:

	Mon	Tue	Wed	Thur	Fri	Sat	Sun
WK1							
B							
L							
D							
Notes							

	Mon	Tue	Wed	Thur	Fri	Sat	Sun
WK2							
B							
L							
D							
Notes							

	Mon	Tue	Wed	Thur	Fri	Sat	Sun
WK3							
B							
L							
D							
Notes							

Appendix I. Example of completed activity recording sheet



example

Name: Nikki Cummings Date: 10/9/04 ≈ 420 mins

In each time allocation write how many minutes of each activity you have completed.

Activity	7-8am	8-9am	9-10am	10-11am	11-12am	12-1pm	1-2pm	2-3pm	3-4pm	4-5pm	5-6pm	6-7pm	7-8pm	8-9pm	9-10pm	10-11pm	11-12mid	12-7am
Basic activity-lying, sitting, TV, talking	60					60	60		60	10			30	60	60	→	→	360
Walking																		
slow		30			30								30					50
brisk, hilly, bush		20																
moderate, slight hills										50								
stairs																		
Sport & leisure																		
back exercise, riding in car, driving																		
exercise bike, sailing																		
light calisthenics																		
Tai Chi, bowls, leisurely cycling																		
square dancing, table tennis, golf, doubles tennis																		
exercise class, rebounder, hill cycling																		
badminton																		
tennis (singles)																		
other																		
Housework																		
machine sewing																		
child minding, sweeping, polishing											60	60						
kitchen duties, vacuuming, shopping																		
dusting, tidying, light work																		
heavy housework, scrubbing floors																		
making beds, cleaning windows																		
scrubbing																		
Gardening																		
watering																		
pruning, potting																		
raking leaves, weeding																		
power mowing																		
digging, hand mowing, chopping wood																		
Office work		10	60	60	30			30										
sitting at desk, writing, painting, typing, assistant								30										
carrying objects, stacking shelves (uni work)																		10

Appendix J: Correlations between weight loss and confounders (SPSS for Windows (Version 11, SPSS Inc. USA) statistical software package

Correlations

Correlations

		WEIGHT	WAIST	TOTFAT	TOT_FFM	TRK_FFM	TRK_FM
WEIGHT	Pearson Correlation	1	.474**	.537**	.607**	.477**	.745**
	Sig. (2-tailed)	.	.001	.000	.000	.000	.000
	N	50	48	50	50	50	50
WAIST	Pearson Correlation	.474**	1	.333*	.479**	.329*	.420**
	Sig. (2-tailed)	.001	.	.021	.001	.022	.003
	N	48	48	48	48	48	48
TOTFAT	Pearson Correlation	.537**	.333*	1	-.165	-.177	.891**
	Sig. (2-tailed)	.000	.021	.	.253	.219	.000
	N	50	48	50	50	50	50
TOT_FFM	Pearson Correlation	.607**	.479**	-.165	1	.832**	.087
	Sig. (2-tailed)	.000	.001	.253	.	.000	.548
	N	50	48	50	50	50	50
TRK_FFM	Pearson Correlation	.477**	.329*	-.177	.832**	1	.053
	Sig. (2-tailed)	.000	.022	.219	.000	.	.717
	N	50	48	50	50	50	50
TRK_FM	Pearson Correlation	.745**	.420**	.891**	.087	.053	1
	Sig. (2-tailed)	.000	.003	.000	.548	.717	.
	N	50	48	50	50	50	50
ROI_FFM	Pearson Correlation	.217	.048	.152	.196	.258	.155
	Sig. (2-tailed)	.130	.745	.292	.172	.070	.282
	N	50	48	50	50	50	50
ROI_FM	Pearson Correlation	.389**	.276	.613**	-.045	-.043	.563**
	Sig. (2-tailed)	.005	.058	.000	.759	.768	.000
	N	50	48	50	50	50	50
WT_START	Pearson Correlation	-.512**	-.208	.163	-.512**	-.502**	-.119
	Sig. (2-tailed)	.000	.156	.259	.000	.000	.409
	N	50	48	50	50	50	50
FOX_bline	Pearson Correlation	-.266	-.034	.152	-.287*	-.155	.018
	Sig. (2-tailed)	.062	.817	.291	.044	.281	.903
	N	50	48	50	50	50	50
RMR	Pearson Correlation	-.603**	-.443**	-.144	-.586**	-.546**	-.328*
	Sig. (2-tailed)	.000	.002	.320	.000	.000	.020
	N	50	48	50	50	50	50
pth_start	Pearson Correlation	-.050	.046	-.004	.001	-.026	-.021
	Sig. (2-tailed)	.735	.757	.976	.994	.862	.889
	N	49	47	49	49	49	49
FT3_start	Pearson Correlation	-.217	.089	-.391**	.126	.189	-.342*
	Sig. (2-tailed)	.135	.551	.005	.389	.194	.016
	N	49	47	49	49	49	49
HOMA_start	Pearson Correlation	-.185	.223	.026	-.029	-.165	-.043
	Sig. (2-tailed)	.198	.127	.860	.840	.253	.764
	N	50	48	50	50	50	50
trigs_start	Pearson Correlation	.065	.022	.179	-.133	-.277	.130
	Sig. (2-tailed)	.655	.882	.214	.355	.052	.367
	N	50	48	50	50	50	50

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Correlations

		ROI_FFM	ROI_FM	WT_START	FOX_bline	RMR
WEIGHT	Pearson Correlation	.217	.389**	-.512**	-.266	-.603**
	Sig. (2-tailed)	.130	.005	.000	.062	.000
	N	50	50	50	50	50
WAIST	Pearson Correlation	.048	.276	-.208	-.034	-.443**
	Sig. (2-tailed)	.745	.058	.156	.817	.002
	N	48	48	48	48	48
TOTFAT	Pearson Correlation	.152	.613**	.163	.152	-.144
	Sig. (2-tailed)	.292	.000	.259	.291	.320
	N	50	50	50	50	50
TOT_FFM	Pearson Correlation	.196	-.045	-.512**	-.287*	-.586**
	Sig. (2-tailed)	.172	.759	.000	.044	.000
	N	50	50	50	50	50
TRK_FFM	Pearson Correlation	.258	-.043	-.502**	-.155	-.546**
	Sig. (2-tailed)	.070	.768	.000	.281	.000
	N	50	50	50	50	50
TRK_FM	Pearson Correlation	.155	.563**	-.119	.018	-.328*
	Sig. (2-tailed)	.282	.000	.409	.903	.020
	N	50	50	50	50	50
ROI_FFM	Pearson Correlation	1	.643**	-.030	-.043	-.160
	Sig. (2-tailed)	.	.000	.836	.766	.266
	N	50	50	50	50	50
ROI_FM	Pearson Correlation	.643**	1	-.026	-.019	-.250
	Sig. (2-tailed)	.000	.	.860	.898	.080
	N	50	50	50	50	50
WT_START	Pearson Correlation	-.030	-.026	1	.553**	.783**
	Sig. (2-tailed)	.836	.860	.	.000	.000
	N	50	50	50	50	50
FOX_bline	Pearson Correlation	-.043	-.019	.553**	1	.559**
	Sig. (2-tailed)	.766	.898	.000	.	.000
	N	50	50	50	50	50
RMR	Pearson Correlation	-.160	-.250	.783**	.559**	1
	Sig. (2-tailed)	.266	.080	.000	.000	.
	N	50	50	50	50	50
pth_start	Pearson Correlation	-.222	-.128	.047	-.036	.060
	Sig. (2-tailed)	.126	.382	.746	.807	.680
	N	49	49	49	49	49
FT3_start	Pearson Correlation	-.166	-.221	-.068	.004	.063
	Sig. (2-tailed)	.256	.128	.645	.979	.668
	N	49	49	49	49	49
HOMA_start	Pearson Correlation	-.344*	-.084	.395**	.147	.275
	Sig. (2-tailed)	.014	.561	.004	.307	.053
	N	50	50	50	50	50
trigs_start	Pearson Correlation	-.152	-.025	.343*	.191	.427**
	Sig. (2-tailed)	.291	.862	.015	.183	.002
	N	50	50	50	50	50

Correlations

		pth_start	FT3_start	HOMA_start	trigs_start
WEIGHT	Pearson Correlation	-.050	-.217	-.185	.065
	Sig. (2-tailed)	.735	.135	.198	.655
	N	49	49	50	50
WAIST	Pearson Correlation	.046	.089	.223	.022
	Sig. (2-tailed)	.757	.551	.127	.882
	N	47	47	48	48
TOTFAT	Pearson Correlation	-.004	-.391**	.026	.179
	Sig. (2-tailed)	.976	.005	.860	.214
	N	49	49	50	50
TOT_FFM	Pearson Correlation	.001	.126	-.029	-.133
	Sig. (2-tailed)	.994	.389	.840	.355
	N	49	49	50	50
TRK_FFM	Pearson Correlation	-.026	.189	-.165	-.277
	Sig. (2-tailed)	.862	.194	.253	.052
	N	49	49	50	50
TRK_FM	Pearson Correlation	-.021	-.342*	-.043	.130
	Sig. (2-tailed)	.889	.016	.764	.367
	N	49	49	50	50
ROI_FFM	Pearson Correlation	-.222	-.166	-.344*	-.152
	Sig. (2-tailed)	.126	.256	.014	.291
	N	49	49	50	50
ROI_FM	Pearson Correlation	-.128	-.221	-.084	-.025
	Sig. (2-tailed)	.382	.128	.561	.862
	N	49	49	50	50
WT_START	Pearson Correlation	.047	-.068	.395**	.343*
	Sig. (2-tailed)	.746	.645	.004	.015
	N	49	49	50	50
FOX_bline	Pearson Correlation	-.036	.004	.147	.191
	Sig. (2-tailed)	.807	.979	.307	.183
	N	49	49	50	50
RMR	Pearson Correlation	.060	.063	.275	.427**
	Sig. (2-tailed)	.680	.668	.053	.002
	N	49	49	50	50
pth_start	Pearson Correlation	1	.050	.338*	-.013
	Sig. (2-tailed)	.	.731	.017	.929
	N	49	49	49	49
FT3_start	Pearson Correlation	.050	1	.151	-.165
	Sig. (2-tailed)	.731	.	.302	.257
	N	49	49	49	49
HOMA_start	Pearson Correlation	.338*	.151	1	.438**
	Sig. (2-tailed)	.017	.302	.	.001
	N	49	49	50	50
trigs_start	Pearson Correlation	-.013	-.165	.438**	1
	Sig. (2-tailed)	.929	.257	.001	.
	N	49	49	50	50

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

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