EFFECTS OF DAIRY PROTEINS AND CALCIUM ON DIET-INDUCED OBESITY IN MICE

TARU PILVI

Institute of Biomedicine, Pharmacology
University of Helsinki

Foundation for Nutrition Research
Helsinki, Finland

ACADEMIC DISSERTATION

To be presented by kind permission of the Medical Faculty of the University of Helsinki for public examination in Lecture Hall 2, Biomedicum Helsinki, Haartmaninkatu 8, on October 31st, 2008, at 12 noon.

Helsinki 2008
SUPERVISORS
Professor Eero Mervaala, MD, PhD
Institute of Biomedicine, Pharmacology
University of Helsinki
Helsinki, Finland

Professor Riitta Korpela, PhD
Institute of Biomedicine
University of Helsinki
Helsinki, Finland

REVIEWERS
Docent Kirsi Pietiläinen, MD, PhD
Obesity Research Unit
Helsinki University Central Hospital
Helsinki, Finland

Professor Ilkka Pörsti, MD, PhD
Medical School
Internal Medicine
University of Tampere
Tampere, Finland

OPPONENT
Professor Hannu Mykkänen, PhD
Department of Clinical Nutrition,
Food and Health Research Centre
University of Kuopio
Kuopio Finland

GRAPHIC DESIGN AND LAYOUT
Pinja Meretoja

ISBN 978-952-10-5020-6 (PDF)
http://ethesis.helsinki.fi

Helsinki University Print
Helsinki 2008
“One day is all we need to change the way we see”
**TABLE OF CONTENTS**

Main abbreviations .............................................................. 9

Abstract ...................................................................................... 10

1 Introduction ............................................................................ 13

2 Review of the literature .......................................................... 17
   2.1 Dairy proteins and calcium .............................................. 17
       2.1.1 Dairy proteins ..................................................... 17
       2.1.2 Calcium ........................................................... 20
   2.2 Obesity and dairy products .............................................. 21
       2.2.1 Obesity ............................................................ 21
       2.2.2 Obesity and the intake of dairy products: Epidemiological studies . 23
       2.2.3 Obesity and the intake of dairy products: Randomised clinical trials . 30
       2.2.4 Obesity and the intake of dairy products: Experimental studies . 31
   2.3 Dairy obesity and components .......................................... 33
       2.3.1 Calcium ........................................................... 33
       2.3.2 Dairy proteins .................................................... 38
       2.3.3 Other dairy components ....................................... 38
   2.4 Possible mechanisms of action ........................................ 40
       2.4.1 Calcium and fat absorption ................................... 40
       2.4.2 Calcium, 1,25(OH)₂-vitamin D₃ and intracellular calcium ....... 41
       2.4.3 Calcium and fat oxidation ................................... 44
2.4.4  Dairy proteins and satiety ........................................ 45
2.4.5  Dairy proteins and insulin action .............................. 46

3  Aims of the study............................................................ 49

4  Materials and methods................................................... 51

  4.1  Study design ........................................................... 51

  4.2  Materials ............................................................. 52

  4.2.1  Experimental animals ......................................... 52

  4.2.2  Diets and groups ............................................... 52

  4.3  Methods .............................................................. 54

  4.3.1  Body weight and body fat measurements ...................... 54

  4.3.2  Collection and analysis of faecal samples (Studies I, II and IV) . 54

  4.3.3  Calorimetry and metabolic performance (Study IV) ........... 54

  4.3.4  Collection of the tissue samples .............................. 55

  4.4  Biochemical determinations ...................................... 55

  4.4.1  Blood glucose, serum insulin and serum lipids (Studies I, II and IV) . 55

  4.4.2  Serum 1,25(OH)₂D₃ and PTH (Study I) ....................... 56

  4.5  Histology, immunohistochemistry and adipocyte cross-sectional area . 56

  4.5.1  Histological analysis (Study IV) .............................. 56

  4.5.2  Immunohistochemical staining of F4/80 (Study III) .......... 56

  4.5.3  Adipocyte cross-sectional area (Studies II and III) .......... 57

  4.6  Microarray procedure and gene expression analysis (Study III) .... 57

  4.6.1  Microarray procedure ........................................... 57

  4.6.2  Data processing ............................................... 57

  4.6.3  Gene expression analysis ..................................... 58

  4.7  Liver metabolomic profile (Study IV) ............................ 58

  4.8  Statistical analysis ............................................... 58
5 Results .......................................................... 61
  5.1 Body weight .................................................. 61
    5.1.1 Weight gain (Study I) ................................ 61
    5.1.2 Weight loss (Study II, IV and unpublished data) ............ 61
    5.1.3 Weight re-gain (Study II) ............................. 62
  5.2 Amount of adipose tissue and adipocyte size .................... 62
    5.2.1 Weight gain (Study I and III) ......................... 62
    5.2.2 Weight loss (Study II and IV) ........................... 63
    5.2.3 Weight re-gain (Study II) ............................. 65
  5.3 Energy intake and metabolic performance ........................ 65
  5.4 Fat absorption ............................................. 66
  5.5 Blood glucose and serum insulin (Studies I, II and IV) ......... 66
  5.6 Adipose tissue gene expression (Study III) .................... 68
  5.7 Liver (Study IV). .......................................... 69
    5.7.1 Liver histology ..................................... 69
    5.7.2 Liver metabolomic profile ............................. 69
  6 Discussion .................................................... 75
    6.1 Methodological aspects ................................... 75
    6.2 Effect of dairy proteins and calcium intake on body weight and
        the amount of adipose tissue ............................. 78
    6.3 Effect of dairy proteins and calcium on adipose tissue and the liver .... 81
    6.4 Clinical relevance ...................................... 84
  7 Summary and conclusions .................................... 87
Acknowledgements .............................................. 89
References ..................................................... 93
Original publications ........................................ 113
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications (Studies I-IV) and some unpublished data.


II Pilvi TK, Harala S, Korpela R, Mervaala EM. Effects of high calcium diet with different whey proteins on weight loss and weight re-gain in high-fat fed C57Bl/6J mice. Br J Nutr In Revision.


The original publications are reprinted with the kind permission of the copyright holders.
## MAIN ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CLA</td>
<td>Conjugated Linoleic Acid</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-Energy X-ray Absorptiometry</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>HPLC/MS/MS</td>
<td>High Performance Liquid Chromatography Tandem</td>
</tr>
<tr>
<td></td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MIAME</td>
<td>Minimum Information About a Microarray Experiment</td>
</tr>
<tr>
<td>UPLC/MS</td>
<td>Ultra Performance liquid Chromatography coupled to</td>
</tr>
<tr>
<td></td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>$1,25(OH)_2D_3$</td>
<td>1,25-dihydroxy-vitamin-D$_3$</td>
</tr>
</tbody>
</table>
ABSTRACT

Diet high in dairy products is inversely associated with body mass index, risk of metabolic syndrome and prevalence of type 2 diabetes in several populations. Also a number of intervention studies support the role of increased dairy intake in the prevention and treatment of obesity. Dairy calcium has been suggested to account for the effect of dairy on body weight, but it has been repeatedly shown that the effect of dairy is superior to the effect of supplemental calcium. Dairy proteins are postulated to either enhance the effect of calcium or have an independent effect on body weight, but studies in the area are scarce. The aim of this study was to evaluate the potential of dairy proteins and calcium in the prevention and treatment of diet-induced obesity in C57Bl/6J mice. The effect of dairy proteins and calcium on the liver and adipose tissue was also investigated in order to characterise the potential mechanisms explaining the reduction of risk for metabolic syndrome and type 2 diabetes.

A high-calcium diet (1.8%) in combination with dietary whey protein inhibited body weight and fat gain and accelerated body weight and fat loss in high-fat-fed C57Bl/6J mice during long-term studies of 14 to 21 weeks. α-lactalbumin, one of the major whey proteins, was the most effective whey protein fraction showing significantly accelerated weight and fat loss during energy restriction and reduced the amount of visceral fat gain during ad libitum feeding after weight loss. The microarray data suggest sensitisation of insulin signalling in the adipose tissue as a result of a calcium-rich whey protein diet. Lipidomic analysis revealed that weight loss on whey protein-based high-calcium diet was characterised by significant decreases in diabetogenic diacylglycerols and lipotoxic ceramide species.

The calcium supplementation led to a small, but statistically significant decrease in fat absorption independent of the protein source of the diet. This augments, but does not fully explain the effects of the studied diets on body weight. A whey protein-containing high-calcium diet had a protective effect against a high-fat diet-induced decline of D3-adrenergic receptor expression in adipose tissue. In addition, a high-calcium diet with whey protein increased the adipose tissue leptin expression which is
decreased in this obesity-prone mouse strain. These changes are likely to contribute to the inhibition of weight gain. The potential sensitisation of insulin signalling in adipose tissue together with the less lipotoxic and diabeticogenic hepatic lipid profile suggest a novel mechanistic link to explain why increased dairy intake is associated with a lower prevalence of metabolic syndrome and type 2 diabetes in epidemiological studies.

Taken together, the intake of a high-calcium diet with dairy proteins has a body weight-lowering effect in high-fat-fed C57Bl/6J mice. High-calcium diets containing whey protein prevent weight gain and enhance weight loss, α-lactalbumin being the most effective whey protein fraction. Whey proteins and calcium have also beneficial effects on hepatic lipid profile and adipose tissue gene expression, which suggest a novel mechanistic link to explain the epidemiological findings on dairy intake and metabolic syndrome. The clinical relevance of these findings and the precise mechanisms of action remain an intriguing field of future research.
1 INTRODUCTION

Obesity is a major risk factor for severe chronic disease, such as type 2 diabetes, metabolic syndrome, other cardiovascular disorders and certain types of cancers (for review, see Haslam and James 2005). The worldwide prevalence of obesity is increasing both in adults and in children, and the significant burden of obesity-related complications is expected to grow. Investing resources in the prevention and treatment of obesity are thus valuable ways to reduce the overall risk of cardiovascular morbidity and mortality.

Nutrition has a crucial role both in the prevention and treatment of obesity. Epidemiological studies suggest that milk consumption and a diet high in dairy products are one of the few known dietary components which are inversely related to body mass index (BMI) (Mirmiran et al. 2005, Marques-Vidal et al. 2006, Vareenna et al. 2007). Epidemiological evidence also indicates that the intake of dairy products is related to a lower risk of type 2 diabetes and metabolic syndrome (Pereira et al. 2002, Liu et al. 2005, Liu et al. 2006). Dairy calcium has been suggested to account for the body weight-regulating effects of dairy products, and some intervention studies have indeed shown dairy products and calcium to accelerate weight loss during energy restriction (Zemel et al. 2004, Zemel et al. 2005a, Zemel et al. 2005b). The results are nevertheless conflicting (Harvey-Berino et al. 2005, Thompson et al. 2005, Wagner et al. 2007). Interestingly, both clinical and experimental intervention studies indicate that the effect of dairy-derived calcium is superior to that of calcium supplementation (Shi et al. 2001a, Sun and Zemel 2004a, Zemel et al. 2004).

Dairy proteins, especially whey proteins, have been suggested to play a role in the anti-obesity effect of dairy products (for review, see Zemel 2005). However, the mechanism of action by which dairy products affect
body weight is still unknown. Calcium binds fatty acids in the gastrointestinal tract and thereby decreases the absorption of dietary fat (Jacobsen et al. 2005, Lorenzen et al. 2007). Animal and in vitro studies also suggest an alternative mechanism whereby dietary calcium may control the adipocyte size via 1,25-dihydroxy-vitamin D$_3$ (1,25(OH)$_2$D$_3$) and the concentration of intracellular calcium (for review, see Zemel and Miller 2004, Zemel 2005). However, the effect of dairy proteins on obesity and the consequent health risks have not been evaluated on a mechanistic level.

The link between obesity and other key elements of the metabolic syndrome has been under intensive investigation in recent years. According to current understanding, the inflammatory status of adipose tissue is an important factor contributing to the overall cardiometabolic risk associated with obesity (for review, see Hotamisligil 2006). Also the development of fatty liver is a crucial phenomenon which links obesity to insulin resistance and type 2 diabetes (for review, see Kotronen and Yki-Järvinen 2007).

The purpose of the present study was to investigate the potential of dairy protein and calcium intake in the prevention and treatment of diet-induced obesity in C57Bl/6J mice, a well-established model of obesity. Another aim was to evaluate the effect of dairy protein and calcium intake on the liver and adipose tissue in order to characterise the potential mechanisms explaining the reduction of risk for metabolic syndrome and type 2 diabetes.
2 REVIEW OF THE LITERATURE

2.1 DAIRY PROTEINS AND CALCIUM

Dairy products are a fundamental part of a healthy diet. This is elucidated by the current Finnish dietary recommendation, which suggests that dairy products should be consumed every day in the form of low-fat liquid dairy products, yoghurt or cheese (National Nutrition Council, 2005). Dairy products are an essential source of protein, vitamins and minerals and, according to FINDIET2002 report, the majority of calcium, phosphorus, iodine, riboflavin and vitamin B₁₂ intake in the Finnish diet is covered by dairy products (Ovaskainen et al. 2003). In addition to proteins, minerals and vitamins, milk contains lactose and fat (Fox and McSweeney 1998).

2.1.1 DAIRY PROTEINS

On average, bovine milk contains 3.5% of protein (Fox and McSweeney 1998). The protein is divided into casein and whey fractions which separate when the pH of milk is lowered to 4.6. The acidification induces coagulation of casein, whereas whey proteins remain soluble. The relative amount of whey proteins and casein in milk varies during lactation and between species. On average, the whey to casein ratio in bovine milk is 20:80, whereas in human milk the ratio is 60:40. Interestingly, the whey to casein ratio in human milk changes during different phases of lactation (Kunz and Lønnerdal 1990, 1992). The amount of whey proteins in relation to casein decreases from about 90:10 in early lactation to 60:40 in mature milk and 50:50 in late lactation. In addition, the relative proportion of the different casein subunits (Kunz and Lønnerdal 1990) as well as whey proteins (Sanchez-Pozo et al. 1986) has been found to vary throughout lactation.
Since the first report of the American Dairy Science Association Committee on the Nomenclature, Classification, and Methodology of Milk Proteins (Jenness et al. 1956), the classification of dairy proteins has been updated every 5 to 10 years (Farrell et al. 2004). The committee reports the most significant findings related to nomenclature and methodology of dairy proteins and suggests changes in nomenclature where appropriate. This shows that the field of dairy proteins is constantly evolving along with the technological and methodological development of protein research.

**CASEINS**

Caseins are defined as those phosphoproteins that precipitate from raw skim milk by acidification to pH 4.6 at 20°C (Farrell et al. 2004). Caseins are further divided into different subclasses: $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$- and $\kappa$-casein, according to the homology of their amino acid sequences. The different subclasses represent approximately 37% ($\alpha_{s1}$-), 10% ($\alpha_{s2}$-), 35% ($\beta$-) and 12% ($\kappa$-) of total casein (Fox and McSweeney 1998). The caseins are synthesised and secreted by mammary epithelial cells as large aggregates called micelles (Bouguyon et al. 2006), which bind minerals, such as calcium, inorganic phosphate and magnesium (for review, see Gaucheron 2005). Caseins and casein-derived peptides are suggested to have various physiological functions ranging from antibacterial and immunomodulatory activities (for review, see Meisel 2005) to their capacity to enhance the bioavailability of minerals (for review, see Vegard et al. 2000, Bouhallab and Bougle 2004). Even though most of the suggested properties of casein-derived peptides are based on experimental and in vitro studies, their antihypertensive effect has been demonstrated in clinical studies as well (Seppo et al. 2003, Tuomilehto et al. 2004, Jauhiainen et al. 2005).

**WHEY PROTEINS**

Whey is the fluid that remains after caseins are removed from milk (for review, see Krissansen 2007). Thus, whey contains also minerals, vitamins, lactose and traces of fat in addition to proteins. The whey proteins are a mixture of different proteins and peptides, some of which are derived from casein during the cheese-making process and end up in whey.
Therefore, the composition of total whey protein varies according to the manufacturing process and there are several types of whey protein products on the market: sweet and acid whey powders, reduced lactose whey, demineralised whey, whey protein concentrates with different protein contents and whey protein isolate, which has the highest protein concentration of these products (US Dairy Export Council 2004). Traditionally, β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins, and proteose-peptone fractions have been considered the major characterised components of whey proteins (Farrell et al. 2004). Whey also contains lactoferrin, lactoperoxidase, insulin-like growth factor and other minor proteins (for review, see Yalcin 2006). β-lactoglobulin is the major whey protein accounting for approximately half of the total whey protein in bovine milk while α-lactalbumin accounts for roughly 25%.

In vitro studies suggest that whey proteins have various physiological functions from immunomodulatory and anti-inflammatory effects (for review, see Krissansen 2007) to anticancer activity (Hakkak et al. 2000, Dave et al. 2006). The importance of whey proteins has also been under investigation in the infant formula industry, since the amount of whey protein is substantially greater in human milk than in bovine milk (for review, see Lien 2003).

**BIOACTIVE PEPTIDES**

Bioactive peptides are formed from dietary proteins either in the gastrointestinal tract during digestion or during the food manufacturing process by, for example, fermentation (for review, see Meisel 2005). In the gastrointestinal tract, the proteins are subjected to hydrolysis by a variety of proteases and peptidases which are either secreted from the stomach and pancreas or bound to the brush border membrane of enterocytes (for review, see Daniel 2004). The protein hydrolysis generates a spectrum of short- and medium-size peptides, as well as free amino acids. Most proteins and oligopeptides are rapidly degraded. However, the extent and the rate by which dietary proteins are broken down are dependent on the proteins’ amino acid sequence and post-translational modifications, such as glycosylation, which render peptides that are more resistant to hydrolysis. Certain whey proteins, such as lactoferrin, have been demonstrated to be absorbed intact, at least in mice (Fischer et al. 2007). α-lactalbumin and
ß-lactoglobulin are also partly resistant to in vitro digestion with human gastric and duodenal juice (Almaas et al. 2006). Even though a wide range of potential health effects of dairy protein-derived peptides are suggested by in vitro data (for review, see Meisel 2005, Severin and Wenshui 2005), the exact spectrum of peptides formed in vivo after the ingestion of dairy products remains unresolved.

2.1.2 CALCIUM

Dairy products account for about two thirds of the calcium intake in the Finnish diet (Ovaskainen et al. 2003). Besides the amount of calcium in the diet, the absorption is a critical factor in determining the bioavailability of dietary calcium. In milk, the majority of calcium is bound to casein micelles, a small proportion is bound to α-lactalbumin and a part of the calcium is in the aqueous phase of milk (for review, see Gaucheron 2005). It has been suggested that certain dairy components, such as casein phosphopeptides, may positively influence the absorption of calcium (for review, see Bouhallab and Bougle 2004).

Calcium is absorbed in the intestine by two distinct mechanisms (Bronner et al. 1986). An active transcellular mechanism is regulated by 1,25-di-hydroxyvitamin D (1,25(OH)₂D₃) and is predominant in the duodenum and upper jejunum. The 1,25(OH)₂D₃ is formed in the kidneys from 25-hydroxyvitamin D by a renal 1α-hydroxylase (for review, see Dusso et al. 2005). The synthesis of active vitamin D is regulated by various factors including PTH and serum phosphate and calcium levels, but new regulators, such as fibroblast growth factor member 23 and klotho, play a role as well (Renkema et al. 2008). The 1,25(OH)₂D₃ stimulates epithelial calcium transport by enhancing calcium entry into the cell, facilitating thus calcium movement across the cell and activating the basolateral plasma membrane ATPase that pumps calcium from the cell (for review, see Hoenderop et al. 2000). This active absorption mechanism is predominant when dietary calcium intake is low. A passive paracellular absorption process is based on concentration-dependent diffusion and takes place throughout the length of the intestine especially during high dietary calcium intake.

The importance of calcium intake for bone health is widely recognised (Cashman 2002). Sufficient calcium intake is also considered to be an
important part of the dietary treatment of hypertension (Champagne 2006). The role of dietary calcium in the regulation of body weight is a relatively new area of research. The first observational finding on the inverse relationship between calcium intake and body weight in humans was published in 1984 (McCarron et al. 1984), and the next report on the subject appeared only 8 years ago (Zemel et al. 2000).

2.2 OBESITY AND DAIRY PRODUCTS

2.2.1 OBESITY

Obesity is a major risk factor for a number of chronic diseases, including diabetes, cardiovascular diseases and cancer (for review, see Haslam and James 2005). Obesity is estimated to decrease the life expectancy by 7 years at the age of 40 years (Peeters et al. 2003) and according to the World Health Organisation (WHO), obesity is already responsible for 2–8% of health care costs and 10–13% of deaths in different parts of Europe (2008). The current estimates suggest that around 2.3 billion adults will be overweight and more than 700 million obese by 2015.

According to WHO, overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (2008). The classification of obesity and overweight is commonly based on a simple weight-for-height index, the body mass index (BMI). BMI is defined as the weight in kilograms divided by the square of the height in meters (kg/m²). BMI provides the most useful population-level measure of overweight and obesity. It is the same for both sexes and can be used for all adult age groups. BMI correlates well with the amount of fat tissue in the body and also with the increased health risks of obesity (Revicki and Israel 1986, Calle et al. 1999). WHO divides the definition of obesity into subclasses which correspond to the Finnish Current care guidelines (2006). The subclasses are presented in Table 1.
Overweight results from continuous excess energy intake in relation to energy expenditure (for review, see Flier 2004). Therefore, food and nutrition have a crucial role in the development of obesity. If the energy content of food is excluded, only few dietary factors have been identified to be related to lower BMI. These factors include increased intake of fibre and whole grains (for review, see Flight and Clifton 2006), high intake of fruit and vegetables (for review, see Tohill et al. 2004) and high intake of dairy products (Table 2). On average, dairy products account for 15% (men) to 16% (women) of daily energy intake in the Finnish diet (Ovaskainen et al. 2003).

Obesity and particularly abdominal obesity, is a well-established risk factor for cardiovascular diseases, type 2 diabetes and metabolic syndrome (for review, see Kahn et al. 2006), and the primary goal for obesity treatment is to prevent and treat these chronic diseases. Metabolic syndrome is a cluster of cardiovascular risk factors including abdominal obesity, elevated plasma glucose, dyslipidemia, hypertension and prothrombotic/proinflammatory state (Grundy 2008). Depending on the diagnostic criteria, at worst, every fifth middle-aged man and every sixth woman in Finland can be diagnosed as having metabolic syndrome (Hu et al. 2004). At present, patients with metabolic syndrome are often treated with multiple drugs. Multiple drugs are needed to target the different comorbidities of metabolic syndrome, because none of the approved drugs on the market can reliably reduce all of the metabolic risk factors in the long term.


<table>
<thead>
<tr>
<th>CLASSIFICATION</th>
<th>CURRENT CARE GUIDELINES</th>
<th>WHO DEFINITION</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORMAL RANGE</td>
<td>18.50 - 24.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OVERWEIGHT</td>
<td></td>
<td></td>
<td>≥ 25.00</td>
</tr>
<tr>
<td>SLIGHTLY OBESE</td>
<td></td>
<td>PRE-OBESE</td>
<td>25.00 - 29.99</td>
</tr>
<tr>
<td>OBESE</td>
<td></td>
<td></td>
<td>≥ 30.00</td>
</tr>
<tr>
<td>SIGNIFICANTLY OBESE</td>
<td>OBESE CLASS I</td>
<td></td>
<td>30.00 - 34.99</td>
</tr>
<tr>
<td>SEVERELY OBESE</td>
<td>OBESE CLASS II</td>
<td></td>
<td>35.00 - 39.99</td>
</tr>
<tr>
<td>MORBIDLY OBESE</td>
<td>OBESE CLASS III</td>
<td></td>
<td>≥ 40.00</td>
</tr>
</tbody>
</table>
Thus, there is growing interest in therapeutic strategies that might target multiple factors more efficiently, thereby minimising the problems of polypharmacy. Life-style therapies are currently the recommended first-line treatment for metabolic syndrome and the nutritional approaches are especially crucial in the preventive management of this growing epidemic.

2.2.2 OBESITY AND THE INTAKE OF DAIRY PRODUCTS: EPIDEMIOLOGICAL STUDIES

The intake of dairy products correlates negatively with BMI or body weight in several, but not all, of the studied populations (Table 1). The largest (n=37,513) of the population studies is based on the Portuguese National Health Interview survey (Marques-Vidal et al. 2006) which showed that BMI increased with decreased milk intake. In addition to the Portuguese population, the inverse association of dairy product intake and body weight has been demonstrated in Italian postmenopausal women (Varema et al. 2007), in Canadian (Drapeau et al. 2004) and Iranian adults (Mirmiran et al. 2005, Azadbakht and Esmailzadeh 2007). No relationship was detected in a Japanese study on 1,905 female dietetic students (Murakami et al. 2006). The subjects in that study were within a very narrow BMI range, 78% having BMI of 18.5 to 24.9 kg/m² and only 6% having BMI ≥ 25 kg/m². Also the intake of dairy products was low even in the highest quartile of dairy intake, where it was comparable with the lowest quartile of US (Rajpathak et al. 2006) or European studies (Snijder et al. 2007).

Interestingly, some of the studies have found an inverse association between BMI and high-, but not low-fat, dairy products (Rajpathak et al. 2006, Rosell et al. 2006, Snijder et al. 2007). Unfortunately, the type of dairy product has not been reported in all of the studies. This is a critical confounding factor which is likely to explain the inconsistency between different studies. In addition to the dairy product type, the assortment and the traditional use of dairy products vary greatly between different populations. For example, the intake of chocolate milk and non-flavoured milk were treated as comparable in the analysis of a longitudinal study of 9- to 14-year-old adolescents (Berkey et al. 2005) and in the Bogalusa Heart Study (Brooks et al. 2006). An estimate of dairy intake from foods, such
as pizza, nachos, lasagne and puddings, was also included in the analysis of the Bogalusa Heart Study. On the other hand, some studies have only recorded the fluid milk intake (Marques-Vidal et al. 2006) which complicates the comparison of the epidemiological data.

A recent review of dietary patterns and the risk of metabolic syndrome concluded that even though there is some inconsistency in the literature regarding the risk of obesity, high dairy intake is generally associated with a reduced risk for components of the metabolic syndrome (Baxter et al. 2006). Indeed, the epidemiological data on dairy product intake and the risk of metabolic syndrome are more consistent along with the data on high dairy intake and lower risk of type 2 diabetes (Table 2).

Taken together, there is growing line of epidemiological evidence of the protective effect of dairy product intake against overweight and obesity. The inverse relationship has been demonstrated in different countries and age groups as well as in both sexes. In addition to obesity, dairy product intake is associated with a reduced risk of metabolic syndrome and type 2 diabetes in several epidemiological datasets.
### Table 2. Epidemiological Studies on Dairy Intake and BMI and/or Body Weight. Studies are Listed According to the Size of the Study Population.

<table>
<thead>
<tr>
<th>Country (Database)</th>
<th>Population</th>
<th>Design</th>
<th>Main Result</th>
<th>Inverse Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Portugal (National Health Interview Survey 1998-1999)</td>
<td>37,513 (17,771 men and 19,742 women, over 18 y of age)</td>
<td>Cross-sectional</td>
<td>Milk intake decreased with increasing BMI.</td>
<td>Yes</td>
<td>(Marques-Vidal et al., 2006)</td>
</tr>
<tr>
<td>Japan</td>
<td>1,905 female Japanese dietetic students (18-20 y)</td>
<td>Cross-sectional</td>
<td>Intakes of calcium and dairy products were not significantly associated with BMI.</td>
<td>No</td>
<td>(Murakami et al., 2006)</td>
</tr>
<tr>
<td>The Netherlands (The Hoorn Study)</td>
<td>1,896 (852 men, 1044 women, aged 50-75 y)</td>
<td>Cross-sectional</td>
<td>Higher dairy consumption was not associated with lower weight. High-fat dairy showed inverse associations between BMI and waist circumference, and low-fat dairy was positively associated with BMI and waist circumference.</td>
<td>No</td>
<td>(Snijder et al., 2007)</td>
</tr>
<tr>
<td>Italy</td>
<td>1,771 post-menopausal women</td>
<td>Cross-sectional, retrospective, observational</td>
<td>BMI and prevalence of overweight showed significant inverse trends with increasing dairy intake.</td>
<td>Yes</td>
<td>(Varenna et al., 2007)</td>
</tr>
<tr>
<td>USA (Bogalusa Heart Study)</td>
<td>1,306 (505 men, 801 women, 952 whites/354 blacks, 19-38 y)</td>
<td>Cross-sectional</td>
<td>No significant association between dairy product consumption, calcium intake and BMI or waist circumference. A significant inverse association between calcium intake, low-fat dairy product consumption and waist-to-hip ratio in white males.</td>
<td>Yes/No</td>
<td>(Brooks et al., 2006)</td>
</tr>
<tr>
<td>Iran</td>
<td>926 women (aged 40-60 y)</td>
<td>Cross-sectional</td>
<td>Dairy consumption was inversely correlated with central fat accumulation.</td>
<td>Yes</td>
<td>(Azadbakht and Esmailzadeh, 2007)</td>
</tr>
<tr>
<td>Iran (Tehran Lipid and Glucose Study)</td>
<td>462 (223 men, 239 women, over 16 y of age)</td>
<td>Cross-sectional</td>
<td>A significant inverse correlation between the servings of dairy consumption per day and BMI.</td>
<td>Yes</td>
<td>(Miriran et al., 2005)</td>
</tr>
<tr>
<td>COUNTRY (DATABASE)</td>
<td>POPULATION</td>
<td>DESIGN</td>
<td>MAIN RESULT</td>
<td>INVERSE ASSOCIATION</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>--------------------</td>
<td>------------</td>
<td>--------</td>
<td>-------------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>USA (Health Professionals Follow-up Study)</td>
<td>19,615 men (40-75 y at baseline)</td>
<td>Longitudinal, 12-year follow-up</td>
<td>The men with the largest increase in total dairy intake gained slightly more weight than did the men who decreased intake the most. This association was primarily due to an increase in high-fat dairy intake. Low-fat dairy intake was not significantly associated with weight change.</td>
<td>No</td>
<td>Rajpathak et al., 2006</td>
</tr>
<tr>
<td>Sweden (Swedish Mammography Cohort)</td>
<td>19,352 women (40-55 y at baseline)</td>
<td>Longitudinal, 9-year follow-up</td>
<td>The constant (≥ 1 serving/d) intakes of whole milk, sour milk and cheese were inversely associated with weight gain during the 9-year follow-up.</td>
<td>Yes</td>
<td>Rosell et al., 2006</td>
</tr>
<tr>
<td>USA (Baltimore Longitudinal Study of Aging)</td>
<td>459 (219 women, 240 men, 40-75 y)</td>
<td>Longitudinal, 7-year follow-up</td>
<td>Consuming a diet high in fruit, vegetables, reduced-fat dairy, and whole grains and low in red and processed meat, fast food, and soda was associated with smaller gains in BMI and waist circumference.</td>
<td>Yes</td>
<td>Newby et al., 2003</td>
</tr>
<tr>
<td>Canada (Quebeck Family Study)</td>
<td>248 (116 women, 112 men, 18-65 y)</td>
<td>Cross-sectional, longitudinal, 6-year follow-up</td>
<td>Skimmed milk and partly skimmed milk correlated negatively with the changes of body weight, percentage body fat, subcutaneous skinfold thicknesses, and waist circumference.</td>
<td>Yes</td>
<td>Drapeau et al., 2004</td>
</tr>
<tr>
<td>COUNTRY (DATABASE)</td>
<td>POPULATION</td>
<td>DESIGN</td>
<td>MAIN RESULT</td>
<td>INVERSE ASSOCIATION</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------------------------------------------------</td>
<td>---------------------------------</td>
<td>------------------------------------------------------------------------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>USA</td>
<td>12,829 children (9-14 y at baseline)</td>
<td>Longitudinal, 3-year follow-up</td>
<td>Children who drank the most milk gained more weight, but the added calories appeared responsible.</td>
<td>NO</td>
<td>(Berkey et al., 2005)</td>
</tr>
<tr>
<td>USA</td>
<td>342 non-obese children (4-10 y at baseline)</td>
<td>Cross-sectional, longitudinal, 1-year follow-up</td>
<td>An inverse relation between the intake of dairy foods and measures of obesity at baseline and over 1 year in the 7- to 10-year-old normocholesterolemic children. Calcium or dairy intake was not associated with measures of obesity in hypercholesterolemic children or in the 4- to 6-year-old normocholesterolemic children.</td>
<td>YES/NO</td>
<td>(Dixon et al., 2005)</td>
</tr>
<tr>
<td>USA (Framingham Children’s Study)</td>
<td>92 children (56 boys, 36 girls), 3-6 y at baseline</td>
<td>Longitudinal, 7-year follow-up</td>
<td>Children in the lowest sex-specific tertile of dairy intake during preschool had significantly greater gains in body fat during childhood.</td>
<td>YES</td>
<td>(Moore et al., 2006)</td>
</tr>
<tr>
<td>USA</td>
<td>53 children (29 boys, 24 girls, 2 y at baseline)</td>
<td>Longitudinal, 2-to-96-month follow-up</td>
<td>Higher mean longitudinal calcium (mg/day) intakes and more servings/day of dairy products were associated with lower body fat.</td>
<td>YES</td>
<td>(Carruth and Skinner, 2001)</td>
</tr>
</tbody>
</table>
TABLE 3. EPIDEMIOLOGICAL STUDIES ON DAIRY INTAKE AND THE METABOLIC SYNDROME, TYPE 2 DIABETES AND OTHER DISORDERS OF GLUCOSE AND INSULIN METABOLISM. STUDIES ARE LISTED ACCORDING TO THE SIZE OF THE STUDY POPULATION.

<table>
<thead>
<tr>
<th>COUNTRY (DATABASE)</th>
<th>POPULATION</th>
<th>DESIGN</th>
<th>MAIN RESULT</th>
<th>INVERSE ASSOCIATION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA (WOMEN’S HEALTH STUDY)</td>
<td>10 066 (45 Y)</td>
<td>CROSS-SECTIONAL</td>
<td>LOWER PREVALENCE OF METABOLIC SYNDROME WAS ASSOCIATED WITH TOTAL DAIRY PRODUCTS, LOW-FAT DAIRY PRODUCTS, AND TOTAL MILK INTAKE.</td>
<td>NO</td>
<td>(LIU ET AL., 2005)</td>
</tr>
<tr>
<td>FRANCE (DESR STUDY)</td>
<td>4 976 (2537 WOMEN AND 2439 MEN)</td>
<td>CROSS-SECTIONAL</td>
<td>DAIRY INTAKE WAS INVERSESLY RELATED TO THE FREQUENCY OF THE METABOLIC SYNDROME IN MEN, BUT NOT IN WOMEN. MEN WHO ATE MORE THAN 1 PORTION OF DAIRY PRODUCTS PER DAY HAD AT LEAST A 40% LOWER PREVALENCE OF THE METABOLIC SYNDROME.</td>
<td>YES</td>
<td>(MENNEN ET AL., 2000)</td>
</tr>
<tr>
<td>USA (CARDAI- STUDY)</td>
<td>3 157 BLACK AND WHITE ADULTS (18-30 Y)</td>
<td>PROSPECTIVE, 15-YEAR FOLLOW-UP</td>
<td>AMONG OVERWEIGHT INDIVIDUALS THE RISK OF DEVELOPING METABOLIC SYNDROME WAS LOWER IN THE HIGHEST COMAPRED WITH THE LOWEST CATEGORY OF DAIRY CONSUMPTION. EACH DAILY OCCASION OF DAIRY CONSUMPTION WAS ASSOCIATED WITH A 21% LOWER ODDS OF METABOLIC SYNDROME.</td>
<td>YES</td>
<td>(PEREIRA ET AL., 2002)</td>
</tr>
<tr>
<td>UK (CAERPHILLY COHORT)</td>
<td>2 375 MEN (45-59 Y)</td>
<td>PROSPECTIVE COHORT STUDY, 20-YEAR FOLLOW-UP</td>
<td>THE CONSUMPTION OF MILK AND DAIRY PRODUCTS IS ASSOCIATED WITH A MARKEDLY REDUCED PREVALENCE OF THE METABOLIC SYNDROME.</td>
<td>YES</td>
<td>(ELWOOD ET AL., 2007)</td>
</tr>
<tr>
<td>THE NETHERLANDS (THE HOORN STUDY)</td>
<td>1 124 (50-75 Y AT BASELINE)</td>
<td>PROSPECTIVE COHORT STUDY, 6.4-YEAR FOLLOW-UP</td>
<td>BASELINE DAIRY CONSUMPTION WAS NOT ASSOCIATED WITH CHANGES IN fasting POST-LOAD GLUCOSE CONCENTRATIONS, SERUM LIPOID LEVELS OR BLOOD PRESSURE, NOR WITH THE RISK OF DEVELOPING THE METABOLIC SYNDROME IN 6.4 YEARS.</td>
<td>NO</td>
<td>(SNIJDER ET AL., 2008)</td>
</tr>
<tr>
<td>IRAN (TEHRAN LIPID AND GLUCOSE STUDY)</td>
<td>827 (337 MEN AND 470 WOMEN, 18-74 Y)</td>
<td>CROSS-SECTIONAL</td>
<td>DAIRY CONSUMPTION IS INVERSESLY ASSOCIATED WITH THE RISK OF HAVING METABOLIC SYNDROME.</td>
<td>YES</td>
<td>(AZADBakh et al., 2005)</td>
</tr>
</tbody>
</table>
### Type 2 Diabetes

<table>
<thead>
<tr>
<th>Country (Database)</th>
<th>Population</th>
<th>Design</th>
<th>Main Result</th>
<th>Inverse Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA (Black Women’s Health Study)</td>
<td>41,186 women (21–69 y at baseline)</td>
<td>Prospective cohort study, 8-year follow-up</td>
<td>Daily consumption of low-fat dairy and whole grains was associated with a lower risk of type 2 diabetes compared with a consumption less than once a week.</td>
<td>Yes</td>
<td>Van Dam et al., 2006</td>
</tr>
<tr>
<td>USA (Health Professionals Follow-up Study)</td>
<td>41,254 men (40–75 y at baseline)</td>
<td>Prospective, 12-year follow-up</td>
<td>Dairy intake was associated with a modestly lower risk of type 2 diabetes. Each serving-per-day increase in total dairy intake was associated with a 9% lower risk for type 2 diabetes.</td>
<td>Yes</td>
<td>Choi et al., 2005</td>
</tr>
<tr>
<td>USA (Women’s Health Study)</td>
<td>37,183 women</td>
<td>Prospective study, 10-year follow-up</td>
<td>Each serving-per-day increase in dairy intake was associated with a 4% lower risk of type 2 diabetes. The inverse association with type 2 diabetes appeared to be mainly attributed to low-fat dairy intake.</td>
<td>Yes</td>
<td>Liu et al., 2006</td>
</tr>
</tbody>
</table>

### Other Disorders of Glucose and Insulin Metabolism

<table>
<thead>
<tr>
<th>Country (Database)</th>
<th>Population</th>
<th>Design</th>
<th>Main Result</th>
<th>Inverse Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK (British Women’s Heart and Health Study)</td>
<td>4,024 women (60–79 y)</td>
<td>Cross-sectional</td>
<td>Women who never drank milk had lower homeostasis model assessment insulin resistance scores, triglyceride concentrations and body mass indices, and higher HDL-cholesterol concentrations, than those who drank milk.</td>
<td>No</td>
<td>Lawlor et al., 2005</td>
</tr>
<tr>
<td>Japan (Self-Defense Forces Health Study)</td>
<td>2,106 men (47–59 y)</td>
<td>Cross-sectional</td>
<td>A dietary pattern characterized by frequent consumption of dairy products and fruits and vegetables but low alcohol intake may be associated with a decreased risk of developing a glucose tolerance abnormality.</td>
<td>Yes</td>
<td>Mizoue et al., 2006</td>
</tr>
<tr>
<td>USA (Insulin Resistance Atherosclerosis Study)</td>
<td>1,036 (43.6% male, 56.4% female, 40–69 y)</td>
<td>Cross-sectional, longitudinal, 5-year follow-up</td>
<td>This study suggests that magnesium and calcium intake specifically, but not dairy intake, is associated with insulin sensitivity.</td>
<td>No</td>
<td>Ma et al., 2006</td>
</tr>
</tbody>
</table>
2.2.3 OBESITY AND THE INTAKE OF DAIRY PRODUCTS: RANDOMISED CLINICAL TRIALS

WEIGHT LOSS

Most of the randomised clinical trials designed to investigate the effect of dairy product intake on body weight have been weight loss trials. Intake of dairy products has been found to significantly increase weight loss during energy restriction (Zemel et al. 2004, Zemel et al. 2005a, Zemel et al. 2005b). In these 12- to 24-week trials, a 500 kcal daily energy restriction has been combined with either low (0–1 portions) or high (3 portions) intake of dairy products. High dairy intake has resulted in 1.5 to 2.3-fold greater body weight loss than low dairy intake. However, despite comparable study designs, a similar effect has not been found in all of the studies (Harvey-Berino et al. 2005, Thompson et al. 2005). Wagner and co-workers (2007) studied the differences between placebo and calcium lactate, calcium phosphate and low-fat milk supplementation during weight loss, but none of the calcium supplementations or milk increased weight or fat loss in comparison with placebo. This is in contrast with the study by Zemel et al. (Zemel et al. 2004), which demonstrated that the effect of dairy calcium was significantly greater than the effect of supplemental calcium.

One of the factors differentiating the clinical trials is the BMI of the study subjects. All of the studies which support the effect of dairy in weight loss have been conducted with obese subjects (BMI>30), whereas studies by Wagner (2007) and Harvey-Berino et al. (2005) included overweight subjects (BMI 25–30) as well. Also Bowen and colleagues (2005) studied subjects with BMI 25 or above. They could not detect any difference during a 12-week weight loss trial on two high-protein diets; one with high dairy protein and high calcium and the other with mixed protein and low calcium.

The effect of dairy products on body weight has also been reported in intervention trials, which have not been primarily designed to study the association of dairy intake and body weight. Data from a randomised 6-month clinical trial assessing the effect of three isocaloric diets in type 2 diabetic patients showed an association of dairy calcium intake with weight
loss (Shahar et al. 2007). Besides weight, serum triglyceride levels were significantly improved in line with the consumption of dairy calcium.

**WEIGHT GAIN**

A few studies have addressed the issue of weight gain or alteration of body composition as a consequence of increased dairy calcium intake, but the results are contradictory (Gunther et al. 2005a, Zemel et al. 2005b, Eagan et al. 2006). A 24-week increased dairy calcium intake on isocaloric diet significantly decreased the fat mass of obese African-American subjects (Zemel et al. 2005b) but did not have an effect on body weight or fat mass in healthy young women during a 1-year follow up (Gunther et al. 2005a). The African-American subjects also had significantly improved insulin and blood pressure values as a result of increased dairy calcium intake and subsequent decrease in fat mass. Eagan and colleagues continued the study of Gunther et al. and analysed the fat mass of the subjects six months after the end of the trial (Eagan et al. 2006). They found that the subjects had maintained the level of dairy calcium intake after the trial and that the calcium intake over 18 months predicted a negative change in body fat mass.

Taken together, the randomised clinical trials aiming at investigating the effect of dairy products or dairy calcium on body weight are not totally conclusive. Most of the studies which have investigated the effect of dairy products on weight loss in obese subjects support the hypothesis that dairy products augment the effect of weight loss. The effect of dairy products on weight gain or modification of body composition is less clear and more studies are needed in that area.

**2.2.4 OBESITY AND THE INTAKE OF DAIRY PRODUCTS: EXPERIMENTAL STUDIES**

Experimental studies investigating the effect of dairy on body weight have mainly concentrated on the effect of calcium and body weight instead of the effect of dairy products. Zemel and co-workers have successfully in-
cluded also dairy-based high-calcium diets in their studies and the results repeatedly demonstrate that calcium in combination with dairy is more effective than supplemental calcium alone. The results from animal models support the role of calcium in inhibiting body weight gain in male rats (Papakonstantinou et al. 2003) and mice (Zemel et al. 2000, Sun and Zemel 2006, Parra et al. 2007). A high-calcium diet also increased weight loss in two different mouse models (Shi et al. 2001a, Sun and Zemel 2004a, Parra et al. 2007) and attenuated weight regain after weight loss (Sun and Zemel 2004a).

However, the experimental evidence does not support the role of calcium deficiency in the development of obesity (Paradis and Cabanac 2005). Also the effect of calcium on body weight in high-or normal-fat diet-fed female C57Bl/6J mice and Sprague Dawley rats is questionable (Zhang and Tordoff 2004).

In addition to the effects on body weight, high-calcium diet has been shown to decrease the expression of pro-inflammatory factors tumour necrosis factor-alpha and interleukin-6 and to stimulate the expression of the anti-inflammatory factors interleukin-15 and adiponectin in mouse visceral fat (Sun and Zemel 2007b). An increased expression of interleukin-15 was also observed in the soleus muscle of these mice in comparison with control mice on a low-calcium diet. The high-calcium diet has also been shown to reduce the production of reactive oxygen species in visceral and subcutaneous adipose tissue in mice (Sun and Zemel 2006). Adipose tissue inflammation and oxidative stress are considered to contribute to the development of insulin resistance and metabolic syndrome (Hotamisligil 2006). Hence, these preliminary findings suggest that dietary calcium not only modulates body weight but has beneficial effects on the overall cardiometabolic risk of obesity. In addition, studies in the diabetic rat model suggest that a fermented dairy product, Dahi, alleviates the development of type 2 diabetes (Yadav et al. 2006a, 2006b, 2007). Additionally, a whey protein-containing high-protein diet has been shown to increase insulin sensitivity and lower weight gain in comparison with red meat-fed control animals (Belobrajdic et al. 2004). These results suggest that not only calcium but also other dairy components may affect the risk of obesity-related co-morbidities.
To sum up, the experimental evidence on the effect of dairy products on body weight is fairly conclusive, but the results are based on a limited number of studies and an even more limited number of different animal models. The preliminary evidence suggests that calcium and other dairy components may play a role in the prevention of obesity-related co-morbidities, such as type 2 diabetes. However, there is an undisputed need for experimental studies designed for clarifying the role and mechanisms of dairy components in the prevention and treatment of obesity and metabolic syndrome.

2.3 OBESITY AND DAIRY COMPONENTS

The idea that dairy foods have a role in the regulation of body weight was first set out when McCarron and colleagues (1984) studied the relationship between the intake of dietary minerals and hypertension. As a secondary finding they observed that calcium intake was inversely related to body mass index. Since dairy is the major dietary source of calcium, the role of dairy was subsequently assessed. Based on this history, calcium is by far the most studied dairy component in the field of obesity, whereas studies on the contribution of the other nutritional components of dairy are limited.

2.3.1 CALCIUM

The relationship of calcium intake and body weight has been investigated in several epidemiological studies (Table 4). Since dairy products are the major dietary source of calcium, the role of dairy in these studies cannot be ruled out. On the whole, epidemiological data on the relationship of calcium intake and obesity are based on smaller population samples than the data on dairy consumption and obesity (Table 2). The studies on children and adolescents are more inconclusive than the studies on adults. In accordance with the epidemiological data on dairy intake and markers of metabolic syndrome, the epidemiological data support the inverse association between calcium intake and central adiposity, glucose disorders and serum lipid profile.
The effect of dietary calcium on body weight has recently been evaluated in a review by Lanou and Barnard (2008) and in a meta-analysis by Trowman et al. (2006). After evaluating 48 (Lanou and Barnard 2008) and 13 (Trowman et al. 2006) randomised controlled trials using calcium supplementation and/or dairy products as an intervention, the authors concluded that calcium supplementation has no significant association with a reduction in body weight. However, only one of the studies reviewed by Trowman et al. (Zemel et al. 2004) was specifically designed and powered to examine whether calcium supplementation affects body weight while the other studies were designed to investigate bone mass during calcium treatment. Of the 48 trials evaluated by Lanou and Barnard, only five studies (Lappe et al. 2004, Reid et al. 2005, Zemel et al. 2005b, Lorenzen et al. 2006, Caan et al. 2007) were designed to investigate the effect of calcium/dairy supplementation on weight gain, whereas eight were designed to investigate the effect of calcium/dairy supplementation on weight loss (Shapses et al. 2004, Zemel et al. 2004, Bowen et al. 2005, Harvey-Berino et al. 2005, Thompson et al. 2005, Zemel et al. 2005a, Zemel et al. 2005b, Major et al. 2007). Most of the remaining studies included in the above review were designed to study the effect of calcium supplementation on bone-related outcomes.

Of the studies specifically designed and powered to investigate the effect of calcium supplementation on body weight, only three studies on weight gain and two on weight loss were conducted using calcium supplements. Calcium supplementation did not enhance weight loss in either of the weight loss trials (Shapses et al. 2004, Major et al. 2007) and weight gain was inhibited in only one of the three trials (Caan et al. 2007). Keeping in mind that dairy supplementation has been shown to affect body weight in several trials (Zemel et al. 2004, Zemel et al. 2005a, Zemel et al. 2005b, Eagan et al. 2006), it can be argued that the effect of dairy is superior to that of calcium supplementation, as has been shown in an intervention study (Zemel et al. 2004).
### Table 4. Epidemiological Studies on Calcium Intake and BMI, Body Weight and Obesity-Related Disorders. Studies are Listed According to the Size of the Study Population.

<table>
<thead>
<tr>
<th>Country (Database)</th>
<th>Population</th>
<th>Design</th>
<th>Main Result</th>
<th>Inverse Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA (NHANES I)</td>
<td>10,372 (18-74 Y)</td>
<td>Cross-sectional</td>
<td>Body mass index was inversely correlated with calcium intake.</td>
<td>Yes</td>
<td>(McCarron et al., 1984)</td>
</tr>
<tr>
<td>USA (Strong Heart Study)</td>
<td>American Indians (1,823 women, 1,152 men, 47-79 Y)</td>
<td>Cross-sectional</td>
<td>BMI and body fat were lower in participants with higher vs. lower calcium intake.</td>
<td>Yes</td>
<td>(Eilat-Adar et al., 2007)</td>
</tr>
<tr>
<td>USA (NHANES III)</td>
<td>1,391 women, 1,364 men (47-79 Y)</td>
<td>Cross-sectional</td>
<td>Calcium intake did not correlate with BMI or body fat in either gender.</td>
<td>No</td>
<td>(Eilat-Adar et al., 2007)</td>
</tr>
<tr>
<td>USA (Heritage Family Study)</td>
<td>362 men (109 blacks/253 whites), 462 women (201 blacks/261 whites), 17-65 Y</td>
<td>Cross-sectional</td>
<td>The strongest inverse associations between calcium intake and BMI and body fat appeared in black men and white women. No significant associations were found in black women. The percentage of fat of white men in the highest CA intake group was significantly lower than in the lowest CA group.</td>
<td>Yes</td>
<td>(Loos et al., 2004)</td>
</tr>
<tr>
<td>Spain</td>
<td>647 (261 men, 383 women, aged 18-70 Y)</td>
<td>Cross-sectional</td>
<td>Negative relationship between calcium intake and BMI in a Mediterranean community.</td>
<td>Yes</td>
<td>(Garcia-Lordia et al., 2007)</td>
</tr>
<tr>
<td>USA</td>
<td>65 Pima Indians (35 men, 30 women, mean age 33 Y)</td>
<td>Cross-sectional</td>
<td>No relationship between calcium intake and BMI or adiposity.</td>
<td>No</td>
<td>(Venti et al., 2005)</td>
</tr>
<tr>
<td>COUNTRY (DATABASE)</td>
<td>POPULATION</td>
<td>DESIGN</td>
<td>MAIN RESULT</td>
<td>INVERSE ASSOCIATION</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>--------</td>
<td>-------------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>CALCIUM INTAKE AND BMI IN CHILDREN AND ADOLESCENTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PORTUGAL</strong></td>
<td>1 503 GIRLS AND 1541 BOYS (7-9 Y)</td>
<td>CROSS-SECTIONAL</td>
<td>AN INVERSE RELATIONSHIP BETWEEN CALCIUM INTAKE AND BMI ONLY IN GIRLS.</td>
<td>YES/NO</td>
<td>(MOREIRA ET AL., 2005)</td>
</tr>
<tr>
<td><strong>VENEZUELA</strong></td>
<td>100 ADOLESCENTS (13-18 Y)</td>
<td>CROSS-SECTIONAL</td>
<td>A SIGNIFICANT NEGATIVE ASSOCIATION BETWEEN CALCIUM INTAKE AND BMI IN THE BOYS (13-15 Y), BUT NOT IN OTHER AGE GROUPS OR GIRLS.</td>
<td>YES/NO</td>
<td>(PALACIOS ET AL., 2007)</td>
</tr>
<tr>
<td><strong>USA</strong></td>
<td>78 PIMA INDIANS (36 MEN, 42 WOMEN, MEAN AGE 10 Y)</td>
<td>CROSS-SECTIONAL</td>
<td>NO RELATIONSHIP BETWEEN CALCIUM INTAKE AND BMI OR ADIPOSITY.</td>
<td>NO</td>
<td>VENTI ET AL., 2005</td>
</tr>
<tr>
<td><strong>CALCIUM INTAKE, BODY COMPOSITION AND MARKERS OF METABOLIC SYNDROME</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FRANCE (DESIR)</strong></td>
<td>2 235 MEN, 2 137 WOMEN (30-65 Y AT BASELINE)</td>
<td>LONGITUDINAL 9-YEAR FOLLOW-UP</td>
<td>INCREASED CALCIUM INTAKE WAS INVERSELY ASSOCIATED WITH CENTRAL ADIPOSITY. ALSO A BENEFICIAL ASSOCIATION BETWEEN DIETARY CALCIUM AND ARTERIAL BLOOD PRESSURE, INSULIN AND HDL-CHOLESTEROL LEVELS WERE FOUND IN WOMEN, WHEREAS IN MEN ONLY A BENEFICIAL ASSOCIATION WITH DIASTOLIC BLOOD PRESSURE WAS FOUND.</td>
<td>YES</td>
<td>(DROUILLET ET AL., 2007)</td>
</tr>
<tr>
<td><strong>THE NETHERLANDS (AMSTERDAM GROWTH AND HEALTH LONGITUDINAL STUDY)</strong></td>
<td>296 MEN, 333 WOMEN (13 Y AT BASELINE)</td>
<td>LONGITUDINAL 23-YEAR FOLLOW-UP</td>
<td>A SLIGHT INDICATION OF A WEAK INVERSE RELATION OF CALCIUM INTAKE WITH BODY COMPOSITION WAS FOUND. THESE DATA SUGGEST A THRESHOLD OF APPROXIMATELY 800 MG/DAY ABOVE WHICH CALCIUM INTAKE HAS NO ADDITIONAL BENEFICIAL EFFECT ON BODY COMPOSITION.</td>
<td>YES</td>
<td>(BOON ET AL., 2005)</td>
</tr>
<tr>
<td>COUNTRY (DATABASE)</td>
<td>POPULATION</td>
<td>DESIGN</td>
<td>MAIN RESULT</td>
<td>INVERSE ASSOCIATION</td>
<td>REFERENCE</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------</td>
<td>--------</td>
<td>-------------</td>
<td>---------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CANADA (Québec Family Study)</td>
<td>235 men, 235 women, 20-65 y</td>
<td>CROSS-SECTIONAL</td>
<td>A low daily calcium intake is associated with greater adiposity, particularly in women. In both sexes, a high calcium intake is associated with a plasma lipoprotein-lipid profile predictive of a lower risk of coronary heart disease risk compared with a low calcium intake.</td>
<td>YES</td>
<td>(Jacqmain et al., 2003)</td>
</tr>
<tr>
<td>SOUTH AFRICA</td>
<td>BLACK (102) and WHITE (106) women (20-60 y of age)</td>
<td>CROSS-SECTIONAL</td>
<td>The association between calcium intake and percentage body fat, BMI, fasting glucose, and insulin were significant only with high intake of fat and calcium, which is not characteristic of the habitual diet of African women.</td>
<td>YES</td>
<td>(Kruger et al., 2007)</td>
</tr>
<tr>
<td>BRAZIL</td>
<td>96 post-pubertal adolescents (mean age 16.6 y)</td>
<td>CROSS-SECTIONAL</td>
<td>Calcium intake inversely associated with body trunk fat, insulin and HOMA-IR in the obese group.</td>
<td>YES</td>
<td>(Dos Santos et al., 2008)</td>
</tr>
</tbody>
</table>
2.3.2 DAIRY PROTEINS

In vitro studies suggest that dairy proteins have various physiological functions (Yalcin 2006). Dairy proteins are also a source of various bioactive peptides with for example ACE-inhibitory properties, opioid-like activities and mineral-binding and antithrombotic properties (for review, see Jauhiainen and Korpela 2007). Thus the hypothesis that dairy protein-derived peptides might modulate also energy metabolism-related pathways is plausible, but studies on the area are scarce.

Many of the currently known dairy-derived peptides with ACE-inhibitory properties, such as Ile-Pro-Pro and Val-Pro-Pro, are derived from casein (Jauhiainen and Korpela 2007). The local renin-angiotensin-aldosterone system in adipose tissue is postulated to play a role in the development of type 2 diabetes and metabolic syndrome but the molecular mechanisms are not yet fully understood (Sharma 2006). Hence, milk-derived ACE-inhibitory peptides may in theory contribute to the decreased risk of metabolic syndrome and type 2 diabetes in high dairy consumers.

Whey has been reported to inhibit body weight gain in Wistar rats in comparison with red meat (Belobrajdic et al. 2004) and casein (Royle et al. 2007). A whey protein-rich diet has also been shown to have an influence on liver and muscle lipogenic enzyme activities in rats (Morifuji et al. 2005a, Morifuji et al. 2005b). Whey proteins may also have satiety-increasing properties (Luhovyy et al. 2007), which may influence obesity.

In addition to various bioactive protein components, the amount of branched-chain amino acids; leucine, isoleucine and valine, is high in total whey protein. Leucine, in particular, is known to have various metabolic functions including the initiation of muscle protein synthesis (Anthony et al. 2001) and modulation of the insulin/PI3-kinase signalling (Patti et al. 1998), and long-term leucine supplementation has been shown to increase body fat loss during food restriction in Wistar rats (Donato et al. 2006).

2.3.3 OTHER DAIRY COMPONENTS

With regard to calcium intake, dairy products represent the major dietary source, which is not the case with other minerals or vitamins in dairy
(Ovaskainen et al. 2003). Hence, the inverse relationship between dairy intake and body weight is unlikely to be explained by other minerals or vitamins.

The intake of dairy fat has been traditionally linked to an increased risk of cardiovascular disease. This is mainly due to the high amount of saturated fatty acids which have unfavourable effects on serum lipids (Matthan et al. 2004), (Tholstrup et al. 2004). Interestingly, a Swedish longitudinal study found the increased intake of whole milk and cheese, in particular, to be inversely associated with weight gain during a 9-year follow-up (Rosell et al. 2006). A similar association was found in two other epidemiological studies as well (Rajpathak et al. 2006, Snijder et al. 2007). In addition, cheese consumption was associated with a more favourable cardiovascular risk profile in a recent publication (Houston et al. 2008). Most of the epidemiological studies do not distinguish between the types of dairy products and hence the contribution of high-fat versus low-fat dairy products to the risk of obesity and metabolic disorders is difficult to comprehend. However, a possibility of dairy containing bioactive lipid components that contribute to the regulation of body weight and cardiometabolic risk, cannot be ruled out.

One lipid component of dairy, which has been suggested to have favourable effects on body weight, is conjugated linoleic acid (CLA) (for review, see Silveira et al. 2007). Despite numerous studies on CLA’s effects on body composition for nearly a decade, the mechanisms by which CLA isomers elicit their effects remain largely unknown. Intervention studies suggest that the potentially effective form of CLA is in particular the trans-10,cis-12 isomer (for review, see Wang and Jones 2004), whereas CLA in dairy products is mainly in the form of the cis-9,trans-11 isomer. Additionally, some studies raise concern about the possibility of deleterious effects of trans-10,cis-12 CLA on lipid profile, glucose metabolism and insulin sensitivity. The dietary intake of CLA has been estimated to be around 200 mg/day in US adults, dairy products accounting for approximately 90% of the intake (Ritzenthaler et al. 2001). However, the amount of CLA in the intervention studies has been 3.5 to 34-fold in comparison with the average intake (for review, see Silveira et al. 2007). Therefore, it is not likely that the potential body weight–regulating effects of dairy would be explained by CLA.
To sum up, the epidemiological data do support the role of calcium in reducing obesity-related co-morbidities, such as insulin resistance, but the data on BMI are more inconclusive, especially in children and adolescents. Randomised clinical studies designed and powered to study changes in body weight suggest that calcium supplementation is not as effective as dairy in regulating body weight. Scattered experimental evidence support the multiple health benefits of dairy proteins and dairy protein-derived peptides. The beneficial amino acid composition also supports the potential health effects of dairy proteins.

2.4 POSSIBLE MECHANISMS OF ACTION

2.4.1 CALCIUM AND FAT ABSORPTION

One of the mechanisms by which calcium may modulate body weight is by binding fatty and bile acids in the gastrointestinal tract (Van der Meer et al. 1990, Denke et al. 1993, Welberg et al. 1994, Papakonstantinou et al. 2003, Jacobsen et al. 2005). Calcium forms insoluble fat-calcium soaps in the intestine and thereby increases fat excretion. In humans, the increased fat excretion has only been measured in short-term studies, and the potential effect of this increased fat excretion on body weight has been estimated without taking into account the actual fat intake during/prior to the collection of faeces. Depending on the study, the fat excretion has increased from 1.6 (Boon et al. 2007b) to 2.5-fold (Jacobsen et al. 2005) on a high-calcium diet in comparison with a low-calcium diet. It has been argued that even though the effect of calcium on fat excretion is significant in certain clinical settings, it is too small to explain the effects on body weight (Zemel 2005, Major et al. 2008). Indeed, the only study which has demonstrated that increased fat excretion in the high-calcium fed group accounted for a substantial amount of body weight decrease was a study in rats, in which the apparent fat absorption was calculated from fat intake and fat excretion data (Papakonstantinou et al. 2003).

In order for calcium to bind dietary fatty acids, it should be present in the gastrointestinal tract at the same time with the dietary fat. In this regard, dairy products have a clear advantage, since they are more likely
to be ingested simultaneously with dietary fat than supplemental calcium. However, even when ingested simultaneously with a meal, supplemental calcium has a smaller effect on postprandial lipid absorption than dairy calcium (Lorenzen et al. 2007). The reason for this is currently not understood, but the differences in the chemical form of calcium or yet unknown cofactors in dairy products have been suggested to play a role in this.

To summarise, dietary calcium increases the faecal fat excretion in both humans and animals by formation of insoluble fat-calcium soaps. This mechanism presumes calcium and fatty acids to be present in the intestine at the same time, whereby dairy calcium has a clear advantage against supplemental calcium, since it is more likely provided with the meal. Preliminary evidence suggests that dairy calcium also suppresses postprandial lipid absorption significantly more than supplemental calcium, but the mechanisms explaining the phenomena are unclear. Most of the clinical evidence on calcium’s effect on faecal fat excretion is based on acute studies and hence the long-term effects on body weight need further studies.

2.4.2 CALCIUM, 1,25(OH)₂-VITAMIN D₃ AND INTRACELLULAR CALCIUM

One of the key regulators of calcium homeostasis in the body is 1,25(OH)₂D₃ (for review, see Dusso et al. 2005). During low dietary calcium intake, the level of 1,25(OH)₂D₃ increases, which in turn stimulates the absorption of calcium in the small intestine. Since dietary calcium intake is one of the regulators of serum 1,25(OH)₂D₃ levels, it has been hypothesised that the effect of calcium on body weight would also be induced via 1,25(OH)₂D₃.

The actions of 1,25(OH)₂D₃ in the adipocytes are in part mediated via intracellular calcium (Figure 1). A high level of 1,25(OH)₂D₃ increases intracellular calcium concentration in isolated human adipocytes by stimulating the influx of calcium into the adipocytes (Zemel et al. 2000, Shi et al. 2001b). The high concentration of intracellular calcium subsequently increases lipogenesis through increased fatty acid synthase expression and
activity (Kim et al. 1996). An increase in intracellular calcium leads also to inhibition of lipolysis via activation of phosphodiesterase, which reduces the level of cyclic AMP and decreases phosphorylation of hormone-sensitive lipase. (Xue et al. 2001).

**Figure 1.** An integrated summary of the mechanism by which dietary calcium may modulate adipocyte size via 1,25(OH)$_2$D$_3$ and intracellular calcium (modified from Zemel 2005).

The high-calcium diet has been shown to decrease intracellular calcium concentration in adipocytes during weight gain (Zemel et al. 2000), weight loss (Shi et al. 2001a) and weight re-gain (Sun and Zemel 2004a) in mice that express the agouti gene in adipose tissue under the control of the aP2-promoter. The decrease in fatty acid synthase expression and activity and the increase in lipolysis have been observed along with the decreased intracellular calcium levels in these studies. However, these findings have not been confirmed in any other animal model. Clinical
studies do not support the theory that increased calcium intake or changes in \(1,25(\text{OH})_2\text{D}_3\) would affect fatty acid synthase expression (Boon et al. 2005a, Boon et al. 2006, Boon et al. 2007b), but there is preliminary evidence supporting the effect of intracellular calcium modification on lipolysis (Boon et al. 2007a).

The studies on cultured adipocytes suggest additional mechanisms by which \(1,25(\text{OH})_2\text{D}_3\) may mediate the effects of dietary calcium. These actions include inhibition of the uncoupling protein 2 expression through nuclear vitamin D receptors and subsequent regulation of thermogenesis (Shi et al. 2002) and attenuation of adipocyte apoptosis through uncoupling protein 2 inhibition (Sun and Zemel 2004b). On the other hand, \(1,25(\text{OH})_2\text{D}_3\) has been shown to inhibit adipocyte differentiation (Kong and Li 2006) and therefore decreasing PTH and subsequently \(1,25(\text{OH})_2\text{D}_3\) by dietary calcium would lead to increased adipogenesis. It should be noted that activation of vitamin D receptor mediates the expression of numerous genes. For example, nearly 200 genes in human smooth muscle cells were affected as a result of vitamin D receptor activation (Wu-Wong et al. 2007). Therefore, the hypothesis by Zemel et al. may be an oversimplified representation of the situation. Furthermore, the epidemiological studies on the relationship between serum \(1,25(\text{OH})_2\text{D}_3\) levels and obesity are conflicting as well (Bell et al. 1985, Zamboni et al. 1988, Parikh et al. 2004).

In addition to obesity, \(1,25(\text{OH})_2\text{D}_3\) may be associated with obesity-related comorbidities. Preliminary findings from cell culture studies and aP2 transgenic mice suggest that \(1,25(\text{OH})_2\text{D}_3\) promotes inflammatory cytokine expression, inhibits anti-inflammatory cytokine expression (Sun and Zemel 2007b) and modulates formation of reactive oxygen species (ROS) via uncoupling protein 2 and intracellular calcium (Sun and Zemel 2006, 2007a). Hence, decreasing the level of \(1,25(\text{OH})_2\text{D}_3\) by dietary calcium would decrease the inflammatory cytokine production and ROS formation in the adipose tissue and consequently contribute to the decreased risk of metabolic syndrome and type 2 diabetes. However, \(1,25(\text{OH})_2\text{D}_3\) has also been shown to protect against oxidative stress (Bao et al. 2008) and to have anti-inflammatory properties (Zittermann et al. 2005, Giulietti et al. 2007). It is therefore obvious that the role of \(1,25(\text{OH})_2\text{D}_3\) is not yet clear in the modulation of inflammatory and oxidative stress.
Taken together, 1,25(OH)₂D₃ offers an intriguing mechanistic explanation for the effects of dietary calcium. There is accumulating evidence on the role of 1,25(OH)₂D₃ in adipose tissue metabolism, but the results are mainly based on in vitro data and are yet inconclusive. The theory on the effects of 1,25(OH)₂D₃ on adipose tissue is partly connected to adipocyte intracellular calcium levels. However, the significance of adipocyte intracellular calcium as a regulator of adipocyte size and metabolism is supported by a limited amount of experimental data and their relevance remains to be elucidated. In addition, the epidemiological and clinical evidence on the role of 1,25(OH)₂D₃ in obesity warrants further research.

2.4.3 Calcium and Fat Oxidation

The effects of increased calcium and dairy product intake on body weight have also been explained by increased fat oxidation. Fat oxidation has been studied after either short-term (Melanson et al. 2003, Gunther et al. 2005b, Cummings et al. 2006) or chronic calcium intake (Boon et al. 2005a, Gunther et al. 2005b, Jacobsen et al. 2005, Melanson et al. 2005, Teegarden et al. 2008) and the results from both settings are inconclusive. Interestingly, some studies have found a difference in the effect between dairy and supplemental calcium (Teegarden et al. 2008) while others have not (Melanson et al. 2003, Cummings et al. 2006). Most of the studies have investigated the effect of calcium on fat oxidation during isocaloric conditions, but also acute (Melanson et al. 2005) and long term energy restriction (Teegarden et al. 2008) approaches have been used. Both energy restriction studies support the hypothesis that calcium intake increases fat oxidation. However, only supplemental, but not dairy, calcium was found to increase fat oxidation after a long-term (12-week) calorie restriction (Teegarden et al. 2008).

One possible mechanism by which dietary calcium could increase fat oxidation is via 1,25(OH)₂D₃ and uncoupling protein 2 (Shi et al. 2002). The 1,25(OH)₂D₃ inhibits uncoupling protein 2 expression via nuclear vitamin D receptor in human adipocytes in vitro and hence suppressing 1,25(OH)₂D₃ levels through increased dietary calcium intake may increase...
uncoupling protein 2 expression and subsequently fat oxidation. Actions mediated by serum PTH levels have also been suggested (Teegarden et al. 2008).

2.4.4 DAIRY PROTEINS AND SATIETY

The role of satiety-related signals and pathways in the regulation of body weight has been an area of intensive research in the past years. Increased intake of dietary protein is known to increase satiety and decrease food intake more than the intake of fat or carbohydrates (Porrini et al. 1997, Latner and Schwartz 1999, Lejeune et al. 2006), resulting in both improved weight loss and weight loss maintenance (Skov et al. 1999, Dumesnil et al. 2001, Westerterp-Plantenga et al. 2004, Lejeune et al. 2005). However, it is still not fully understood how increased protein intake increases satiety and hence also the role of different proteins in satiety-mediated weight management remains unclear. Since dairy products are an important source of dietary protein (Ovaskainen et al. 2003), their effect on body weight may be related to their satiety-inducing properties.

Whey has been demonstrated to have greater satiety effect than casein (Hall et al. 2003), soy or egg albumin (Anderson et al. 2004) in some but not all of the studies (Bowen et al. 2006a, Bowen et al. 2006b). The digestion of whey and absorption of its amino acids is faster than that of casein, whereby these proteins are classified as “fast” and “slow” protein, respectively (Boirie et al. 1997, Dangin et al. 2002). In humans, the intake of whey results in fast, but short and transient increase in plasma amino acids. In contrast, casein results in a slower and lower rise in plasma amino acid concentrations but sustains a prolonged plateau after consumption. Therefore, it has been suggested that milk-induced satiation and satiety are a result of synergistic action of whey proteins providing early and casein providing overlapping but later signals (for review, see Luhovyy et al. 2007).

In the controlled clinical trials which have investigated the effects of dairy intake on body weight, the average energy or protein intake has not differed between the high and low dairy groups (Zemel et al. 2004, Zemel et al. 2005a, Zemel et al. 2005b). Accordingly, at least in these studies, the potential satiety effect of dairy proteins is not likely to play a role. The same can be concluded from the experimental studies studying dairy protein-
based diets (Zemel et al. 2000, Shi et al. 2001a, Sun and Zemel 2004a). In the epidemiological studies reporting the inverse relationship between BMI or body weight and dairy intake, the correction for protein intake has not been widely used. Therefore, it is possible that the satiety effect of dairy proteins plays a role in the regulation of body weight in the long run.

To summarise, diets high in protein are considered to increase satiety and support weight management. Dairy products are an important source of dietary protein and dairy proteins may have a greater satiety effect than other protein sources. Therefore, the satiety effect of dairy proteins may play a role in the epidemiological findings on dairy intake and body weight, but this has not been studied. The subjective satiety or levels of satiety hormones have not been studied during the experimental and clinical interventions which have focused on the effect of dairy product intake on body weight. However, energy and protein intakes have not differed between the intervention groups in these studies and hence the results cannot be explained by a decreased energy intake which would have resulted from greater satiety.

2.4.5 DAIRY PROTEINS AND INSULIN ACTION

Insulin resistance is a central element of obesity, metabolic syndrome and type 2 diabetes, and recent data demonstrate that chronic, low-grade inflammation associated with obesity is an important mechanism decreasing insulin signalling (for review, see de Luca and Olefsky 2008). Dairy products increase the postprandial insulin response, which is reduced in type 2 diabetic subjects (Liljeberg Elmståhl and Björck 2001, Östman et al. 2001). Whey protein, in particular, has been demonstrated to contribute to better glycaemic control due to its greater postprandial insulino-trophic effect as compared with casein (Nilsson et al. 2004, Frid et al. 2005, Tessari et al. 2007).

The insulino-trophic effect of whey protein is likely to be mediated through several mechanisms. The rapid amino acid absorption and the substantial amount of certain insulino-trophic amino acids (isoleucine, leucine, lysine, threonine, valine) result in great postprandial insulino-
mic response (Nilsson et al. 2007). Whey proteins also increase the concentration of incretin hormones by inhibiting the activity of dipeptidyl peptidase IV, the enzyme that catalyses the inactivation of the incretin hormones in the gastrointestinal tract (Gunnarsson et al. 2006). Incretin-based therapies, including glucagon-like peptide 1 analogues and dipeptidyl peptidase IV inhibitors, have been shown to restore glucose homeostasis and improve glycaemic control and are thus considered a promising new treatment option for patients with type 2 diabetes (for review, see Drucker and Nauck 2006, for review, see Pratley and Salsali 2007). The recent meta-analysis of incretin therapies also concluded that treatment with dipeptidyl peptidase IV inhibitors resulted in some improvements in triglycerides and low- and high-density lipoprotein cholesterol levels (Amori et al. 2007). The postprandial insulinotropic effect of dairy, combined with the possible dipeptidyl peptidase IV inhibitory activity of whey, may thus in theory contribute to the decreased risk of metabolic syndrome and type 2 diabetes in high dairy consumers.

**Taken together, dairy products and especially whey protein may favourably affect the postprandial glycaemic control. However, the current data only support the acute postprandial increase of insulin response. Furthermore, it has not been established whether long term consumption of diet high in dairy products has a stable effect on glycaemic control.**
3 AIMS OF THE STUDY

Consumption of a diet high in dairy products is associated with lower BMI, reduced risk of type 2 diabetes and lower incidence of metabolic syndrome. Earlier studies have shown that dietary calcium modifies body weight and adipose tissue metabolism and that the effect of calcium from dairy sources is superior to the effect of supplemental calcium. Dairy proteins have been suggested to play a role in the modulation of body weight, but the effects of dairy proteins in combination with calcium at different stages of obesity are not well understood. Furthermore, the effect of calcium and dairy proteins on adipose tissue and liver metabolism, which play a central role in the development of metabolic syndrome and type 2 diabetes, is not known. The present study investigated these effects of calcium and dairy proteins in more detail by using a well established animal model of diet-induced obesity.

The specific aims of the study were:
1. To evaluate the potential of dairy protein and calcium intake in the prevention and treatment of diet-induced obesity (I, II, III, IV).
2. To study the effect of dairy protein and calcium intake on adipose tissue and the liver in order to characterise the potential mechanisms explaining the reduction of risk for metabolic syndrome and type 2 diabetes (III, IV).
4 MATERIALS AND METHODS

4.1 STUDY DESIGN

All of the studies were conducted with male C57Bl/6J mice. The C57Bl/6J mouse is a well-established model of diet-induced obesity (Surwit et al. 1988, Collins et al. 2004, Koza et al. 2006). This inbred mouse strain develops obesity and insulin resistance when fed a high-fat diet and thus serves as a human-like experimental model of obesity research.

The studies evaluated the effect of dairy proteins and calcium at different phases of obesity. Study I concentrated on the prevention of weight gain (Figure 2), whereas Study II investigated the effect of dairy proteins and calcium on weight loss and subsequent weight re-gain. Study III evaluated the changes in adipose tissue gene expression as a result of weight gain and Study IV concentrated on the hepatic effect of dairy proteins and calcium during weight loss.

Figure 2. The illustrative body weight curve describing the different phases of body weight studied in this thesis.
4.2 MATERIALS

4.2.1 EXPERIMENTAL ANIMALS

All of the studies were carried out on 8-week-old male C57Bl/6J mice, which were purchased from Harlan (Horst, The Netherlands). The mice were housed five in a cage in a standard experimental animal laboratory, illuminated from 6.30 a.m. to 6.30 p.m., temperature 22±1°C. The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland, permission number HY 22-05. The mice had free access to feed and tap water during the experiments. After a 1-week acclimatisation period on a normal chow diet (Harlan Tekland 2018, Harlan Holding, Inc., Wilmington, DE, USA), the mice were put on a high-fat diet for 21 (Studies I, III and IV) or 28 weeks (Study II).

In Studies I and III, the body weight-matched mice were divided into treatment groups in the beginning of the high-fat feeding (weight gain) phase (Figure 2). In Studies II and IV, the body weight-matched mice were divided into treatment groups after 14-week ad libitum feeding on a high-fat diet and subjected to energy restriction for 7 weeks (weight loss phase). During the energy restriction period, the energy intake of the mice was limited to 70% of the energy intake of the ad libitum feeding. In Study II, part of the mice were sacrificed after the weight loss phase and the remaining mice were fed ad libitum for another 7 weeks (weight re-gain phase).

4.2.2 DIETS AND GROUPS

All of the high-fat diets were manufactured by Research Diets Inc. (New Brunswick, NJ, USA). The diets were based on the standard DIO-diet containing 60% of energy in fat (D12492). In the study diets, the amount of calcium and the source of protein were modified according to the study design. The specifications of the diets are presented in Table 5. The lean control group in Study IV was fed a regular rodent chow (Harlan Teklad 2018, Harlan Holding, Inc., Wilmington, DE, USA) instead of a high-fat diet. The powdered diets were moistened with tap water (200 ml/kg in whey and beta-lactoglobulin, 205 ml/kg in alpha-lactalbumin, 190 ml/kg in lactoferrin, 100 ml/kg in casein and 110 ml/kg in control and
700 ml/kg in the normal chow diet) using an industrial dough mixer and packed in one-day portions and stored at -20°C.

The labelling of the groups varies in the original publications. In this thesis, the groups are named as follows:

### Table 5. Diets and Labelling of the Groups Used in This Thesis.

<table>
<thead>
<tr>
<th>Name of the Group</th>
<th>Diet Characteristics (Fat, Ca, Protein)</th>
<th>Diet Reference (Producer)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>High-fat (60% of energy), low-ca (0.4%), casein (18% of energy)</td>
<td>D05031101M (Research Diets Inc.)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>High-ca Whey</td>
<td>High-fat (60% of energy), high-ca (1.8%), whey (18% of energy)</td>
<td>D05031104M (Research Diets Inc.)</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>High-ca</td>
<td>High-fat (60% of energy), high-ca (1.8%), casein (18% of energy)</td>
<td>D05031102M (Research Diets Inc.)</td>
<td>I, unpublished</td>
</tr>
<tr>
<td>Whey</td>
<td>High-fat (60% of energy), low-ca (0.4%), whey (18% of energy)</td>
<td>D06050901M (Research Diets Inc.)</td>
<td>Unpublished</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>High-fat (60% of energy), high-ca (1.8%), α-lactalbumin (18% of energy)</td>
<td>D07041804M (Research Diets Inc.)</td>
<td>II</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>High-fat (60% of energy), high-ca (1.8%), β-lactoglobulin (18% of energy)</td>
<td>D07041803M (Research Diets Inc.)</td>
<td>II</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>High-fat (60% of energy), high-ca (1.8%), lactoferrin (18% of energy)</td>
<td>D07041805M (Research Diets Inc.)</td>
<td>II</td>
</tr>
<tr>
<td>Lean</td>
<td>Normal fat (10% of energy), normal ca (0.6%), not a purified diet, i.e. protein source cannot be separated (soy, maize, wheat etc.) (18% of energy)</td>
<td>20185 (Harlan)</td>
<td>IV</td>
</tr>
</tbody>
</table>
4.3 METHODS

4.3.1 BODY WEIGHT AND BODY FAT MEASUREMENTS

Body weight was monitored weekly during the weight gain period and one to two times per week during the calorie restriction period using a standard table scale (Ohaus Scout™ Pro, SP4001, Näikon, Switzerland). The consumption of feed per cage was monitored daily using the same table scale. Body fat content was analysed by dual-energy X-ray absorptiometry (DEXA, Lunar PIXImus, GE Healthcare, Chalfont St. Giles, UK) at the end of the weight gain, weight loss and weight re-gain phases.

4.3.2 COLLECTION AND ANALYSIS OF FAECAL SAMPLES (STUDIES I, II AND IV)

At end of the weight gain, weight loss and weight re-gain periods, the mice were housed in metabolic cages for 72 hours. All faeces were collected during housing in the metabolic cages. Fat content of the faecal samples was determined by the SBR (Schmid-Bondzynski-Ratzlaff) method (International standard 1986), modified for faecal sample analysis and the calcium content was determined using the ICP-MS (inductively-coupled plasma mass spectrometry) (Elan 6100, Perkin Elmer, Boston, MA, USA). The apparent fat absorption was calculated from the amount of feed consumed and the amount of fat excreted during housing in the metabolic cages. The apparent fat absorption (%) was determined as 100 x [(fat intake - faecal fat)/(fat intake)].

4.3.3 CALORIMETRY AND METABOLIC PERFORMANCE (STUDY IV)

The possibility of different dietary proteins influencing metabolic performance, energy expenditure, physical activity as well as drinking and feeding behaviour, was analysed by housing a group of animals (n=4/whey and 3/casein group) in a home cage-based monitoring system for laboratory animals (LabMaster®, Bad Homburg, Germany). The mice were housed in this system over 7 subsequent days after 5 days of adaptation to the system and the
study diets in identical training cages. The instrument consists of highly sensitive feeding and drinking sensors for automated online measurement, a calorimetry system that determines $O_2$ consumption, $CO_2$ production, and respiratory quotient ($RQ = \frac{V_{CO_2}}{V_{O_2}}$, where $V$ is volume), respiratory exchange rate and a heat and photobeam-based activity monitoring system which detects and records every ambulatory movement.

4.3.4 COLLECTION OF THE TISSUE SAMPLES

At the end of each treatment period, the mice were rendered unconscious with $CO_2/O_2$ (95%/5%) (AGA, Riihimäki, Finland), and decapitated. The blood samples were taken in plastic tubes and the serum separated by centrifugation at +4°C for 15 minutes. The livers (Study IV) and the subcutaneous, epididymal, abdominal and retroperitoneal fat pads (Studies II, III and IV) were removed, washed with saline, blotted dry and weighed. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until assayed.

The samples for histology and immunohistochemistry were fixed in 10% formaline and embedded in paraffin with routine techniques (Studies II and IV). The liver samples for Oil Red O staining were frozen in isopentane (-38°C) and stored at -80°C until further processed.

4.4 BIOCHEMICAL DETERMINATIONS

4.4.1 BLOOD GLUCOSE, SERUM INSULIN AND SERUM LIPIDS (STUDIES I, II AND IV)

Blood glucose (Studies I, II and IV) was analysed from the truncal blood samples taken straight after sacrificing the animals. Blood glucose was determined by glucometer (Super Glucocard™II, GT-1630, Arkray Factory Inc., Shiga, Japan).

Serum insulin (Study IV) was determined by ELISA kit for mouse insulin (Ultra sensitive Mouse Insulin ELISA kit 90080, Crystal Chem Inc., IL, USA).

Serum lipids (Study I) were analysed by an accredited laboratory, HUSLAB, Helsinki University Central Hospital, Helsinki, Finland (Hitachi 912 Automatic Analyser, Hitachi Ltd., Tokyo, Japan).
4.4.2 SERUM 1,25(OH)$_2$D$_3$ AND PTH (STUDY I)

The serum 1,25(OH)$_2$D$_3$ was determined by RIA-kit (IDS Ltd, Boldon, UK) and serum intact PTH was determined by ELISA (Immutopics Inc., San Clemente, CA, USA) according to the instructions of the manufacturer.

4.5 HISTOLOGY, IMMUNOHISTOCHEMISTRY AND ADIPOCYTE CROSS-SECTIONAL AREA

4.5.1 HISTOLOGICAL ANALYSIS (STUDY IV)

Sections (4 μm) of paraffin-embedded liver samples were cut with a microtome, stained with standard haematoxylin/eosin staining and examined with a light microscopy. The severity of the observed lesions was graded according to Herbert et al. (2002).

The relative amount of lipids in the liver samples was determined from Oil Red O stained frozen sections (4 μm) with AnalySIS Pro-software (Soft Imaging System, Münster, Germany).

4.5.2 IMMUNOHISTOCHEMICAL STAINING OF F4/80 (STUDY III)

As a marker of macrophage infiltration into the adipose tissue, sections (5 μm) of paraffin-embedded adipose tissue samples were stained for the expression of F4/80 with an anti-F4/80 monoclonal antibody (F4/80 antibody [CI:A3-1] ab6640, Abcam, Cambridge, UK) according to the indirect peroxidase-conjugated streptavidin procedure. Three different microscope fields were analyzed for each individual mouse adipose depot. The total number of nuclei and the number of nuclei of F4/80-expressing cells were counted for each field. The fraction of F4/80-expressing cells for each sample was calculated as the sum of the number of nuclei of F4/80-expressing cells divided by the total number of nuclei in sections of a sample.
4.5.3 Adipocyte Cross-sectional Area (Studies II and III)

Adipocyte cross-sectional area was determined for each adipocyte in three (Study III) to six fields (Study II) per sample using Leica QWin Standard software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

4.6 Microarray Procedure and Gene Expression Analysis (Study III)

4.6.1 Microarray Procedure

Five μg of RNA from the epididymal fat pads of two control mice, and of two high-calcium whey protein-fed mice were reverse-transcribed to cDNA, hybridised according to the standard protocol with Mouse Genome 430 2.0 array (Affymetrix) and scanned with GeneChip Scanner 3000 (Affymetrix). The complete data set is available from the NCBI’s Gene Expression Omnibus (GEO) database and the gene expression profiling data comply with the MIAME standard (accession number GSE9280).

4.6.2 Data Processing

The data were pre-processed with the robust multichip algorithm (RMA) (Irizarry et al. 2003), normalised per chip to the median, and analyzed with Genespring 7.2. (Agilent, USA). The genes detected to be present in the data from all four microarrays were passed to further analysis. Differentially expressed probe sets were selected based on filtering by the fold change (±1.2-fold). The lists of the obtained up- and down-regulated probe sets were inspected for the enriched Gene Ontology (GO) terms and the KEGG pathways among the genes by using the "David 2006" program (Dennis et al. 2003), and the genes were clustered based on the GO terms in order to detect possible subgroups of co-expressed genes with certain functions using the "TAFFEL" (Kurki et al. 2008).
4.6.3 GENE EXPRESSION ANALYSIS

The increased expression of leptin and β₃ adrenergic receptor (Adrb3) in the adipose tissue of high-calcium whey protein-fed mice was verified by quantitative real-time PCR. One μl of cDNA was subjected to a quantitative real-time polymerase chain reaction by the LightCycler instrument (Roche diagnostics, Neuilly-sur-Seine, France) for detection of leptin, Adrb3 and 18S mRNAs. The 18S served as a housekeeping gene. The quantities of leptin, Adrb3 and 18S PCR products were quantified with an external standard curve amplified from purified PCR product in the presence of 0.5 μM of the following primers: leptin forward AGAC-CGGGAAAGAGTG, reverse GCCATAGTGCAAGGTT; Adrb3 forward ACCAACGTGTTCGTGACT, reverse CAGCTAGGATCGGGTCCA; 18S forward ACATCCAAGGAAGGCAGCAG, reverse TTTCGTCAC-TACCTCCCG.

4.7 LIVER METABOLOMOMIC PROFILE (STUDY IV)

In order to detect energy restriction-induced changes in hepatic metabolism, the metabolomic profiling of liver samples was performed. Characterisation of changes in lipid species was performed with a lipidomic strategy using ultra performance liquid chromatography coupled to mass spectrometry (UPLC/MS). Additionally, a set of 13 primary metabolites (glucose-6-phosphate, fructose-6-phosphate, mannose-6-phosphate, fructose bisphosphate, glycerate-3-phosphate, ribose-5-phosphate, succinate, malate, citrate, pyruvate, phosphoenolpyruvate, 6-phosphogluconate and fumarate) was analysed in order to investigate the changes in energy metabolism. The analysis was conducted by high performance liquid chromatography tandem mass spectrometer (HPLC/MS/MS).

4.8 STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Statistically significant difference in mean values were tested by ANOVA, followed by Tukey’s test excluding the microarray and metabolomics data. Statistical analyses of metabolomics data (Study IV) were performed using the R statistical software (www.r-
and a Welch type t-test was used to analyse the microarray data (Study III).

P-values below 0.05 were considered statistically significant. The GraphPad Prism, version 4.02 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS, version 10.1 (SPSS Inc. Chicago, IL, USA) were used for the statistical analyses.
5 RESULTS

5.1 BODY WEIGHT

5.1.1 WEIGHT GAIN (STUDY I)

The effect of dairy proteins and calcium on weight gain differed signifi-
cantly depending on the type of dairy protein (Figure 3). The addition of
calcium in the casein-based diet did not affect weight gain during the 21-
week treatment period, but high calcium intake combined with whey pro-
tein inhibited weight gain (p<0.05). The final body weight in the High-
Ca Whey group (44.1±1.1g) was 7.9% lower than in the High-Ca group
(47.9±1.0g) and 8.3% lower than in the Control group (48.1±0.8g).

5.1.2 WEIGHT LOSS (STUDY II, IV AND UNPUBLISHED DATA)

Weight loss was induced by a 30% energy restriction from the ad libitum
energy intake. In Study II, the whey protein components (α-lactalbumin,
β-lactoglobulin and lactoferrin) enhanced weight loss in comparison with
the Control diet (p<0.001), whereas the weight loss in the High-Ca Whey
group did not reach statistical significance (Figure 3). In Study IV how-
ever, the High-Ca Whey diet accelerated weight loss significantly in com-
parison with the Control diet (p<0.001). The High-Ca or Whey diets did
not induce significant enhancement of weight loss.
5.1.3 WEIGHT RE-GAIN (STUDY II)

Even though whey protein-containing diets efficiently inhibited weight gain and accelerated weight loss, they were not able to attenuate the significant increase in body weight resulting from the *ad libitum* feeding after energy restriction (Figure 3).

![Figure 3](image)

**Figure 3.** Difference in mean body weight in comparison with the Control group (%).

5.2 AMOUNT OF ADIPOSE TISSUE AND ADIPOCYTE SIZE

5.2.1 WEIGHT GAIN (STUDY I AND III)

The body fat content of the mice increased significantly during the weight gain phase. However, when high calcium intake was combined with whey protein, the increase in body fat content was significantly smaller ($p<0.05$).
than in the High-Ca and Control groups (Figure 4). In addition, the mean adipocyte cross-sectional area was significantly smaller in the High-Ca Whey group than in the Control group (7458±147 μm² vs. 8012±156 μm², p=0.01) at the end of the weight gain period.

Figure 4. Difference in mean fat percentage in comparison with the Control group (%).

5.2.2 WEIGHT LOSS (STUDY II AND IV)

In addition to attenuating fat tissue gain, the High-Ca Whey diet also increased the fat tissue loss during weight loss (Figure 4). From the whey protein components, α-lactalbumin and lactoferrin increased the loss of fat tissue significantly in comparison with the Control diet (p<0.001), the fat percentage of the Lactoferrin group (24.5±1.6%) being even significantly lower than the fat percentage of the High-Ca Whey (31.0±1.9%) and the β-lactoglobulin (30.4±1.2%) groups (p<0.05) after weight loss. Even though the mean fat percentage in the High-Ca and Whey groups reached
the same level than in High-Ca Whey group, the reduction in fat percentage during weight loss was only significant in High-Ca Whey group.

The localisation of fat tissue loss was evaluated by weighing the fat pads (subcutaneous, epididymal, abdominal and perirenal). Weight loss effectively reduced the weight of the fat pads in all groups except for the epididymal fat, which was reduced significantly only in the α-lactalbumin (p<0.01) and Lactoferrin groups (p<0.001) (Figure 5). In Study IV, the High-Ca Whey diet significantly increased the reduction of all the fat pads in comparison with the Control group, whereas the effect of the High-Ca or Whey diets was not statistically significant.

Figure 5. The decrease in mean fat pad weight during weight loss in comparison with the mean fat pad weight before weight loss (%).

Adipocyte size after weight loss was analysed only in Study II. In the Control (4583±276 μm²) and β-lactoglobulin (4045±220 μm²) groups adipocyte size was not significantly different from the values obtained before
Results

weight loss (5340±400 μm²). However, adipocyte size after weight loss was significantly decreased in the α-lactalbumin (2381±209 μm², p<0.001 vs. before weight loss), Lactoferrin (3147±284 μm², p<0.05 vs. before weight loss) and High-Ca Whey (3824±408 μm², p<0.01 vs. before weight loss) groups. Also the distribution of adipocyte size varied between the groups, the variation in size being greatest before weight loss and after weight loss in the Control group.

5.2.3 WEIGHT RE-GAIN (STUDY II)

During the weight re-gain phase, the body weight of the mice returned to the level observed before weight loss. However, the amount of body fat was from 4.4±6.6% (Lactoferrin group) to 15.3±3.7% (α-lactalbumin) lower after weight re-gain (ANOVA p-value 0.331). The fat pad weights revealed that the α-lactalbumin diet significantly inhibited the accumulation of visceral fat during weight re-gain (p<0.05).

The adipocyte size increased in all of the groups during the weight re-gain phase. The largest adipocytes after weight re-gain were found in the Control group (5337±359 μm²) while the size of adipocytes was slightly, but non-significantly, smaller in the High-Ca Whey (4879±454 μm²), α-lactalbumin (4567±204 μm²), β-lactoglobulin (4407±175 μm²) and Lactoferrin groups (4391±336 μm²) (ANOVA p-value 0.232). Also the distribution of adipocyte size was more similar between the groups after weight re-gain than after weight loss.

5.3 ENERGY INTAKE AND METABOLIC PERFORMANCE

There were no differences in energy intake between the groups in any of the studies. The mean *ad libitum* energy intake during weight gain (13.4±0.2 kcal/mouse/day in the High-Ca Whey; 13.3±0.5 kcal/mouse/day in the High-Ca and 12.5±0.01 kcal/mouse/day in the Control group, ANOVA p-value 0.290, Study I) was slightly, but not significantly, lower than the mean energy intake during weight re-gain (14.0±0.02 kcal/mouse/day in α-lactalbumin, 12.6±0.4 kcal/mouse/day in the β-lactoglobulin, 15.0±1.1 kcal/mouse/day in the Lactoferrin, 15.2±1.2 kcal/mouse/day in the High-
Ca Whey and 14.1±0.6 kcal/mouse/day in the Control group, ANOVA p-value 0.419).

In order to investigate the differences in drinking and feeding behaviour, activity and metabolic performance, the groups of Control and High-Ca Whey diet-fed mice were housed in a home cage-based monitoring system. A 7-day monitoring did not reveal differences in cumulative feed or water intake, respiratory exchange rate, heat production, O₂ consumption, CO₂ production, total or rearing activity or ambulatory movements during the observation period.

5.4 FAT ABSORPTION

The effect of different diets on fat excretion was analysed and the apparent fat absorption calculated based on the fat intake and fat excretion data. The fat absorption in the Control group did not differ significantly between the studies or between the weight gain, weight loss or weight re-gain phases (Table 6). The fat absorption in the High-Ca Whey group varied between 96.1±0.9% in the weight gain (Study I) and 98.0±0.3% in the weight re-gain phase (Study II), which resulted in a significant difference in comparison with the Control group in some (Studies I and IV), but not all studies (Study II). The addition of calcium in the study diets decreased fat absorption significantly in some, but not all, of the studies.
### Table 6. Apparent Fat Absorption in Different Groups During Weight Gain, Weight Loss and Weight Re-Gain (% of Fat Intake)

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
<th>Control</th>
<th>High-CA Whey</th>
<th>High-CA $\alpha$-Lactalbumin</th>
<th>High-CA $\beta$-Lactoglobulin</th>
<th>High-CA Lactoferrin</th>
<th>Whey</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Weight Gain</td>
<td>98.6±0.2</td>
<td>96.1±0.9*</td>
<td>97.6±0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight Loss</td>
<td>98.2±0.2</td>
<td>98.0±0.2</td>
<td>97.6±0.2</td>
<td>97.1±0.4</td>
<td>93.2±2.0**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight Re-Gain</td>
<td>98.4±0.1</td>
<td>96.7±0.3</td>
<td>95.8±1.3</td>
<td>96.6±0.3</td>
<td>96.9±0.6</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Weight Gain</td>
<td>98.0±0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight Loss</td>
<td>98.4±0.1</td>
<td>96.9±0.3*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Weight Gain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Weight Loss</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UN-PUBLISHED</td>
<td>Weight Loss</td>
<td>96.6±0.6**</td>
<td></td>
<td></td>
<td></td>
<td>98.1±0.2#</td>
<td></td>
</tr>
</tbody>
</table>

Data is presented as mean±SEM (n=8-10/group). * p<0.05, ** p<0.01 in comparison with control, # p<0.05 in comparison with high-CA whey and p<0.01 in comparison with high-CA $\alpha$-lactalbumin and $\beta$-lactoglobulin.
5.5 BLOOD GLUCOSE AND SERUM INSULIN (STUDIES I, II AND IV)

Blood glucose was measured at the end of the weight gain (Studies I and IV), weight loss (Studies II and IV) and weight re-gain phases (Study II). The changes in body weight did not alter blood glucose significantly in the Control group either in Study II or Study IV. Weight loss on the High-Ca Whey diet resulted in significantly decreased blood glucose level in Study IV (p<0.001 in comparison with the values observed before weight loss) but not in Study II. The level of serum insulin was also significantly lowered as a result of weight loss in the High-Ca Whey (p<0.01 in comparison with the values observed before weight loss and energy restricted Control group) but not in the Control group (Study IV).

5.6 ADIPOSE TISSUE GENE EXPRESSION (STUDY III)

In order to investigate the effects of the High-Ca Whey diet on adipose tissue metabolism, the gene expression profiling of epididymal adipose tissue was carried out using the Affymetrix Mouse Genome 430 2.0 array. The microarray analysis of two representative samples per group (Control and High-Ca Whey) revealed significant changes in the expression of 129 genes, with a similar amount of up- and down-regulated genes in the High-Ca Whey group. The largest number of up-regulated genes was enriched in insulin signalling pathway, which contained five reporters with over 1.2-fold changes and fourteen genes with a smaller or insignificant difference in the expression. The second largest number of up-regulated genes was found in the adipocytokine signalling pathway, the down-regulated genes being clustered in the fatty acid metabolism pathway.

Based on the expression data and the pathways associated with altered genes, two interesting up-regulated genes that may transmit alterations of metabolism in the fat tissue were identified in the microarray data. The putative targets were adrenergic β3-receptor and leptin, which could be related to the inhibition of fat tissue gain in the High-Ca Whey group. The qRT-PCR analysis revealed a significant, 2.3-fold up-regulation (p=0.0002) in the expression of adrenergic β3-receptor in the High-Ca Whey group vs. the Control, and 2.1 times greater leptin mRNA expression (p=0.02).
5.7 LIVER (STUDY IV)

5.7.1 LIVER HISTOLOGY

With a view to investigating the potential mechanisms by which dairy products might lower the risk of insulin resistance and type 2 diabetes, the effect of weight loss on liver histology and fat accumulation was studied. The effect of weight loss on the Control diet was compared to that on the High-Ca whey diet.

After weight loss, the fat droplets were smaller and mainly present in the perivenular regions, while slight to severe macrovesicular fatty change of a diffuse pattern was observed in the obese mice. Minimal foci of inflammatory cells and necrotic hepatocytes were occasionally noted after weight loss, but fibrosis was absent.

Oil Red O staining revealed that weight loss significantly reduced hepatic lipid accumulation independent of the diet, even though the amount of lipids still failed to reach the level observed in the lean mice. Oil Red O staining demonstrated that weight loss on the Control and High-Ca Whey diet significantly reduced hepatic lipid accumulation (40.5±3.3% before weight loss vs. 23.8±3.5% in the Control and 17.5±2.9 in the High-Ca Whey group) and lipid droplet size (348.9±75.7, 98.9±18.6 and 58.8±7.8 arbitrary units before weight loss and in the Control and High-Ca Whey group, respectively), but the amount of fat did not reach the level observed in the lean mice (lipid accumulation 4.4±1.2% and lipid droplet size 21.3±1.3 arbitrary units).

5.7.2 LIVER METABOLOMIC PROFILE

UPLC/MS and HPLC/MS/MS -based metabolomic platforms were used to assess the effect of weight loss on liver metabolomic profile. Samples from the Control (n=10) and High-Ca Whey (n=10) weight loss groups were analysed and the results were compared against the Lean group (n=10) as well as the samples from animals that were sacrificed before weight loss (n=10, Obese group).

Weight loss significantly reduced the amount of triacylglycerols (TAG) (p>0.05 in the Control and p<0.01 in the High-Ca Whey vs. values before weight loss) and ceramides (p<0.05 in the Control and p<0.001 in the High-
Ca Whey vs. values before weight loss), while the amount of cholesterol esters was significantly increased (p<0.001 in the Control and p<0.01 in the High-Ca Whey vs. values before weight loss) in both weight loss groups. The distinct features of weight loss on the High-Ca Whey diet were the restoration of the level of lipotoxic ceramides to the level in lean mice, the significant decrease in the potentially diabetogenic diacylglycerol and the significant increases in the level of total phosphatidylserines, phosphatidylethanolamines and sphingomyelins (Figure 6). The High-Ca Whey diet specifically affected certain ceramide species (Cer(d18:0/22:5), Cer(d18:0/22:6), Cer(d18:1/23:3), Cer(d18:1/23:5), Cer(d18:1/26:4)), whose level was reduced to that of lean mice, but which was unaffected by weight loss on the control diet.

**Figure 6.** Fold change of top 15 up- and down-regulated metabolites after weight loss in the High-Ca Whey group in comparison with the Control group.
The primary metabolite analysis revealed that weight loss on the High-Ca Whey diet was associated with significant increase of succinate, belonging to the TCA cycle, and of ribose-5-phosphate, a product of the pentose phosphate pathway. The High-Ca Whey diet also decreased the level of glycolytic metabolites: glucose-6-phosphate, fructose-6-phosphate and fructose bisphosphate in contrast with weight loss on the control diet, which did not affect the level of these metabolites. The overall effect of the treatments on primary metabolite pathways is presented in Table 7 and Figure 7.

**TABLE 7. CONCENTRATIONS OF PRIMARY METABOLITES IN LIVER SAMPLES (NMOL/G TISSUE).**

<table>
<thead>
<tr>
<th>ENERGY RESTRICTION</th>
<th>LEAN</th>
<th>OBESE</th>
<th>CONTROL</th>
<th>HIGH-CA WHEY</th>
<th>THE EFFECT OF HIGH-CA WHEY IN COMPARISON WITH CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28.6±2.7</td>
<td>19.2±3.5</td>
<td>22.7±1.7º</td>
<td>11.2±3.2</td>
<td>↓</td>
</tr>
<tr>
<td>GLUCOSE-6-PHOSPHATE</td>
<td>7.0±0.7</td>
<td>4.9±0.6º</td>
<td>6.9±0.7º</td>
<td>3.2±0.9</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>1.7±0.2º</td>
<td>1.7±0.2º</td>
<td>1.4±0.1º</td>
<td>0.8±0.2</td>
<td>↓</td>
</tr>
<tr>
<td>MANNOSE-6-PHOSPHATE</td>
<td>3.2±0.6ºb</td>
<td>8.7±2.0ºc</td>
<td>9.5±2.4º</td>
<td>0.5±0.1</td>
<td>↓</td>
</tr>
<tr>
<td>FRUCTOSE BISPHOSPHATE</td>
<td>26.2±3.7</td>
<td>21.0±0.8</td>
<td>17.4±1.2</td>
<td>15.1±1.4</td>
<td>← ↔ →</td>
</tr>
<tr>
<td>GLYCERATE-3-PHOSPHATE</td>
<td>0.3±0.02º</td>
<td>0.7±0.1º</td>
<td>0.7±0.1º</td>
<td>1.3±0.1</td>
<td>↑</td>
</tr>
<tr>
<td>RIBOSE-5-PHOSPHATE</td>
<td>24.5±3.3º</td>
<td>5.3±1.1º</td>
<td>6.7±2.4º</td>
<td>24.0±4.0-promoter</td>
<td>↑</td>
</tr>
<tr>
<td>SUCCINATE</td>
<td>42.0±5.2º</td>
<td>54.5±3.7º</td>
<td>61.3±4.7º</td>
<td>40.5±5.1-promoter</td>
<td>↓</td>
</tr>
<tr>
<td>MALATE</td>
<td>4.9±0.7º</td>
<td>2.0±0.3º</td>
<td>1.6±0.2º</td>
<td>1.8±0.3</td>
<td>↓ ↔ →</td>
</tr>
<tr>
<td>CITRATE</td>
<td>5.6±0.8º</td>
<td>0.9±0.2º</td>
<td>2.6±0.4º</td>
<td>1.5±0.3</td>
<td>↓ ↔ →</td>
</tr>
<tr>
<td>PYRUVATE</td>
<td>2.8±0.8ºa</td>
<td>2.3±0.3º</td>
<td>1.4±0.2º</td>
<td>1.2±0.2</td>
<td>↓ ↔ →</td>
</tr>
<tr>
<td>PHOSPHOENOLPYRUVATE</td>
<td>3.5±0.2ºb</td>
<td>2.4±0.2ºc</td>
<td>3.7±0.2º</td>
<td>2.6±0.4ºc</td>
<td>↓</td>
</tr>
<tr>
<td>6-PHOSPHOGLUCONATE</td>
<td>9.6±1.0ºa</td>
<td>10.4±1.1º</td>
<td>17.8±1.7º</td>
<td>11.9±1.3º</td>
<td>↓</td>
</tr>
</tbody>
</table>

_DATA ARE PRESENTED AS MEAN±SEM (N=10/GROUP). MEANS WITHOUT A COMMON LETTER DIFFER (P<0.05)._
Figure 7. The presentation of the effect of Whey+Ca diet and energy restriction (ER) on primary metabolism.
6 DISCUSSION

Previous epidemiological and experimental studies have shown that dairy intake has beneficial effects on body weight and the amount of body fat. Population studies have also indicated that increased dairy consumption lowers the risk of developing insulin resistance and type 2 diabetes. However, the mechanisms and other potential active components other than calcium, are not well known. Moreover, the effectiveness and mechanisms of calcium intake on body weight is a highly controversial area of research. This study investigated the effects of dietary intake of calcium and dairy proteins on body weight and fat tissue in an experimental model of diet-induced obesity. The potential effects of dairy protein and calcium intake on liver metabolism, the central element in the development of metabolic syndrome and type 2 diabetes, was also studied.

6.1 METHODOLOGICAL ASPECTS

ANIMAL MODEL

The animal model used in these studies is a well-established model of diet-induced obesity (Surwit et al. 1988, Collins et al. 2004, Koza et al. 2006). The C57Bl/6J mouse strain is often used as a healthy control animal and as a basis for genetically modified animal models (Rivera and Tessarollo 2008). When fed a standard laboratory rodent chow, the mean body weight of these mice stays below 35g and the body fat percentage is on average 25% (Denier et al. 2008). Therefore, this mouse strain serves as a better model of typical human obesity than other widely used commercial obese mouse models, such as ob/ob (leptin-deficient) or db/db (leptin-resistant), which are characterised by hyperphagia (Carroll et al. 2004).
Despite the wide use of C57Bl/6J mice in obesity research, the exact mechanisms explaining their weight gain are not completely understood. The weight gain cannot be simply explained by changes in energy intake (Lin et al. 2000, Collins et al. 2004, Koza et al. 2006). Other mechanisms, such as reduced locomotor activity (Bjursell et al. 2008), decreased leptin (Surwit et al. 1997) and $\beta_3$-adrenergic responsiveness (Collins et al. 1997, Collins et al. 1999) and altered Wnt signalling (Koza et al. 2006), are likely to explain part of the obesity-prone phenotype in these mice.

The length of the weight gain period in the current studies is relatively long when compared with what has been reported in the literature (Zemel et al. 2000, Sun and Zemel 2004a, 2006). This ensures that at the end of the weight gain phase, the mice have significantly higher body weight and more body fat than the parallel group of mice on a low-fat diet (unpublished data, Study I), and that the effects really indicate obesity, not a normal growth of the animals. This has not always been the case in previous studies on dietary calcium intake and body weight (Sun and Zemel 2004a).

**COMPOSITION OF THE STUDY DIETS**

In all of the studies, the mice were fed with a high-fat diet in order to induce obesity. The protein source and calcium content of the high-fat diet was modified. The amount of protein in the diet (18% of energy) was the same as in a standard laboratory chow (Harlan Teklad 2018, Harlan Holding, Inc., Wilmington, DE, USA) in all of the diets. Thus the effects of the diets are not confounded by an increase in protein intake, which is known to affect body weight and the amount of fat tissue (Halton and Hu 2004). In these experimental diets, all protein was replaced by the protein of interest. This approach clearly separates the effects of different protein sources and helps to distinguish between the potential differences in the proteins in an experimental setting. However, the obvious disadvantage of this setting is that the relative amount of a certain dietary protein type is only a fraction of this in real-life conditions.

Since calcium intake is suggested to explain at least part of the effect of dairy intake on body weight (Zemel 2001), we studied the effect of whey and casein in both low- (0.4%) and high-calcium (1.8%) diets. In case of
α-lactalbumin, β-lactoglobulin and lactoferrin, the effect of proteins and calcium cannot be separated, since these proteins were not studied within low-calcium matrices. However, when investigating the effect of dairy-derived components, the presence of calcium in the diet is natural. In milk, calcium is mainly integrated with caseins, not whey proteins, but whey-derived peptides are also known to bind calcium (Vegarud et al. 2000). Calcium may also interact with various dairy protein-derived peptides and affect calcium metabolism and bioavailability (Narva et al. 2004, Jauhiainen and Korpela 2007). It can therefore be reasoned that the presence of calcium in the diet gives a more reliable estimate on the effects of dairy proteins, even though the effect of calcium and protein cannot be separated.

CHARACTERISATION OF METABOLIC CHANGES IN ADIPOSE TISSUE AND THE LIVER

Affymetrix microarray procedure was utilised to determine the High-Ca Whey diet-induced changes in adipose tissue. Microarrays provide a practical approach for screening the expression of thousands of genes simultaneously (Schena et al. 1995). Since the understanding of calcium and whey protein intake-activated pathways in adipose tissue is scarce, the genome-wide screening provides a good basis for creating hypothesis of the possible mechanisms. The microarray procedure was performed with the high-accuracy Affymetrix Mouse Genome 430 2.0 array (Alberts et al. 2007) with two representative samples per group in order to avoid the possible bias resulting from pooling the samples before hybridisation (Mary-Huard et al. 2007). However, the restricted number of replicates is the principal limitation of the microarray results (2/group), and consequently the results should be interpreted with caution. Despite the fact that the use of an inbred and highly homogenous animal model under strictly controlled research conditions decreases inter-individual variation in the results, the enormous data matrices produced by the microarray method inevitably contain noise and uncertainty, which have to be filtered and modified by different computer-based techniques. This also limits the accuracy of interpreting significant results and, without further confirmation of the results with traditional techniques, such as qRT-PCR, the results can only be considered preliminary.
Hepatic lipid and primary metabolite profile was investigated by two high-throughput metabolomic platforms. A UPLC/MS-based method was used for screening the lipid species and an HPLC/MS/MS-based platform for the detection of primary metabolites. MS-based methods are considered sensitive for characterising, identifying, and quantifying a large number of compounds in a biological sample in which metabolite concentrations might cover a broad range (Kaddurah-Daouk et al. 2008). Metabolomics, even though a novel technique, is seen as a particularly valuable tool especially in the area of nutrition (Whitfield et al. 2004) and multifactorial diseases, such as obesity and metabolic syndrome (Griffin and Nicholls 2006). Nevertheless, the most critical shortcoming of the method is the limited identification of spectral peaks and the lack of universal metabolomic databases.

Taken together, the present study has been conducted by using a wide array of methods suitable for the research question. The animal model and study design are appropriate for investigating the effect of nutritional components in complex diseases, such as obesity and metabolic syndrome. Furthermore, the long duration of the interventions corresponds well with the chronic nature of the diseases. Novel methods have been utilised in studies for screening the genome- and metabolome-wide effects of dairy proteins but especially microarray analysis may have benefited from having a larger number of replicates.

6.2 EFFECT OF DAIRY PROTEINS AND CALCIUM INTAKE ON BODY WEIGHT AND THE AMOUNT OF ADIPOSE TISSUE

Effect of protein type
Dairy proteins have been postulated to explain the more pronounced effect of dairy on body weight in comparison with calcium supplementation (for review, see Zemel 2004, 2005). However, the effect of dairy proteins on body weight has not been compared prior to the present study. The current results indicate that whey proteins and especially α-lactalbumin and
lactoferrin have body weight and body composition-modulating properties when administered in combination with a high-calcium diet.

Whey proteins may suppress food intake in humans by increasing satiety (Hall et al. 2003, Anderson et al. 2004, Bellissimo et al. 2008), but no differences in the food intake of the mice were detected between the treatment groups in this study. The amino acid composition of whey proteins or the substantial amount of branched-chain amino acids has often been suggested to explain their health effects (for review, see Ha and Zemel 2003, for review, see Yalcin 2006). Especially an increased intake of leucine, which stimulates muscle protein synthesis (Stipanuk 2007) and has a role as an energy substrate, may be beneficial in the treatment of obesity and metabolic syndrome (for review, see Layman and Walker 2006, Zhang et al. 2007b). The amount of leucine in the High-Ca Whey diet was nearly 1.5-fold in comparison with the Control diet, and leucine intake may explain the differences in body weight and fat between these two groups. However, the amount of leucine in the High-Ca Whey diet was 1.2 to 1.3-fold greater than in the α-lactalbumin and Lactoferrin diets which enhanced weight loss more effectively than the High-Ca Whey protein diet. This suggests the effect of whey proteins on body weight and fat tissue to be dependent on factors other than leucine intake alone.

Whey protein-derived bioactive peptides have also been hypothesised to mediate the body weight-regulating effects of dairy proteins. Dairy-derived peptides have beneficial effects for example on blood pressure (for review, see Jauhiainen and Korpela 2007), bone metabolism (Narva et al. 2007) and gastrointestinal health (for review, see Severin and Wenshui 2005), and therefore the peptide hypothesis serves as an appealing explanation in the field of obesity as well. Bioactive peptides are formed during digestion in the gastrointestinal tract, but the precise peptide profile which is absorbed after the intake of different dairy proteins, remains unknown. Detecting the potential peptides with adipose tissue metabolism-modulating properties would require the development of a specific peptidomics platform (Schulz-Knappe et al. 2005) for postprandial peptides of dairy origin. Currently, the key focus of peptidomics platforms is still on the identification of endogenous peptides (Schrader and Selle 2006, Tammen et al. 2007) and thus the identification of potential dairy-derived anti-obesity peptides represents a major challenge.
EFFECT OF CALCIUM INTAKE

The findings of this study support the previous epidemiological and clinical evidence of the body weight-modulating effect of dairy intake. However, in this series of studies the role of dietary calcium was not in line with previous experimental data. The High-calcium diet did not significantly inhibit weight gain or enhance weight loss in contrast to earlier findings (Zemel et al. 2000, Shi et al. 2001a, Parra et al. 2007), and the role of 1,25(OH)₂D₃ in the regulation of body weight was not supported by the results of this study. Instead, the current findings give more evidence to the hypothesis that whey proteins are a source of body weight-modulating components (for review, see Zemel 2005).

The discrepancy between the findings of this study and the previous experimental results may be influenced by the animal model. The data from Zemel and co-workers are based on experiments on aP2-agouti-transgenic mice. This mouse expresses the agouti gene in adipose tissue under the control of aP2-promoter and thus resembles human adipocytes in the expression pattern of the agouti gene (Mynatt et al. 1997). The agouti protein stimulates calcium influx into the adipocytes as well as the expression and activity of fatty acid synthase (Jones et al. 1996, Xue and Zemel 2000). Similarly to other mice, the C57BL/6J-mice lack the expression of agouti in their adipocytes and hence the basal expression and activity of fatty acid synthase are likely to differ from those in aP2-agouti-transgenic mice. Since fatty acid synthase expression and activity represent a key variable in the 1,25(OH)₂D₃-centered theory on the body weight-regulating effect of calcium, the aP2-agouti-transgenic mice might be more sensitive to the effects of calcium.

Increased faecal fat excretion has been suggested to explain at least part of the effects of calcium and dairy on body weight. This study confirmed that high-calcium diets have an effect on fat excretion resulting in a small, and in some circumstances significant, reduction of fat absorption. In addition, these results pointed out the importance of measuring the fat ingestion during faecal collection, a variable which has not been determined in all of the studies (Jacobsen et al. 2005, Boon et al. 2007b, Lorenzen et al. 2007). Even though fat absorption was decreased in the High-Ca Whey group and not in the High-Ca group during weight gain, an equivalent
difference between these diets was not seen in all of the studies. Neither consistent differences in the fat absorption between the individual whey protein components during weight loss and weight re-gain were detected. Hence the question of why dairy calcium affects fat absorption more efficiently than supplemental calcium (Lorenzen et al. 2007) cannot be answered by the effect of dairy proteins.

To summarise, whey protein-containing high-calcium diets inhibited body weight gain and accumulation of fat tissue. Whey protein-containing high-calcium diets also accelerated body weight and fat tissue loss during energy restriction in high-fat-fed C57Bl/6J mice. Calcium supplementation alone did not significantly affect body weight or the amount of fat tissue. High-calcium diets decreased fat absorption slightly independent of the protein source of the diet, which augments, but does not fully explain, the effect on body weight. The amino acid content of whey proteins and the amount of leucine may play a role in the effects on body weight, but the significant enhancement of weight loss on the α-lactalbumin and lactoferrin diets cannot be explained by the leucine content of the diets. The formation of anti-obesity bioactive peptides during protein digestion is a plausible, yet unverified, possibility and an interesting area of future research.

6.3 EFFECT OF DAIRY PROTEINS AND CALCIUM ON ADIPOSE TISSUE AND THE LIVER

ADIPOSE TISSUE GENE EXPRESSION

Microarray analysis revealed significant changes in the expression of 129 genes, with similar amount of up- and down-regulated genes in the High-Ca Whey group in comparison with the Controls. The qRT-PCR analysis confirmed the significant up-regulation of the β3-adrenergic receptor and leptin expression in the High-Ca Whey diet-fed mice. Interestingly, the expression of the β3-adrenergic receptor is down-regulated in response to
high-fat feeding in C57Bl/6J mice (Collins et al. 1997, Collins et al. 1999, Moraes et al. 2003), which is considered to be one of the factors contributing to diet-induced obesity. Leptin expression has also been found to be decreased in these mice in comparison with obesity-resistant mouse strains (Surwit et al. 1997, Watson et al. 2000). Thus, the High-Ca Whey diet showed a protective effect against high-fat diet-induced decline of the β3-adrenergic receptor expression and corrected the leptin expression in the direction normally seen in an obesity-resistant mouse strain. These changes are likely to contribute to the inhibition of weight gain. The significant up-regulation of leptin and the β3-adrenergic receptor expression is also connected with the insulin signalling pathway, which was enriched with the up-regulated genes.

Whey protein intake has been associated with insulin metabolism already previously, but this study showed the effect of whey protein on the level of adipose tissue gene expression for the first time. In comparison with casein, whey protein has a greater post-prandial insulinotropic effect (Nilsson et al. 2004, Tessari et al. 2007) resulting from the rapid amino acid absorption, the substantial amount of certain insulinotropic amino acids (leucine, isoleucine, valine, lysine, threonine) and the inhibition of dipeptidyl peptidase IV in the intestine, which leads to an increased concentration of incretin hormones, glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 (Gunnarsson et al. 2006, Nilsson et al. 2007). It is also of note that an increase in adipocyte size results in increased insulin resistance, at least in vitro (Olefsky 1977, Molina et al. 1989). Thus, smaller adipocyte size in the High-Ca Whey group could also partly explain the clustering of up-regulated genes in the insulin signalling pathway.

The development of adipose tissue insulin resistance limits the triglyceride storage capacity of adipose tissue, which results in ectopic fat accumulation in non-adipose tissue (for review, see Lewis et al. 2002). This, in turn, leads to a complex array of metabolic abnormalities characteristic of insulin-resistant states. The sensitisation of insulin and adipocyte-tokine signalling pathways as a result of the High-Ca Whey diet represents a mechanistic link between an increased intake of dairy products and a lower risk of metabolic syndrome and type 2 diabetes (Pereira et al. 2002, Azadbakht et al. 2005, Choi et al. 2005, Liu et al. 2005, Liu et al. 2006, Mi-
In line with this, the serum insulin and blood glucose values were improved to the level of healthy controls during weight loss on the High-Ca Whey diet but not on the Control diet. However, the lowering effect on blood glucose was not seen consistently in all of the studies.

LIVER METABOLISM

The liver lipidomic and primary metabolite profile was investigated after weight loss. In accordance with the previous studies, we found increased levels of triacylglycerols, diacylglycerols and specific ceramide species and observed down-regulation of sphingomyelins in the obese mice (Yetukuri et al. 2007). Weight loss on the High-Ca Whey diet significantly increased the level of sphingomyelins and decreased the level of diacylglycerol, restoring the relative level of ceramides and diacylglycerols to the level of lean animals. The accumulation of both ceramides and diacylglycerols in peripheral tissues contribute to insulin resistance (Stratford et al. 2004, Summers 2006, Holland et al. 2007b, Zhang et al. 2007a) and lowering ceramide levels in obese rodents, in particular, has been shown to ameliorate insulin resistance and prevent the onset of diabetes (Holland et al. 2007a). Therefore, the decrease of these lipids can be considered particularly beneficial. The level of the analysed primary metabolites was particularly affected in the High-Ca Whey group. Energy restriction in normal-weight, healthy mice enhances hepatic gluconeogenesis (Hagopian et al. 2003, Selman et al. 2006) and suppresses glycolysis (Dhahbi et al. 1999). The level of primary metabolites indicated that this effect was pronounced in the High–Ca Whey group, together with an enhanced flux through the pentose phosphate pathway. The metabolic changes in the liver were accompanied by a decrease in hepatic lipid content, which was clearly, but not significantly, greater in the High-Ca Whey than in the Control group.

Lipid accumulation in the liver is currently considered to be one of the cornerstones in the development of insulin resistance and metabolic syndrome (for review, see Yki-Järvinen 2005, for review, see Kotronen and Yki-Järvinen 2007). These results clearly suggest that a High-Ca Whey diet may beneficially modulate the hepatic lipid profile targeting specifically the lipotoxic ceramide and diacylglyceride species.
Taken together, these results demonstrate that a High-Ca Whey diet has beneficial effects on both adipose tissue and liver metabolism. The microarray data suggest sensitisation of insulin signalling as a result of High-Ca Whey feeding, which, in theory, limits the ectopic fat storage. Together with the less lipotoxic and diabetogenic hepatic lipid profile, these findings provide a novel mechanistic link to explain the epidemiological findings which support the role of increased dairy intake in the prevention of metabolic syndrome and type 2 diabetes. Since the microarray findings are based on only two representative samples per group, they should be interpreted with caution.

6.4 CLINICAL RELEVANCE

The aim of obesity treatment in healthcare is to prevent and treat other chronic diseases, such as type 2 diabetes, metabolic syndrome and hypertension (Finnish Current Care guidelines, 2006). The goal in obesity treatment is a permanent 5–10% weight loss, whereby the significantly lower body weight by 5 to 10% in the High-Ca Whey group after weight gain and weight loss corresponds well with the current recommendations. However, the results of an animal experiment are not by any means directly applicable to a clinical situation. In the clinical studies investigating the effect of dairy products on weight loss, the amount of weight loss in the high dairy group has been nearly two-fold in comparison with the controls (Zemel et al. 2004, Zemel et al. 2005a, Zemel et al. 2005b). In these studies, subjects in the high dairy group have been given yogurt or some other type of traditional dairy products in which whey proteins account for 20% of the milk proteins (Fox and McSweeney 1998). The results of this study suggest that increasing the amount of whey proteins could enhance the effect of a high dairy diet.
7 SUMMARY AND CONCLUSIONS

The present study investigated the effects of dairy protein and calcium intake in the prevention and treatment of diet-induced obesity as well as the associated effects on adipose tissue and liver metabolism in order to characterise the potential mechanisms explaining the reduction of the risk for metabolic syndrome and type 2 diabetes that have been reported in previous observational studies.

The main findings of the present study are as follows:

1. OBESITY

- The intake of calcium in combination with whey protein inhibits weight and fat gain and accelerates weight and fat loss in high-fat diet-fed C57Bl/6J mice. α-lactalbumin was the most beneficial protein showing significantly accelerated fat and weight loss and significantly reduced amount of visceral fat after weight re-gain period.

- The intake of calcium slightly decreases fat absorption, but this does not fully explain the results on body weight and body fat. A whey protein-containing high-calcium diet modifies the adipose tissue gene expression, having a protective effect against a high-fat diet-induced decline of β3-adrenergic receptor expression and increase of leptin expression. These changes are likely to contribute to the inhibition of weight gain.

2. ADIPOSE TISSUE AND LIVER METABOLISM

- A High-Ca Whey diet modifies the metabolism of both adipose tissue and the liver. The potential sensitisation of insulin signalling in adipose tissue, together with the less lipotoxic and diabetogenic hepatic lipid profile suggest a mechanistic link to explain the epidemiological findings on increased dairy intake in the prevention of metabolic syndrome and type 2 diabetes.
ACKNOWLEDGEMENTS

This study was carried out in 2005–2008 at the Institute of Biomedicine, Pharmacology, University of Helsinki.

I owe my deepest gratitude to my supervisors Professor Eero Mervaala, MD, PhD and Professor Riitta Korpela, PhD. It has been a privilege to work under their guidance. I sincerely admire Professor Eero Mervaala’s ability to see the big picture without losing the focus on molecular details and I am genuinely grateful for his endless support and advice during this thesis. His open-mindedness and enthusiasm towards scientific challenges has been an essential cornerstone of this thesis. I wish to express my sincerest gratefulness to Professor Riitta Korpela for inspiring and encouraging me to step on the path of science. Her infinite passion towards science has guided my way through this work. She has been a fundamental source of inspiration and energy during this thesis and her never-ending support can not be thanked enough.

Professor Esa Korpi, the Head of the Institute of Biomedicine, is sincerely acknowledged for the opportunity to work at the Institute of Biomedicine, Pharmacology. I also owe my deepest appreciation to Professor Tiina Mattila-Sandholm, Senior Vice President of Valio R&D, for the opportunity to finalise this thesis alongside my permanent work at Valio R&D.

I am most grateful to the official reviewers of this thesis, Docent Kirsii Pietiläinen and Professor Ilkka Pörsti, for the rapid and fluent review process and for their constructive suggestions to improve my thesis.

My co-authors are warmly acknowledged for their expertise and fruitful collaboration. I owe my warmest thanks to Markus Storvik, PhD at the University of Kuopio, whose expertise in bioinformatics was a crucial resource during this thesis. Professor Matej Oresic, Docent Tuulikki Seppänen-Laakso and Helena Simolin, MSc at VTT are sincerely acknowledged for introducing me to the fascinating world of metabolomics. I am grateful to Professor Karl-Heinz Herzig at the University of Oulu and Anne Huo-
tari, MSc at the University of Kuopio for the efficient collaboration in the metabolic performance study. I also wish to thank Piet Finckenberg PhD, Minna Huttunen PhD and Saara Harala MSc for their expertise and valuable cooperation in this thesis.

I owe my warmest thanks to Professor (emeritus) Heikki Vapaatalo. His advice and support during these years have been an essential source of encouragement.

My special thanks go to my co-authors and closest co-workers at the Institute of Biomedicine, Saara Merasto MSc and Marjut Louhelainen MSc for all their advice and support during these years and the memorable moments in and outside of the laboratory. I also owe my warmest gratitude to Juha Ketonen MSc for sharing the enjoyable moments and discussions in the laboratory at the end of each experimental series. Erik Vahtola MSc, Teemu Aitta-Aho MSc, Kim Lemberg MD, Martin Ranna MD and all the other PhD students and co-workers at the Institute of Biomedicine, Pharmacology are warmly thanked for the joyful moments, help and advice during these years. Ms Sari Laakkonen is greatly acknowledged for her valuable contribution in the animal facilities; without her this work would not have been possible. Mrs Anneli von Behr, Mrs Nada Bechara-Hirvonen, Mrs Sonja Latvakoski and Mrs Anne-Maria Riihimäki are also warmly thanked for their expertise and technical assistance during this thesis.

I owe my warmest thanks to the Nutraceuticals research group and my colleagues at Valio R&D. The support and company of the girls, Kajsa Kajander PhD, Tiina Jauhiainen PhD, Riina Kekkonen PhD, Eveliina Myllyluoma PhD, Katja Hatakka PhD and Laura Piirainen PhD, has been the most valuable source of motivation and encouragement during the best and worst times of this process. Thank you for this unforgettable experience, the lists and procedures you created, and all the unforgettable moments during this journey. I am truly thankful to all my other co-workers at Valio R&D, Nutrition and Health unit: Anikki, Tuula, Netta, Mikko, Minna, Maarit, Leena, Anu, and Hanna. It has been a great joy to share the final steps of this thesis in such a wonderful company. I also wish to thank Matti Harju PhD, Olli Tossavainen PhD, Outi Kerojoki MSc and Pia Ollikainen MSc at Valio R&D for guiding me into the fascinating world.
of dairy proteins. Your expertise and advice have been valuable throughout this thesis.

I owe my whole-hearted gratefulness to my friends outside of the academic and Valio worlds. Maiju & Happis, Ira & Sami, Anna-Mari, Kisse, Laura & Esko, Köpi, Hanko girls and many others – thank you for being there for me during these years and reminding me of the joyful life outside of science. The cheerful moments with you have been a crucial source of energy, without which this book would never had been completed. My sincerest gratitude belongs also to the Team Kissala for the understanding and encouragement during the final stages of this thesis.

Last, I owe my deepest and warmest gratefulness to my family, my mother Marja, my brothers Eero and Timo with their families Aulikki, Laura, Lilli and Nuppu. Your love and existence has been the empowering force throughout this process. My parents-in-law Ritva & Rami are thanked for their endless support and encouragement during these years. Finally, I wish to express my deepest gratefulness to my dear husband Tero for his loving support, patience and admirable understanding – thank you for standing by my side.

This thesis was primarily supported by the Foundation for Nutrition Research, Helsinki, Finland and Valio Ltd, Helsinki, Finland. It was also supported by the Sigrid Juselius Foundation, Academy of Finland and the grants from the Finnish Association of Academic Agronomists, the Finnish Association for the Study of Obesity and the Finnish Pharmacological Society.

Helsinki, September 2008

Taru Pilvi
REFERENCES


BOON N, GOOSSENS GH, BLAAK EE, SARIS WH. The effects of hydralazine on lipolysis in subcutaneous adipose tissue in humans. Metabolism 2007a: 56; 1742–1748.


BOWEN J, NOAKES M, CLIFTON PM. Appetite regulatory hormone responses to various dietary proteins differ by body mass index status despite similar reductions in ad libitum energy intake. J Clin Endocrinol Metab 2006a: 91; 2913-2919.


CARRUTH BR, SKINNER JD. The role of dietary calcium and other nutrients in moderating body fat in preschool children. Int J Obes Relat Metab Disord 2001: 25; 559-566.


GRIFFIN JL, NICHOLLS AW. Metabolomics as a functional genomic tool for understanding lipid dysfunction in diabetes, obesity and related disorders. Pharmacogenomics 2006; 7; 1095-1107.


GUNNARSSON PT, WINZELL MS, DEACON CF, LARSEN MO, JELIC K, CARR RD, AHREN B. Glucose-induced incretin hormone release and inactivation are differently modulated by oral fat and protein in mice. Endocrinology 2006; 147; 3173-3180.


hu g, qiao q, tuomilehto j, balkau b, borch–johnsen k, pyörälä k, group ds. prevalence of the metabolic syndrome and its relation to all-cause and cardiovascular mortality in non-diabetic european men and women. arch intern med 2004: 164; 1066–1076.

irizarry ra, hobbs b, collin f, beazer–barclay yd, antonellis kj, scherf u, speed tp. exploration, normalization, and summaries of high density oligonucleotide array probe level data. biostatistics 2003: 4; 249–264.

international standard idf 5b, iso 1735. determination of fat content. 1986.


jacqmain m, doucet e, despres jp, bouchard c, tremlay a. calcium intake, body composition, and lipoprotein–lipid concentrations in adults. am j clin nutr 2003: 77; 1448–1452.

jauhiainen t, korpela r. milk peptides and blood pressure. j nutr 2007: 137; 825s–829s.

jauhiainen t, vapaatalo h, poussa t, kyronpalo s, rasmussen m, korpela r. lactobacillus helveticus fermented milk lowers blood pressure in hypertensive subjects in 24–h ambulatory blood pressure measurement. am j hypertens 2005: 18; 1600–1605.

jenness r, larson bl, mcmeekin tl, swanson am, whitnah ch, whitney rm. nomenclature of the proteins of bovine milk: report of the committee on milk protein nomenclature, classification, and methodology of the manufacturing section of a.d.s.a. j dairy sci 1956: 39; 536–541.

jones bh, kim jh, zemel mb, woychik rp, michaud ej, wilkinson wo, moustaid n. upregulation of adipocyte metabolism by agouti protein: possible paracrine actions in yellow mouse obesity. am j physiol 1996: 270; e192–e196.


kahn se, hult rl, utzschneider km. mechanisms linking obesity to insulin resistance and type 2 diabetes. nature 2006: 444; 840–846.

kanehisa m, goto s, hattori m, aoki–kinoshita kf, itoh m, kawashima s, katayama t, araki m, hirakawa m. from genomics to chemical genomics: new developments in kegg. nucleic acids res 2006: 34; d354–d357.


LATNER JD, SCHWARTZ M. The effects of a high-carbohydrate, high-protein or balanced lunch upon later food intake and hunger ratings. Appetite 1999: 33; 119–128.


LIEN EL. Infant formulas with increased concentrations of alpha-lactalbumin. Am J Clin Nutr 2003: 77; 1555S–1558S.


MOORE LL, BRADLEE ML, GAO D, SINGER MR. Low dairy intake in early childhood predicts excess body fat gain. Obesity (Silver Spring) 2006: 14; 1010-1018.


ORIGINAL PUBLICATIONS