METABOLIC EFFECTS OF WHEY PROTEINS IN AN EXPERIMENTAL MODEL OF DIET-INDUCED OBESITY

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ACADEMIC DISSERTATION

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To my family
TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS .................................................................................. 6
MAIN ABBREVIATIONS ................................................................................................. 7
ABSTRACT ...................................................................................................................... 8
1 Introduction ................................................................................................................. 10
2 Review of the literature .............................................................................................. 11
  2.1 Obesity .................................................................................................................. 11
    2.1.1 Obesity and its classification ............................................................................. 11
    2.1.2 The consequences of obesity .......................................................................... 11
    2.1.3 The treatment of obesity ................................................................................ 13
  2.2 Whey proteins ...................................................................................................... 13
    2.2.1 Whey proteins and its components ................................................................. 14
    2.2.2 The digestion and absorption of whey proteins ............................................. 20
  2.3 Health effects of whey proteins .......................................................................... 21
    2.3.1 Obesity and whey proteins .............................................................................. 21
      2.3.1.1 Clinical studies ......................................................................................... 21
      2.3.1.2 Preclinical studies .................................................................................... 22
    2.3.2 Metabolic syndrome and whey proteins ....................................................... 31
    2.3.3 Type 2 diabetes and whey proteins ............................................................... 32
    2.3.4 Other health effects of whey proteins ............................................................ 33
    2.3.5 Possible mechanisms behind the anti-obesity effect of whey proteins .......... 33
      2.3.5.1 Whey proteins and satiety ........................................................................ 33
      2.3.5.2 The insulinotropic property of whey proteins .......................................... 34
  2.4 Whey proteins manufacturing ............................................................................. 35
    2.4.1 Traditional method ........................................................................................ 35
    2.4.2 Microfiltration method .................................................................................. 35
3 Aims of the study ...................................................................................................... 36
4 Materials and methods ............................................................................................ 37
  4.1 Study design ....................................................................................................... 37
  4.2 Experimental animals ........................................................................................ 37
  4.3 Diets and groups .................................................................................................. 38
  4.4 Body weight and energy intake measurements ............................................... 43
  4.5 Dual-energy X-ray absorptiometry (DEXA) measurement ............................. 43
  4.6 Oral glucose tolerance test .................................................................................. 43
  4.7 Faecal sample collection (Study I, II and III) .................................................... 43
LIST OF ORIGINAL PUBLICATIONS

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


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**MAIN ABBREVIATIONS**

ACE  
Angiotensin-converting-enzyme

AMPK  
Adenosine monophosphate (AMP)-activated protein kinase

AUC  
Area under the curve

BCAA  
Branched-chain amino acid

BMI  
Body mass index

BSA  
Bovine serum albumin

CMP  
Caseinomacropeptide

DEXA  
Dual-energy X-ray absorptiometry

ER  
Energy restriction

GMP  
Glycomacropeptide

IGF  
Insulin-like growth factor

LF  
Lactoferrin

LBM  
Lean body mass

LPS  
Lipopolysaccharide

LRP1  
Low-density lipoprotein-receptor-related protein 1

MCP-1  
Monocyte chemoattractant protein-1

MFNW  
Microfiltered native whey

mTOR  
Mammalian target of rapamycin

NAD⁺  
Nicotinamide adenine dinucleotide

NASH  
Non-alcoholic steatohepatitis

NAFL  
Non-alcoholic fatty liver

NAFLD  
Non-alcoholic fatty liver disease

OGTT  
Oral glucose tolerance test

PAI-1  
Plasminogen activator inhibitor-1

PP3  
Proteose-peptone component 3

qRT-PCR  
Quantitative real-time polymerase chain reaction

SIRT1  
Silent mating type information regulation-2 homolog 1

SIRT3  
Silent mating type information regulation-2 homolog 3

WPI  
Whey protein isolate
Milk proteins which make up 3.5% of the bovine milk are classified into casein and whey proteins. A high intake of milk proteins, especially whey proteins, has been shown to exert the beneficial effects on obesity and obesity related diseases in both humans and animals via unknown mechanisms. The aim of the present study was to investigate the potential of different whey proteins, i.e. whey protein isolate (WPI), alpha-lactalbumin (α-lac), lactoferrin (LF) and microfiltered native whey (MFNW), and their mechanisms of actions to prevent and treat diet-induced obesity and its consequences in C57Bl/6J mice.

In the present study, all of the tested whey proteins were given as the only protein source in high-fat diets with a constant protein (18 % of the energy): carbohydrate (21 % of the energy): fat (61 % of the energy) ratio. We used weekly body weight measurements, daily food intake monitoring, apparent fat digestibility, dual-energy X-ray absorptiometry, oral glucose tolerance test, monitoring of fat pad weights, as well as biochemical measurements in order to assess the metabolic effects of whey proteins.

Compared to casein, WPI (rich in lactoperoxidase, LF, growth factors and immunoglobulins) and LF accelerated weight and fat loss under energy restriction, and inhibited weight and fat regain during the ad libitum feeding after energy restriction without interfering with energy intake or apparent fat digestibility in C57Bl/6J mice. Both WPI and LF ameliorated fatty liver formation, and exerted beneficial effects on glucose tolerance under high-fat-feeding. The beneficial effects of WPI occurred in a dose-dependent manner. In addition, LF reduced the adipose tissue inflammation after weight regain, a property not shared with WPI. The further biochemical analysis indicated that these effects of both WPI and LF are mediated, at least partly, via the inhibition of mTOR nutrient sensing pathway and the activation of SIRT3 in the liver. Alpha-lac has been reported as one of the most effective whey protein fractions for accelerating weight and fat loss during energy restriction in the same mouse model. It was observed that the effects of α-lac on body weight and fat under energy restriction could be reproduced by supplying an amino acid mixture with an identical amino acid profile, which indicates that the anti-obesity effects of α-lac were mainly mediated by its individual amino acid composition. The MFNW produced by polymeric membranes using novel microfiltration technology, prevented weight gain and fat accumulation without interfering with energy intake or glucose homeostasis during ad libitum high-fat-feeding. The findings also suggest that the beneficial effects of MFNW are largely due to its rich α-lac content.

In summary, the intake of whey proteins exerts anti-obesity effects in C57Bl/6J mice during high-fat-feeding. WPI and LF enhance weight loss, prevent weight regain and ameliorate obesity induced fatty liver formation. The anti-obesity effects of WPI are attributable, to a large extent, to its LF content. The anti-obesity effects of α-lac are mainly due to its amino acid composition.
beneficial effects of MFNW point to a possible method to generate whey proteins with high bioactive value on a large scale.
1 Introduction

The worldwide prevalence of obesity, a condition that substantially elevates the risk of morbidity from hypertension, dyslipidemia, type 2 diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea and respiratory problems, as well as endometrial, breast, prostate, and colon cancers, poses a major public health challenge (National Institutes of Health, 1998). Obesity is also strongly associated with a chronic low-grade inflammation and a state of insulin resistance, which is a primarily result of the non-alcoholic fatty liver disease (NAFLD) (Angulo, 2007; Guillett et al., 2012). Weight loss via life style modification, i.e. the combination of dietary therapy, physical activity and behavioral therapy, is the mainstay of treatments for obesity (National Institutes of Health, 1998). Nutrition therefore plays a crucial role in the prevention and treatment of obesity and its consequences.

Epidemiological studies have shown that the intake of dairy products is related to reduced body mass index (BMI) (Mirmiran et al., 2005; Marques-Vidal et al., 2006; Varenna et al., 2007), and the risk of type 2 diabetes and metabolic syndrome (Crichton et al., 2011). Dietary calcium has been proposed to play a role in the effect of dairy products on body weight by increasing fat excretion (Christensen et al., 2009) and 1,25-dihydroxy-vitamin D₃-mediated alterations in adipocyte metabolism (Zemel, 2000; Shi et al., 2001b, 2002; Zemel & Miller, 2004). In addition, dairy proteins, especially whey proteins, have been postulated to account at least in part for the anti-obesity effect of dairy products (Zemel, 2005; Pilvi et al., 2007, 2009; Frestedt et al., 2008; Royle et al., 2008).

Normally, whey proteins are acquired by the ultrafiltration of whey produced during cheese making (Heino et al., 2007). However, the additives used in the manufacture of cheese may impair the functional properties and decrease the nutritional value of whey proteins. Microfiltration has been suggested as an excellent technique to remove the native whey from milk in order to enhance the quality of whey proteins (Brans et al., 2004). It has been reported that the MFNW exerts superior functional properties as compared with cheese whey protein (Heino et al., 2007).

The present study aimed to investigate the potential effects of different whey protein fractions and their mechanisms of actions in the prevention and treatment of diet-induced obesity and its consequences in an experimental model of diet-induced obesity.
2 Review of the literature

2.1 Obesity

2.1.1 Obesity and its classification

Obesity has been described as a chronic condition characterized by abnormal or excessive body fat accumulation (Gortmaker et al., 2011). The prevalence of obesity has markedly increased since the 1970s in the United States (Flegal et al., 1998). In the United States, the National Health and Nutrition Examination Survey (NHANES) revealed that the obesity prevalence in adults was 33.8% in 2007-2008, a number which had more than doubled as compared to that in 1976-1980 (Flegal et al., 1998, 2010). According to the World Health Organization (WHO), 2.8 million people die each year because they are overweight or obese (WHO, 2012). In global terms, the prevalence of obesity almost doubled between the years 1980 to 2008 (WHO, 2012).

There are various measures of obesity, among which body mass index (BMI) is most commonly used (Luke et al., 1997; National Institutes of Health, 1998). BMI is defined as a person’s weight (in kilograms) divided by the square of his or her height (in meters) (National Institutes of Health, 1998). Since BMI is the same for both genders and all ages of adults and significantly correlated with total body fat content, it provides the most useful population-level measure of overweight and obesity (Revicki & Israel, 1986; National Institutes of Health, 1998). The WHO defines a BMI greater than or equal to 25.0 kg/m\(^2\) as overweight; a BMI greater than or equal to 30.0 kg/m\(^2\) as obesity (National Institutes of Health, 1998). There are three grades of obesity, which are defined according to the different BMI levels (Table 1) (National Institutes of Health, 1998).

Table 1 Obesity grades.

<table>
<thead>
<tr>
<th>Obesity grades</th>
<th>BMI (kg/m(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>30≤BMI&lt;35</td>
</tr>
<tr>
<td>Grade II</td>
<td>35≤BMI&lt;40</td>
</tr>
<tr>
<td>Grade III</td>
<td>BMI≥40</td>
</tr>
</tbody>
</table>

2.1.2 The consequences of obesity

Obesity has been found to reduce average life expectancy; its major consequences are cardiovascular disease, type 2 diabetes, and the development of several cancers (Fig. 1) (Haslam & James, 2005). It substantially increases the risk of morbidity from gallbladder disease, osteoarthritis, sleep apnea and respiratory problems, hypertension, stroke, and non-alcoholic fatty liver disease (Fig. 1) (National
Institutes of Health, 1998; Haslam & James, 2005). Obesity is also associated with psychological and reproductive disorders (Fig. 1) (National Institutes of Health, 1998). In particular, visceral obesity, which is an excess intra-abdominal adipose tissue accumulation, is closely linked with diabetes mellitus, cardiovascular disease especially hypertension, and some cancers (Tchernof & Despres, 2013).

Fig. 1 The consequences of obesity.

As long ago as the 1970s, Sims and the colleagues (1973) observed that there were reversible increases in fasting concentration of glucose, insulin, triglycerides and impaired glucose tolerance in young men without a family history of diabetes, with a BMI of 28.0 kg/m² due to six months overfeeding. Weight gain has been reported to associate with enhanced insulin resistance (Swinburn et al., 1991) and glucose intolerance (Berger et al., 1975), which are the key factors in the development of diabetes. Approximately 90 % of patients with type 2 diabetes have BMI higher than 23.0 kg/m² (Stevens et al., 2001), and the risk of diabetes markedly increases if there has been early weight gain (Wannamethee & Shaper, 1999).

Non-alcoholic fatty liver disease is a disease spectrum including hepatic steatosis (non-alcoholic fatty liver, (NAFL)), non-alcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (Adams et al., 2005; Bjornsson & Angulo, 2007; Chiang et al., 2011). It refers to excessive fat accumulation, which is stored as triglycerides, in hepatocytes such that it exceeds 5 % of the liver weight (Angulo, 2007). NAFLD is closely linked to obesity (Marchesini et al., 1999), which is considered as one of the aetiological factors for NAFLD (Haslam & James, 2005). It has been proposed that in obese subjects,
the increased adiposity and insulin resistance contribute to the progression from NASH to fibrosis via the development of a profibrotic environment in liver (Chiang et al., 2011). In the general population, the prevalence of NAFLD has been reported as being between 10 - 24 % in different countries, but it is present in the majority (57.5 - 74 %) of obese people (Tarantino et al., 2007). NAFLD may even be detected in children: the overall incidence is 2.6 %, increasing up to 22.5 - 58.5 % in obese children (Tarantino et al., 2007).

2.1.3 The treatment of obesity

An energy imbalance resulting from a combination of an excessive energy intake and a lack of physical activity is considered to be the fundamental cause of overweight and obesity, although there are a limited number of cases which are due primarily to genetics, medical reasons, and psychiatric illness (National Institutes of Health, 1998; Bleich et al., 2008). Therefore, dietary and physical activity patterns play a pivotal role in the development of obesity. Life-style therapy, i.e. the combination of dietary therapy, physical activity and behavioral therapy, is the recommended treatment for obesity (National Institutes of Health, 1998). Pharmacotherapy and surgery are only considered as referral treatments for severe and resistant obesity (National Institutes of Health, 1998). At the individual level, by limiting energy intake from total fats, increasing consumption of fruit, vegetables, legumes, whole grains and nuts, limiting the sugar intake, and enhancing the amount of regular physical activity, individuals can achieve energy balance and this will prevent them from becoming overweight and obese (WHO, 2012). In the food industry, nutritional approaches are attracting more and more attention as valuable ways of enhancing the health-promoting quality of food in order to prevent the growing global epidemic of obesity.

2.2 Whey proteins

Bovine milk contains about 3.5 % proteins (Yalcin, 2006). These milk proteins are divided into casein and whey proteins; these separate when the pH of milk is lowered to 4.6 (Yalcin, 2006). Casein proteins are the phosphoproteins which precipitate from raw skimmed milk with acidification to pH 4.6 at 20 °C (Farrell et al., 2004). The proteins which remain in the supernatant of milk after precipitation of casein are defined as whey proteins (Yalcin, 2006). According to the homology of the amino acid sequences, the casein fractions can be further divided into α-s1- and α-s2-caseins, β-casein and κ-casein (Yalcin, 2006). Casein proteins are postulated to have various physiological functions, such as antibacterial, immunomodulatory, properties as well as enhancing the bioavailability of certain minerals (for reviews, see Vegarud et al., 2000; Bouhallab & Bougle, 2004; Meisel, 2005). In bovine milk, the average whey to casein ratio is 20: 80 (Yalcin, 2006). Although the amount of whey proteins in bovine milk is less than the amount of casein, the inexpensive source and high nutritional
quality of whey proteins have encouraged extensive investigation into its functional effects in humans (described in detail later).

2.2.1 Whey proteins and its components

Traditionally, whey is considered as a by-product of the cheese-making process (Krissansen, 2007). It is the fluid that remains after milk has been curdled and strained to remove the caseins. Whey includes proteins, lactose, vitamins, minerals, and traces of fat. Whey proteins consist of five different major proteins, including β-lactoglobulin, α-lactalbumin, glycomacropeptide (GMP) (depending on the manufacturing methods), proteose peptone 3, immunoglobulins, and bovine serum albumin (Krissansen, 2007). In addition, whey proteins contain lactoferrin (LF), lactoperoxidase, natural growth factor, and other minor proteins (Krissansen, 2007). It has been claimed that whey proteins can exert many different biological activities. These range from effects on bone (Takada et al., 1997; Aoe et al., 2001; Yamamura et al., 2002), muscle (Buckley et al., 2010; Pennings et al., 2011; Kanda et al., 2013), blood (Pins & Keenan, 2006; Petersen et al., 2009; Pal & Ellis, 2010b; Aldrich et al., 2011), immune system (Otani et al., 1995; Kayser & Meisel, 1996; Monnai M, 1997; Wong et al., 1997b; Ward et al., 2002; Legrand et al., 2005), cancer (Wang et al., 2000; Sternhagen & Allen, 2001; Varadhachary et al., 2004; Parodi, 2007), satiety (Bowen et al., 2006a, 2007; Diepvens et al., 2008), combatting infections (Brody, 2000; Campagna et al., 2004; Weinberg, 2007; Jenssen & Hancock, 2009), lipid metabolism (Moreno-Navarrete et al., 2009; Fernandez-Real et al., 2010; Ono et al., 2010), wound healing (Rayner et al., 2000), mood control (Markus et al., 2002; Orosco et al., 2004) and oxidative stress (Bouthegourd et al., 2002; Kent & Bomser, 2003). Whey proteins are also present as ingredients in different forms of pharmaceuticals, nutraceuticals and cosmeceuticals (for reviews, see Marshall, 2004; Yalcin, 2006; Krissansen, 2007). Due to the wide spectrum of bioactive effects of whey proteins and the current advances in processing technologies including ultrafiltration, microfiltration, reverse osmosis, and ion-exchange, whey proteins have been incorporated into various commercialized products (Table 2) such as dietary protein supplements (Marshall, 2004). The whey proteins of special therapeutic importance are α-lactalbumin, β-lactoglobulin, bovine serum albumin, immunoglobulins, lactoferrin and lactoperoxidase (Table 3).
Table 2 Commercialized whey proteins. a

<table>
<thead>
<tr>
<th>Products</th>
<th>Protein concentration</th>
<th>Lactose, fat and mineral content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein isolate</td>
<td>90 - 95 %</td>
<td>Little if any</td>
</tr>
<tr>
<td>Whey protein concentrate</td>
<td>25 - 89 %</td>
<td>Some lactose, fat and minerals</td>
</tr>
<tr>
<td></td>
<td>Most commonly available as 80 %</td>
<td>When protein concentration increases, fat, lactose, and mineral content decreases.</td>
</tr>
<tr>
<td>Hydrolyzed whey protein</td>
<td>Variable</td>
<td>Varies with protein concentration</td>
</tr>
<tr>
<td></td>
<td>Hydrolysis used to cleave peptide bonds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Larger proteins become smaller peptide fractions</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduces allergic potential compared to non-hydrolyzed</td>
<td></td>
</tr>
<tr>
<td>Undenatured whey concentrate</td>
<td>Variable</td>
<td>Some lactose, fat and minerals</td>
</tr>
<tr>
<td></td>
<td>Usually ranges from 25 - 89 %</td>
<td>When protein concentration increases, fat, lactose, and mineral content decreases.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Processed for preserving native protein structures; typically have higher amounts of immunoglobulins and lactoferrin</td>
</tr>
</tbody>
</table>

Table 3 Whey protein components. *

<table>
<thead>
<tr>
<th>Whey protein components</th>
<th>Approximate percentage contributions of the major proteins in whey (%)</th>
<th>Molecular weight (kDa)</th>
<th>Benefits and biological activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta-lactoglobulin</td>
<td>50-55</td>
<td>18.4</td>
<td>Source of essential and branched chain amino acids; Retinal carrier; Binding of fatty acids; Antioxidant</td>
</tr>
<tr>
<td>Alpha-lactalbumin</td>
<td>20-25</td>
<td>14</td>
<td>Source of essential and branched chain amino acids; Ca carrier; Immunomodulation; Anticarcinogenic; Antiulcer; Health effects on mood; Anti-inflammatory</td>
</tr>
<tr>
<td>Glycomacropeptide</td>
<td>10-15</td>
<td></td>
<td>Source of branched chain amino acids; Lacks the aromatic amino acids phenylalanine, tryptophan, and tyrosine; Inhibitory effect on acid gastric secretions; Immunomodulation; Antiviral</td>
</tr>
<tr>
<td>Proteose Peptone 3</td>
<td>12</td>
<td></td>
<td>Enhances monoclonal antibody production; Antibacterial</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>10-15</td>
<td></td>
<td>Immune protection</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>5-15</td>
<td>66.5</td>
<td>Source of essential amino acids; Anti-cancer activity in some cell lines; Opioid agonist activity; Food intake regulation</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>1</td>
<td>80</td>
<td>Antioxidant; Antimicrobial, wound healing; Antiviral; Promoting growth of beneficial bacterial; Anticarcinogenic; Antitoxin; Anti-inflammatory; Antithrombotic; Fe absorption; Immunomodulation</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>0.25-0.5</td>
<td></td>
<td>Antimicrobial, wound healing</td>
</tr>
<tr>
<td>Natural growth factor</td>
<td></td>
<td></td>
<td>Wound healing</td>
</tr>
</tbody>
</table>

**Beta-lactoglobulin**

Beta-lactoglobulin, a member of the lipocalin family, is the most abundant protein in bovine milk, accounting for approximately half of the total proteins in bovine whey, while it is absent in human milk (Perez and Calvo, 1995; Sawyer & Kontopidis, 2000; Kontopidis et al., 2004; Marshall, 2004; Krissansen, 2007). It is a noncovalently linked dimer containing two internal disulfide bonds and one free thiol group (Kontopidis et al., 2004; Yalcin, 2006). Primarily, β-lactoglobulin serves as a source of essential and branched chain amino acids (Marshall, 2004). In addition, it has binding sites for calcium, zinc, minerals, fat-soluble vitamins and it displays partial sequence homology to retinol binding proteins (Perez and Calvo, 1995; Kontopidis et al., 2004; Yalcin, 2006; Krissansen, 2007). Beta-lactoglobulin has been reported to bind retinol, triglycerides and long-chain fatty acids in order to enhance their intestinal uptake in preruminant calves (Perez & Calvo, 1995; Kushibiki et al., 2001), and it is also a major allergen in bovine milk and thus is responsible for milk allergy (Krissansen, 2007; Tsabouri et al., 2014).

**Alpha-lactalbumin**

Alpha-lactalbumin, which is found in both human and bovine milk, accounts for roughly 20-25 % of whey proteins (Lonnerdal & Lien, 2003; Marshall, 2004). It is a single-chain polypeptide of 123 amino acids with a molecular mass of approximately 14 kDa (Brew & Grobler, 1992). It is recognized as a part of the lactose synthase complex and it is a rich source of amino acids, especially in infant nutrition, due to the wide spectrum of its amino acids composition which allows it to a large extent to meet the essential amino acid requirements of newborn infants (Lonnerdal & Lien, 2003). Alpha-lactalbumin is a calcium binding protein which enhances the absorption of calcium (Lonnerdal & Glazier, 1985; Yalcin, 2006). Several in vitro and in vivo studies have indicated that α-lactalbumin can exert a variety of physiological functions from immune-stimulating (Gattegno et al., 1988; Jaziri et al., 1992; Migliore-Samour et al., 1992; Kayser & Meisel, 1996; Wong et al., 1997b), antiulcer (Matsumoto et al., 2001), anti-inflammatory (Yamaguchi et al., 2009) to anticancer activity (Sternhagen & Allen, 2001) and it may even have a beneficial effect on mood (Markus et al., 2002; Orosco et al., 2004).

**Glycomacropeptide**

Glycomacropeptide (GMP) is a protein accounting for 10-15 percent of whey (Saito et al., 1991; Marshall, 2004). It originates from the action of chymosin on casein in the cheese-making process (Eigel et al., 1984; van Hooydonk et al., 1984). Thus, GMP is only present when chymosin is used in
the processing (therefore cottage cheese which is not made with chymosin does not produce GMP). GMP is rich in branched chain amino acids, but lacks the aromatic amino acids such as phenylalanine, tryptophan, and tyrosine (Brody, 2000; Krissansen, 2007). It has been reported to inhibit the secretion of acid gastric and modifies the blood concentration of regulatory digestive peptides (Yvon et al., 1994). GMP has also been claimed to possess immunosuppressive (Otani et al., 1995), immunostimulatory (Monnai M, 1997), and anti-infective (Brody, 2000) properties.

**Proteose Peptone 3**

The proteose peptones are the proteins remaining in solution after milk has been heated at 95°C for 20 mins and then acidified to pH 4.7 (Rowland, 1937, 1938). These consist of four major components, of which the proteose-peptone component 3 (PP3) fragment represents 25% by weight (Sorensen & Petersen, 1993). PP3 is absent in humans, and only found in whey which is produced by the fermentation of fat-free bovine milk. PP3 has been reported to enhance monoclonal antibody production by human hybridoma cells (Sugahara et al., 2005) and it has the potential to inhibit the growth of both gram positive and negative bacteria (Campagna et al., 2004).

**Immunoglobulins**

Immunoglobulins (Ig) are antibodies or gamma-globulins, which contain five classes of antibodies i.e. IgA, IgD, IgE, IgG, and IgM (Woof & Burton, 2004). In whey, immunoglobulins represent 10-15 percent of the total proteins (Marshall, 2004). Not do they only provide passive immunity for the neonate, but they also may potentially be powerful agents which could be incorporated in diets to remove toxic and undesirable dietary factors (Krissansen, 2007). Furthermore, the hyperimmune whey from milk, which is acquired by immunizing cows with a pathogen or its antigens, can potentially provide prophylactic protection against many different infectious gut microbes such as rotavirus and Helicobacter pylori (Korhonen et al., 2000).

**Bovine Serum Albumin**

Bovine serum albumin is a large protein which accounts for approximately 5-15 percent of total whey protein (Marshall, 2004; Krissansen, 2007). In addition to being a source of essential amino acids, its potential therapeutical activity is largely unexplored. It has displayed anti-cancer activity in a cell line (Laursen I, 1990). In addition, some BSA-derived peptides have been reported to exert opioid agonist activity (Meisel, 2005) and are maybe involved in the regulation of food intake (Ohinata et al., 2002).
**Lactoferrin**

Lactoferrin is a single-chain iron-binding glycoprotein of mammary origin which can be found in the milk of most species (Lonnerdal & Iyer, 1995). It is an 80-kDa protein with a globular protein folded into two highly homologous iron-binding lobes (Metz-Boutigue et al, 1984; Anderson et al., 1987, 1989). In adults, LF is generated by glandular epithelial cells and secreted into mucosal fluids (Ward et al., 2005). LF is highly detected in colostrum and milk (Masson et al., 1966; Levay & Viljoen, 1995). It is also found with lower levels in tears, nasal fluids, saliva, pancreatic, gastrointestinal and reproductive tissue secretions (Masson et al., 1966; Levay & Viljoen, 1995). LF is the most abundant protein of all of the hundreds of low-abundance whey proteins (about 1%) (Krissansen, 2007). The evidence is accumulating that LF affects iron homeostasis, cellular growth and differentiation, and exerts anti-bacterial, anti-viral, anti-inflammatory and anti-cancer properties (for reviews, see Levay & Viljoen, 1995; Brock, 2002; Ward et al., 2002; Legrand et al., 2005; Weinberg, 2007; Gonzalez-Chavez et al., 2009; Jenssen & Hancock, 2009). In addition, LF has recently been reported to exert a regulatory effect on lipid metabolism in humans (Moreno-Navarrete et al., 2009; Fernandez-Real et al., 2010; Ono et al., 2010). It has been postulated that one of potential mechanism to explain the observed biological functions of LF is the ability of LF to modulate cellular signaling pathways after binding to a wide range of epithelial and immune cells (Ward et al., 2005).

**Lactoperoxidase**

Lactoperoxidase is the most abundant enzyme in whey protein fractions; it ends up in whey after the curding process (Marshall, 2004). It represents a mere 0.25-0.5 percent of total protein in whey (Marshall, 2004). Lactoperoxidase has been reported to be able to catalyze the peroxidation of thiocyanate and some halides (such as iodine and bromium), which eventually produce products that inhibit growth and even kill bacterial species (Kussendrager & van Hooijdonk, 2000). Oral administration of lactoperoxidase has shown to be able to attenuate pneumonia in mice with influenza virus infection by inhibiting the infiltration of inflammatory cells into the lung (Shin et al., 2005). The treatment of sheep neutrophils with lactoperoxidase revealed a dose-dependent enhancement of superoxide production (Wong et al., 1997a).

**Natural growth factor**

Whey contains proteins which exert a dramatic impact on cell growth; they promote the synthesis of DNA and protein and inhibit the degradation of protein in a series of mammalian cell lines in culture (Smithers et al., 1996). These growth factors can be acquired from cheese whey if it is prepared by membrane and chromatographic techniques (Ballard, 1991). Several growth factor activities including
insulin-like growth factor (IGF)-I, IGF-II, platelet-derived growth factor (PDGF), transforming growth factor, acidic and basic fibroblast growth factors have been detected in whey extract (Francis et al., 1995; Rogers et al., 1995; Rogers, 1995). These growth factors have been observed to reverse the healing deficit associated with diabetes (Greenhalgh et al., 1990), cytotoxic therapy (Lawrence et al., 1986), administration of steroids (Pierce et al., 1989), radiation (Mustoe et al., 1989; Cromack et al., 1993), and ischemia (Uhl et al., 1993).

2.2.2 The digestion and absorption of whey proteins

After their oral intake and processing, whey proteins are hydrolyzed by an array of proteases and peptidases which are secreted from the stomach, pancreas or bound to the brush border membrane of enterocytes (Daniel, 2004). These enzymatic degradation generates a number of short- and medium-sized peptides as well as free amino acids (Daniel, 2004). Most of the peptides formed after hydrolysis are rapidly and ultimately degraded into amino acids, which are taken up into the blood circulation from the small intestine, whereas certain whey proteins and their derived peptides are more resistant to hydrolysis. For instance, both α-lactalbumin and β-lactoglobulin are partly resistant to the digestion with human gastric and duodenal juice in vitro (Almaas et al., 2006). Furthermore, lactoferrin has been demonstrated to be absorbed intact in mice (Fischer et al., 2007). Whey proteins derived peptides have been postulated to exert a variety of physiological functions; these have been reported to range from opioid agonist and antagonist effects to ACE-inhibitory and antimicrobial effects (for review, see Meisel, 2005). However, due to the complexity of protein digestion in vivo, a detailed characterization of the components formed after digestion of whey proteins needs to be conducted in order to explore the role of bioactive peptides in the beneficial effects of their precursor proteins.

Amino acids are organic substances which contain both amino and acid groups (Wu, 2009). They are primarily considered to serve as building blocks of proteins and polypeptides. There is growing evidence to suggest that there are some amino acids which can regulate key metabolic pathways that are necessary for maintenance, growth, reproduction and immunity in organisms (Wu et al., 2007a, b, c; Suenaga et al., 2008). These amino acids are defined as functional amino acids and they include arginine, cysteine, glutamine, leucine, proline and tryptophan (Wu, 2009). According to the key regulatory roles of functional amino acids in nutrition and metabolism (for review, see Wu, 2009), dietary supplementation with the functional amino acids may be beneficial for ameliorating certain health problems (e.g. neonatal morbidity and mortality, fetal growth restriction, weaning-associated intestinal dysfunction and wasting syndrome, cardiovascular disease, obesity, diabetes, metabolic syndrome, and infertility) and maximizing the efficiency of metabolic transformation to improve health in both humans and animals.
2.3 Health effects of whey proteins

2.3.1 Obesity and whey proteins

2.3.1.1 Clinical studies

Based on a search in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) data-base for randomized controlled trials concerning whey proteins, it was found that the studies had focused on growth and allergy of infants, body weight, body composition, muscle hypertrophy, protein synthesis, gastrointestinal function, blood pressure, insulin responses, appetite, satiety, thermogenesis, and bone health. Those trials which had been designed to investigate the health effects of whey proteins in overweight/obese subjects are described in Table 4.

The effects of whey protein intake have been tested under different conditions including free-living style, energy restriction (ER), and energy restriction (weight loss) followed by ad libitum energy intake (weight regain). In a 12-month study, GMP-enriched whey protein isolate showed effects on overall sustained weight loss (10 kg) in both obese men and women (Keogh & Clifton, 2008). The consumption of whey supplement achieved a significant reduction in body weight in overweight women, while no difference was observed while consuming a fortified collagen hydrolysate protein supplement (Hays et al., 2009). Free-living overweight or obese individuals consuming a daily supplementation of whey proteins (56 g protein/day) had a decrease in body weight and fat mass after 23 weeks (Baer et al., 2011). During the caloric restriction-induced weight loss, the supplementation of whey proteins with an essential amino acids formulation promoted the loss of adipose tissue in elderly, obese individuals (Coker et al., 2012). In a weight loss followed by weight regain study, although an elevated intake of whey protein achieved similar effects on weight and fat loss as compared with mixed proteins, significantly improved regional fat loss was observed in whey protein group (Aldrich et al., 2011). Furthermore, Mojtahedi and colleagues (2011) observed that a higher whey protein intake during weight loss could maintain muscle relative to body weight lost in overweight and obese women. However, in a study comparing the effects of casein and whey proteins in obese subjects, the intake of whey proteins was unable to change either the body weight or the body composition as compared with casein after 12 weeks supplementation (Pal et al., 2010a). Interestingly, when combined with resistance exercise, the supplementation of casein proteins achieved greater mean fat loss and a significant strength increase as compared with whey proteins in overweight police officers (Demling & DeSanti, 2000). In addition to the effects on body weight and body composition, the intake of whey proteins also exerted beneficial effects on the consequences and other indicating parameters of obesity including blood pressure, blood glucose, insulin level, triglyceride concentration and satiety (Table 4) (Bowen et al., 2006a, 2006b, 2007; Pins & Keenan, 2006; Diepvens et al., 2008; Petersen et al., 2009; Pal et al., 2010a, b; Pal & Ellis, 2010b; Aldrich et al.,
2011; Baer et al., 2011; Berthold et al., 2011; Holmer-Jensen et al., 2011, 2012, 2013; Gouni-Berthold et al., 2012; Lorenzen et al., 2012; Sheikholeslami Vatani & Ahmadi Kani Golzar, 2012). However, controversial evidence exists (Claessens et al., 2009; Keogh et al., 2010; Pal & Ellis, 2011; Ang et al, 2012; Arnberg et al., 2012; Weinheimer et al., 2012; Arnarson et al., 2013).

2.3.1.2 Preclinical studies

Whey protein intake has had beneficial effects on obesity and obesity related consequences in animal models. In one study concerning the effects of dietary whey proteins in rats, whey protein diet inhibited body weight and fat gain after ten weeks’ feeding period as compared with control diet (Zhou et al., 2011). Interestingly, the whey protein fed rats showed decreased food intake and increased fat oxidation, which might contribute to the effects of whey proteins on fat gain. As compared with red meat, a high-whey-protein diet reduced body weight gain, energy intake, adiposity and increased insulin sensitivity in Wistar rats (Belobrajdic et al., 2004). Royle et al. (2008) reported that whey protein isolate combined with GMP decreased body weight gain and body fat accumulation in Wistar rats without interfering with the food intake, and furthermore the whey protein isolate alone appeared to have the major influence accounting for 70% of the overall effect on body weight gain. Shertzer et al. (2011) observed that whey protein isolate inhibited body weight and fat gain, increased lean body mass, but did not change energy consumption in mice fed a high fat diet. There is a report that a whey protein diet could effectively suppress body weight and fat gain as compared with a casein diet in combination with calcium supplementation in mice receiving ad libitum high-fat diet feeding (Pilvi et al., 2007). However, under low-fat (fat containing 11.8 % of the energy in the diets) feeding, neither the high whey proteins nor a corresponding leucine supplementation significantly affected body weight gain or body composition in mice (Noatsch et al., 2011). Interestingly, it has been reported that the complete dairy protein showed better effects against body weight gain than whey or casein alone in obese rats (Eller & Reimer, 2009).

Beta-lactoglobulin enriched high protein diet reduced body weight and the adiposity index in obese rats as compared with whey and milk proteins (Pichon et al., 2008). Both consumption of β-lactoglobulin and whey proteins reduced energy intake, body weight and adiposity in rats fed with high-protein high-fat diet as compared with milk proteins (Pichon et al., 2008). In an obese mouse model, β-lactoglobulin also modestly enhanced the body weight and fat loss under energy restriction (Pilvi et al., 2009). Interestingly, Pilvi et al. (2009) showed that α-lactalbumin effectively improved the outcomes of weight loss and weight regain in mice fed with a high-fat diet as compared with whey protein isolate. In the same study, LF also significantly accelerated both body weight and fat loss during energy restriction as compared to the consumption of whey protein isolate (Pilvi et al., 2009).
Table 4 Clinical studies on whey proteins and overweight/obesity. *

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Study design</th>
<th>Time range</th>
<th>Intervention</th>
<th>Main results</th>
<th>References</th>
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<tbody>
<tr>
<td>38 overweight police officers</td>
<td>randomized controlled, prospective</td>
<td>12 weeks</td>
<td>nonlipogenic, hypocaloric diet</td>
<td>1) control diet + RE + casein protein hydrolysate 1.5 g/kg/day; 2) control diet + RE + whey protein hydrolysate 1.5 g/kg/day</td>
<td>Casein group exerted greater mean fat loss and significant strength increase.</td>
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<td>72 men with a BMI range 20.6 - 39.9 kg/m² (range)</td>
<td>randomized controlled, cross-over</td>
<td>pre-loads before meal</td>
<td>Liquid preloads (1.1 MJ, 450 ml) containing 50 g items as follows: 1) whey; 2) soy; 3) gluten; 4) glucose.</td>
<td>Whey, soy, and gluten inhibited EI in both lean and overweight men in comparison with glucose. Both fasting and postprandial GLP-1 concentrations were higher in overweight subjects than in lean ones.</td>
<td>Bowen et al., 2006a</td>
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<tr>
<td>19 overweight men</td>
<td>randomized controlled, cross-over</td>
<td>pre-loads before meal</td>
<td>Liquid preloads (1 MJ) containing: 1) whey (55 g); 2) casein (55 g); 3) lactose (56 g); 4) glucose (56 g).</td>
<td>Acute appetite and EI were equally decreased after lactose, casein, whey preloads as compared to glucose. These were consistent with differences in plasma ghrelin. The higher CCK responses found after whey and casein preloads correlated with satiety without interfering with EI.</td>
<td>Bowen et al., 2006b</td>
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<td>30 prehypertensive or stage 1 hypertensive subjects with BMI (kg/m²): control (29.6±4.4); active (28.3±4.5)</td>
<td>randomized controlled, double-blinded</td>
<td>6 weeks</td>
<td>20 g/d unmodified whey protein (UMWP)</td>
<td>HWP decreased both systolic blood pressure and diastolic blood pressure in a population of prehypertensive or stage 1 hypertensive overweight men and women. HWP significantly improved both low-density lipoprotein cholesterol and high-sensitivity C-reactive protein.</td>
<td>Pins &amp; Keenan, 2006</td>
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<td>28 obese men with BMI; 32.5±0.6 kg/m²</td>
<td>randomized controlled, cross-over, double-blinded</td>
<td>After beverage loading</td>
<td>4 beverages (1.1 MJ) containing: 1) whey (50 g); 2) fructose (50 g); 3) glucose (50 g); 4) whey (25 g) + fructose (25 g)</td>
<td>Whey proteins caused a prolonged suppression of ghrelin and elevation of GLP-1 and CCK, which were decreased in combination with fructose, but glucose and insulin responses were similar in both groups.</td>
<td>Bowen et al., 2007</td>
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<td>39 subjects with BMI; 27.6±1.7 kg/m²</td>
<td>randomized controlled, cross-over</td>
<td>experiment 1: 4 h; experiment 2: 7 h</td>
<td>Shakes containing: 1) 15 g whey protein (WP); 2) 15 g pea protein hydrolysate (PPH); 3) WP (7.5 g) + PPH (7.5 g); 4) 15 g milk protein (MP)</td>
<td>Both WP and PPH induced greater satiety and fullness than MP and WP+PPH. A positive correlation between insulin and both CCK and GLP-1 was noted i in WP group.</td>
<td>Diepvens et al., 2008</td>
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<td>127 subjects (95 women, 32 men; BMI 33.4±3.4 kg/m²), 72 completed the 12-month study.</td>
<td>randomized controlled, parallel-design, double-blinded</td>
<td>12 months</td>
<td>Meal replacements containing 900 kJ/sachet and : 1) 15 g GMP-enriched whey protein isolate (GMP-WPI); 2) 15 g skim milk powder (SMP)</td>
<td>Both meal replacements exerted similar effects on the overall sustained 12 months weight loss and improvements in cardiovascular disease risk markers.</td>
<td>Keogh &amp; Clifton, 2008</td>
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<td>48 subjects (31 women) with BMI ≥ 27 kg/m²</td>
<td>randomized controlled</td>
<td>5-6 weeks of ER period followed by a 12 weeks of weight maintenance period</td>
<td>Weight loss was induced by a very low-calorie diet; during weight maintenance: 1) maltodextrin (HC group); 2) casein (HPC group); 3) whey (HPW group) supplements (2 x 25 g/d) were served respectively, with a low-fat diet.</td>
<td>The low-fat, high protein (both whey and casein) displayed a better maintenance of weight loss than the low-fat, high carbohydrate diet, and did not adversely affect metabolic and cardiovascular risk factors in weight-reduced moderately obese subjects without metabolic or cardiovascular complications.</td>
<td>Claessens et al., 2009</td>
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<td>9 healthy women with BMI: 29.2±1.3 kg/m²</td>
<td>randomized controlled, cross-over, double-blinded</td>
<td>two 15-day trials separated by a ≥ 1-week washout period</td>
<td>Each trial provided a total dietary protein intake about 0.8 g/kg body weight/day; about half of the protein was administered as a supplement and consisted of either Protein A (a whey protein concentrate) or Protein B (a concentrated, fortified, collagen protein hydrolysate)</td>
<td>The consumption of whey supplement reduced body weight. No changes were observed on body weight after consumption of the collagen supplement. The nitrogen balance was not different between the groups.</td>
<td>Hays et al., 2009</td>
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<td>10 subjects (3 men) with BMI: 33.6±4.8 kg/m²</td>
<td>randomized controlled, acute</td>
<td>After meal</td>
<td>50 g glucose 1) 50 g glucose + 5 g GILP protein; 2) 50 g glucose + 10 g GILP protein; 3) 50 g glucose + 20 g GILP protein</td>
<td>Adding GILP to an oral glucose bolus dose-dependently decreased blood glucose iAUC and averaged 4.6±1.4 mmol.min/L per gram of GILP.</td>
<td>Petersen et al., 2009</td>
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<td>20 men with BMI: 31.5 (SD 3.0) kg/m²</td>
<td>randomized controlled, double-blinded, acute</td>
<td>protein preloads first, followed by ad libitum lunch</td>
<td>glucose control</td>
<td>The protein fractions in the dose used did not cause any reduction in food intake at the subsequent meal. In addition, they did not change the postprandial CCK concentrations.</td>
<td>Keogh et al., 2010</td>
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<td>70 subjects with BMI: 31.3±0.8 kg/m²</td>
<td>randomized controlled, parallel-design</td>
<td>12 weeks</td>
<td>glucose control (27 g glucose)</td>
<td>Supplement sachets: 1) whey protein isolate (27 g protein); 2) sodium caseinate (27 g protein)</td>
<td>Pal et al., 2010a</td>
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<td>20 overweight or obese, postmenopausal women with BMI: 25-40 kg/m² (range)</td>
<td>randomized controlled, single-blinded, three-way cross-over</td>
<td>3 weeks with a 4-week washout period before commencement</td>
<td>glucose control (45 g glucose)</td>
<td>Supplements containing: 1) whey protein isolate (45 g protein); 2) sodium caseinate (45 g protein)</td>
<td>Pal et al., 2010b</td>
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<tr>
<td>70 subjects with BMI: 31.3±0.8 kg/m²</td>
<td>randomized controlled, single-blinded, parallel-design</td>
<td>12 weeks period with a 4-week washout period before commencement</td>
<td>glucose control (27 g glucose)</td>
<td>1) sodium caseinate (27 g protein); 2) whey protein isolate (27 g protein)</td>
<td>Pal &amp; Ellis, 2010b</td>
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<td>18 healthy subjects with BMI: 27-32 kg/m² (range)</td>
<td>randomized controlled, parallel-design</td>
<td>5-month study of 8-week controlled food intake followed by 12-week ad libitum intake</td>
<td>control diet (15 % energy from protein, 55 % energy from carbohydrate)</td>
<td>1) mixed protein diet (30 % energy from mixed protein, 40 % energy from carbohydrate); 2) whey protein diet (15 % energy from mixed protein, 15 % energy from whey protein, 40% energy from carbohydrate)</td>
<td>Aldrich et al., 2011</td>
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<td>73 overweight or obese subjects with BMI: 28-38 kg/m²</td>
<td>randomized controlled, double-blinded</td>
<td>23 weeks</td>
<td>an isoenergetic amount of carbohydrate 1) whey protein (56 g protein per day); 2) soy protein</td>
<td>The subjects from the whey protein group exerted lower body weight, fat mass and smaller waist circumference and fasting ghrelin level after 23 weeks free-living period.</td>
<td>Baer et al., 2011</td>
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<td>(range)</td>
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<td>(56 g protein per day)</td>
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<td>161 subjects with BMI: 26.3±3.6 kg/m²</td>
<td>randomized, placebo-controlled, multi-centre, double-blinded, parallel-design</td>
<td>6-week run-in phase, followed by 12 weeks treatment phase</td>
<td>placebo malleable protein matrix (MPM)</td>
<td>The whey fermentation product MPM showed significant triglyceride-lowering properties in subjects with combined hypercholesterolemia and higher triglyceride levels.</td>
<td>Berthold et al., 2011</td>
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<td>11 subjects with BMI: 30.3-42.0 kg/m² (range)</td>
<td>randomized controlled, acute, cross-over</td>
<td>After meal up to 4 h</td>
<td>a fat rich mixed meal with one of the following dietary protein supplements: 1) cod protein; 2) whey isolate; 3) gluten; 4) casein</td>
<td>CCL5/RANTES initially increased after all meals, while the whey meal caused the smallest overall postprandial increase. MCP-1 was initially suppressed after all meals and the smallest overall postprandial suppression was noted after the whey meal.</td>
<td>Holmer-Jensen et al., 2011</td>
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<td>31 overweight or obese, postmenopausal women with BMI: 33.7±4.9 kg/m²</td>
<td>randomized controlled, double-blinded, parallel-design</td>
<td>6 months</td>
<td>a reduced calorie diet (1,400 kcal/day) with either of the supplements containing: 1) 2 x 25 g/day whey protein; 2) 2 x 25 g/day maltodextrin (CARB)</td>
<td>During caloric restriction, a higher whey protein intake maintained muscle relative to weight lost. This effect helped enhance physical function in older women.</td>
<td>Mojtabahedi et al., 2011</td>
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<td>Patient groups</td>
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<td>20 overweight or obese, postmenopausal women with BMI: 32.5±1.03 kg/m²</td>
<td>randomized controlled, three-way cross-over</td>
<td>Over 3 weeks with a 4-week washout period before commencement</td>
<td>glucose control (45 g glucose)</td>
<td>Supplements containing: 1) whey protein isolate (45 g protein); 2) sodium caseinate (45 g protein)</td>
<td>No significant difference was observed in augmentation index, systolic or diastolic blood pressure within or between the glucose control, casein or whey protein groups. No significant group effects were noted on plasma inflammatory markers. This indicated that the health effects previously seen with chronic whey protein ingestion were better observed with the long-term consumption of whey proteins.</td>
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<td>30 subjects with BMI: 29.0±0.7 kg/m²</td>
<td>randomized controlled, cross-over</td>
<td>After drink loading. 3 days washout period</td>
<td>Drink containing only 50 g isomaltulose (ISO)</td>
<td>1) Drink containing 50 g ISO and 21 g whey/soy 2) Drink containing 50 g ISO and 21g casein</td>
<td>The combination of carbohydrate with whey proteins increased postprandial insulin levels, but reduced the actions of insulin in comparison with supplementing with casein proteins.</td>
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<tr>
<td>203 overweight adolescents with BMI: 25.4 (SD 2.3) kg/m²</td>
<td>randomized controlled</td>
<td>12 weeks</td>
<td>Drink containing 1) skim milk; 2) whey; 3) casein; 4) water</td>
<td>The high intakes of skim milk, whey, and casein increased BMI-for-age Z-scores in overweight adolescents. The high intakes of whey and casein increased insulin secretion in the same subjects.</td>
<td>Arnberg et al., 2012</td>
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<tr>
<td>12 subjects with BMI: 31.3±0.5 kg/m²</td>
<td>randomized controlled</td>
<td>8 weeks</td>
<td>Daily 400 kcal solid food combined with 800 kcal of: 1) whey protein + essential amino acid meal replacement (EAAMR) 2) competitive meal replacement (CMR)</td>
<td>The whey proteins containing EAAMR promoted preferential adipose tissue reduction with modest loss of lean mass in elderly subjects during energy restriction.</td>
<td>Coker et al., 2012</td>
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<td>171 subjects with BMI: 31.0 (SD 4.1) kg/m²</td>
<td>randomized, placebo-controlled, multi-center, double-blinded, parallel-design</td>
<td>3 months</td>
<td>placebo yoghurt whey malleable protein matrix (MPM) yoghurt</td>
<td>The fasting triglyceride concentrations in patients with the metabolic syndrome were significantly decreased after the consumption of whey MPM yoghurt twice a day over 3 months. This treatment also improved multiple other cardiovascular risk factors i.e. fasting plasma glucose, blood pressure and body weight.</td>
<td>Gouni-Berthold et al., 2012</td>
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<td>11 obese non-diabetic subjects with BMI: 30.41.4 kg/m² (range)</td>
<td>randomized controlled, acute, single-blinded, cross-over</td>
<td>After meal up to 8 h</td>
<td>45 g proteins: 1) alpha-lactalbumin; 2) whey isolate; 3) caseinoglycomacropeptide; 4) whey hydrolysate</td>
<td>Whey hydrolysate was associated with a significantly smaller postprandial suppression of non-esterified free fatty acids compared with other tested proteins.</td>
<td>Holmer-Jensen et al., 2012</td>
</tr>
<tr>
<td>17 slightly overweight male subjects with BMI: 29±4 kg/m²</td>
<td>randomized controlled, single-blinded, cross-over</td>
<td>After meal up to 4 h</td>
<td>isocaloric meals containing one of the following drinks: 1) whey drink; 2) casein drink; 3) skimmed milk</td>
<td>Milk inhibited subsequent EI as compared with isocaloric drinks containing only whey or casein. There was a significant increase in lipid oxidation after casein drink intake as compared with whey.</td>
<td>Lorenzen et al., 2012</td>
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<tr>
<td>30 men with BMI: 25-30 kg/m² (range)</td>
<td>randomized controlled, single-blinded</td>
<td>6 weeks</td>
<td>No training or heavy physical activity</td>
<td>1) Resistance training with whey consumption; 2) Resistance training with placebo consumption</td>
<td>The combination of resistance training with whey consumption enhanced the beneficial effects of exercise alone on the antioxidant system in overweight subjects.</td>
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</table>
### Patient groups and Study design

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Study design</th>
<th>Time range</th>
<th>Intervention</th>
<th>Main results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>327 overweight and obese subjects with BMI: 30.0±2.8 kg/m²</td>
<td>randomized placebo-controlled, double-blind</td>
<td>36 weeks</td>
<td>Powder supplements containing: 1) 0 g; 2) 10 g; 3) 20 g; 4) 30 g whey proteins</td>
<td>The whey protein supplementation did not affect the exercise induced responses in body composition or indices of metabolic syndrome in overweight and obese adults.</td>
<td>Weinheimer et al., 2012</td>
</tr>
<tr>
<td>161 subjects BMI: 28.1±4.4 kg/m² in Whey protein group; 29.4±4.8 kg/m² in Carbohydrates group</td>
<td>randomized controlled, double-blind</td>
<td>12 weeks</td>
<td>Liquid supplementation taken after training containing: 1) whey protein 20 g; 2) isocaloric carbohydrate 20 g</td>
<td>Compared to isocaloric carbohydrate, the whey protein supplementation did not lead to greater gains in lean body mass, strength and physical function in elderly people in this study.</td>
<td>Arnarson et al., 2013</td>
</tr>
<tr>
<td>11 obese non-diabetic subjects with BMI: 30.3-42.0 kg/m² (range)</td>
<td>randomized controlled, acute, cross-over</td>
<td>After meal, 8-h postprandial period, 2 weeks washout period</td>
<td>Fat rich mixed meal containing 45 g proteins. The protein sources: 1) cod; 2) whey isolate; 3) gluten; 4) casein</td>
<td>The supplementation with whey proteins caused lower postprandial lipemia compared to the supplementation with cod and gluten, which indicated that long-term supplementation with whey proteins might be beneficial in the prevention of cardiovascular disease in obese non-diabetic subjects.</td>
<td>Holmer-Jensen et al., 2013</td>
</tr>
</tbody>
</table>

*RE represents resistance exercise. EI represents energy intake. GILP represents glycemic index lowering peptide fraction. Data of BMI are presented either as mean±SEM, or mean (SD), or range.*
It has been reported that whey proteins with calcium supplementation accelerated body weight and fat loss under energy restriction in mice as compared to casein (Pilvi et al., 2008). Consumption of whey proteins with calcium supplementation also decreased the serum insulin level and normalized the relative level of potential diabetogenic ceramides and diacylglycerols in mice subjected to energy restriction (Pilvi et al., 2008). In accordance with the results of Pilvi et al. (2008), it was reported that the mice fed the diet containing whey protein isolate had higher basal metabolic rates and less hepatosteatosis and insulin resistance as compared with controls (Shertzer et al., 2011). There is also a report that oral administration of whey proteins achieved an improvement in fatty infiltration in hepatocytes and a reduction of oxidative stress parameters in rats (Hamad et al., 2011). These findings were considered to indicate that a diet with whey protein isolate may be effective in slowing the development of fatty liver and type 2 diabetes.

2.3.2 Metabolic syndrome and whey proteins

The metabolic syndrome is a cluster of metabolic abnormalities including abdominal obesity, dyslipidemia, insulin resistance, and increased blood pressure, which reflects overnutrition, sedentary lifestyles, and resultant excess adiposity (Cornier et al., 2008). It is associated with other comorbidities, including non-alcoholic fatty liver disease, a prothrombotic state, a pro-inflammatory condition, and reproductive disorders (Cornier et al., 2008). It has been reported that a patient with metabolic syndrome has an increased risk of developing cardiovascular disease and type 2 diabetes (Grundy et al., 2005; Grundy, 2008). According to U.S. government health surveys conducted between 1999 and 2006, about 34 % of the adults in U.S. meet the criteria of the metabolic syndrome (Ervin, 2009; Mozumdar & Liguori, 2011). The worldwide increased prevalence of metabolic syndrome is largely due to the epidemic of obesity (Bruce & Hanson, 2010). Although the genetic background affects the development of metabolic syndrome, lifestyle intervention, including dietary control, play the main role in the long-term prevention (Grundy et al., 2005; Bruce & Hanson, 2010; Lindstrom et al., 2013; Orchard et al., 2013).

The intake of dairy products exerted beneficial effects on the risk of developing the metabolic syndrome (Grundy et al., 2005). It has been reported that a high consumption of dairy products (3-4 servings per day) was associated with a 29 % decreased risk of developing the metabolic syndrome as compared with low consumption of dairy products (less than 2 servings per day) (Pittas et al., 2007). Recently a systematic review was conducted on 13 observational studies; seven of the studies detected an inverse association between dairy intake and metabolic syndrome, three found no association, and the other three reported mixed relationships between specific dairy foods and metabolic syndrome (Crichton et al., 2011). The intake of dairy products not only exerted the beneficial effects on the incidence of metabolic syndrome, but also on the risk factors for metabolic syndrome such as impaired fasting glucose level, diastolic blood pressure and BMI (Fumeron et al., 2011). In addition,
adequate dairy intake during weight maintenance reduced oxidative and inflammatory stress, which are commonly observed in association with the metabolic syndrome (Stancliffe et al., 2011).

Although the physiological mechanism behind the effects of dairy products on the metabolic syndrome have yet to be fully elucidated, several components, such as milk-fat, vitamin D, magnesium, and whey proteins, have been postulated to individually or collectively provide protection against this disease by modulating its individual components such as blood lipid levels, blood pressure, fasting glucose levels, and body composition (Pfeuffer & Schrezenmeir, 2007; Scholz-Ahrens & Schrezenmeir, 2006; Rice et al., 2011). There are studies revealing that whey protein intake was able to improve blood lipid profile, blood pressure control, insulin sensitivity and body composition, which were the risk factors associated with the metabolic syndrome (Westerterp-Plantenga et al., 2009b; Hulmi et al., 2010; Pal et al., 2010a; Pal & Ellis, 2010b; Rice et al., 2011).

2.3.3 Type 2 diabetes and whey proteins

Diabetes is defined as a disability of the body to regulate its blood glucose level, which increases the risk of blindness, kidney disease, coronary heart disease, leg and foot amputations, stroke, hypertension, and nerve damage (Centers for Disease Control and Prevention, 2011). It has been classified into two main types i.e. type 1 diabetes, the insulin-dependent diabetes mellitus, and type 2 diabetes, the non-insulin dependent diabetes mellitus (Centers for Disease Control and Prevention, 2011). About 90-95% of all diagnosed diabetics suffer from type 2 diabetes mellitus (Centers for Disease Control and Prevention, 2011). Type 2 diabetes is in association with older age, family history of diabetes, impaired glucose metabolism, obesity, physical inactivity, and race/ethnicity (Centers for Disease Control and Prevention, 2011). Although the cause of type 2 diabetes remains unclear, genetic, environmental and lifestyle factors are considered to be risk factors for this disease (Centers for Disease Control and Prevention, 2011). It has been proposed that diet (or medical nutrition therapy) can play an important role in successful management of type 2 diabetes (American Diabetes Association, 2008; Franz et al., 2010). In several studies, dairy food intake has been shown to display an inverse association with type 2 diabetes (Pittas et al., 2007; Tremblay & Gilbert, 2009; Elwood et al., 2010). In one meta-analysis of observational studies, the highest dairy intake (3 to 5 servings per day) lowered the risk of type 2 diabetes by 14% as compared with the lowest dairy intake (less than 1.5 servings per day) (Pittas et al., 2007). Recently, data from one systematic review and a meta-analysis of seven prospective studies showed similar effects of dairy consumption on type 2 diabetes: the highest intake of dairy products achieved a significant reduction in the risk in adults as compared with the lowest intake (Tong et al., 2011). The highest dairy consumption reduced by 47% the risk of type 2 diabetes in men in the Australian Diabetes, Obesity and Lifestyle study as compared with the lowest consumption (Grantham et al., 2013). A high dairy intake in adolescence may also
lower risk of type 2 diabetes in adulthood according to a prospective study in adult women (Malik et al., 2011).

Several dairy nutrients and components, including calcium, vitamin D, magnesium, trans-palmitoleic acid and whey proteins, have been proposed to be involved in the reduction of risk of type 2 diabetes achieved by high consumption of dairy products (Larsson & Wolk, 2007; Pittas et al., 2007; Tremblay & Gilbert, 2009; Akhavan et al., 2010; Mozaffarian et al., 2010; Gagnon et al., 2011). Whey protein supplementation at both breakfast and lunch increased insulin secretion and improved blood glucose control in type 2 diabetes (Frid et al., 2005). In comparison with casein, the ingestion of whey proteins induced greater post-prandial aminoacidemia and higher beta-cell secretion in type 2 diabetes mellitus patients (Tessari et al., 2007). Preloading with whey proteins also markedly reduced post-prandial glycemia in type 2 diabetes by stimulating insulin, incretin hormone secretion, and slowing gastric emptying (Ma et al., 2009). However, in one study investigating the effects of co-ingestion of carbohydrate and protein on glucose and insulin responses in type 2 diabetic subjects, it was observed that the combination of carbohydrate with whey proteins increased postprandial insulin levels, but reduced the actions of insulin in comparison with supplementing with casein proteins (Ang et al., 2012). The authors proposed that the fast-absorbing protein mixture (whey) might not be recommended for glycemic control in type 2 diabetes (Ang et al., 2012).

2.3.4 Other health effects of whey proteins

Whey proteins have also shown other different physiological properties and biological activities: these range from antioxidant and immunomodulation to anti-carcinogenic and anti-inflammatory effects (Table 3) (for reviews, see Marshall, 2004; Yalcin, 2006; Krissansen, 2007).

2.3.5 Possible mechanisms behind the anti-obesity effect of whey proteins

2.3.5.1 Whey proteins and satiety

An increased protein intake has been reported to enhance satiety and reduce energy intake in comparison with an intake of fats or carbohydrates (Anderson & Moore, 2004). Furthermore, a high protein diet has been shown to induce more feelings of satiety than the normal protein diet (Lejeune et al., 2006; Westerterp-Plantenga et al., 2006; Veldhorst et al., 2008; Westerterp-Plantenga et al., 2009a). Thus, there has been a great interest in studying the relationship between milk proteins and satiety.

Several studies have investigated the effects of different protein sources on satiety (Hall et al., 2003; Anderson et al., 2004; Bowen et al., 2006b; Veldhorst et al., 2009; Pal & Ellis, 2010a). Whey proteins have been claimed to be more satiating than casein, soy, turkey and egg proteins (Hall et al., 2003;
Anderson et al., 2004; Veldhorst et al., 2009; Pal & Ellis, 2010a). However, some workers failed to observe any difference between whey and casein in terms of their effects on feelings of satiety (Bowen et al., 2006b). Whey and casein have been classified as fast and slow proteins respectively due to their digestion and absorption speed (Boirie et al., 1997; Dangin et al., 2002). It has been postulated that the satiety effect of milk products is a synergistic effect of whey (acting as ‘fast’ proteins) providing early and casein (acting as ‘slow’ proteins) providing overlapping but later signals (for review see Luhovyy et al., 2007). When compared with casein, the greater satiating effect of whey proteins could be explained by the higher plasma concentration of cholecystokinin (CCKs) and glucagon-like peptide-1 (GLP-1) found after whey digestion (Hall et al., 2003). Furthermore, the high concentration of leucine in whey may also contribute to its satiety effects. An intracerebroventricular administration of leucine or an increase in the amount of leucine in the diet inhibited food intake and body weight gain and enhanced glucose and cholesterol metabolism in both rat and mouse models (Cota et al., 2006; Zhang et al., 2007; Morrison et al., 2007); this effect was mediated through the activation of mTOR pathway in the hypothalamus (Morrison et al., 2007).

2.3.5.2 The insulinotropic property of whey proteins

The consumption of dairy products has been shown to reduce the risk of the metabolic syndrome and type 2 diabetes (see 2.3.2 and 2.3.3). It has been reported that an intake of whey protein in particular improves post-prandial glycaemic control (Nilsson et al., 2004; Frid et al., 2005; Tessari et al., 2007; Ma et al., 2009; Akhavan et al., 2010). The insulinotropic property of whey proteins has been proposed to be involved in several mechanisms.

Since they are classified as ‘fast’ proteins, the rapid digestion and absorption of whey proteins results in higher concentrations of amino acids in the blood stream after ingestion, and this can stimulate the secretion of insulin. The rapid absorption of certain insulinotropic amino acids (isoleucine, leucine, lysine, threonine, and valine) leads to a clear post-prandial glycaemic and insulinemic response (Nilsson et al., 2004). In comparison with other proteins, whey proteins contain a high concentration of branched-chain amino acids (BCAAs) e.g. isoleucine, leucine and valine, and this may contribute to its insulinotropic effect (Marshall, 2004). Whey proteins also increase the concentration of incretin hormones which enhance insulin secretion in a glucose-dependent manner (Drucker, 2006). The incretin hormones, such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP), are released after food ingestion; they are rapidly degraded via dipeptidyl peptidase IV (DPP-IV) in the gastrointestinal tract (Gunnarsson et al., 2006). Whey proteins have been reported to inhibit the activity of DPP-IV and to increase GLP-1 level, which leads to an overall increased insulin response (Gunnarsson et al., 2006).
2.4 Whey proteins manufacturing

2.4.1 Traditional method

Whey was previously considered simply as a by-product of cheese manufacture. Traditionally, cheese production starts with milk fat-to-protein standardization, including pasteurization or some other heat treatment. Additives, such as calcium chloride and copper sulphate, are added to milk since they confer different properties (Heino, 2009). After the addition of chymosin, the milk is coagulated to form a curd (coagulated milk). Then, the curd is cut and whey is partly removed from the cheese vat. After the addition of water to curd to dilute the lactose content, the curd is cooked and curd syneresis removes whey from the pieces of curd. The collected raw whey is then subjected to clarification (removal of casein fines), separation (removal of residual fat), pasteurization, and concentration (by a variety of methods e.g. reverse osmosis, nanofiltration, evaporation) (Outinen, 2010). This resulting product is then processed to generate different whey products (including whey proteins) in different ways such as ultrafiltration, demineralization, chromatography, and protein fractionation (Outinen, 2010).

2.4.2 Microfiltration method

The microfiltration technique is a type of pressure driven membrane filtration: this is used to separate molecules, particles, or microbes from liquids (Heino, 2009). This technique has been widely used in mining, chemical, pharmaceutical, and food industry. Microfiltration has been postulated as being an excellent technique to remove whey from milk and it differs from the traditional cheese making process (Brans et al., 2004). When micellar casein micelles are concentrated via microfiltration, the milk microfiltrate permeate is called native whey and it contains whey proteins which are called native whey proteins (Heino, 2009). The native whey has a higher technological and economic value than the standard cheese whey (Maubois, 2002). Furthermore, when compared to cheese whey, the microfiltered native whey is free of fat, cheese fines, bacteria, somatic cells, lactic acid, κ-casein and β-lactoglobulin complexes and cheese chymosin (Maubois, 2002; Ardisson-Korat & Rizvi, 2004), and it contains only a minimal amount of BSA, GMP and caseinomacropeptide (CMP) (Maubois, 2002; Ardisson-Korat & Rizvi, 2004; Heino et al., 2007).
3 Aims of the study

Previous studies have shown that the intake of dairy products is related to a lower risk of type 2 diabetes, metabolic syndrome and obesity. Whey proteins have been postulated to play an important role in the beneficial effects of dairy products on weight management, but the mechanisms behind this phenomenon remain unknown. Non-alcoholic fatty liver disease has been reported to be strongly associated with obesity. There is growing evidence to suggest that the weight loss by energy restriction decreases liver fat and adipose tissue inflammation but again the mechanisms remain unclear. The aim of the present study was to investigate the potential of different whey proteins and their mechanisms of actions to prevent and help in the treatment of diet-induced obesity and its consequences in an experimental model of diet-induced obesity.

The special aims of the study were the following:

1. To investigate the metabolic effects of a novel whey protein isolate, rich in lactoperoxidase, lactoferrin, growth factors and immunoglobulins on diet-induced obesity and obesity related disorders in C57Bl/6J mice during weight loss and weight regain.

2. To evaluate the effects of lactoferrin supplementation on diet-induced obesity and its consequences in C57Bl/6J mice during weight loss and weight regain.

3. To compare the effects of α-lactalbumin and amino acid mixtures with equivalent amino acid profiles on diet-induced obesity in C57Bl/6J mice during weight loss and weight regain, and to attempt to characterize the mechanisms which could explain the anti-obesity property of α-lactalbumin.

4. To assess the metabolic effects of a novel microfiltered native whey protein, acquired by filtration through polymeric membranes, on diet-induced obesity in C57Bl/6J mice during weight gain and weight loss, and furthermore to compare these effects with those obtained with α-lactalbumin in the same animal model.
4 Materials and methods

4.1 Study design

All of the four studies were conducted in C57Bl/6J male mice, a well-established animal model of diet-induced obesity (Surwit et al., 1988; Collins et al., 2004; Koza et al., 2006; Kanasaki & Koya, 2011). This mouse strain develops obesity and insulin resistance when subjected to high-fat feeding. Therefore, these mice can serve as a diet-induced obesity experimental animal model of obesity research.

All of the four studies investigated the effects of different whey proteins on diet-induced obesity during three different phases of obesity (Table 5), i.e. weight gain (Study IV (i)), weight loss (Studies I, II, III, and IV (ii)), and weight regain (Studies I, II, and III). Study I investigated the effects of whey protein isolate (WPI) on obesity as well as the development of fatty liver during the weight loss and the weight regain phases. Study II evaluated the effects of lactoferrin on obesity and fatty liver formation during the weight loss and the weight regain phases. Study III compared the effects of α-lactalbumin to the amino acid mixture with equal amino acid composition on obesity during the weight loss and the weight regain phases. Study IV focused on the effects of microfiltered native whey on obesity during the weight gain (Study IV(i)) and the weight loss (Study IV(ii)) phases.

Table 5 The tested proteins and study phases in all four studies in the thesis.

<table>
<thead>
<tr>
<th>Tested whey proteins</th>
<th>Study phases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight gain</td>
</tr>
<tr>
<td>Study I</td>
<td>Whey protein isolate (WPI)</td>
</tr>
<tr>
<td>Study II</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>Study III</td>
<td>α-Lactalbumin</td>
</tr>
<tr>
<td>Study IV</td>
<td>Microfiltered native whey</td>
</tr>
</tbody>
</table>

4.2 Experimental animals

All the studies were carried out in C57Bl/6J male mice at the age of 6 weeks (Studies I, II and III) or 7 weeks (Study IV (i) and (ii)), which were purchased from Charles River Europe (Sulzfeld, Germany). The mice were housed five per cage in a standard experimental animal laboratory, illuminated from 7:00 to 19:00, temperature 22 ± 1 °C. The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland and the principles of laboratory animal care (NIH
publication no. 85-23, revised 1985) were followed. The mice had free access to tap water during the experiments.

After a one-week acclimatization period, the mice were fed with the high-fat diets for 100 days (Study IV(i)), 150 days (Study IV(ii)), 200 days (Studies I and II) or 250 days (Study III). In Studies I, II, III and IV(ii), the mice were first put on a standard high-fat diet (fat containing 60% of the diet energy and casein serving as the only protein source) (D12492, Research Diets Inc., New Brunswick, NJ, USA) (Table 6) for 100 days (Studies I, II, and IV(ii)) or 150 days (Study III) ad libitum feeding to induce obesity. Then, the body weight-matched mice were divided into treatment groups, fed different high-fat diets (Table 6) and subjected to energy restriction for 50 days (weight loss phase), in which the energy intake was reduced to 70% of the energy intake of the ad libitum feeding. In Studies I, II and III, some of the mice were sacrificed after the weight loss phase and the rest of the mice were kept on the same diets fed ad libitum for another 50 days (weight regain phase). In Study IV (i), the body weight-matched mice were fed different high-fat diets from the beginning of ad libitum feeding for 100 days (weight gain phase). Reference values for some parameters used in the studies were taken from additional age-matched obese mice fed casein-based high-fat diet and lean mice fed normal low-fat chow ad libitum starting immediately after weaning.

4.3 Diets and groups

All the diets were manufactured by Research Diets Inc. (New Brunswick, NJ, USA). The diets were produced on a standard high-fat diet (D12492), where fat contains 60% of the diet energy. The protein source and calcium amount (in Study III) of the testing diets were modified based on the study designs. The microfiltered native whey protein was produced by microfiltration technology with polymeric membranes and its composition was analyzed in Valio Ltd. (Helsinki, Finland). All test diet compositions and protein amino acid profiles are given in Tables 6 and 7. The protein source of the AA+Ca diet was an amino acid mixture with an equivalent amino acid profile compared to α-lactalalbumin (Table 6). Leucine (Leu), aspartate (Asp) and tryptophan (Trp) were enriched in the α-lac(2xLeu)+Ca and the α-lac(2xAsp and Trp)+Ca diets. In the α-lac(2xLeu)+Ca diet, the final amino acid concentration of Leu was 2-fold as compared to the α-lac+Ca diet. In the α-lac(2xAsp and Trp)+Ca diet, the final amino acid concentration of Asp and Trp was 2-fold as compared to the α-lac+Ca diet. The composition of WPI and microfiltered native whey protein is described in Table 8. The lean control groups in Studies I and II were fed with a low-fat rodent chow diet (RMI (E) FG SQC 811004, SDS, NOVA-SCB, Sweden). The powdered diets were all moistened with tap water (50 ml/kg in casein, 60 ml/kg in WPI 5%, 90 ml/kg in WPI 50%, 90 ml/kg in WPI 100%, 140 ml/kg in α-lac + Ca, 80 ml/kg in AA + Ca, 130 ml/kg in α-lac(2xAsp and Trp) + Ca, 155 ml/kg in α-lac(2xLeu) + Ca, 60 ml/kg in lactoferrin 15%, 155 ml/kg in microfiltered native whey, 145 ml/kg in α-lactalalbumin), mixed in an industrial dough mixer and packed in one-day portions and stored at -20°C.
Table 6 Diet composition in the thesis.

<table>
<thead>
<tr>
<th></th>
<th>Protein (g)</th>
<th>Casein</th>
<th>WPI 5%</th>
<th>WPI 50%</th>
<th>WPI 100%</th>
<th>Lactoferrin supplementation</th>
<th>α-Lac + Ca</th>
<th>AA + Ca</th>
<th>α-Lac (2xAsp and Trp) + Ca</th>
<th>α-Lac (2xLeu) + Ca</th>
<th>MFNW</th>
<th>α-Lactalbumin</th>
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<tbody>
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<td>WPI 50%</td>
<td>WPI 100%</td>
<td>Lactoferrin supplementation</td>
<td>α-Lac + Ca</td>
<td>AA + Ca</td>
<td>α-Lac (2xAsp and Trp) + Ca</td>
<td>α-Lac (2xLeu) + Ca</td>
<td>MFNW</td>
<td>α-Lactalbumin</td>
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<td>5.2</td>
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<tr>
<td>Protein (% energy)</td>
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<td>Carbohydrate (% energy)</td>
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<tr>
<td>Fat (% energy)</td>
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<tr>
<td>P (%)</td>
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<td>0.59</td>
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<td>0.59</td>
<td>0.59</td>
<td>0.59</td>
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<td>0.6</td>
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<td>III</td>
<td>III</td>
<td>IV</td>
<td>IV</td>
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Table 7 Amino acid profile of the proteins used in the thesis.

<table>
<thead>
<tr>
<th></th>
<th>Casein (g/100 protein)</th>
<th>WPI (g/100 protein)</th>
<th>Lactoferrin (g/100 protein)</th>
<th>α-Lactalbumin (g/100 protein)</th>
<th>Microfiltered native whey (g/100 protein)</th>
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<tr>
<td>Alanine</td>
<td>2.9</td>
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<td>Arginine</td>
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<td>Aspartic acid</td>
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<td>11.6</td>
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<td>Glutamic acid</td>
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<td>Glycine</td>
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<td>4.3</td>
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<td>α-Histidine</td>
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<td>α-Isoleucine</td>
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<td>11</td>
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<tr>
<td>Proline</td>
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<td>Serine</td>
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<td>5.2</td>
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<td>α-Tryptophan</td>
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<td>Tyrosine</td>
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<td>α-Valine</td>
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</table>

*Essential Amino Acids
Table 8 The composition of WPI and microfiltered native whey protein.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
<th>Unit</th>
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</thead>
<tbody>
<tr>
<td><strong>WPI</strong></td>
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<td></td>
</tr>
<tr>
<td>Protein (TNx6.38)</td>
<td>97.9</td>
<td>% dry matter</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>49.5</td>
<td>% dry matter</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>15.5</td>
<td>% dry matter</td>
</tr>
<tr>
<td>IgG</td>
<td>7.2</td>
<td>% dry matter</td>
</tr>
<tr>
<td>IgA</td>
<td>6.2</td>
<td>% dry matter</td>
</tr>
<tr>
<td>Minor proteins</td>
<td>19.6</td>
<td>% dry matter</td>
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<tr>
<td>IgF-1</td>
<td>2.1</td>
<td>mg/100g dry matter</td>
</tr>
<tr>
<td>TGF-beta-2</td>
<td>5.2</td>
<td>mg/100g dry matter</td>
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<tr>
<td>Fat</td>
<td>0.3</td>
<td>% dry matter</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>0.1</td>
<td>% dry matter</td>
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<td>Ash</td>
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<td>Sodium</td>
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<td>Potassium</td>
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<td>Calcium</td>
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<td>Chloride</td>
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<tr>
<td><strong>Microfiltered native whey</strong></td>
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<tr>
<td>Protein ((N-NPN)x6.38)</td>
<td>97.2</td>
<td>% dry matter</td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>23.9</td>
<td>% of protein</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
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<td>% of protein</td>
</tr>
<tr>
<td>Fat</td>
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<td>% of dry matter</td>
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<tr>
<td>Lactose</td>
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<td>% of dry matter</td>
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<td>Ash</td>
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<td>% of dry matter</td>
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<td>Sodium</td>
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<tr>
<td>Magnesium</td>
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</tr>
<tr>
<td>Phosphorus</td>
<td>192.4</td>
<td>mg/100g dry matter</td>
</tr>
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</table>
4.4 Body weight and energy intake measurements

Body weight was monitored once per week and food consumption on a daily basis with a standard table scale (Ohaus Scour™ Pro, SP4001, Nänikon, Switzerland). The energy intake was calculated according to food consumption and nutritional data. In Study IV, weight gain and weight loss were determined from body weight before and after the weight gain (WG) and the weight loss (WL) phases, using Eq. (1) and (2).

Weight gain (%) = 100 x [(weight after WG - weight before WG)/weight before WG] (1)
Weight loss (%) = 100 x [(weight before WL - weight after WL)/weight before WL] (2)

4.5 Dual-energy X-ray absorptiometry (DEXA) measurement

The body fat content and lean body mass (LBM) were measured by dual-energy x-ray absorptiometry (DEXA, Lunar PIXIImus, GE Healthcare, Chalfont St. Giles, UK) after the weight gain, before and after the weight loss, and after the weight regain phases. During the measurement, the mice were under anaesthesia. In Study IV, fat loss and lean body mass maintenance (LBMM) were determined from body fat and LBM before and after weight loss phase, using Eq. (3) and (4).

Fat loss (%) = 100 x [(fat content before WL - fat content after WL)/fat content before WL] (3)
LBMM (%) = 100 x (LBM after WL/LBM before WL) (4)

4.6 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) was performed on the mice after 6 hours of fasting at the end of the weight gain, the weight loss and the weight regain phases. Glucose (glucose dosage 1g/kg given as 20% glucose solution) was administered to the mice by gavage. Blood glucose was measured with a glucose meter (Super Glucocard™ II, GT-1630, Arkray Factory Inc., Shiga, Japan) on blood samples taken from the tail vein at time points 0, 15, 30, 60 and 90 min after gavage. The areas under the curve (AUC) (blood glucose x time) were calculated as well.

4.7 Faecal sample collection (Studies I, II and III)

At the end of both the weight loss and the weight regain phases, the mice were housed in metabolic cages for 24 h. Food and water intakes were recorded. The faecal samples were collected. The faeces were weighed and stored at -70°C until further assayed.
4.8 Tissue sample preparation

At the end of weight gain, weight loss and weight regain phases, the mice were rendered unconscious with CO\textsubscript{2}/O\textsubscript{2} (95%/5%) (AGA, Riihimäki, Finland) and decapitated. The subcutaneous, epididymal, abdominal and perirenal fat pads (in Studies I, II, III and IV), liver (in Studies I and II) and skeletal muscle (musculus quadriceps femoris) (in Study II) were removed, washed with saline, blotted dry and weighted. The total and visceral fat weights were calculated using Eq. (5) and (6) individually. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until further analyzed. In Studies I and II, the distal end of perirenal fat pad and the liver samples for histology were fixed in 10% formalin and embedded in paraffin with routine techniques. In Study I, blood samples were taken into plastic tubes, and serum was separated by centrifugation at 960 x g at 4°C for 20 min.

Total fat weight = subcutaneous + abdominal + epididymal + perirenal fat weights (5)
Visceral fat weight = abdominal + epididymal + perirenal fat weights (6)

4.9 Liver histology (Studies I and II)

In Study I and II, formalin fixed, paraffin embedded liver sections (4 μm) were cut with a microtome. Liver sections were stained with hematoxylin and eosin (H&E) stain and evaluated under a conventional light microscope in a ‘blinded’ fashion. The samples were subjected to a semi-quantitative histological scoring which was slightly modified from the non-alcoholic steatohepatitis (NASH) Clinical Research Network scoring system for Non-alcoholic fatty liver disease (Kleiner et al., 2005; Pilvi et al., 2008; Tiniakos et al., 2010). The scoring system is feature based and derived from individual scores for lobular inflammation, fibrosis, hepatocellular ballooning, and steatosis, which reflect the major histological lesions encountered in NASH (Kleiner et al., 2005; Tiniakos et al., 2010).

4.10 Adipocyte cross-sectional area (Studies I, II and IV)

In Studies I, II and IV, the perirenal fat sections (4 μm) were stained with Hematoxylin and eosin (H&E) stain. The adipocyte cross-sectional area was determined in eight fields from each sample (In Studies I and II, n=5 in each group; In Study IV, n=8-10 in each group) by Leica QWin Standard-software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).
4.11 Biochemical measurements

4.11.1 Serum lipopolysaccharide (LPS) (Study I)

In Study I, serum lipopolysaccharide (LPS) concentration was measured by a Limulus amoebocyte lysate chromogenic end point assay from 1:5 diluted samples according to the instructions of the manufacturer (Hycult Biotechnology, Uden, The Netherlands).

4.11.2 Apparent fat absorption (Studies I, II and III)

In Studies I, II and III, the faecal fat content was determined by the Schmid-Bondzynski-Ratzlaff method ((NMKL), 1989). The apparent fat absorption (%) was calculated based on fat intake and faecal fat content using Eq. (7).

\[
\text{Apparent fat absorption } = 100 \times \frac{\text{fat intake} - \text{faecal fat content}}{\text{fat intake}} \tag{7}
\]

4.12 Western Blotting (Studies I and II)

In Studies I and II, samples from liver were homogenized using a Bertin Precellys 24 homogenizer (Bertin Technologies, Aix en Provence, France) and ceramic beads (Precellys CK14, Bertin Technologies) according to a protocol consisting of 5000 rpm for 50 s repeated twice. The proteins were isolated with lysis buffer (NaCl 136mM, Na₂HPO₄ 8 Mm, KCl 2.7mM, KH₂P₄ 1.46 Mm, Tween 20 0.001%, and complete protease inhibitors (Roche Diagnostics, Neuilly-Sur-Seine, France)). During the immunoblotting, 20 μg of the total protein per lane was loaded and separated by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P®, Millipore, Bedford, MA, USA). After blocking in 5 % non-fat milk-TBS-0.01 % Tween-20® buffer, the membranes were probed with the dilutions of the primary antibodies including anti-SIRT1, S6, phospho-S6, AMP-activated protein kinase α (AMPKα), phospho-AMPKα, anti-SIRT3 and α-tubulin (Table 9). Alpha-tubulin (Abcam, Cambridge, UK) served as a loading control at dilution 1: 2000. The horseradish peroxidase-conjugated anti-rabbit secondary antibody (Chemicon, Temecula, CA, USA) was performed at dilution 1:5000. The localization of horseradish peroxidase was detected with the enhanced chemiluminescence solution (ECLplus, Amersham Biosciences, Buckinghamshire, UK). The protein expression was visualized by using FLA-9000 fluorescent image analyzer (Fujifilm Corp, Tokyo, Japan).
4.13 Quantitative real-time RT-PCR (Studies I and II)

In Studies I and II, total RNA from the perirenal fat pads and skeletal muscle were collected with TRIzol (Invitrogen, Carlsbad, Calif., USA), treated with DNase I (deoxyribonuclease I, Sigma Chemicals, St Louis, MO., USA). One µg total RNA was reverse transcribed to cDNA in the presence of transcription enzyme by ImProm-II™ Reverse Transcription System (Promega, Madison, WI, USA). One µl of cDNA was subjected to a quantitative real time polymerase chain reaction by Lightcycler instrument (Roche Diagnostics, Neuilly sur Seine, France) for the detection of leptin, plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP-1) and 18S mRNAs. The ribosomal 18S served as a housekeeping gene. The samples were amplified with FastStart DNA Master SYBR Green I(Roche Diagnostics) based on the protocol of the manufacturer in the presence of 0.5 µM of the primers listed in Table 10. The quantities of the PCR products were quantified with an external standard curve which was amplified from the purified PCR product.

4.14 Statistical analysis

Data are presented as mean ± SEM. Statistically significant difference in mean values were tested by ANOVA followed by Dunnett’s test (Study I), Newman-Keuls test (Studies III and IV), and Student’s t-test (Study II). The difference was considered significant when P<0.05. The data were analyzed using GraphPad Prism, version 4.02 (GraphPad Software, Inc., San Diego, CA, USA).
<table>
<thead>
<tr>
<th>Name</th>
<th>Producer</th>
<th>Dilution</th>
</tr>
</thead>
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<td>Primary antibodies</td>
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<td></td>
</tr>
<tr>
<td>anti-SIRT1</td>
<td>Upstate, Lake Placid, New York, USA</td>
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</tr>
<tr>
<td>S6</td>
<td>Cell Signaling, Beverly, MA, USA</td>
<td>1:2000</td>
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<tr>
<td>phospho-S6 (Ser240/244)</td>
<td>Cell Signaling, Beverly, MA, USA</td>
<td>1:2000</td>
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<tr>
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<td>phospho-AMPKα (Thr172)</td>
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<td>1:1000</td>
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<tr>
<td>anti-SIRT3</td>
<td>Abcam, Cambridge, UK</td>
<td>1:500</td>
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<td>Loading control</td>
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<tr>
<td>α-tubulin</td>
<td>Abcam, Cambridge, UK</td>
<td>1:2000</td>
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</tbody>
</table>

Table 10 Quantitative real-time RT-PCR primer sequences used in the thesis (Studies I and II).

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<th>Direction</th>
<th>Sequence</th>
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<tr>
<td></td>
<td>reverse</td>
<td>GCCATAGTGCAAGGTT</td>
</tr>
<tr>
<td>plasminogen activator inhibitor 1 (PAI-1)</td>
<td>forward</td>
<td>ACAGCCTTTTGTACATCTGAGCC</td>
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<tr>
<td></td>
<td>reverse</td>
<td>CCGAACCACAAAGAGAAAGGA</td>
</tr>
<tr>
<td>monocyte chemoattractant protein 1 (MCP-1)</td>
<td>forward</td>
<td>CGGAACCAAATGAGATCAG</td>
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<tr>
<td></td>
<td>reverse</td>
<td>TCACAGTCCGAGTCAC</td>
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<td>18S</td>
<td>forward</td>
<td>ACATCCAAAGGAAGGCAGCAG</td>
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<tr>
<td></td>
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</table>
5 Results

5.1 Changes in body weight

Weight gain phase (Study IV(i))

In Study IV, both MFNW and α-lactalbumin inhibited body weight gain during 100 days high-fat feeding as compared to casein (Fig. 2). The final body weight in the α-lactalbumin group (31.9±1.0 g) was 14.3% lower than in MFNW group (37.2±1.1 g). The weight gain in α-lactalbumin group was approximately only 1/3 of the weight gain observed in MFNW group (Fig. 2).

Weight loss phase (Studies I, II, III and IV(ii))

In Study I, WPI dose-dependently enhanced the body weight loss under ER in comparison to casein (Fig. 2). In Study II, the final body weight in the LF supplementation group was 9.5 % lower than in the casein group after weight loss (Fig. 2). In Study III, the AA+Ca diet achieved the greatest effect on accelerating weight loss under ER (Fig. 2). After weight loss, the final body weights in the AA+Ca (P<0.001) and the α-lac+Ca (P<0.05) groups were 21.1 % and 15.1 % lower than that in the casein group, respectively (Fig. 2). In Study IV, no significant differences were observed between groups with respect to body weight at the end of weight loss phase (ANOVA P=0.566).

Weight regain phase (Studies I, II, and III)

In Study I, WPI dose-dependently prevented weight regain as compared to casein (Fig. 2), although only the difference of final body weight between WPI 100 % (35.2±1.9 g) and casein groups (43.4±0.8 g) reached statistical significance (P<0.01). In Study II, LF supplementation inhibited the weight regain as compared to casein during the weight regain phase (Fig. 2). No significant differences were observed in any of the groups with respect to body weight after weight regain in Study III (ANOVA P=0.679).
5.2 Changes in amount of body fat, different adipose tissue and adipocyte size

Weight gain phase (Study IV(i))

In Study IV, both MFNW ($P<0.05$) and $\alpha$-lactalbumin ($P<0.001$) efficiently decreased the body fat content as compared to the casein diet at the end of weight gain phase (Fig. 3). The body fat percentage after weight gain in the $\alpha$-lactalbumin group was also lower than in the MFNW group ($P<0.05$). The effects of MFNW and $\alpha$-lactalbumin on total fat pad weights were in line with those observed for body fat content (Fig. 4). Both MFNW and $\alpha$-lactalbumin decreased the size of adipocytes as compared to casein after weight gain, when measured via the adipocyte cross-sectional area (Fig. 5). As compared with MFNW ($4951\pm 597 \mu m^2$), the adipocyte size was $38.3$ % smaller in the $\alpha$-lactalbumin ($3057\pm 257 \mu m^2$) group ($P<0.05$).
Fig. 3 Difference in mean body fat percentage in comparison with casein control group (%) at the end of the weight gain, the weight loss and the weight regain phases.

**Weight loss phase (Studies I, II, III, and IV(ii))**

In Study I, the body fat content was dose-dependently decreased by WPI as compared to casein after ER (Fig. 3). The body fat content was reduced in all treatment groups as compared with the casein group in both Studies II and III (Fig. 3), although the differences between casein (30.3±3.4 %) and the amino acid supplementation groups (α-lac(2xAsp and Trp)+Ca: 22.5±2.8 %; α-lac(2xLeu)+Ca: 22.7±2.2 %) in Study III in the final body fat percentage did not reach statistical significance.

In Study I, the weights of total fat pad were significantly lower in all WPI groups as compared to that in the casein group (Fig. 4) (ANOVA $P=0.009$). In Study III, all of the tested whey protein diets successfully decreased the total fat pad accumulation as compared to casein (Fig. 4) (ANOVA $P=0.019$).

Adipocyte size after weight loss was measured as adipocyte cross-sectional area in Studies I, II and IV. In Study I, the adipocyte size was significantly smaller in all WPI groups as compared with that in
the casein group after weight loss (Fig. 5) \((P<0.01\) in all WPI groups when compared to the casein group). In Study IV, both MFNW and \(\alpha\)-lactalbumin tended to decrease the adipocyte size in comparison to the casein diet after weight loss, but the difference was not statistically significant (Fig. 5) (Study IV ANOVA \(P=0.051\)).

![Graph showing differences in mean total body fat pad weights in comparison with casein control (%)](image)

**Fig. 4** Difference in mean weight of total fat pad in comparison with casein control group (%) at the end of the weight gain, the weight loss and the weight regain phases.

**Weight regain phase (Studies I, II, and III)**

In Study I, WPI dose-dependently attenuated fat accumulation as compared to casein during weight regain phase (Fig. 3). The total fat percentage in the casein group \((40.7\pm1.7\ %)\) was even higher than the level found in age-matched obese mice \((33.3\pm3.3\ %)\) fed continuously *ad libitum* with the casein diet after the weight regain phase. It is interesting that in both Studies I and II, the total fat percentage in WPI 100 % group (after the weight loss phase: \(21.4\pm1.9\ %\); after the weight regain phase: \(19.2\pm3.4\ %\)) and LF supplementation group (after the weight loss phase: \(24.7\pm2.8\ %\); after the weight regain phase: \(23.8\pm4.4\ %\)) remained at the same level as that observed after the weight loss phase.
The weights of total fat pad were lower in the WPI groups than in the casein group after the weight regain phase in Study I (ANOVA $P=0.001$), although the difference between WPI 50% and casein group was not statistically significant (Fig. 4). In Study II, LF supplementation markedly reduced total fat pad weights as compared to casein (Fig. 4) (t-test $P=0.005$).

**Fig. 5** Difference in mean adipocyte size in comparison with casein control group (%) at the end of the weight gain, the weight loss and the weight regain phases.

Adipocyte size after weight regain was measured as the adipocyte cross-sectional area in Studies I and II (Fig. 5). Adipocyte size was significantly smaller in all WPI and LF groups compared with that in the casein group after weight regain (Study I $P<0.05$ in all WPI groups when compared to the casein group; Study II t-test $P<0.0001$).

**5.3 Changes in lean body mass (LBM)**

*Weight gain phase (Study IV(i))*
The LBM in the MFNW group (26.55±0.73 g) tended to be higher than in either α-lactalbumin (25.54±0.67 g) or casein (25.48±0.58 g) groups after the weight gain phase in Study IV.

**Weight loss phase (Studies I, II, III, and IV(ii))**

In Studies I, II, and IV, no significant difference was observed in LBM in any of the treatment groups as compared to casein group at the end of weight loss phase (Fig. 6) (Study I ANOVA $P=0.243$; Study II t-test $P=0.299$; Study IV ANOVA $P=0.734$). In Study III, as compared with age-matched obese mice fed *ad libitum* with casein-based high-fat diet, energy restriction decreased the LBM in all groups at the end of weight loss phase (ANOVA $P<0.01$). It is of great interest that only AA+Ca and MFNW decreased the LBM, whereas the other diets increased the LBM as compared to casein under ER (Fig. 6).

![Fig. 6 Difference in mean LBM in comparison with casein control group (%) at the end of the weight gain, the weight loss and the weight regain phases.](image)
Weight regain phase (Studies I, II, and III)

A dose-dependent manner towards increased LBM by WPI was noted after weight regain phase in Study I, although the difference was not statistically significant (Fig. 6) (ANOVA P=0.090). In Studies II and III, no significant differences were found in all treatment groups in LBM when compared to casein group, although the LBM in the LF supplementation group tended to be higher than in the casein group (Fig. 6) (Study II t-test P=0.067; Study III ANOVA P=0.913). Interestingly, only the amino acid supplementation groups (α-lac(2xAsp and Trp)+Ca; α-lac(2xLeu)+Ca) decreased the LBM, whereas the other diets increased the LBM as compared to casein after weight regain (Fig. 6).

5.4 Oral glucose tolerance test (OGTT)

OGTT was performed on the mice at the end of the weight gain, the weight loss and the weight regain phases. The area under curve (AUC) (blood glucose level x time) was also calculated.

Weight gain phase (Study IV(i))

In Study IV, no differences among the groups were observed in fasting blood glucose level (ANOVA P=0.90) or the AUC at the end of weight gain phase (ANOVA P=0.33).

Weight loss phase (Studies I, II, III, and IV(ii))

In Study IV, the mice in ER MFNW group displayed lower fasting blood glucose level than in the ER α-lactalbumin group (ANOVA P=0.036). No differences were detected among the groups in the AUC (blood glucose level x time) at the end of weight loss phase in any of the studies (Study I ANOVA P=0.538; Study II t-test P=0.987; Study III ANOVA P=0.085; Study IV ANOVA P=0.385).

Weight regain phase (Study I, II, and III)

In Study I, the mice in WPI 5 % (P<0.05) and 100 % (P<0.05) groups had lower fasting blood glucose levels than the mice in the casein group at the end of the weight regain phase (ANOVA P=0.022). In Study II, an increase in the AUC in LF supplementation group was observed as compared to casein at the end of the weight regain phase (t-test P=0.029). In Study III, leucine supplementation increased the AUC measured in OGTT as compared with α-lac without amino acids supplementation (P<0.05).
An important result to emerge from Study I was, when comparing the AUC of the same treatment group measured after the weight loss phase and the weight regain phase, there was an increase in the AUC in casein group (t-test $P=0.003$) whereas the value in WPI 100 % group remained unaltered (t-test $P=0.527$).

### 5.5 Energy intake

#### Weight gain phase (Study IV(i))

In Study IV, no difference was observed in mean daily energy intake between MFNW, α-lactalbumin and casein groups in the weight gain phase (Table 11).

#### Weight loss phase (Studies I, II, III, and IV(ii))

The mean daily energy intake, measured during the 100 days pre-feeding period, was used in the calculation of 70% energy intake at the beginning of the weight loss phase (in Study III, the pre-feeding period was 150 days). The energy intake measured from additional age-matched obese mice fed ad libitum with casein-based high-fat diet during the weight loss phase from day 100 to day 150 (in Study III, the value was measured from day 150 to day 200) did not differ from the values observed from the pre-feeding period (Study I: t-test $P=0.236$; Study II: t-test $P=0.172$; Study III: t-test $P=0.744$; Study IV: t-test $P=0.063$) in any of the studies. There was no difference in daily energy intake among the groups during the weight loss phase in Studies I, II, and IV. In Study III, no differences were observed between the casein, α-lac+Ca, and AA+Ca groups in daily energy intake during the weight loss phase (Table 11). Neither Asp and Trp nor Leu supplementation significantly influenced the effects of α-lac on daily energy intake during the weight loss phase (Table 11).

#### Weight regain phase (Studies I, II, and III)

No difference was observed among the groups in daily energy intake in Studies I or II during the weight regain phase. In Study III, when compared to the casein group, no significant differences were detected either in the α-lac+Ca group or in the AA+Ca group in daily energy intake during the weight regain (Table 11). Neither Asp and Trp nor Leu supplementation significantly influenced the effects of α-lac on daily energy intake during the weight regain phase (Table 11).
5.6 Apparent fat absorption

In Studies I, II, and III, the mice were housed individually in metabolic cages for 24 h at the end of the weight loss and the weight regain phases. The fecal fat content was analyzed and the apparent fat absorption was calculated based on the fat intake and excretion data. There was no significant difference in the apparent fat absorption between the groups in Studies I or II (Table 11). In Study III, the apparent fat absorption was significantly decreased in AA+Ca group compared to both α-lac+Ca and casein groups at the end of the weight loss phase (Table 11). A similar statistically non-significant difference was also observed at the end of the weight regain phase (Table 11).

5.7 Serum LPS activity

In Study I, serum levels of LPS, a marker of metabolic endotoxemia and low-grade inflammation, were analyzed after the weight loss phase. No difference was found among the groups in serum LPS concentration (casein: 47.8±5.4 pg/ml, WPI 5 %: 41.0±2.7 pg/ml, WPI 50 %: 56.5±8.3 pg/ml, WPI 100 %: 36.0±2.2 pg/ml, ANOVA $P=0.07$). There was also no difference between the age-matched obese mice (casein diet ad libitum feeding) and lean mice (normal rodent chow diet ad libitum feeding) (obese controls: 45.3±4.6 pg/ml vs. lean controls: 47.4±8.8 pg/ml, t-test $P=0.82$).

5.8 Liver histology

In Studies I and II, prominent steatosis was present in the obese mice fed ad libitum. The liver histology showed variable degrees of fat accumulation ranging from focal midzonal and perivenular vesicle clusters to diffuse macrovesicular fatty changes. Minimal foci of inflammatory cells were noted in some samples from the obese mice, but fibrosis was absent in all samples. After the weight regain phase, the samples of WPI 100 % group and LF group exhibited less steatosis and improved tissue morphology. It is important that the samples of WPI 100 % group showed a complete absence of steatosis and displayed histology similar to the lean controls, i.e. the mice which were age-matched and had been constantly fed with a normal chow diet.
Table 11 Energy intake and apparent fat absorption in different groups during weight gain, weight loss and weight regain.

<table>
<thead>
<tr>
<th>Study</th>
<th>Casein control</th>
<th>Weight gain</th>
<th>Weight loss</th>
<th>Weight regain</th>
<th>Apparent fat absorption (%)</th>
<th>Weight loss</th>
<th>Weight regain</th>
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<tr>
<td></td>
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<td>Daily energy intake (kcal/mouse/day)</td>
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<td>Weight loss</td>
<td>Weight regain</td>
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<tr>
<td>Study I</td>
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<td>WPI 5%</td>
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<td>WPI 100%</td>
<td>10.33±0.11</td>
<td>17.10±1.36</td>
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<tr>
<td></td>
<td>ANOVA P</td>
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<td>Study II</td>
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<td>Lactoferrin supplementation</td>
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<td>Study III</td>
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<td>α-Lac + Ca</td>
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<td>20.15±0.28</td>
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<tr>
<td></td>
<td>AA + Ca</td>
<td>11.36±0.13</td>
<td>24.03±2.41</td>
<td>81.61±3.42*,**</td>
<td>90.95±1.35</td>
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<td>α-Lac (2xAsp and Trp) + Ca</td>
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<td>α-Lac (2xLeu) + Ca</td>
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<td>91.23±2.09**</td>
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### Study IV

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<th>Apparent fat absorption (%)</th>
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<tr>
<td></td>
<td>Weight gain</td>
<td>Weight loss</td>
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<tr>
<td>(i) Casein control</td>
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<td>MFNW</td>
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<td>α-Lactalbumin</td>
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<td>ANOVA P</td>
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<tr>
<td>(ii) Casein control</td>
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<td>ER MFNW</td>
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<tr>
<td>ER α-Lactalbumin</td>
<td>10.17±0.03</td>
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</tr>
<tr>
<td>ANOVA P</td>
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<td></td>
</tr>
</tbody>
</table>

*a Data is presented as mean ± SEM. The asterisk (*) denotes the significant difference in comparison with the casein control group (*P<0.05). #Denotes the significant difference in comparison with the α-Lac + Ca group (#P<0.01). ‡Denotes the significant difference in comparison with the AA + Ca group (‡‡P<0.01, ‡‡‡P<0.01).
5.9 Adipose tissue and muscle inflammation

In Study I, in comparison with the age-matched lean control mice, obese mice fed *ad libitum* with casein diet showed increased expression of leptin mRNA (obese 1.00±0.17 vs. lean 0.15±0.06, t-test *P*=0.002), MCP-1 mRNA (obese 1.00±0.19 vs. lean 0.17±0.06, t-test *P*=0.003), and PAI-1 mRNA (obese 1.00±0.22 vs. lean 0.11±0.03, t-test *P*=0.003) in the adipose tissue. Although ER per se evoked a decrease in the expression levels of MCP-1 and leptin mRNA in the adipose tissue in all groups as compared to age-matched obese mice fed *ad libitum* in Study I, no significant difference was observed between casein and WPI groups after the weight loss or the weight regain phase. In Study II, there was no significant difference in adipocyte MCP-1 mRNA expression between casein and LF groups after the weight loss phase, whereas this difference did achieve statistical significance after the weight regain phase (t-test *P*=0.032).

5.10 Hepatic S6 ribosomal protein, AMPK, SIRT1 and SIRT3 expressions

In both Studies I and II, the phosphorylation of S6 ribosomal protein in the liver was markedly reduced in WPI (Fig. 7A) and LF (Fig. 7B) groups as compared with the casein group after weight loss, whereas these differences did not reach statistical significance after the weight regain phase (Study I ANOVA *P*=0.966; Study II t-test *P*=0.368). WPI at 50 %, 100 % concentration (Fig. 7C) and LF (Fig. 7D) significantly enhanced the hepatic SIRT3 expression at the protein level at the end of the weight loss phase. However, these differences were attenuated after the weight regain phase (Study I ANOVA *P*=0.493; Study II t-test *P*=0.430). In Study I, there were no differences between the casein and WPI groups in the phosphorylation level of AMPK or SIRT1 expression after the weight loss (AMPK ANOVA *P*=0.280; SIRT1 ANOVA *P*=0.321) or the weight regain phase (AMPK ANOVA *P*=0.150; SIRT1 ANOVA *P*=0.464).
Fig. 7 Hepatic S6 ribosomal protein phosphorylation and SIRT3 expression in the liver of C57Bl/6J mice after energy restriction in Studies I and II. Data are presented as mean ± SEM. The asterisk (*) denotes the significant difference in comparison with casein control group (*$P<0.05$, **$P<0.01$).
6 Discussion

6.1 Methodological aspects

Animal Model

All studies were conducted in the C57Bl/6J mouse strain, which is a well-established model of diet-induced obesity (Surwit et al., 1988; Collins et al., 2004; Koza et al., 2006; Kanasaki & Koya, 2011). This mouse strain is often used as a healthy control animal and as the basis for generating genetically modified animal models (Rivera & Tessarollo, 2008), since these mice remain lean and physiologically normal when they are fed with a standard rodent diet. After being fed a high-fat diet, this mouse strain develops obesity and displays the consequences of obesity i.e. insulin resistance, hyperinsulinemia, hypertension, and fatty liver (Collins et al., 2004). Furthermore, the C57Bl/6J mouse strain has shown the capacity to develop obesity and its consequences in a relatively short time as compared with humans. An increase in body weight was observed after 2 weeks’ high-fat feeding and hyperglycemia developed after 4 weeks’ high-fat feeding (Collins et al., 2004; Wang & Liao, 2012). After 16-20 weeks of high fat feeding, the mice had gained 20-30 % more body weight than normally fed mice. These results demonstrate that this mouse strain can serve as a better human-like model of obesity and metabolic syndrome than the other widely used obese mouse models, such as leptin-deficient ob/ob or leptin-resistant db/db mice.

Diet modification

In all of the studies, the mice were fed with a high-fat diet with constant energy ratio of carbohydrate (21 %), protein (18 %) and fat (61 %). The protein sources of the diets in all studies were modified by replacing casein with the protein of interest. This approach helps to identify the distinctive effects of proteins and allows a direct comparison of the potential of different whey proteins to modify diet-induced obesity in the experimental mice model. Energy restriction, which was used in all of the studies, has been shown to exert beneficial effects on health and to safely induce weight loss in animal models (for review see Speakman and Mitchell, 2011).

The novel WPI examined in Study I mainly contains lactoperoxidase (about 50 %) and lactoferrin (about 15 %), which is different from the normal WPI (lactoperoxidase 0.5 %; lactoferrin 1 %) (Krissansen, 2007). It is also rich in immunoglobulins and growth factors. The normal WPI has a high BCAA content; the major differences on the amino acids profile between the novel WPI and casein are the levels of cysteine, glutamic acid, methionine and proline. The effects of novel WPI observed in Study I were further investigated by replacing 15 % of the casein by lactoferrin in the high-fat diet.
used in Study II. Compared with the normal whey protein which is obtained as a by-product of cheese making, the MFNW protein in Study IV is manufactured by a microfiltration technique using a polymeric membrane. The MFNW protein is rich in β-lactoglobulin (about 65 %), α-lactalbumin (about 24 %) and free of CMP. Based on the amino acid profile, MFNW contains a higher level of two BCAAs, leucine and isoleucine, than casein. In comparison with casein, the most enriched amino acids in MFNW are cystine and tryptophan.

In Study III, the amount of calcium in the diets was modified from 0.79 % to 1.8 %. Since α-lactalbumin has shown its prominent anti-obese effects in the same mouse strain under high-calcium feeding (Pilvi et al., 2009), the effects of α-lactalbumin and an amino acid mixture with an identical amino acid profile as in high-calcium diets were evaluated in Study III. In milk, as compared with whey proteins, calcium is mainly integrated with casein. However, some whey-derived peptides are also known to bind calcium (Vegarud et al., 2000). Therefore, calcium might interact with whey protein-derived peptides during digestion, and this may affect the metabolism of both calcium and whey proteins.

6.2 Effects of whey proteins on body weight and body fat content

In previous studies, the intake of whey proteins with high calcium supplementation has conferred beneficial effects on body weight and fat content in diet-induced obese animal model under high-fat feeding (Pilvi et al., 2007; Pilvi et al., 2008; Pilvi et al., 2009). The current results of Studies I, II, and IV indicate that without calcium supplementation, whey proteins themselves, including novel WPI, α-lactalbumin and LF, exert beneficial effects on body weight and fat content in the same model under high-fat feeding. The effects of novel WPI in the present study were displayed in a dose-dependent manner. The functional component of novel WPI was further studied in Study II by examining the effects of LF alone with the same LF concentration (15 %) in novel WPI. It was noted that LF supplementation and novel WPI had comparable effects on body weight and fat content, which evidences that the effects of novel WPI are largely due to its LF component. In Study IV, the observed effects of MFNW on body weight and fat content were about 1/3 of that obtained with α-lactalbumin. In addition, α-lactalbumin accounted for 24 % of MFNW. Thus the present results suggest that the beneficial effects of MFNW are largely due to its α-lactalbumin content.

LF has been reported to exert a modulatory effect on lipid metabolism. Previous studies had revealed that the plasma LF level was inversely related to hyperglycemia and positively to insulin sensitivity in subjects with insulin resistance (Moreno-Navarrete et al., 2008; Moreno-Navarrete et al., 2009). Ono et al. (2010) noted that the enteric-coated LF could decrease visceral fat accumulation in obese patients possibly by binding and inactivating LPS and LRP1 to block incorporation of lipid in the visceral fat. Thus, these mechanisms may partly explain the effects of novel WPI and LF on body weight and fat content found in the present studies.
Whey proteins have been shown to inhibit food intake in humans by increasing feelings of satiety (see review Luhovyy et al., 2007). However, the present studies did not detect any differences in energy intakes in Studies I, II and IV. The optimal amino acid composition of whey proteins has been proposed to account for their potential health effects, especially the high amount of BCAAs, which play important roles as both energy substrates and in muscle protein synthesis (Zemel, 2005; Zemel & Miller, 2004; Layman & Walker, 2006; Halford et al., 2007). It was found in the present studies that there was a higher amount of two BCAAs, leucine and isoleucine, in α-lactalbumin and in the MFNW than in casein. Interestingly, although MFNW contains a higher amount of BCAAs than α-lactalbumin, α-lactalbumin achieved better anti-obesity effects than MFNW. Furthermore, the amounts of BCAAs in novel WPI and isoleucine in LF were even less than in casein, but both WPI and LF displayed prominent anti-obesity effects in the present studies. Therefore, there are other non-BCAAs factors which mediate these beneficial metabolic effects of whey proteins.

In comparison with casein, the most enriched amino acids in novel WPI were cystine and arginine, whereas in LF they were cystine and glycine, in α-lactalbumin cystine and aspartic acid, and in MFNW cystine and tryptophan. If one considers these amino acids, arginine has been shown to reduce adiposity in both genetically (Fu et al., 2005; Wu et al., 2007c) and diet-induced obese rats (Jobgen et al., 2009), pigs (He et al., 2009) and in obese human subjects with type 2 diabetes (Lucotti et al., 2006). There are recent studies indicating that arginine can stimulate mitochondrial biogenesis and brown adipose tissue development possibly by enhancing the synthesis of cell-signaling molecules and triggering the expression of genes which promote whole-body oxidation of energy substrates (McKnight et al., 2010), which may partly explain the effects of the novel WPI on body weight and fat content noted in Study I. Tryptophan, the precursor of the neurotransmitter serotonin, has been suggested to be involved in the regulation of appetite through the anorexic effects of serotonin in the central nervous system (Halford et al., 2007). Cysteine has been reported to be a precursor for the most abundant intracellular antioxidant glutathione (Wu et al., 2004). It is of great interest that cystine is the most enriched amino acid in all whey proteins tested here as compared with casein. Therefore, the role of amino acid composition in the anti-obesity effects of whey proteins remains to be clarified.

Alpha-lactalbumin has been reported to exert beneficial effects on obesity and obesity related metabolic disorders (Nieuwenhuizen et al., 2008; Pilvi et al., 2009; Hursel et al., 2010). There is a report that, when combined with calcium supplementation, α-lactalbumin is the most promising whey protein fraction (i.e. out of normal WPI, α-lactalbumin, β-lactoglobulin, and LF) for improving the outcome of weight loss and weight regain during high-fat feeding (Pilvi et al., 2009). The present studies investigated the effects of α-lactalbumin and an amino acid mixture with an equivalent amino acid profile on diet-induced obesity in the same mouse model and with calcium supplementation in order to explore the anti-obesity effects of α-lactalbumin on a molecular basis. It was found that the effects of α-lactalbumin could be reproduced by providing an amino acid mixture with an identical amino acid profile during ER, which indicates that the anti-obesity effects of α-lactalbumin were
mainly being mediated by its amino acid composition rather than by any peptides formed during digestion.

Dietary calcium intake has been shown to have beneficial effects on body weight by increasing fat excretion (Christensen et al., 2009) as well as promoting 1,25-dihydroxy-vitamin D₃-mediated alterations in adipocyte metabolism (Zemel, 2000; Shi et al., 2001a; Shi et al., 2002; Zemel & Miller, 2004). Therefore, this may explain the only difference in fat excretion found with calcium supplementation in Study III, which in turn at least partly accounts for the observed effects on body weight and fat content detected in Study III. There did not appear to be any difference in apparent fat absorption in Studies I and II, which indicates that an intake of whey proteins without calcium supplementation does not interfere with the fat digestibility during high-fat feeding. In a previous study, Pilvi et al. (2007) observed that whey proteins increased the fat excretion as compared with casein during high-calcium high-fat feeding, which suggests that milk proteins may affect the calcium binding fatty acids properties. Interestingly, in Study III, it was also found that the increased fat excretion in the amino acid mixture with an identical amino acid profile of α-lactalbumin group as compared with α-lactalbumin group. However, it is unclear whether the changes in calcium binding properties between α-lactalbumin and amino acids mixture actually contribute to the differences in fat excretion.

Although a large proportion of the whey proteins, and their peptides which are bioavailable after hydrolysis in the intestine, are ultimately degraded into amino acids, which are taken up into the blood circulation from the small intestine, it does seem that there are certain whey proteins and their derived peptides which are resistant to hydrolysis. Whey-derived peptides have shown several bioactivities including opioid agonist (α-lactorphin), opioid antagonists (lactoferroxins), ACE-inhibitory (lactokinins), and antimicrobial (lactoferricin) (Jones et al., 1994; Teschemacher et al., 1997; FitzGerald & Meisel, 1999; Meisel, 2005; Korhonen & Pihlanto, 2006; Yalcin, 2006; Hartmann & Meisel, 2007). Interestingly, a novel hypocholesterolemic peptide derived from β-lactoglobulin clearly affected serum cholesterol level in rats in comparison with β-sitosterol (Nagaoka et al., 2001). It is possible that certain whey protein-derived peptides may also play an important role in mediating the anti-obesity effects of whey proteins (Torres-Fuentes et al., 2014). The potential whey-derived peptides with anti-obese effects still need to be studied in future.

6.3 Effects of whey proteins on fatty liver formation, glucose homeostasis and adipose tissue

In Studies I and II, the obesity-induced fatty liver formation was based on a previously published and validated histological scoring system (Kleiner et al., 2005; Tiniakos et al., 2010). Although this system was intended to detect NAFLD in clinical trials, one could propose that it is also suitable for
screening fatty liver formation in animal models. It was noted that both novel WPI and LF ameliorated the ectopic fat accumulation after the weight loss and inhibited fatty liver formation after the weight regain, especially WPI which in Study I completely blocked fatty liver formation after the weight regain. Insulin resistance is clearly associated with ectopic fat accumulation in muscle and liver. The decreased body fat content can improve insulin sensitivity, which is consistent with the lower fasting blood glucose levels found in WPI and LF groups after the weight regain and the improved insulin sensitivity as reflected in blood glucose-time-area under curves from OGTT after the weight loss and the regain phases in the present studies. In addition, it was observed that the improved insulin sensitivity obtained with LF was associated with a reduction in skeletal muscle leptin mRNA expression pointing to an amelioration of obesity-induced ectopic fat accumulation within muscle. It is of great interest that there was more severe liver damage after the weight regain in the casein control group as compared with the age-matched group which was consuming casein diet during the whole follow-up period ad libitum in Study II. This severe liver damage is likely to be mediated, at least partly, via weight gain and fat accumulation i.e. the rebound effects on body weight and fat content in the casein group after the weight regain phase.

Chronic adipose tissue inflammation has been postulated to play a key role in the development of obesity-related insulin resistance (Weisberg et al., 2003; Xu et al., 2003; Tilg & Moschen, 2006). The release of cytokines is known to trigger the recruitment and activation of immune cells to sites of inflammation. LF has been reported to modulate the inflammatory process mainly by preventing the release of cytokines (Legrand et al., 2005), which explains the findings in Study II that LF ameliorated obesity-induced low grade inflammation measured as MCP-1 mRNA expression in the visceral fat. It has been postulated that high-fat feeding induced low grade inflammation is at least partly due to the increased intestinal permeability leading to endotoxemia (Cani et al., 2007). Unlike other whey protein fractions, LF is partially resistant against proteolytic degradation, and thus it is retained at least to some degree as undigested molecule or as lactoferricin-containing peptides in the gastrointestinal tract (Kuwata et al., 2001; Tomita et al., 2009). Previous research has found evidence for the down-regulation of cytokines by LF to be partly related to the LPS-binding properties of LF through its lactoferricin domain (Appelmelk et al., 1994; Elass-Rochard et al., 1995; Brandenburg et al., 2001). However, the present studies were unable to detect any difference in the concentration of serum LPS between obese and lean control groups. Thus, it is not clear whether the anti-inflammatory effects of LF found in the present studies were linked to its LPS binding properties and this will need to be evaluated in the future.
6.4 Effects of whey proteins and energy restriction on nutrient sensing signaling pathways

The nutrient sensor links regulating energy intake and metabolism are complex. AMPK is known to be a highly conserved ultrasensitive energy sensor and it participates in the regulation of energy-generating and energy-consuming pathways, i.e. it integrates nutritional and hormonal signals in both peripheral tissues and in the hypothalamus (Kahn et al., 2005). AMPK has been proposed to mediate the effects of adipokines to regulate food intake, body weight and lipid, glucose metabolism (Kahn et al., 2005). The activation of AMPK in the peripheral tissues reduces the risk of obesity and insulin resistance by stimulating fatty acid oxidation and enhancing glucose transport and metabolism (Kahn et al., 2005). The beneficial effects of AMPK activation are largely mediated via the inhibition of the mTOR-S6 kinase (S6K1) pathway, which is chronically activated by nutrient excess (Kahn et al., 2005; Korsheninnikova et al., 2006). Previous research has revealed that high-fat feeding can increase S6 phosphorylation in the liver (Korsheninnikova et al., 2006), whereas S6K1-deficient mice were protected against the obesity attributable to enhanced β-oxidation (Um et al., 2004). In agreement with the above findings, the present studies found that weight loss under ER in both novel WPI and LF fed mice was associated with decreased hepatic S6 ribosomal protein phosphorylation. Sirtuins, a highly conserved family of NAD⁺-dependent enzymes regulating life span in lower organisms, have been claimed to act as metabolic sensors and mediators of the cellular effects of caloric restriction (Finkel et al., 2009; Haigis & Sinclair, 2010). SIRT3, which is a member of this family, has been reported to regulate mitochondrial function, thermogenesis and mitochondrial fatty acid oxidation. SIRT3 is the only sirtuin for which there is a reported association with human lifespan (Finkel et al., 2009; Haigis & Sinclair, 2010). Previous research has demonstrated that mice fed with high-fat diet exhibited a reduced level of SIRT3 activity in the liver, decreased hepatic NAD⁺ levels and increased mitochondrial function (Kendrick et al., 2011). In good agreement with these findings, it was found that there was increased hepatic SIRT3 expression in both novel WPI and LF fed mice after ER. These findings indicate that the metabolic effects of novel WPI and LF might be partly mediated via inhibition of the mTOR nutrient sensing pathway and activation of SIRT3 in the liver. However, this is speculation since the enzyme activity of SIRT3 was not determined. Therefore, the cellular mechanism mediating the dietary alteration effects on the above mentioned nutrient sensors will need to be clarified in the future.

6.5 Clinical relevance

The aim of obesity treatment is to prevent and treat other obesity associated diseases such as type 2 diabetes, cardiovascular diseases, sleep apnoea and osteoarthritis by achieving a permanent at least 5 %
reduction of body weight (Tsigos et al., 2008). In the present studies, whey proteins inhibited weight gain under *ad libitum* energy intake, as well as enhancing the weight loss due to 30% decreased energy intake below *ad libitum* intake and the whey proteins also prevented weight regain with subsequent *ad libitum* energy intake. Although the results of an animal experiment cannot be directly applicable to human beings, the present study confirms the anti-obesity effects of different whey proteins and indicates that increasing the amount of whey protein content in diets could be beneficial during both weight loss and in subsequent weight maintenance.

The current treatment of NAFLD is primarily aimed to slow the progression of NAFLD, and to prevent liver-related illness and death (Adams et al., 2005; Bjornsson & Angulo, 2007). Life style management has been suggested as the first step towards improving NAFLD (Adams et al., 2005; Bjornsson & Angulo, 2007). In the present studies, whey proteins ameliorated fat accumulation in liver in conditions of energy restriction and prevented fatty liver formation after weight regain. Although these results cannot be directly extrapolated to humans, it is tempting to recommend that whey proteins supplementation could become a part of the life style management in the treatment of NAFLD.
7 Conclusions

The aim of the present study was to investigate the potential of different whey proteins and their mechanisms of actions, including whey protein isolate (WPI), alpha-lactalbumin (α-lac), lactoferrin (LF) and microfiltered native whey (MFNW), in the prevention and treatment of diet-induced obesity and its consequences in C57Bl/6J mice. The main findings and conclusions of the present study were as follows:

1. WPI dose-dependently accelerated weight and fat loss and prevented weight and fat regain during high-fat-feeding in C57Bl/6J mice. WPI ameliorated obesity induced fatty liver formation and improved glucose tolerance without influencing energy intake, apparent fat digestibility, or adipose tissue inflammation. These findings indicate that the effects of WPI are mediated, at least partly, via the inhibition of the mTOR nutrient sensing pathway and the activation of SIRT3 in liver.

2. LF enhanced weight and fat loss and inhibited weight and fat regain in C57Bl/6J mice fed a high fat diet. LF exerted beneficial effects on glucose tolerance, adipose tissue inflammation and it ameliorated the obesity induced fatty liver formation without interfering with energy intake or apparent fat digestibility. It is suggested that the effects of LF are mediated, at least partly, via the inhibition of the mTOR nutrient sensing pathway and the activation of SIRT3 in liver.

3. The anti-obesity effects of α-lac under energy restriction could be reproduced by feeding an amino acid mixture with an equivalent amino acid profile. The findings indicate the anti-obesity effects of α-lac are mainly attributable to its amino acid composition.

4. The MFNW produced by polymeric membranes, was able to prevent weight gain and fat accumulation without interfering with glucose homeostasis or energy intake in high-fat-fed C57Bl/6J mice. This may be a possible method to generate whey proteins with high bioactive value in a large scale. The findings also indicate these effects of MFNW are largely due to its abundant α-lac content.
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83


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