METABOLIC AND CELLULAR EFFECTS OF CALORIE RESTRICTION AND
WHEY PROTEINS IN EXPERIMENTAL OBESITY

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University of Helsinki

ACADEMIC DISSERTATION

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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>ACVR2B</td>
<td>Activin receptor member II</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALDH1A7</td>
<td>Aldehyde dehydrogenase 1 family, member A1</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate (AMP)-activated protein kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CHREBP</td>
<td>Carbohydrate-responsive-element-binding protein</td>
</tr>
<tr>
<td>CR</td>
<td>Calorie restriction</td>
</tr>
<tr>
<td>CXCL16</td>
<td>Chemokine (C-X-C motif) ligand 16</td>
</tr>
<tr>
<td>C5a</td>
<td>Complement component 5a</td>
</tr>
<tr>
<td>DPPIV</td>
<td>Dipeptidyl peptidase-IV</td>
</tr>
<tr>
<td>EIF4EBP1</td>
<td>Eukaryotic translation initiation factor 4e</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FBXO32</td>
<td>F-box protein 32</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein 01</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin like growth factor-1</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Insulin-like growth factor-binding protein 3</td>
</tr>
<tr>
<td>IL-6</td>
<td>Inteleukin 6</td>
</tr>
<tr>
<td>LBM</td>
<td>Lean body mass</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MIG</td>
<td>Chemokine (C-X-C motif) ligand 9</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metallopeptidase</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NAM</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NOV</td>
<td>Nephroblastoma overexpressed</td>
</tr>
<tr>
<td>NR4A3</td>
<td>Nuclear receptor subfamily 4, group A, member 3</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor-γ coactivator 1 alpha</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPS6</td>
<td>Ribosomal protein S6</td>
</tr>
<tr>
<td>RANTES</td>
<td>Chemokine (C-C motif) ligand 5</td>
</tr>
<tr>
<td>SCD1</td>
<td>Stearoyl-coenzyme A desaturase 1</td>
</tr>
<tr>
<td>sICAM</td>
<td>Soluble intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Silent mating type information regulation-2 homolog 1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>STACS</td>
<td>Sirtuin activating compounds</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Tissue inhibitor of metalloproteinases 1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UCP-2</td>
<td>Uncoupling protein 2</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WPI</td>
<td>Whey protein isolate</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>1,25-dihydroxy-vitamin-D₃</td>
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</tbody>
</table>
ABSTRACT

Obesity, an epidemic problem in the world, is associated with higher mortality and increase in the risk of diabetes, cardiovascular diseases, and certain forms of cancer. Calorie restriction (CR) with adequate nutrition is the most effective method to induce weight loss, though compliance with low-caloric diets is often poor among obese individuals. Compounds capable of mimicking the effects of CR therefore hold great promise as novel anti-obesity drugs. The main aim of the present study was to investigate the molecular and signaling pathways mediating the effects of CR with special emphasis on the sirtuin, AMPK and mTOR pathways. As data from recent clinical and experimental studies suggest, milk-derived whey proteins could enhance the anti-obesity effects of CR by yet unknown mechanisms, the study also aimed to clarify the anti-obesity effects of whey proteins and their mechanisms of action.

High-fat diet induced C57Bl/6J mice were used as a model of experimental obesity. The metabolic effects of dietary regimens were examined by daily recording of food and energy intake, body weight monitoring three times weekly, in vivo calorimetry, and analysis of body fat percentage and lean body mass by dual-energy X-ray. The cellular effects were investigated by immunohistochemistry, Western blot and qRT-PCR analyses, as well as by protein arrays and microarray genechips.

CR (energy intake 70% of ad libitum intake) protected against obesity and fatty liver, induced physical activity and ameliorated adipose tissue inflammation. These effects were associated with an increased SIRT1 expression in the liver and skeletal muscle as well as the SIRT3 expression in the liver, skeletal muscle and adipose tissue. CR also increased the SIRT4 expression in the skeletal muscle. In contrast, the SIRT1 activating compound resveratrol did not prevent obesity although it partially prevented fatty liver and modestly increased skeletal muscle SIRT1 and SIRT4 expressions. CR exerted distinct effects on adipose tissue cytokine and angiogenesis profiles in obese and lean mice. Obesity induced cytokine and angiogenesis-related protein expressions, and these changes were largely ameliorated by CR, while in lean mice, CR increased the expression of several cytokines and angiogenesis-related proteins.

High-calcium whey protein (WPI) and α-lactalbumin diets enhanced the anti-obesity effects of CR. These diets produced marked alteration in the skeletal muscle gene expression profile compared to the casein diet, with the Wnt signaling being the most highly altered pathway. Unlike casein, WPI and α-lactalbumin diets induced SIRT3 protein expressions in muscle and decreased the Aldh1a7, Fasn, leptin, Nr4a3 and Scd1 mRNA expressions, indicating alterations in lipid and fatty acid metabolism. A novel WPI rich in lactoperoxidase, lactoferrin, growth factors and immunoglobulins dose-dependently enhanced weight loss during CR, prevented weight re-gain and protected against fatty liver formation during the
ad libitum phase. WPI increased hepatic SIRT3 expression more than casein and decreased hepatic S6 ribosomal protein phosphorylation, suggesting inhibition of the mTOR pathway.

In conclusion, the present study showed that CR increases the expression of sirtuins, in particular SIRT3, in metabolically important tissues suggesting their central role as mediators of the metabolic and cellular effects of CR. The present study also provides evidence that CR ameliorates obesity-induced cytokine and angiogenesis protein overexpression in adipose tissue. Finally, the present study underlined that whey protein-based diets enhance the anti-obesity effects of CR via mechanisms linked to sirtuins and altered skeletal muscle gene expression profile.
1. Introduction

Obesity has become a major worldwide health problem that is associated with several metabolic abnormalities contributing to the risk of chronic diseases, such as type 2 diabetes, cardiovascular diseases, and certain types of cancer (Mitchell et al. 2011). Development of obesity is associated with adipose tissue remodeling, which leads to adipocyte dysfunction, abnormal cytokine secretion and chronic low-grade inflammation (Guilherme et al. 2008, Hajer et al. 2008). Obesity also contributes fat deposition to non-adipose tissues as an ectopic fat, and it is a major risk factor for nonalcoholic fatty liver disease (NAFLD) (Parekh and Anania 2007, Dowman et al. 2010). Fatty liver is insulin resistant and it overproduces glucose, very-low density lipoprotein, C-reactive protein and coagulation factors (Kotronen and Yki-Järvinen 2008). Both chronic low-grade inflammation and NAFLD are important mediators in development of obesity-linked metabolic diseases.

Weight loss is the primary treatment for obesity and its consequences. Calorie restriction (CR) with adequate nutrition effectively induces weight loss and ameliorates obesity-induced metabolic disturbances. In lower organisms, reducing the caloric intake below the usual levels decreases the incidence of aging-related diseases and is associated with longevity (Fontana et al. 2010). Although the results are still insufficient to show the longevity impact of CR in humans, CR has been shown to reduce the risk of type 2 diabetes and cardiovascular diseases and induce similar adaptive responses as in lower organisms (for review see Holloszy and Fontana 2007). However, the mechanism underlying the beneficial effects of CR is not well understood. Accumulating evidence indicates an important role for highly conserved nutrient sensing pathways; sirtuin, AMPK and mTOR pathways in mediating the effects of CR on health and lifespan (for review see Guarente 2005, Fontana et al. 2010, Canto and Auwerx 2011).

Compliance to a CR lifestyle is low and therefore there has been great interest in finding compounds capable of regulating the activity of nutrient sensing pathways and to mimic the effects of CR. Several sirtuin activating compounds (STACS) have been developed (Howitz et al. 2003, Milne et al. 2007). The polyphenolic compound resveratrol (3,5,4′-trihydroxystilbene) is one of the STACS that has been shown to extend lifespan in yeast in a Sir2-dependent manner (Howitz et al. 2003). The beneficial effects of resveratrol are also demonstrated in mammalian cells (for review see Baur and Sinclair 2006). In vivo studies with diet-induced obese mice have shown that resveratrol improves health and prevents premature mortality associated with obesity (Baur et al. 2006, Lagouge et al. 2006). The effects of resveratrol are generally believed to happen through mammalian SIRT1, which is the closest homologue of the yeast Sir2 protein.

Nutrition also has a crucial role in the prevention and treatment of obesity. Epidemiological studies have shown that a diet high in dairy products is inversely associated
with body mass index (BMI) (Mirmiran et al. 2005, Marques-Vidal et al. 2006, Varenieta et al. 2007, Azadbakht and Esmaizadeh 2008), and the intake of dairy products is related to a lower risk of type 2 diabetes and metabolic syndrome (Crichton et al. 2011). In addition, clinical trials have shown that high dairy intake facilitates weight and fat loss during CR (Abargouei et al. 2012). Dairy calcium has been suggested to account for part of the anti-obesity effects of dairy products via increased fat excretion (Christensen et al. 2009) and 1,25-dihydroxy-vitamin D3 (1,25(OH)2D3)–mediated changes in adipocyte lipid metabolism (Zemel 2003). In addition, dairy proteins, especially whey proteins, have been shown to prevent weight gain and enhance weight loss during CR via as yet unknown mechanisms (Pilvi et al. 2007, Frestedt et al. 2008, Royle et al. 2008, Pilvi et al. 2009).

The better understanding of the mechanism of how CR mediates its effects could reveal new targets for anti-obesity drug development. Therefore, the present study aimed to investigate the molecular and signaling pathways mediating the effects of CR with special emphasis on the sirtuin, AMPK and mTOR pathways. As milk-derived whey proteins have been shown to augment weight loss effects of CR via as yet unknown mechanisms, the study also aimed to clarify the anti-obesity effects of whey proteins and their mechanisms of action.
2. Review of literature

2.1 Obesity

Obesity is defined as abnormal or excessive fat accumulation in adipose tissue to the extent that health may be impaired (WHO 2012a). The fundamental cause of obesity is a positive imbalance between energy intake and energy expenditure. However, the etiology of obesity is multifactorial, and it involves the interaction between genetic, environmental, psychosocial, physiological and metabolic factors (Mitchell et al. 2011). Body mass index (BMI) is the most useful population-level measure of obesity to classify underweight, overweight and obesity in adults (WHO 2000). BMI is defined as the weight in kilograms divided by the square of the height in meters (kg/m²). Overweight and obesity in adults is based on various BMI cutoffs that are associated with the risk of co-morbidities (Table 1).

Both overweight and obese persons with excess fat are associated with many risks of medical conditions that can lead to further morbidity and mortality. Numerous epidemiological studies have shown that the main health consequences of being overweight and obese are type 2 diabetes, cardiovascular diseases, certain types of cancer and musculoskeletal disorders (Guh et al. 2009). In addition, excess weight is an important factor in the development of other illnesses and metabolic disorders, including respiratory diseases, chronic kidney diseases, gastrointestinal and hepatic disorders, lower physical performance and psychological problems (Tsigos et al. 2008). Obesity is estimated to decrease life expectancy by 7 years at the age of 40 years (Peeters et al. 2003). Being overweight or obese is estimated to be the fifth leading risk factor for global deaths (WHO 2012a).

The prevalence of obesity has increased dramatically worldwide, and the rate of obesity has more than doubled since 1980 (WHO 2012a). According to the WHO, more than 1.4 billion adults were overweight in the year 2008, and of those, over 200 million men and nearly 300 million women were obese (WHO 2012a). In Europe, obesity has increased 10-40% in the past 10 years, and 10-25% of men and 10-30% of women are obese depending on

Table 1. Definition of obesity and risk of co-morbidities according to WHO (2000).

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI (kg/m²)</th>
<th>Risk of co-morbidities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt;18.5</td>
<td>Low (but risk for other clinical problems increased)</td>
</tr>
<tr>
<td>Normal weight</td>
<td>18.5-24.9</td>
<td>Average</td>
</tr>
<tr>
<td>Overweight:</td>
<td>≥25.0</td>
<td></td>
</tr>
<tr>
<td>Preobese</td>
<td>25.0-29.9</td>
<td>Increased</td>
</tr>
<tr>
<td>Obese Class I</td>
<td>30.0-34.9</td>
<td>Moderate</td>
</tr>
<tr>
<td>Obese Class II</td>
<td>35.0-39.9</td>
<td>Severe</td>
</tr>
<tr>
<td>Obese Class III</td>
<td>≥40.0</td>
<td>Very severe</td>
</tr>
</tbody>
</table>
the European country (Tsigos et al. 2008). The cost to society from obesity is enormous, and up to 8% of the total health-care costs in Europe are due to obesity in adults (WHO 2013). Of alarming concern is the increase in the prevalence of obesity in childhood which is associated with a higher chance of obesity, premature death and disability in adulthood (WHO 2012a).

The increased intake of energy-dense foods which are high in fat, salt and sugars, together with reduced physical activity at work and during leisure time are considered to be the main reason for the dramatic growth in obesity (WHO 2012a). Both the European (Tsigos et al. 2008) and Finnish (Aikuisten lihavuus, Käypä hoito –suositus, 2011) evidence-based guidelines for management and treatment of adult obesity aim to improve health and prevent and alleviate obesity associated co-morbidities which can be achieved by 5-10% permanent weight loss. Guidelines involve lifestyle counseling on eating and physical activity behavior as well as on thoughts and attitudes that guide eating and physical activity habits. The guidelines emphasize that weight management should be lifelong and in addition to weight loss, it involves weight maintenance and prevention of weight re-gain. In some cases weight reduction can be supported by a very-low-calorie diet, drug therapy and obesity surgery.

According to Finnish and European guidelines, drug therapy is recommended for patients with a BMI ≥30 kg/m² or a BMI ≥27 kg/m² with obesity-related diseases (Tsigos et al. 2008, Aikuisten lihavuus, Käypä hoito-suositus, 2011). At present, orlistat is the only anti-obesity drug on the market in Europe. The appetite suppressants sibutramine and phentermine are no longer licensed in Europe due to adverse cardiac effects. Orlistat, a pancreatic lipase inhibitor, reduces intestinal digestion and absorption of approximately 30% of dietary fat (Bray and Ryan 2007). A one year orlistat treatment has shown to induce an additional 2.9 kg weight loss compared to a placebo (Rucker et al. 2007). Several combination therapies targeting hypothalamic pathways that regulate appetite and body weight are under investigation for development of novel pharmacologic treatments for obesity (Vetter et al. 2010). The most promising of those is phentermine/topiramate (sympatomimetic amine/anti-epileptic agent) combination which is currently approved in the US as an obesity treatment.

Obesity surgery is considered for severely obese patients aged 18-60 years with a BMI ≥40 kg/m² or a BMI ≥35 kg/m² with a obesity-related co-morbidities which can be expected to be alleviated by the surgery (Tsigos et al. 2008, Aikuisten lihavuus, Käypä hoito-suositus, 2011). Bariatric surgery has been shown to decrease body weight an average of 40 kg, and at the moment, it is the most effective treatment against obesity in severely obese patients (Aikuisten lihavuus, Käypä hoito-suositus, 2011).
2.1.1 Adipose tissue metabolism and obesity

The main physiological function of white adipose tissue (WAT) is to store excess energy as triglycerides and release them as fatty acids when energy expenditure exceeds energy intake to ensure a sufficient energy status (Rosen and Spiegelman 2006). In addition, WAT is a highly active endocrine organ that secretes large number of hormones, cytokines and other proteins involved in specific biological function such as glucose and lipid metabolism, inflammation, coagulation, and blood pressure and food intake control (Rosen and Spiegelman 2006, Hajer et al. 2008). Due to the major role of WAT in the whole-body energy homeostasis, WAT metabolism has a central role in the development of obesity-associated metabolic disorders.

Obesity is associated with visceral adipose tissue accumulation and the expansion of adipose tissue is characterized by adipocyte hypertrophy (an increase in adipocyte volume) and hyperplasia (an increase in adipocyte cell number) (for review see Bays et al. 2008). However, it is believed that the number of adipocytes is largely set by early adulthood and adipocyte hypertrophy is the dominant feature of obese adipose (Spalding et al. 2008). Adipose tissue is also a highly vascularized organ and therefore, new blood vessel formation, angiogenesis is a necessity for adipose tissue growth (Christiaens and Lijnen 2010, Daquinag et al. 2011, Sun et al. 2011). Adipocyte hypertrophy is known to cause hypoxia in cells which induces expressions of angiogenic factors (Hosogai et al. 2007). Overall, adipocyte hypertrophy leads to dysfunctional adipocytes that metabolic and secretory activities are changed (Figure 1). Dysfunctional adipocytes are known to produce chemoattractant peptides (e.g. MCP-1) that enhance macrophage infiltration into adipose tissue (Weisberg et al. 2003, Xu et al. 2003). Macrophages are responsible for most of the pro-inflammatory cytokine (e.g. IL-6, TNF-α) production in obese adipose tissue contributing to the progression of chronic low-grade inflammation (Guilherme et al. 2008, Ouchi et al. 2011). This is accompanied by the diminished insulin action in adipocytes which results in increased lipolysis and fatty acids release (Guilherme et al. 2008). High levels of circulating free fatty acids (FFAs) increase depositions in non-adipose tissues, primarily in the liver and skeletal muscle (Guilherme et al. 2008). Both inflammation and ectopic fat accumulation is associated with reduced skeletal muscle and liver insulin sensitivity, and the development of type 2 diabetes and cardiovascular diseases (Hajer et al. 2008).
Figure 1. **Obesity induced adipocyte dysfunction and development of insulin resistance.** Adipocyte hypertrophy (increased adipocytes size) increases cytokine and free fatty acid secretion leading to inflammation and ectopic fat accumulation which contributes to the development of insulin resistance, type 2 diabetes and cardiovascular diseases. IL-6; interleukin 6, MCP-1; monocyte chemoattractant protein-1, TNF-α; tumor necrosis factor-alpha (Adapted from Galic et al. 2010).

### 2.1.2 Skeletal muscle metabolism and obesity

Skeletal muscle is responsible for the major part of insulin-stimulated whole body glucose disposal, and hence plays an important role in the pathogenesis of insulin resistance and development of type 2 diabetes. Much evidence suggests that FFAs, which circulating levels are markedly increased in obesity (see section 2.1.1), play a crucial role in the development of skeletal muscle insulin resistance (for review see Phielix and Mensink 2008). It has been shown that prolonged exposure of skeletal muscle and myocytes to high levels of fatty acids, especially to saturated fatty acids, leads to severe insulin resistance (Griffin et al. 1999, Yu et al. 2002, Hirabara et al. 2010).

Several mechanisms have been shown to explain the insulin resistance induced by fatty acids (for review see Martins et al. 2012). The first mechanistic explanation for the fatty acid-induced insulin resistance was proposed by Randle et al. (1963), who assumed that there is an inverse relationship between fatty acid availability and glucose utilization. According to
that glucose fatty-acid cycle theory, elevated fatty acids supply and oxidation lead to reduced glucose uptake and metabolism. Later, it was been found that FFAs primarily inhibit glucose transport, but glucose metabolism remains unchanged (Dresner et al. 1999, Griffin et al. 1999). In addition, the increased accumulation of FFAs within skeletal muscle (intramyocellular lipids) is strongly associated with insulin resistance (for review see Moro et al. 2008). However, intramyocellular lipid per se does not cause muscle insulin resistance, but rather the accumulation of fatty acid-derived metabolites, such as diacylglycerol and ceramides (Schmitz-Peiffer et al. 1999, Yu et al. 2002). Evidence has shown that reduced muscle oxidative capacity in obese subjects, which is assumed be due to mitochondrial dysfunction, results in muscle lipid accumulation (for review see Martins et al. 2012). Chronic elevation of FFA levels has been shown to reduce the expression of genes involved in mitochondrial biogenesis and oxidative capacity (Schmid et al. 2004, Sparks et al. 2005), and the production of reactive oxidative species (ROS) is increased by FFAs (Bonnard et al. 2008). As a result, mitochondrial biogenesis and function are impaired, which reduce mitochondrial mass and impair mitochondrial oxidative capacity leading to an accumulation of lipid metabolites and further increased ROS production (for review see Martins et al. 2012). Both lipid metabolites and ROS activate several kinases (e.g. JNK (c-Jun N-terminal kinase), NF-κB (nuclear factor-κB), PKC (protein kinase c)). That activation impairs the insulin signaling pathway by inducing serine/threonine phosphorylation of the insulin receptor substrate 1 (IRS-1) and thus decreases glucose uptake and metabolism in response to insulin (for review Martins et al. 2012).

2.1.3  Non-alcoholic fatty liver disease and obesity

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological condition characterized by lipid accumulation in the liver causing liver damage similar to alcohol, but it occurs in individuals without a history of chronic alcohol consumption (Angulo and Lindor 2002). NAFLD represents a wide spectrum of liver diseases ranging from pure steatosis to steatohepatitis (NASH), and fibrosis to irreversible cirrhosis (Machado and Cortez-Pinto 2005, Parekh and Anania 2007). Pure steatosis is defined histological as >5% hepatic lipid accumulation (Neuschwander-Tetri and Caldwell 2003) that rarely progresses to advanced liver diseases, whereas NASH constitutes an inflammation and hepatocellular damage having a strong potential to progress into cirrhosis, end stage liver failure, and hepatocellular carcinoma (Rafiq et al. 2009).

Concomitant with obesity, NAFLD is an increasingly recognized condition and up to 30% of adults in Western countries have NAFLD (Browning et al. 2004, Zelber-Sagi et al. 2006). Obesity and insulin resistant are the key pathogenic abnormalities associated with NAFDL, and it is a common condition among patients with type 2 diabetes (for review see Smith and Adams 2011). A fatty liver is insulin resistant and it overproduces glucose and very-low
density lipoprotein, and also other factors, such as C-reactive protein and coagulation factors, which lead to hyperglycemia, hyperinsulinemia and lipid disorders (for review see Kotronen and Yki-Järvinen 2008). Due to those metabolic consequences, NAFLD is closely linked to metabolic syndrome and the risk of developing type 2 diabetes and cardiovascular diseases. However, not all obese persons deposit fat in the liver and liver fat content is recognized to be independent of age, gender and BMI (Kotronen et al. 2007). The deviation in liver fat accumulation between individuals is thought to explain, at least in part, why some obese and even lean individuals develop metabolic syndrome and insulin resistance whereas other equally obese do not (for review see Kotronen and Yki-Järvinen 2008).

Although the accumulation of triglycerides within the hepatocytes is evident in the pathogenesis of NAFLD, the precise mechanism leading to hepatic lipid accumulation is poorly understood. It has been suggested that it is based on a ‘2-hit hypothesis’ (Day and James 1998). The ‘first hit’ is characterized by hepatic triglyceride accumulation and progression of hepatic steatosis, and the ‘second hit’ involves the emergence and progression of inflammation and development of NASH. Insulin resistance and excess adiposity are associated with increased lipid influx into the liver, increased de novo hepatic lipogenesis by up-regulating hepatic lipogenic transcription factors (e.g. SREBP1c, ChREBP), and decreased hepatic mitochondrial lipid oxidation, promoting the ‘first hit’ in the hypothesis (for review see Browning and Horton 2004, Dowman et al. 2010). In addition, insulin resistance and especially visceral adiposity are associated with increased levels of toxic FFAs, pro-inflammatory cytokines, mitochondrial oxidative stress and endoplasmic reticulum stress which leads to inflammation, cell death and fibrosis, and contribute to the ‘second hit’ of hypothesis (for review see Browning and Horton 2004, Dowman et al. 2010).

### 2.1.4 Type 2 diabetes and obesity

Type 2 diabetes is a complex metabolic and endocrine disease that is characterized by insulin resistance and pancreatic β-cell dysfunction. The early phenomenon of progression of type 2 diabetes is insulin resistance when the biological effects of insulin are less effective, and both glucose disposal in skeletal muscle and suppression of endogenous glucose production primarily in the liver are disturbed. When the pancreatic β-cells are no longer able to produce adequate insulin to overcome insulin resistance, impaired glucose tolerance progresses to type 2 diabetes. (For review see Stumvoll et al. 2005).

Type 2 diabetes is a highly heterogeneous disease that etiology is multifactorial with genetic and environmental factors playing an important role in the pathogenesis (for review see Nolan et al. 2011). As mentioned above (see sections 2.1.1-2.1.3), obesity and subsequent chronic low grade inflammations and ectopic fat accumulation to skeletal muscle and liver (NAFLD) are the major risk factors for type 2 diabetes. Toxic FFAs and
cytokines (e.g. TNF-α and IL-6) can detrimentally affect both insulin signaling and pancreatic β-cells function (for review see Kahn et al. 2006, Hajer et al. 2008). Considering the high prevalence of obesity, it is not surprising that type 2 diabetes is now a pandemic. The estimated worldwide prevalence of diabetes among adults was 285 million (6.4%) in 2010 and the value is predicted to rise to around 439 million (7.7%) by 2030 (Shaw et al. 2010).

Type 2 diabetes causes both macrovascular and microvascular complications and it is a major global cause of premature mortality. It has been estimated that the global excess mortality in 2000 attributable to diabetes in adults was 2.9 million (5.2% of deaths) (Roglic et al. 2005). Diabetes strongly increases the risk of heart disease and stroke and approximately 50% of people with type 2 diabetes die of cardiovascular diseases (Morrish et al. 2001). Diabetes is also a leading cause of kidney failure which explains 10-20% of deaths among people with diabetes (Van Dieren et al. 2010). In addition, diabetes is the most common cause of blindness among adults aged 30-69 years (Klein 2007), as well as non-traumatic lower-limb amputations (Van Dieren et al. 2010). Overall, the risk of dying among people with diabetes is at least double compared to their non-diabetic peers (WHO 2012b).

*Taken together, obesity is associated with visceral fat accumulation and the expansion of adipose tissue, cause adipocyte hypertrophy, which leads to adipocyte dysfunction. In dysfunctional adipocytes, cytokine and free fatty acid secretion are increased resulting in chronic low-grade inflammation and ectopic fat accumulation in skeletal muscle and the liver (NAFLD). Both inflammation and ectopic fat impairs skeletal muscle and liver insulin sensitivity and contribute to the development of cardiovascular diseases and type 2 diabetes.*
2.2  Calorie restriction

While excessive calorie intake and subsequent obesity are associated with the increased risk of several chronic diseases and premature mortality, calorie restriction (CR) with adequate nutrition improves multiple parameters of health and extends lifespan. CR is defined as a dietary intervention where calorie intake is reduced below the usual ad libitum intake while adequate intake of proteins and micronutrients are maintained at sufficient levels to avoid malnutrition. The effects of CR on disease risk and life expectancy is widely studied in model organisms and humans.

2.2.1  Calorie restriction in model organisms

CR in lower organisms

The first evidence that CR can extend the mean and maximum lifespan of rats was published in 1935 by McCay et al. (1935). Subsequent data have shown that CR slows aging and increases maximum lifespan in different species, including yeast, fruit flies, worms, spiders, fish, mice and dogs (Weindruch and Walford 1988, Masoro 2005). However, the only mammals in which CR has clearly shown to slow aging and extend maximum lifespan are rats and mice (Fontana et al. 2010). The magnitude of lifespan extension is shown to be dependent on the age when CR is started, the severity of restriction and strain or genetic background of animals (Cheney et al. 1983, Merry 2002, Liao et al. 2010). In rodents, initiating a 25-60% reduction in calorie intake below ad libitum food intake, started early in life (from shortly after weaning to age 6 months), increases maximum lifespan up to 50% (Koubova and Guarente 2003). When a 44% reduction in calorie intake is started in adulthood (age 12 months), the lifespan is extended by 10-20% (Weindruch and Walford 1982).

Data from studies conducted in laboratory rodent models have shown that CR induces several health benefits such as reduced adiposity and inflammation (Muzumbar et al. 2008, Fontana 2009), decreased oxidative damage and serum IGF-1 levels (Breese et al. 1991, Sohal and Weindruch 1996), and improvements in vascular function, glucose and lipid metabolism (Fontana and Klein 2007, Fontana 2008) (Figure 2). Collectively those physiological changes increase longevity by preventing or delaying the occurrence of chronic diseases, including diabetes, autoimmune and respiratory diseases, cardiovascular diseases (Weindruch and Walford 1988, Guo et al. 2002, Masoro 2005, Fontana 2008), kidney diseases (Lee et al. 2004, Chen et al. 2007), and cancer (Hursting et al. 2003, Longo and Fontana 2010). In addition, CR in mice decreases neurodegeneration, β-amyloid deposition in the brain and enhances neurogenesis in animal models of Alzheimer disease, Parkinson
disease, Huntington disease and stroke (Mattson 2005, Martin et al. 2006). However, approximately one third of these experimental rodents die without any evidence of apparent organ pathology (Shimokawa et al. 1993) suggesting that reduction of chronic diseases does not completely explain the increased lifespan of calorie restricted rodents.

Figure 2. Some of the physiological changes associated with calorie restriction in mammals.

CR in non-human primates
There are two active randomized, non-human primate studies testing the benefits of long-term CR on longevity and disease prevention in rhesus monkeys, one at the University of Wisconsin at Madison (Kemnitz et al. 1993, Ramsey et al. 2000) and the other at the National Institute of Aging in Baltimore (Lane et al. 2001, Mattison et al. 2003). The 20 year data from a group at the University of Wisconsin revealed that moderate CR significantly reduced incidence of aging-related death in rhesus monkeys, even though the overall mortality was unaffected by CR (Colman et al. 2009). Taking only the aging-related death into account, 50% of control monkeys survived compared with 80% survival of CR animals. The group at the University of Maryland found similar beneficial effects of CR on lifespan, though results did not reach statistical significance (Bodkin et al. 2003).

Similar to rodents, CR in rhesus monkeys resulted in lower body weight and adiposity (Bodkin et al. 2003, Mattison et al. 2003, Cefalu et al. 2004, Colman et al. 2009), decreased body temperature and resting energy expenditure (Lane et al. 1996, Blanc et al. 2003), reduced triiodothyronine (T3) concentration (Roth et al. 2002), inflammatory markers and oxidative stress (Kim et al. 1997, Zainal et al. 2000) and delayed immune senescence (Messaoudi et al. 2006). In addition, CR improved cardiometabolic health by decreasing
blood pressure, serum glucose and insulin concentrations, and improving insulin sensitivity and serum lipid profile (Kemnitz et al. 1994, Lane et al. 1995, Verdery et al. 1997, Mattison et al. 2003). The physiological changes in non-human primates are associated with the reduced risk of several chronic diseases, including incidence of diabetes, cancer, cardiovascular diseases and brain atrophy (Colman et al. 2009).

2.2.2 Calorie restriction in humans

The promising evidence from research in monkeys suggests that CR might have beneficial effects in humans as well. There are several studies in the literature evaluating CR in humans, but the evidence is still insufficient to show the longevity impact of CR in humans (for review see Holloszy and Fontana 2007). The Okinawan centenarians are shown as evidence that CR improves human-health. People from the Japanese island of Okinawa have a lower caloric intake and higher prevalence of centenarians than the mainland Japanese population and Americans (Wilcox et al. 2007, 2008).

The role of CR in human health is also evaluated by three different epidemiological studies. One of these studies is Biosphere 2, which included 4 women and 4 men consuming a low-calorie, nutrient dense diet (1750-2100 kcal/day) inside a sealed environment for 2 years (Walford et al. 2002). CALERIE (Comprehensive Assessment of Long-Term Effects of Reducing Calorie Intake) consists of phase 1 studies evaluating the effects of 20-25% CR in humans for 6 or 12 months (for review see Holloszy and Fontana 2007), and a phase 2 study, which is a 2 year multicenter trial of 225 subjects randomized to a 25% CR diet or a weight maintenance diet (Rochon et al. 2011). In addition, data from a series of studies conducted by 18 members of the Calorie Restriction Society (CRS) group that practices self-imposed CR (~30% less calories than age- and sex-matched volunteers consuming a typical Western diet) for an average 6.5 years are published (Fontana et al. 2004, Fontana et al. 2006, Meyer et al. 2006).


Even though CR reduces the risk of cardiovascular diseases and diabetes in humans and induces similar adaptive responses that occur in laboratory animals, the major concern in human studies is the ability to maintain long-term CR. In the Biosphere 2 study, participants
were followed for several months after the 2 year study and their body weight and other variables returned to their pre-study levels after the study, indicating the difficulty of maintaining a CR lifestyle (Walford et al. 2002). In addition, in CALERIE study, subjects were assigned to a 20% CR diet for 1 year, but participants managed to maintain only 10% CR over the study period (Racetta et al. 2006).
2.2.3 Molecular mechanisms of calorie restriction

Although the beneficial effects of CR are well established, the exact mechanism whereby CR exerts its health- and lifespan-extending effects is still quite unclear. Considering the beneficial effects of CR in aging and many chronic diseases, the understanding of how CR exerts these effects could reveal targets for drugs and therapies for broad-spectrum diseases. Recent studies in model systems have revealed that highly conserved nutrient sensing pathways; sirtuin, AMPK and mTOR pathways are connected to CR and longevity regulation (for review see Guarente 2005, Fontana et al. 2010, Canto and Auwerx 2011). Noteworthy is that the nutrient sensing pathways strongly overlap with each other (Figure 3). In addition, it is well-established that down-regulation of insulin and insulin/IGF-1 signaling system are associated with longevity (for review see Berryman et al. 2008). Impaired autophagy is linked to several metabolic- and aging-related diseases (for review see Levine and Kroemer 2008), and all nutrient sensing pathways regulate autophagy.

![Figure 3. Schematic representation of nutrient sensing signaling pathways in calorie restriction.](image)

CR is suggested to activate the SIRT1 (sensitive to high NAD\(^+\) levels) and AMPK (sensitive to high ADP/ATP and AMP/ATP ratios) pathways and suppress insulin/IGF-1/mTOR signaling pathway. Sirtuins and AMPK pathways positively regulate each other through increasing NAD\(^+\) levels and deacetylating LKB1 (upstream kinase of AMPK). AMPK negatively regulates the mTOR pathway through phosphorylating TSC2 (upstream regulator of mTOR) and Raptor (mTOR component). All nutrient sensing pathways regulate autophagy through UNC-51-like kinase (ULK1) and autophagy genes (Atg).
2.2.3.1 Sirtuin pathway

The sirtuin protein family

Sirtuins are the class III histone deacetylase protein family that use oxidized nicotinamide adenine dinucleotide (NAD⁺) as a cofactor. The first member of the sirtuin family of proteins to be identified was histone deacetylase Sir2 (silent information regulator 2) in yeast (Saccharomyces cerevisiae). Sir2 was originally identified as a gene which has importance in the maintenance of the silent chromatin at the mating-type loci, telomeras and rRNA-encoding DNA repeats (Guarente 2000). Later on, Sir2 was recognized as regulating the yeast replicative lifespan as the overexpression of the Sir2 extends lifespan up to 30% and the deletion or mutation of Sir2 gene shortens lifespan by 50% (Kaeberlein et al. 1999). Yeast lifespan can also be increased by CR; reducing the amount of sugar in the growth medium, and the effect has been shown to be dependent on the Sir2 gene (Lin et al. 2000). In addition to yeast, Sir2 orthologous genes found in worms and flies also function to increase lifespan and are required for CR-induced longevity (Tissenbaum and Guarente 2001, Rogina and Helfand 2004, Wang and Tissenbaum 2006).

Sirtuins are highly conserved from prokaryotes to mammals. The first sirtuin identified from mammals was SIRT1 (silent mating type information regulation-2 homolog 1), which is the closest homologue of yeast Sir2 protein. In addition to SIRT1, the mammalian sirtuin family comprises seven proteins (SIRT1 to SIRT7), which can be divided into four classes according to sequence homology to yeast Sir2 protein. SIRT1 - SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III and SIRT6 and SIRT7 to class IV (Frye 2000) (Table 2).

Sirtuins subcellular localization and enzymatic activity

Mammalian sirtuins are localized in numerous compartments within the cell (Table 2). SIRT1 and SIRT6 predominantly localizes in the nucleus, while SIRT7 presents in the nucleolus and SIRT2 predominantly localizes in the cytosol (Haigis and Sinclair 2010, Houtkooper et al. 2012). SIRT3, SIRT4 and SIRT5 localize in the mitochondria (for review see He et al. 2012, Huang et al. 2012). However, subcellular localization of these proteins has been shown to be dependent on the cell type, cellular stress status and molecular interaction. For instance, mainly nuclear SIRT1 can also present in the cytosol, and mainly cytosolic SIRT2 can also present in nucleus (Haigis and Sinclair 2010, Houtkooper et al. 2012). Although the physiological relevance of SIRT1 shuttling is still unclear, SIRT1 has been shown to shuttle to the cytosol upon inhibition of insulin signaling (Tanno et al. 2007).

Sirtuins were originally identified as NAD⁺-dependent class III histone deacetylase enzymes. However, sirtuins do not just deacetylate histones, but also a wide range of proteins in different subcellular compartments (Table 2). In addition to deacetylase activity, SIRT4 and SIRT6 can function as ADP-ribosyltransferases, although SIRT6 also has deacetylase activity (Haigis and Sinclair 2010, Houtkooper et al. 2012). Moreover, SIRT5 was
Table 2. Summary of the mammalian sirtuins.

<table>
<thead>
<tr>
<th>Sirtuin</th>
<th>Class</th>
<th>Localization</th>
<th>Activity</th>
<th>Targets</th>
<th>Functions</th>
<th>Null phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT4</td>
<td>II</td>
<td>Mitochondria</td>
<td>ADP-ribosylation</td>
<td>GHD</td>
<td>Insulin secretion, fatty acid oxidation</td>
<td>Developmentally normal</td>
<td>Haigis et al. 2006</td>
</tr>
<tr>
<td>SIRT5</td>
<td>III</td>
<td>Mitochondria</td>
<td>Deacetylation, demalonylation, desuccinylation</td>
<td>CPS1</td>
<td>Urea cycle</td>
<td>Developmentally normal</td>
<td>Nakagawa et al. 2009, Du et al. 2011, Peng et al. 2011</td>
</tr>
<tr>
<td>SIRT7</td>
<td>IV</td>
<td>Nucleolus</td>
<td>Unknown</td>
<td>Unknown</td>
<td>rDNA transcription</td>
<td>Smaller size, short lifespan, heart defects</td>
<td>Ford et al. 2006, Vakhrusheva et al. 2008</td>
</tr>
</tbody>
</table>

AceCS2; acetyl-CoA synthase 2, CPS1; carbamoyl phosphate synthase 1, CRTC2; CREB regulated transcription coactivator 2, FOXO1; forkhead box O1, FOXO3a; forkhead box O1 3a, FXR; farnesoid X receptor, GDH; glutamate dehydrogenase, H3K9; Histone 3 lysine 9, H3K56; histone 3 lysine 56, H4K16; histone 4 lysine 16, HMGCS2; 3-hydroxy-3-methylglutaryl-CoA synthase 2, IDH2; isocitrate dehydrogenase 2, LCAD; long-chain acyl-CoA dehydrogenase, LXR; liver X receptor α, MnSOD; manganese superoxide dismutase, NF-κB; nuclear factor-κB, PAR3; partitioning defective 3 homologue, PEPCK; phosphoenolpyruvate carboxykinase PGC-1α; peroxisome proliferator-activated receptor-γ coactivator 1α, PPAR-γ; peroxisome proliferator-activated receptor-γ, SOD2; superoxide dismutase 2, SREBP-1c; sterol response element binding protein-1c, STAT3; signal transducer and activator of transcription 3, UCP-2; uncoupling protein 2
recently shown to primarily demalonylate and desuccinylate proteins (Du et al. 2011, Peng et al. 2011). One common feature of all enzymatic reactions of sirtuins is that the enzymatic activities are based on NAD$^+$, which is an indicator of cellular energy and nutrient status (for reviews see Canto and Auwerx 2012, Houtkooper et al. 2012). In the enzymatic reaction, sirtuins convert NAD$^+$ to nicotinamide (NAM) and O-acetyl-ADP-ribose (O-AADPR) (for review see Haigis and Sinclair 2010, Guarente 2012).

**Sirtuins function**

SIRT1 is the best-characterized mammalian sirtuin. SIRT1 has been shown to be important for embryogenesis and reproduction as SIRT1 null mice are small in size, sterile, they have developmental defects, and most die during the early postnatal period (McBurney et al. 2003). SIRT1 deacetylates several transcriptional factors and proteins important for energy metabolism and stress resistance (Table 2) (see in below). However, SIRT1 also has several targets beyond energy metabolism. For instance, SIRT1 has a marked anti-inflammatory effect in diverse tissues and cell models (Pfluger et al. 2008, Purushotham et al. 2009) which is thought to happen through negative regulation of the nuclear factor-κB (NF-κB) (Yeung et al. 2004). In addition, SIRT1 activation was initially linked to increased tumor formation after the finding that SIRT1 deacetylates and inactivates tumour suppressor protein p53 and inhibits p53-dependent apoptosis (Luo et al. 2001, Vaziri et al. 2001). However, contrary to this, in vivo studies have indicated that SIRT1 is in fact a tumour suppressor (for review see Herranz and Serrano 2010).

Of the mitochondrial sirtuins (SIRT3-SIRT5), SIRT3 is the major mitochondrial deacetylase and several targets involved in energy homeostasis have been identified (Table 2) (see below). Although mitochondria are important in energy production and mitochondrial dysfunction is linked to many metabolic and aging-related diseases (for review see Nunnari and Suomalainen 2012), mice lacking mitochondrial sirtuin (SIRT3$^{-/-}$, SIRT4$^{-/-}$ and SIRT5$^{-/-}$ mice) develop normally, but they have several metabolic disturbances and reduced resistance to nutrient stress (for review see Huang et al. 2010, Giralt and Villarroya 2012, He et al. 2012) (see more in detail below).

Compared to SIRT1 and SIRT3, less is known about the physiology of other sirtuins. SIRT2 is known to deacetylate tubulin, but the relevance of it is unknown (North et al. 2003). More importantly, SIRT2 regulates cell cycle by deacetylating histone 4 lysine 16 (H4K16) (Vaquero et al. 2006) and increases cell survival by deacetylating fordkhead box O1 3a (FOXO3a) (Wang et al. 2007). SIRT2 also deacetylates partitioning defective 3 homologue (PAR3) leading to decreased activity of the cell polarity control protein atypical protein kinase C (aPKC), and thereby changes the myelin formation of Schwann cells (Beirowski et al. 2011). In addition, SIRT2 regulates gluconeogenesis through deacetylating phosphoenolpuryvate carboxykinase (PEPCK) (Jiang et al. 2011) and adipogenesis through FOXO1 (Jing et al. 2007).

SIRT6 is the highly specific histone 3 deacetylase that targets H3K9 and H3K56, playing an important role in DNA repair, telomerase function, genomic stability, and cellular senescence (Michishita et al. 2008, Michishita et al. 2009, Yang et al. 2009). SIRT6 deficiency causes the most
striking phenotype. SIRT6 null mice suffer from several metabolic imbalances, postnatal growth retardation and premature death at age one month (Mostoslavsky et al. 2006). SIRT6−/− mice are severely hypoglycaemic which is possibly mediated by hypoxia-inducible factor 1α (HIF1α)-dependent activation of glycolysis (Zhong et al. 2010). Interestingly, neural SIRT6-deleted mice are small at birth which is due to low growth hormone and IGF-1 levels and they reach normal size at age 1 year and develop obesity later in life (Schwer et al. 2010).

SIRT7 is one of the most unknown sirtuins. SIRT7 is reported to activate RNA polymerase I transcription, but the protein substrate is still unknown (Ford et al. 2006). However, SIRT7 null mice have a shorter lifespan, they are smaller, and they develop heart hypertrophy and inflammatory cardiomyopathy, which is linked to p53 hyperacylation (Vakhrusheva et al. 2008).

**Regulation of sirtuin activity**

Regulation of sirtuin activity occurs at four different levels, and as the best-characterized sirtuin, SIRT1 regulation is also the best-described (Figure 4). In general, SIRT1 mRNA expression is higher during low energy status (Nemoto et al. 2004), while high-fat diet (HFD) feeding reduces it (Coste et al. 2008). Various transcription factors are suggested to regulate sirtuin mRNA expression in response to these stimuli (Figure 4a). FOXO1, peroxisome proliferator-activated receptor-α (PPAR-α), PPAR-β and cAMP response element-binding (CREB) induce SIRT1 expression (Hayashida et al. 2010, Okazaki et al. 2010, Noriega et al. 2011, Xiong et al. 2011), whereas PPAR-γ, carbohydrate response element-binding protein (ChREBP), poly(ADP-ribose) polymerase 2 (PARP2) and hypermethylated in cancer 1 (HIC1) repress SIRT1 expression (Chen et al. 2005b, Han et al. 2010, Bai et al. 2011a, Noriega et al. 2011). The HIC1-mediated repression is dependent on the carboxy-terminal binding protein (CTBP) and it is enhanced by NADH (Chen et al. 2005b). MicroRNAs (miRNAs) are post-transcriptional regulators that modulate mRNA levels through the degradation of the primary mRNA transcript or by inhibition of translation. Two miRNAs, miR-34a and miR-199a repress SIRT1 expression (Yamakuchi et al. 2008, Rane et al. 2009), and miR-34a levels are increased during diet-induced obesity (Lee et al. 2010a).

SIRT1 activity is also regulated by post-translational modification (Figure 4b). SIRT1 is phosphorylated by the cyclin-dependent kinase 1 (cyclin B-CDK1), which results in enhanced cell proliferation (Sakaki et al. 2008). JUN N-terminal kinase (JNK) also phosphorylates SIRT1 in response to oxidative stress, which leads to deacetylation of histone H3, but not p53 indicating that phosphorylation directs SIRT1 to specific targets (Nasrin et al. 2009). In addition, dual specificity Tyr-phosphorylated and regulated kinase 1 (DYRK1) and DYRK3 phosphorylate and activate SIRT1 leading to increased cell survival through inhibition of p53-dependent apoptosis (Guo et al. 2010). Genotoxic stress (e.g. UV light and hydrogen peroxide) inactivates SIRT1 by desumoylating through sentrin-specific protease (SENP) (Yang et al. 2007), whereas sumoylation activates SIRT1 (Yang et al. 2007).

Sirtuins are also regulated by complex formation (Figure 4c). AROS is the only identified positive regulator protein of SIRT1 which binding to SIRT1 leads to inhibition of p53-dependent apoptosis.
Figure 4. Regulation of sirtuin expression and activity. Regulation of sirtuins expression and activity occurs at four different levels; modulation of transcription (a), post-translational modifications (b), complex formation (c) and substrate level (d). Cd68i; Cd68 inhibitor, ChREBP; carbohydrate response element-binding protein, CTP; carboxy-terminal binding protein, cyclin B-CDK1; cyclin B-dependent kinase 1, DBC1; deleted in breast cancer 1, DYRK; Tyr-phosphorylated and regulated kinase, H3K4; histone lysine 4, H4K16/26; histone lysine 16/26, HIC1; hypermethylated in cancer 1, CREB; cAMP response element-binding, FOXO1; fordkhead box O1, JNK; JUN N-terminal kinase, LSD1; lys-specific demethylase 1, miRNA; microRNA, NA; nicotinic acid, NAAD; NA adenine dinucleotide, NAD; nicotinamide adenine dinucleotide, NADS; NAD synthase, NAM; nicotinamide, NAMN; NA mononucleotide, NAMPT; nicotinamide phosphoribosyltransferase, NAPT; NA phosphoribosyltransferase, NCoR1; nuclear receptor co-repressor 1, NMN; NAM mononucleotide, NMNAT; NMN adenyltransferase, NR: NAM riboside, NRK; NR kinase, PARP2; poly(ADP-ribose) polymerase 2, PARPi; PARP inhibitor, PPAR-α/-β/-γ; peroxisome proliferator-activated receptor-α/-β/-γ, SENP; sentrin-specific protease, SMRT; silencing mediator of retinoic acid and thyroid hormone receptor. (Adapted from Houtkooper et al. 2012).

(Kim et al. 2007b). However, several negative regulators of SIRT1 have been identified. Nuclear receptor co-repressor 1 (NCoR1) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) form complex with SIRT1 and PPAR-γ, and thus repress the PPAR-γ-mediated adipogenesis during fasting (Picard et al. 2004). Deleted in breast cancer 1 (DBC1) also inhibits SIRT1 activity in vitro during genotoxic stress (Kim et al. 2008c, Zhao et al. 2008). The DBC1-SIRT1 complex formation is dependent on the cellular energy status; fasting inhibits and HFD-feeding induces it, and the deletion of DBC1 protects mice from HFD-induced hepatic steatosis (Escande et al. 2010). Furthermore, lys-specific demethylase 1 (LSD1)-SIRT1 complex represses Notch target gene expression by deacetylating and demethylating specific histones H3K4, H4K16 and H3K26.
(Mulligan et al. 2011), and the repression is reversed by activation of the Notch pathway (Mulligan et al. 2011).

As mentioned above (see page 26), NAD\(^+\) is an important cofactor regulating sirtuin activation (Figure 4d). NAD\(^+\) levels increase in muscle, liver and WAT in response to energy/nutrient stresses such as fasting (Rodgers et al. 2005, Canto et al. 2010), calorie restriction (Chen et al. 2008) and exercise (Canto et al. 2010) and accordingly, SIRT1 activity is enhanced in all of these conditions. In contrast, NAD\(^+\) levels decrease during HFD feeding in mice (Kim et al. 2011a). Sirtuin activity is inhibited by NAD\(^+\)-derived metabolites, nicotinamide (NAM) and NADH (Bitterman et al. 2002, Lin et al. 2004). NAD\(^+\) is synthesized \textit{de novo} from tryptophan mainly in the liver and kidneys (for review see Houtkooper et al. 2010). However, NAD\(^+\) can also be synthesized from nicotinic acid (NA) through the Preiss-Handler pathway and from nicotinamide (NAM) through the salvage pathway (for review see Houtkooper et al. 2010). Both NA and NAM present in the human diet as niacin (vitamin B3). Nicotinamide riboside (NR), found in cow’s milk, is also a precursor of NAD\(^+\), which increases NAD\(^+\) levels through the salvage pathway (Bieganowski and Brenner 2004, Belenky et al. 2007). Recently, it has been demonstrated that treatment with NR activates SIRT1 and SIRT3, enhances oxidative metabolism, and protects against HFD-induced metabolic abnormalities in mice (Canto et al. 2012). In addition, in the salvage pathway NAM is converted to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT) and inhibition or down-regulation of NAMPT has been shown to lead to reduced sirtuin activity (Revollo et al. 2004). HFD and aging have been shown to reduce NAMPT-mediated NAD\(^+\)-biosynthesis contributing to the development of type 2 diabetes in mice, which is reversed by treatment with NMN, a product of the NAMPT reaction, partly through activation of SIRT1 (Yoshino et al. 2011).

NAD\(^+\) levels can also be regulated by manipulating the NAD\(^+\)-consuming enzymes (Figure 4d). The major group of those enzymes is PARPs and the deletion of those enzymes activates SIRT1, increases the number of mitochondria, enhances energy expenditure and protects against diet-induced obesity (Bai et al. 2011a, b). In addition, the deletion of CD38, another NAD\(^+\) consuming enzyme, activates SIRT1 and protects against diet-induced obesity (Barbosa et al. 2007).

\textit{Mammalian sirtuins in calorie restriction}

Results from lower organism and the response of mammalian sirtuins to cellular energy and nutrient status indicator NAD\(^+\) suggest that mammalian sirtuins can be important molecular mediators of CR. The SIRT1 protein levels have been shown to elevate during CR in the brain, WAT, skeletal muscle, liver and kidneys (Cohen et al. 2004, Nisoli et al. 2005). However, cellular NAD\(^+\) levels and the NAD\(^+\)/NADH ratio fluctuate depending on the tissue type suggesting that sirtuins have a tissue-specific response to CR (Chen et al. 2008). Currently, evidence is insufficient to categorize mammalian SIRT1 as a longevity gene as the overexpression of SIRT1 does not extend lifespan (Herranz et al. 2010), and only one report directly shows that SIRT1 is necessary for CR-induced longevity (Boily et al. 2008). However, recent studies strongly suggest that SIRT1 mediates...
adaptive responses to CR in mammals, and it has a crucial role in several metabolic- and aging-associated diseases. One of these pieces of evidence is the observation that CR-induced elevation of physical activity is abolished in SIRT1-deficient mice (Chen et al. 2005a). Other studies have also shown that SIRT1 is required for normal response to CR (Boily et al. 2008, Cohen et al. 2009). Furthermore, SIRT1-overexpressing transgenic mice display phenotypes similar to mice under CR, including reduced body weight, improved glucose tolerance, reduced blood cholesterol, adipokines, insulin and fasted glucose levels, better performance in rotarod challenge, and a delay in reproduction (Bordone et al. 2007). Additionally, whole body SIRT1-overexpressing transgenic mice are protected from adverse effects of HFD- and aging-induced changes in metabolism, such as hepatic steatosis, insulin resistance, diabetes and cancers (Banks et al. 2008, Pfluger et al. 2008, Herranz et al. 2010). Moreover, several studies in mice have shown a protective role of SIRT1 against cardiovascular (Alcendor et al. 2007, Zhang et al. 2008, Stein et al. 2010a, b) and neurodegenerative diseases (Kim et al. 2007a, Donmez 2010).

In addition to SIRT1, mitochondrial SIRT3 associates with CR. SIRT3 expression is induced by CR in the liver and skeletal muscle as well as in WAT and brown adipose tissue (BAT) (Shi et al. 2005, Palacios et al. 2009, Schwer et al. 2009). Interestingly, SIRT3 is the only sirtuin with a reported association with the human lifespan (Rose et al. 2003, Bellizzi et al. 2005). Data from mouse models studies have shown that SIRT3 prevents several pathological conditions that are mediated by cellular oxidative damage, including stress induced tumor progression (Kim et al. 2010), and age-related cardiac hypertrophy and hearing loss (Sundaresan et al. 2009, Hafner et al. 2010, Someya et al. 2010).

**SIRT1 and metabolic regulation**

SIRT1 regulates whole-body metabolism by deacetylating several targets important in a variety of metabolic processes, including in hepatic gluconeogenesis and lipid metabolism, fat cell accumulation and maturation, pancreatic insulin secretion and central nutrient sensing (for review see Li and Kazgan 2011, Chalkiadaki and Guarente 2012) (Figure 5).

During reduced calorie intake, the liver converts lipid and glycogen stores into available energy by increasing fatty acid oxidation and gluconeogenesis, and decreasing glycolysis (van den Berghe 1991). SIRT1 deacetylates and activates peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), which leads to activation of gluconeogenetic genes and inhibition of glycolytic genes (Rodgers et al. 2005). SIRT1 also induces gluconeogenesis by deacetylating FOXO1 (Daitoku et al. 2004, Frescas et al. 2005) and signal transducer and activator of transcription 3 (STAT3) (Nie et al. 2009). On the other hand, SIRT1 has been shown to suppress hepatic glucose production in the late phase of fasting by deacetylating CREB regulated transcription coactivator 2 (CRTC2, also known as TORC2) leading to CRTC2 degradation and reduction in transcription of gluconeogenic genes (Liu et al. 2008). Even though results in mice overexpressing SIRT1 suggest that SIRT1 may function as insulin sensitizer (see above on this page), studies with mice lacking SIRT1 specifically
Figure 5. Metabolic roles of SIRT1 in peripheral tissues and central nervous system. CRTC2; CREB regulated transcription coactivator 2, FOXO1; forkhead box protein O1, FXR; farnesoid X receptor, LXRα, nuclear receptors liver X receptor α, PGC-1α; PPAR-γ coactivator 1α, PPAR-α/γ; peroxisome proliferator activated receptor-α/γ, PTB1B; protein tyrosine phosphatase 1B, SREBP; sterol regulatory element-binding protein, STAT3; signal transducer and activator of transcription 3, UCP-2; mitochondrial uncoupling protein 2. (Adapted from Nogueiras et al. 2012).

in the liver demonstrate conflicting results regarding the role of SIRT1 in gluconeogenesis and insulin sensitivity. Acute adenovirus-mediated SIRT1 knockdown in the liver induced mild hypoglycemia (Rodgers and Puigserver 2007). By contrast, a complete absence of SIRT1 in the liver caused chronic hyperglycemia and insulin resistance (Wang et al. 2011c), whereas in two other studies it showed normal glucose homeostasis (Chen et al. 2008, Purushotham et al. 2009).

SIRT1 promotes hepatic fatty acid oxidation through activating peroxisome proliferator-activated receptor α (PPARα) and PGC-1α (Purushotham et al. 2009). Liver-specific deletion of the hepatic SIRT1 has been shown to lead to reduced expression of fatty acid oxidation genes (Rodgers and Puigserver 2007, Purushotham et al. 2009). Impaired fatty acid oxidation in hepatic SIRT1-deficient mice results in the development of liver steatosis under normal and high-fat diets (Purushotham et al. 2009, Wang et al. 2010). By contrast, adenovirus-mediated overexpression of hepatic SIRT1 attenuates hepatic steatosis and systemic insulin resistance in diet-induced and genetically obese mice (Li et al. 2011a). In one study, however, liver-specific SIRT1-deficient mice were protected against hepatic steatosis but the reason for these conflicting results is unclear (Chen et al. 2008).

SIRT1 also regulates hepatic cholesterol and bile acid homeostasis by deacetylating nuclear receptors liver X receptor α (LXRα) and farnesoid X receptor (FXR) (Li et al. 2007, Kemper et al. 2009). Deletion of SIRT1 in mice leads to decreased LXRα and FXR activity and deleterious
metabolic outcomes, such as decreased HDL cholesterol, liver steatosis and decreased bile output (Li et al. 2007, Kemper et al. 2009). Another way by which SIRT1 regulates hepatic lipid metabolism is through deacetylation of the sterol regulatory element binding protein (SREBP) family of transcription factors (Ponugoti et al. 2010, Walker et al. 2010) that regulate lipid metabolism by promoting expression of lipogenic and cholesterologenic genes involved in lipid storage (Osborne and Espenshade 2009).

Skeletal muscle fatty acid oxidation is also important in preserving glycogen stores and blood glucose levels during reduced calorie intake. In skeletal muscle, SIRT1 promotes mitochondrial biogenesis and fatty acid oxidation by deacetylating PGC-1α (Gerhart-Hines et al. 2007, Amat et al. 2009, Canto et al. 2010). In myotube cells, SIRT1 inhibits the transcription of protein tyrosine phosphatase 1B (PTP1B), the negative regulator of insulin signaling, suggesting that SIRT1 may have role in skeletal muscle insulin sensitivity (Sun et al. 2007).

During reduced calorie intake, energy stored in the WAT is mobilized to be utilized by other tissues. SIRT1 inhibits adipogenesis and improves lipolysis by repressing the nuclear receptor PPAR-γ through promoting the assembly of co-repressors NCoR1 and SMRT on the promoters of PPARγ (Picard et al. 2004) (see page 28). In addition, SIRT1 regulates the production and/or the secretion of insulin-sensitizing factors adiponectin and fibroblast growth factor 21 (FGF21) through the regulation of FOXO1 and PPARγ (Qiao and Shao 2006, Qiang et al. 2007, Banks et al. 2008, Wang et al. 2008). The results, however, are contradictory and further work is needed to understand the role of SIRT1 in adipose tissue insulin sensitivity.

In pancreatic β-cells, SIRT1 enhances glucose-stimulated insulin secretion and improves glucose tolerance which is thought to occur by repressing the expression of uncoupling protein 2 (UCP-2), an inner mitochondrial membrane protein (Moynihan et al. 2005, Bordone et al. 2006). Pancreatic β-cell-specific SIRT1-overexpressing (BESTO) transgenic mice maintain glucose tolerances under the long-term HFD feeding (Ramsey et al. 2008). SIRT1 has been shown to protect β-cells against cellular injuries through deacetylating FOXO1 and NF-κB signaling pathways (Kitamura et al. 2005, Lee et al. 2009).

In the hypothalamus, the anorexigenic POMC expressing neurons produce satiety peptides and thereby inhibit food intake after feeding, and the orexigenic agouti-related protein (AgRP) expressing neurons promotes feeding in response to fasting and CR (Morton et al. 2006). During CR and fasting, hypothalamic SIRT1 expression and activity are increased (Cakir et al. 2009, Satoh et al. 2010). Inhibition of hypothalamic SIRT1 has been shown to decrease food intake and body weight gain by increasing POMC and decreasing AgRP expression through FOXO1 and S6K signaling dependent manners (Cakir et al. 2009). This SIRT1 hypothalamic activity is in contrast to its anti-obesity function in the peripheral tissues, and it appears to be neuron-specific. AgRP neurons-specific deletion of SIRT1 decreases AgRP neuronal activity and alleviates the inhibitory tone on the POMC neurons resulting in decreased food intake and body weight (Dietrich et al. 2010). In contrast, specific deletion of SIRT1 in POMC neurons impairs leptin signaling and reduces energy expenditure leading to a higher risk of obesity (Ramadori et al. 2010). Furthermore, deletion of
SIRT1 in POMC neurons contributes to the development of obesity by reducing sympathetic nerve activity and BAT-like remodeling of perigonadal WAT under HFD (Ramadori et al. 2010).

**Mitochondrial sirtuins and metabolic regulation**

CR improves mitochondrial function by increasing mitochondrial biogenesis and decreasing ROS production (for review see Guarente 2008). CR strongly changes the acetylation of mitochondrial proteins (Kim et al. 2006, Schwer et al. 2009), and the acetylation/deacetylation of proteins within mitochondria is considered to be one mechanisms by which changes in mitochondrial function is mediated in response to CR. Mitochondrial sirtuins SIRT3, SIRT4 and SIRT5 are assumed to function as a metabolic sensors that modulate the activity of metabolic enzymes via protein deacetylation or mono-ADP-ribosylation (for review see Huang et al. 2010, He et al. 2012) (Figure 6).

Among the mitochondrial sirtuins, SIRT3 function has been the most widely studied. SIRT3 has been shown to be responsible for acetylations of several proteins in mitochondria, because the loss of SIRT3, but not SIRT4 or SIRT5, leads to hyperacetylation of mitochondrial proteins (Lombard et al. 2007). The first SIRT3 substrate identified was the mitochondrial enzyme acetyl-CoA synthase 2 (AceCS2) (Hallows et al. 2006, Schwer et al. 2006) which produces acetyl-CoA for the tricarboxylic acid (TCA) cycle to produce ATP and CO₂. SIRT3 also plays an important role in hepatic fatty acid oxidation by activating long-chain acyl-CoA dehydrogenase (LCAD) (Hirschey et al. 2010) and in hepatic ketone bodies production by activating 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) (Shimazu et al. 2010). SIRT3 also deacetylates ROS-scavenging enzymes isocitrate dehydrogenase 2 (IDH2) (Someya et al. 2010), superoxide dismutase 2 (SOD2) (Qiu et al. 2010),

![Figure 6](https://via.placeholder.com/150)

**Figure 6. Metabolic roles of mitochondrial sirtuins SIRT3, SIRT4 and SIRT5 in peripheral tissues.** CPS1; carbamoyl phosphate synthetase 1, GDH; glutamate dehydrogenase, HMGCS2; 3-hydroxy-3-methylglutaryl-CoA synthase 2, IDH2; isocitrate dehydrogenase 2, LCAD; long-chain acyl-CoA dehydrogenase, MnSOD2; manganese superoxide dismutase, NDUFA9; NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9, PGC-1α; peroxisome proliferator-activated receptor-gamma coactivator 1α, SOD2; superoxide dismutase 2, UCP-1; mitochondrial uncoupling protein 1. (Adapted from Nogueiras et al. 2012).
and manganese superoxide dismutase (MnSOD) (Tao et al. 2010). Moreover, SIRT3 facilitates mitochondrial oxidative phosphorylation by deacetylating mitochondrial complex I component NDUFA9 (Ahn et al. 2008, Bao et al. 2010, Kim et al. 2010), as well as other electron transfer components, including complex II (Cimen et al. 2010), III (Kendrick et al. 2011, Kim et al. 2010), IV (Kendrick et al. 2011), and V (Bao et al. 2010). SIRT3 also affects extra-mitochondrial processes and SIRT3 is required for normal PGC-1α expression in BAT and skeletal muscle (Shi et al. 2005, Palacios et al. 2009). SIRT3 promotes PGC-1α expression by stimulating phosphorylation and PGC-1α activating factors, including CREB and AMPK (Shi et al. 2005, Palacios et al. 2009, Pillai et al. 2010). Constitutive expression of SIRT3 in BAT increases the expression of PGC-1α and UCP-1, leading to higher thermogenesis and oxygen consumption (Shi et al. 2005). In muscle cells, SIRT3 is also required for PGC-1α-dependent induction of genes involved in mitochondrial biogenesis and ROS-detoxifying (Kong et al. 2010). Recent studies have revealed that hyperacetylation of mitochondrial proteins in SIRT3-deficient mice leads to metabolic disturbances, including reduced ATP production, decreased rates of fatty acid oxidation and ketone body production, and a propensity to develop fatty liver and metabolic syndrome (Ahn et al. 2008, Hirschey et al. 2010, Shimazu et al. 2010, Hirschey et al. 2011, Kendrick et al. 2011).

The less studied mitochondrial sirtuins are SIRT4 and SIRT5. The only known substrate of SIRT4 is the glutamate dehydrogenase (GHD) enzyme that converts glutamate to α-ketoglutarate and allows it to enter TCA cycle (Haigis et al. 2006). SIRT4 ADP-ribosylates and inactivates GDH leading to repression of amino acid-stimulated insulin secretion in pancreatic β-cells (Haigis et al. 2006). In the liver, SIRT4 expression has been shown to decline during CR (Haigis et al. 2006), whereas in genetic models of diabetes increased SIRT4 protein expression is seen (Nasrin et al. 2010). SIRT4 also plays a role in the regulation of fatty acid oxidation and mitochondrial gene expression in the liver and muscle cells, but the mechanism underlying those effects is still unclear (Nasrin et al. 2010). During CR, amino acid catabolism is activated and up-regulation of urea cycle is a necessity to remove excess amounts of ammonia. SIRT5 deacetylates and activates carbamoyl phosphate synthetase 1 (CPS1), an enzyme catalyzing the first step of the urea cycle (Nakagawa et al. 2009).

2.2.3.2 AMPK pathway

The adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a crucial cellular energy sensor with the ability to regulate the whole body metabolism. It is sensitive to cellular changes in AMP/ATP and ADP/ATP ratios, which reflect the energy status of the cell. AMPK is activated in responses to metabolic stresses that deplete cellular ATP supplies (e.g. hypoxia, ischemia, low nutrient intake), or stimulate ATP consumption (e.g. exercise, fasting) (for review see Hardie et al. 2012a). The ability of AMPK to sense energy stresses and regulate metabolism, suggest that AMPK might also be important in mediating the effects of CR.
AMPK structure and regulation

AMPK is an evolutionarily conserved heterotrimeric serine/threonine protein kinase that is composed of a catalytic α-subunit and regulatory β- and γ-subunits. There are two different forms of α (α₁ and α₂) and β (β₁ and β₂) subunits, and three different γ isoforms (γ₁, γ₂ and γ₃). α subunits contains the Thr172 residue, whose phosphorylation is required for AMPK enzymatic activity. The β subunit contains a carbohydrate-binding domain, which allows association with glycogen particles. The γ-subunits contain four tandem repeats known as cystathione β-synthase (CBS) motifs that bind AMP, ADP or ATP in a mutually exclusive way. (For review see Hardie 2007).

In basal conditions, the binding of ATP keeps the enzymatic activity low. Binding of AMP, which is a signal of low cellular energy status, activates AMPK in two different ways; through allosteric activation and by promoting the phosphorylation of Thr172 (for review see Hardie et al. 2012a). Upstream kinases phosphorylate Thr172 and in mammals, two major upstream kinases are the LKB1-STRAD-MO25 complex (Hawley et al. 2003, Woods et al. 2003, Shaw et al. 2004) and the Ca²⁺/calmodulin-activated protein kinase kinases, especially CaMKKβ (Hawley et al. 2005, Hurley et al. 2005, Woods et al. 2005). The LKB1-STRAD-MO25 complex has high basal activity and it continuously phosphorylates Thr172 that is modulated by the binding of AMP to the AMPK γ-subunit, which promotes phosphorylation and inhibits dephosphorylation (Hawley et al. 1995, Davies et al. 1995, Xiao et al. 2011). Although AMP can only allosterically activate AMPK, the effects on phosphorylation and dephosphorylation can also be produced by ADP (Xiao et al. 2011). Since ADP usually presents in cells at a higher concentration than AMP, it has been assumed that ADP regulates Thr172 phosphorylation during moderate energy stress and allosteric activation by AMP occurs during a more severe stress (for review see Hardie et al. 2012a). CaMKKβ serves as alternative pathway through which AMPK activity is regulated. CaMKKβ activates AMPK in response to increases in cellular Ca²⁺ without necessarily requiring any changes in AMP or ADP levels (Hawley et al. 2005, Hurley et al. 2005, Woods et al. 2005).

As mentioned above, AMPK is activated in response to changes in AMP, ADP and Ca²⁺ levels. Several cytokines, including leptin, adiponectin, ghrelin and interleukin-6, activate AMPK activity (for review see Hardie et al. 2012a). In addition, several drugs, in particular the anti-diabetic drugs metformin and thiazolidinediones (Fryer et al. 2002) and natural plant products (e.g. resveratrol) stimulate AMPK activation (see section 2.2.4.1). Furthermore, AMPK has been shown to be activated by ROS- and DNA-damaging agents without changes in AMP, ADP and Ca²⁺ levels, but the mechanism for that is unclear (for review see Hardie et al. 2012a).

AMPK function

In general, AMPK activation switches on ATP-producing catabolic pathways, such as the uptake and metabolism of glucose and fatty acids, and switches off ATP-consuming anabolic pathways, such as the synthesis of fatty acids, cholesterol, glycogen and proteins (for review see Hardie et al. 2012a) (Figure 7). This happens through a direct phosphorylation of metabolic enzymes or through a phosphorylation of coactivators and transcription factors that regulate gene expression.
Figure 7. AMPK activation switches on ATP-producing catabolic pathways (a) and switches off ATP-consuming anabolic pathways (b). ACC1/2; acetyl-CoA carboxylase 1/2, ChREBP; carbohydrate response element coactivator-2, GPAT; glycerol phosphate acyl transferase, HDACs; histone deacetylases, HMGR; 3-hydroxy-3-methylglutaryl CoA reductase, HSL; hormone-sensitive lipase, PFKFB2/3; 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2/3, PGC-1α; peroxisome proliferator-activated receptor gamma coactivator 1-α, SREBP-1c; sterol response element binding protein-1c, TBC1D1; TBC1 domain family member 1, TSC2; tuberous sclerosis complex protein 2, ULK1; unc-51-like kinase 1. (Adapted from Hardie et al. 2012b).

Catabolic events mediated by AMPK include enhanced glucose uptake in muscle, which is mediated through increasing the translocation of the glucose transporter type 4 (GLUT4) to the plasma membrane (Kurth-Kraczek et al. 1999) (Figure 7a). AMPK phosphorylates TBC1 domain family member 1 (TBC1D1) which is one of the RabGAPs regulating translocation of glucose transporters from storage vehicles to plasma membranes (Frosig et al. 2010). AMPK also promotes glucose uptake into cells expressing only GLUT1 through activation of GLUT1 that already exist in plasma membrane (Barnes et al. 2002). In addition, AMPK promotes fatty acid uptake into cardiac myocytes via translocation of CD36 to the membrane (Habets et al. 2009).

After the glucose and fatty acid enter into the cells, AMPK also promotes their catabolism by enhancing glycolysis and fatty acid β-oxidation. AMPK induces heart glycolysis by phosphorylating and activating PFKFB2 isoform of 6-phosphofructo-2-kinase (PFK-2), the enzyme responsible for the synthesis of fructose 2,6-bisphosphate, a potent stimulator of glycolysis (Marsin et al. 2000). AMPK also phosphorylates PFKFB3 isoform of 6-phosphofructo-2-kinase in monocytes and macrophages (Marsin et al. 2002). In muscle cells, AMPK promotes fatty acid oxidation by phosphorylating and inactivating the mitochondria-associated isoform of acetyl-CoA carboxylase (ACC2) (Merrill et al. 1997). ACC2 inactivation lowers malonyl-CoA, an inhibitor of fatty acid uptake into mitochondria via the carnitine:palmityl-CoA transferase system (Merril et al. 1997). Although AMPK broadly induces catabolism, it still inhibits catabolic pathway lipolysis in rodent and human adipocytes by phosphorylation of hormone-sensitive lipase (HSL) (Daval et al. 2005, Bourron et al. 2010). This is suggested to be due to fact that the recycling of lipolysis-produced fatty acids back to triglycerides consumes large amounts of ATP (for review see Hardie et al. 2012b).
AMPK promotes glucose and fatty acid oxidative catabolism through enhanced mitochondrial biogenesis. AMPKβ1/AMPKβ2 double knockout mice have markedly reduced muscle mitochondrial content (O’Neill et al. 2011). AMPK phosphorylates PGC-1α, a master regulator of mitochondrial biogenesis, both directly and indirectly through SIRT1 (Jäger et al. 2007, Canto et al. 2010). Phosphorylation of PGC-1α by AMPK is suggested to make it more susceptible for deacetylation by SIRT1 (Canto et al. 2009). In addition, AMPK has been shown to increase NAD⁺ concentration, which leads to increased SIRT1 activity and thus promotes PGC-1α deacetylation (Canto et al. 2009, Canto et al. 2010). AMPK also triggers an autophagy cascade by phosphorylating UNC-51-like kinase (ULK1, also called Atg1) (Lee et al. 2010b, Egan et al. 2011, Kim et al. 2011b). In particular, AMPK improves mitochondria turnover via autophagy (mitophagy), suggesting that AMPK is required for the clearance of dysfunctional mitochondria (Egan et al. 2011).

AMPK conserves ATP for cells by switching off anabolic pathways, including the biosynthesis of lipids, carbohydrates and proteins (Figure 7b). AMPK inhibits fatty acid synthesis by phosphorylating and inactivating ACC1 (Davies et al. 1992), and triglyceride and phospholipid synthesis by inactivating glycerol phosphate acyl transferase (GPAT) (Muuoio et al. 1999). AMPK also inhibits cholesterol synthesis through phosphorylating and inactivating 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) (Clarke and Hardie 1990). AMPK phosphorylates carbohydrate response element binding protein (ChREBP) and sterol response element binding protein-1c (SREBP-1c), and thus inhibits transcription of lipogenic genes (Kawaguchi et al. 2002, Li et al. 2011b). Furthermore, AMPK inhibits transcription of hepatic gluconeogenic genes by phosphorylating cyclic AMP response element binding protein (CREB)-regulated transcription coactivator-2 (CRTC2, also known as TORC2) (Koo et al. 2005) and class Ila histone deacetylases (HDAC-4, -5 and -7) (Mihaylova et al. 2011). In addition, AMPK inhibits glycogen synthesis by phosphorylating and inactivating glycogen synthase 1 and 2 in muscle and the liver, respectively (Jorgensen et al. 2004, Bultot et al. 2012). AMPK also reduces protein synthesis by phosphorylating tuberous sclerosis complex protein 2 (TSC2) (an upstream regulator of mTOR) and Raptor (a component of mTORC1 complex) leading to inactivation of mammalian target-of-rapamycin complex 1 (mTORC1) (Inoki et al. 2003, Gwinn et al. 2008).

In addition to the effects of AMPK in peripheral tissues, AMPK also influences metabolism and energy balance on the whole body level through its action in the hypothalamus. An injection of pharmacological activators of AMPK or DNA encoding activated mutants into the hypothalamus promotes feeding (Andersson et al. 2004, Minokoshi et al. 2004). In addition, hormones that inhibit feeding, such as leptin, inhibit AMPK activation and in contrast, hormones that promote feeding, such as ghrelin, activate AMPK (Andersson et al. 2004, Minokoshi et al. 2004). Ghrelin, which is released from the stomach during fasting, has been shown to activate AMPK through growth hormone secretagogue receptor 1 (GHSR) triggering a Ca²⁺ release and thereby lead to the action of AMPK by the CaM KKβ pathway (Andrews 2011).
AMPK also has a role in several processes beyond metabolism, including cell cycle and membrane excitability. AMPK regulates the cell cycle through cyclin-dependent kinase inhibitors p21WAF1 and p27KIP1 (Imamura et al. 2001, Jones et al. 2005, Liang et al. 2007). AMPK also phosphorylates the voltage-gated, delayed rectifier K+ channel, Kv2.1 and reduces membrane excitability and limits the rate of firing of action potentials in neurons (Ikematsu et al. 2011). Because firing of action potentials in the brain is the major consumer of energy, this may protect neurons during energy stress.

**AMPK in calorie restriction**

As a cellular energy sensor and metabolic regulator, AMPK is believed to act as a mediator of CR induced lifespan extension and health benefits. Enhanced AMPK activity has been shown to extend lifespan in worms and flies (Apfeld et al. 2004, Funakoshi et al. 2011). The role, however, of yeast AMPK ortholog Snf1 in longevity is very complex, as both deletion of Snf1 and forced overexpression of Snf1 reduce lifespan (Ashrafi et al. 2000). In worms, reduced glucose intake has been shown to extend lifespan via AMPK ortholog aak-2-dependent manner which is due to increased mitochondrial respiration, suggesting that AMPK is also important for CR-induced lifespan extension (Schulz et al. 2007). However, whether the AMPK activity is involved in the lifespan effect of CR in other species is unclear.

The effect of CR on mammalian AMPK activity is very conflicting. In mice, CR has no effects on AMPK activity in the heart, liver or skeletal muscle (Gonzalez et al. 2004). However, Palacios and colleagues (2009) have shown that CR increases AMPK activity in mice skeletal muscle. Results from studies carried out in rats are even more controversial. One study has shown that CR reduces the AMPK activity in the liver (To et al. 2007), while other studies have shown that AMPK activity is increased in the heart and liver (Shinmura et al. 2005, Jiang et al. 2008, Finckenberg et al. 2012).

Even though evidence that CR activates mammalian AMPK is scarce, AMPK activation still mediates several benefits in glucose and lipid metabolism (Figure 7). As stated above, some of the effects of the anti-diabetic drugs metformin and thiazolidinediones are mediated through AMPK, indicating that AMPK has insulin-sensitizing effects. AMPK has also been shown to be activated by CR-mimetic compound resveratrol (see section 2.2.4.1). In addition, AMPK regulates other known CR mediators. In particular, AMPK and SIRT1 activities are positively linked to each other (see page 37). AMPK enhances SIRT1 activity by increasing NAD+ levels, which leads to enhanced mitochondrial biogenesis through PGC-1α, which is one of the important effects of CR (Canto et al. 2009, Canto et al. 2010). In contrast, SIRT1 has been shown to activate AMPK by deacetylating and activating LKB1 (Hou et al. 2008, Lan et al. 2008), showing that there is a positive feedback loop between AMPK and SIRT1. Furthermore, AMPK induces autophagy and inhibits mTOR pathway (see page 37), the other important mediators of CR. AMPK can also impact on autophagy indirectly through SIRT1, which has been shown to form a complex with several components of autophagy machinery, including Atg5, Atg7 and Atg8 (Lee et al. 2008). The results indicate that AMPK is an
important energy sensor that can mimic several metabolic effects of CR directly or indirectly through other nutrient sensing pathways.

2.2.3.3 mTOR pathway

The target of rapamycin (TOR) is a highly conserved atypical serine/threonine protein kinase that belongs to the phosphoinositide-3-kinase (PI3K)-related kinase family. The TOR pathway controls cell growth and metabolism in response to growth factors (e.g. insulin, IGF-1), nutrients (e.g. amino acids), cellular energy (AMP/ATP ratio) and stress. The name of the kinases refers to the feature that kinases activity is inhibited by immunosuppressant drug rapamycin. The mammalian TOR protein is called mTOR, and it interacts with several proteins to form two distinct complexes termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).

mTOR structure and regulation

mTORC1 is a complex of six proteins and mTORC2 complex of seven proteins. They share catalytic mTOR subunits, and three other subunits called mLST8 (mammalian lethal with sec-13 protein 8), DEPTOR (DEP domain containing mTOR-interacting protein) and Tti1/Tel2 complex. Otherwise, the structure differs and for that reason complexes have different biological responses and different sensitivities to intracellular and extracellular signals and rapamycin. As compared to mTORC1, much less is known about the mTORC2 pathway, and therefore only mTORC1 regulation and function is described below. (For review see Laplante and Sabatini 2012).

The heterodimer consisting of tuberous sclerosis 1 (TSC1) and TSC2 is a key upstream regulator of mTORC1. TSC1/2 complex is a GTPase-activating protein (GAP) for the Ras homolog enriched in the brain (Rheb) GTPase (for review see Huang and Manning 2008). The GTP-bound form of Rheb interacts with mTORC1 and stimulates its kinase activity. TSC1/2 complex induces the conversion of Rheb-GTP into Rheb-GDP, and thereby negatively regulates mTORC1 activity (Tee et al. 2003). TSC1/2 complex transmits many of the upstream signals that impact of mTORC1 activity. Nutrient, growth factors, energy and stress are the major regulators of the mTOR pathway. Insulin and insulin-like growth factor 1 (IGF-1) stimulates mTORC1 through the phosphoinositide-3-kinase (PI3K)/Akt and the Ras–ERK (extracellular signal regulated kinase) pathways, which phosphorylate a tyrosine on TSC2 leading to its inactivation (for review see Manning and Cantley 2007, Ma and Blenis 2009). In addition, proinflammatory cytokines, such as TNF-α, activates mTORC1 through IкB kinase β (IKKβ), which phosphorylates TSC1 and causes TSC1/2 inhibition (Lee et al. 2007). Furthermore, several stresses, including low energy and oxygen levels and DNA damage, activate mTORC1. AMPK responds to low energy levels and inactivates mTORC1 by phosphorylating TSC2 and Raptor (Inoki et al. 2003, Gwinn et al. 2008) (see in section 2.2.3.2). Amino acids, in particularly leucine and arginine, activate mTORC1 independently of TSC1/2 complex. The molecular mechanism of how amino acids stimulate mTORC1 is still unclear, but it has been suggested to be dependent on the Rag GTPases (Kim et al. 2008b, Sancak et al. 2008).
**mTOR function**

The best-characterized substrates of the mTOR pathway are translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), whose phosphorylation by mTORC-1 promotes protein synthesis (for review see Ma and Blenis 2009) (Figure 8). mTORC1 also promotes protein synthesis by activating the element tripartite motif-containing protein 24 (TIF-1A), which enhances its interaction with RNA polymerase I (Pol I) and the expression of ribosomal RNA (rRNA) (Mayer et al. 2004). In addition, mTORC1 phosphorylates and inhibits Maf1, a Pol III repressor, and thereby induces 5S rRNA and transfer RNA (tRNA) transcription (Kantidakis et al. 2010, Shor et al. 2010).

mTORC1 also promotes lipid synthesis that is required for proliferating cells to generate cell membranes (for review see Laplante and Sabatini 2009). Inhibition of mTORC1 represses the expression of SREBP1 and 2 transcription factors that control the expression of numerous genes involved in fatty acid and cholesterol metabolism (Porstman et al. 2008, Duvel et al. 2010, Li et al. 2010, Wang et al. 2011a). mTORC1 regulates SREBP function by several different mechanisms, including through S6K1 (Duvel et al. 2010, Li et al. 2010, Wang et al. 2011a) and lipin-1 (Peterson et al. 2011). mTORC1 also influences lipid metabolism by promoting the expression and activity of PPAR-γ, a master regulator of adipogenesis (Kim and Chen 2004, Zhang et al. 2009).

mTORC1 regulates cellular metabolism and ATP production. mTORC1 activates HIF-1α, which positively regulates several glycolytic genes (Hudson et al. 2002, Brugarolas et al. 2003, Duvel et al. 2010). mTORC1 also promotes mitochondrial biogenesis and oxidative metabolism by mediating the nuclear association between PGC-1α and the transcription factor Ying-Yang 1 (YY1) (Cunningham et al. 2007).

![Figure 8. The key outputs of mTORC1 pathway.](image-url)

4E-BP1; eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1, HIF-1α; hypoxia inducible factor 1α, PGC-1α; PPAR-γ coactivator 1α, PPAR-γ; peroxisome proliferator-activated receptor γ, S6K1; S6 kinase 1, SREBP1/2; sterol regulatory element-binding protein 1/2, ULK1/Atg13/FIP200; unc-51–like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa, YY1; ying-yang 1. (Adapted from Laplante and Sabatini 2012).
mTORC1 is also a major negative regulator of autophagy. mTORC1 directly phosphorylates and suppresses ULK1/Atg13/FIP200 (unc-51–like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDA), a kinase complex important in the initiation of autophagy (Ganley et al. 2009, Hosokawa et al. 2009, Jung et al. 2009). In addition, mTORC1 regulates autophagy also through other mechanisms, including through death-associated protein 1 (DAP1), a negative autophagy regulator (Koren et al. 2010), and through mTOR-dependent phosphoproteome WIPI2 (Atg18 orthologue) (Hsu et al. 2011).

**mTOR in calorie restriction**

TOR signaling controls several cellular processes in response to nutrient availability, suggesting that the TOR pathway might have a role in mediating the effects of CR on health and lifespan. Results from lower organisms support this, and the inhibition of the TOR pathway extends lifespan in yeast (Kaeberlein et al. 2005b, Medvedik et al. 2007), worms (Vellai et al. 2003) and flies (Kapahi et al. 2004, Bjedov et al. 2010). In yeast, CR does not further extend lifespan in the absence of TOR1 gene, which is one of the two TOR genes in yeast (Kaeberlein et al. 2005b). In addition, similar results have been seen in worms, where inhibition of the TOR gene does not extend lifespan in eat-2 mutant worms which have impaired feeding behavior, and represents the genetic model of CR (Hansen et al. 2008), indicating that the TOR pathway is crucial in CR mediated lifespan extension.

In mice, deletion of S6K1 leads to increased lifespan and reduced incidence of age-related pathologies (Selman et al. 2009). In addition, inhibition of mTOR with rapamycin extends lifespan in mice (Harrison et al. 2009, Miller et al. 2011). This is in contrast to other studies, which have unexpectedly shown that inhibition of mTOR with rapamycin causes diabetes-like syndrome by reducing β-cells mass and disrupting glucose and lipid metabolism (Cunningham et al. 2007, Fraenkel et al. 2008, Houde et al. 2010). Therefore, it has been suggested that too intensive mTOR inhibition has detrimental effects and the dose of rapamycin should be more carefully adjusted to get beneficial effects on metabolism and longevity (for review see Laplante and Sabatini 2012).

In mammals, mTOR pathway activity is highly responsive to cellular nutritional status and inhibition of the mTOR pathway mimics several metabolic effects of CR. Obesity, high-fat feeding and insulin resistance increase (Um et al. 2004, Khamzina et al. 2005, Tremblay et al. 2007) and CR decreases mTOR activity in animals (Jiang et al. 2008, Moore et al. 2008, Estep et al. 2009). Mice with adipose-specific loss of mTORC1 have markedly less adipose tissue and they are resistant to HFD-induced obesity and hypercholesterolemia, and they have improved insulin sensitivity (Polak et al. 2008). In skeletal muscle, impaired mTORC1 is associated with reduced muscle mass and oxidative capacity and increased glycogen stores (Ohanha et al. 2005, Aguilar et al. 2007, Bentzinger et al. 2008, Risson et al. 2009). In the liver, inhibition of mTORC1 is required for PPAR-α activation, which induces the production of ketone bodies in response to fasting (Sengupta et al. 2010). Liver-specific deletion of mTORC1 also reduces SREBP1 activity and protects mice against HFD-induced hepatic steatosis and hypercholesterolemia (Peterson et al. 2011).
In all the above mentioned metabolic tissues, mTORC1 has been shown to promote insulin resistance through S6K1-mediated inhibition of insulin substrate 1 (IRS-1) (Um et al. 2004, Khamzina et al. 2005, Tremblay et al. 2007). The impairment of insulin signaling increases lipotoxicity and ectopic fat accumulation by increasing fatty acid release from adipocytes, and increases hyperglycemia and hyperinsulinemia by increasing gluconeogenesis in the liver and decreasing glucose uptake in muscles (Um et al. 2004, Khamzina et al. 2005, Tremblay et al. 2007). All together, these changes contribute to systemic insulin resistance. However, in pancreatic β-cells, mice with the loss of S6K1 have a reduced number and size of β-cells, reduced insulin secretion, and they are glucose intolerant and hypoinsulinemic (Pende et al. 2000, Ruvinsky et al. 2005). This is in contrast to other tissues, where mTORC1 induces insulin resistance. It has been shown, however, that constitutive activation of mTORC1/S6K1 disturbs the insulin signaling pathway through S6K1-mediated inhibition of IRS-1, which leads to β-cells apoptosis and impaired insulin sensitivity (Shigeyama et al. 2008, Elghazi et al. 2010).

In addition to peripheral tissues, leptin and leucine induced activation of hypothalamic mTORC1 reduces food intake (Cota et al. 2006). High-fat feeding is known to induce leptin resistance, which blocks the ability of leptin to activate hypothalamic mTORC1 contributing to increased food intake (for review see Cota 2009). These results indicate that the mTOR pathway has an important role in the whole body metabolism, and in the development of a number of pathological conditions that are due to excess calorie intake, including diet-induced obesity, diabetes and NAFLD. Therefore, CR induced suppression of the mTOR pathway can be important in CR mediated effects on health and lifespan.

2.2.3.4 Insulin/IGF-1 pathway

As previously shown, insulin/insulin like growth factor-1 (IGF-1) signaling is associated with the mTOR pathway, and the reduction in insulin/IGF-1 signaling during CR has been linked to health and lifespan enhancing effects of CR. This proposition is based on the several studies, which have demonstrated that suppression of insulin/IGF-1 signaling improves health and extends lifespan in a variety of animals. In worms (C. elegans) and fruit flies (D. melanogaster), several genes homologue to the mammalian insulin/IGF-1 signaling have been identified, and the loss of function mutations of those genes lead to increased longevity (Kenyon et al. 1993, Kimura et al. 1997, Clancy et al. 2001, Tatar et al. 2001, Giannakou et al. 2004). In mice, mutations in the PROP-1 (Ames dwarf mice) and PIT-1 (Snell dwarf mice) genes, which encodes transcription factors for controlling pituitary development, lead to dwarfism and reduced levels of IGF-1 and insulin (Flurkey et al. 2001. Liang et al. 2003). Both Ames and Snell mice strains are remarkably long-lived, indicating that insulin/IGF-1 signaling regulates lifespan also in mammals. In addition, mice with reduced function in IGF-1 and growth hormone (GH) receptors, as well as defects in downstream molecules of IGF-1 signaling (such as p66^{shc}, klotho, IRS-1 molecules) are associated with longevity (for review see Barbieri et al. 2003, Berryman et al. 2008), further improving the role of
insulin/IGF-1 axis in mammals lifespan. Animals with repression of insulin/IGF-1 share several similar phenotypic characteristics as animals under CR, including reduced body weight and body temperature, improved insulin sensitivity and stress resistance, and lower incidences of cancer (for review see Berryman et al. 2008). Interestingly, CR has been shown to be able to further extend lifespan in Ames mice (Bartke et al. 2001), but not in GH receptor knockout mice (Bonkowsi et al. 2006), indicating that GH signaling appears to be important for the lifespan enhancing effect of CR.

In humans, polymorphisms in the IGF-1 receptor and P13KCB (phosphoinositide-3-kinase), and genotype combination of polymorphisms, which are associated with reduced plasma IGF-1 levels, are more frequent in long-lived people (Bonafe et al. 2003), suggesting that insulin/IGF-1 signaling can be important in human lifespan as well. However, long-term severe CR has not been shown to reduce serum IGF-1 concentration in humans (Fontana et al. 2008).

2.2.3.5 Autophagy

All previously described nutrient sensing pathways regulate autophagy, suggesting that autophagy may function as mechanistic links to how these pathways mediate beneficial effects on health and lifespan. Autophagy is an evolutionarily conserved lysosomal degradation pathway that is essential for cell survival, differentiation, development and energy homeostasis. It is a cellular self-digestion process, which exists in at least three different forms; chaperone-mediated autophagy, microautophagy and macroautophagy (Mizushima et al. 2008). The most prominent and best-studied form of autophagy is macroautophagy (hereinafter referred to as autophagy) in which portions of cytoplasm, including excess or damaged organelles and misfolded and aggregated proteins, is sequestered into double-membrane vesicle called autophagosomes for degradation and recycling in lysosomes. The autophagy pathway is stimulated by multiple forms of cellular stress, and one potent known physiological inducer of autophagy is nutrient deprivation (Kroemer et al. 2010). Under nutrient stress, autophagy is rapidly induced to generate nutrients and substrates for energy production. Impairment of the autophagy process is associated with many human diseases, including cancer, neurodenenerative and cardiac diseases (Levine and Kroemer 2008).

One common feature of all aging-related changes at the tissue level is the accumulation of damaged and harmful modifications of macromolecules and organelles (Terman and Brunk 2006). It has been repeatedly reported that aging decreases autophagy activity contributing to accumulation of intracellular waste products and, in particular, the degradation of defective mitochondria is impaired in senescence cells (Salminen and Kaarniranta 2009, Rubisztein et al. 2011). CR, which is linked to longevity and healthy aging, promotes autophagy and prevents the age-dependent decline of autophagy (Cavallini et al. 2001, Donati et al. 2001, Bergamini et al. 2004, Bergamini et al. 2007). In C. elegans, there is clear evidence that autophagy is linked to longevity (Melendez et al. 2003, Hars et al. 2007), suggesting that autophagy might also be important in mediating the anti-aging effects of CR.
Besides aging, autophagy is also linked to obesity and metabolic diseases. Both genetic and HFD-induced obesity, as well as insulin resistance and hyperinsulinemia, have been shown to decrease hepatic autophagy activity (Liu et al. 2009, Yang et al. 2010). Defective autophagy in obese mice leads to elevated endoplasmic reticulum (ER) stress and insulin resistance (Yang et al. 2010). Autophagy also regulates lipid metabolism, and inhibition of autophagy in hepatocytes and mouse liver has been shown to be associated with increased triglyceride content in lipid droplets (Singh et al. 2009). The role of autophagy in lipid metabolism, insulin sensitivity and cellular injury, which are all contributors to hepatic steatosis, suggest that autophagy may be important in the development of NAFLD and NASH (for review see Amir and Czaja 2011). Taken into account that CR is the best way to activate autophagy in vivo, the recent results suggest that autophagy can be an important mechanism for CR induced health effects.
2.2.4 **SIRT1 activators as calorie restriction mimetics**

Even though CR is known to induce several health benefits, it is unlikely that many would be willing or able to maintain a CR lifestyle. Therefore, there has been intense interest in finding compounds that could affect pathways that mediate CR effects, and thus could act as CR-mimetics. Several plant polyphenols compounds; butein, fisetin, piceatannol, quercetin and resveratrol, have been shown to activate SIRT1, and of them, resveratrol was identified to be the most potent SIRT1 activator (Howitz et al. 2003) (Figure 9). After the findings that resveratrol was able to protect against metabolic stress (see below), there has been a strong effort to find sirtuin activating compounds (STACS) with improved bioavailability and specificity for sirtuin activation. Several synthetic compounds that are structurally unrelated to resveratrol and have more potent SIRT1-activating power in vitro have been developed and of the compounds SRT1720 is the most potent SIRT1 activator (Milne et al. 2007) (Figure 9). Currently, multiple clinical trials have been initiated with three selective SIRT1 activators; SRT2104, SRT2379 and SRT3025 to find out their potential in the treatment of inflammatory, metabolic and cardiovascular diseases (for review see Baur et al. 2012).

![Figure 9. Structure of putative SIRT1 activators resveratrol and SRT1720.](image)
2.2.4.1 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a polyphenol that belongs to the stilbene family of phytoalexins, which are antibiotic compounds produced by plants in response to infection and stress. Resveratrol has been detected at least in 72 different plant species, and the richest dietary sources of resveratrol are grapes, grape juice, red wine and peanuts (Jang et al. 1997, Burns et al. 2002). Originally, resveratrol was believed to account for the ‘French paradox’, the observation that moderate red wine consumption protects against coronary heart diseases despite the consumption of a diet high in saturated fat (for review see Catalgol et al. 2012). Since that, the connection between resveratrol and cardiovascular health was intensively studied, and resveratrol was shown to have several cardiovascular protective properties, including anti-oxidant, anti-inflammatory, anti-proliferative and anti-angiogenic effects (Catalgol et al. 2012). The finding that resveratrol can increase the activity of SIRT1 (Howitz et al. 2003) leads to the assumption that resveratrol could act as a CR-mimetic. After that finding, numerous studies have investigated the potential of resveratrol to mimic CR-induced benefits (Baur 2010a).

Resveratrol has been shown to extend lifespan by 18-56% in yeast (Howitz et al. 2003), worms (Wood et al. 2004) and flies (Bauer et al. 2004), and it functions in a Sir2-dependent manner. Some studies, however, have failed to observe lifespan extension effect in yeast and flies (Kaeberlein et al. 2005a, Bass et al 2007). A study in mammals has shown that resveratrol delays aging-related deterioration and induces similar gene expression to CR in the heart, liver, adipose tissue, skeletal muscle and brain (Barger et al. 2008, Pearson et al. 2008). Resveratrol also increases survival in obese mice such that their lifespans are equivalent to those of lean, untreated mice (Baur et al. 2006). However, resveratrol does not increase survival in lean and healthy mice (Pearson et al. 2008, Miller et al. 2011). Therefore, it has been suggested that metabolic stress is important for the lifespan-extending effect of resveratrol.

Numerous studies have demonstrated the ability of resveratrol to reverse many of the obesity-induced pathologies (for review see Szkudelska and Szkudelska 2010). In HFD fed mice, resveratrol has been shown to increase motor function and change the expression of several genes towards the expression found in mice on a standard diet (Baur et al. 2006, Lagouge et al. 2006). Resveratrol also protects against obesity by reducing the total body fat content, fat pad depots and body weight gain in mice (Lagouge et al. 2006) and rats (Aubin et al. 2008, Rivera et al. 2009) fed a HFD. However, it is remarkable that in some studies, resveratrol has failed to prevent body weight gain (Baur et al. 2006, Rocha et al. 2009). In obese Zucker rats, resveratrol reduced plasma triglycerides, FFAs and total cholesterol levels, and hepatic lipid content (Rivera et al. 2009).

In addition to reduced adiposity and body weight, resveratrol attenuates several obesity-associated disorders. Resveratrol has been shown to improve NAFLD in HFD fed Wistar rats (Shang et al. 2008) and obese Zucker rats (Gòmez-Zorita et al. 2012). Attenuated NAFLD by resveratrol is suggested to be due to reduced fatty acid availability and oxidative stress in the liver (Gòmez-Zorita et al. 2012). Resveratol also improves glucose tolerance in rats with type 2 diabetes, and
increases glucose transport in skeletal muscle (Su et al. 2006, Chi et al. 2007, Park et al. 2007, Deng et al. 2008). In obese Zucker rats, resveratrol has been shown to reduce blood pressure and have anti-inflammatory effects in adipose tissue (Rivera et al. 2009). Preliminary data from clinical trials support the metabolic effects of resveratrol that have been seen in animal studies (Brasnyo et al. 2011, Timmers et al. 2011, Crandall et al. 2012). In general, those trials have shown that resveratrol improves insulin sensitivity and metabolic profile, even though very different subject groups (obese healthy, type 2 diabetics and older adults) were used in trials.

The hypothesis that resveratrol mimics CR effects is based on the finding that resveratrol can activate SIRT1. This is further supported by the findings that many of the effects observed in resveratrol-treated animals are based on the deacetylation of SIRT1 target genes, in particularly PGC-1α (Baur et al. 2006, Lagouge et al. 2006). It has been argued, however, that resveratrol is not a direct activator of SIRT1. The in vitro Fluor-de-Lys assay that was used in the identification of SIRT1 activators requires the presence of fluorescent substrates and when a similar assay is performed using a non-fluorescent substrate, activation of SIRT1 by resveratrol was not observed (Kaeberlein et al. 2005a, Pacholec et al. 2010). Besides SIRT1, resveratrol also has several other targets proteins (for review see Baur 2010b). In fact, many of the metabolic effects of resveratrol are believed to be based on AMPK activation (Canto et al. 2010, Um et al. 2010). Taken into account that AMPK positively regulates NAD⁺ levels and SIRT1 activity (see in section 2.2.3.2), it suggests that resveratrol can also activate SIRT1 indirectly through AMPK. However, the therapeutic potential of resveratrol would be strengthened by further studies to clarify the role of SIRT1 and other target proteins on resveratrol metabolic benefits.

2.2.4.2 SRT1720

SRT1720 is structurally unrelated to resveratrol, but it was identified to be a 1000-fold more potent activator of SIRT1 (Milne et al. 2007). However, SRT1720, like resveratrol, exhibits a substrate-specific effect on SIRT1 activity in vitro (Pacholec et al. 2010) (see above), which arouses the suspicion of its mechanism of action. Unlike resveratrol, SRT1720 does not acutely activate AMPK (Feige et al. 2008), indicating a more specific activity.

Similar to resveratrol, SRT1720 mitigates various negative effects of obesity in both mice and rats. SRT1720 has been shown to improve whole-body glucose homeostasis and insulin sensitivity in diet-induced obese mice and Zucker rats (Milne et al. 2007). Furthermore, SRT1720 has been shown to increase endurance performance and protect against diet-induced obesity and insulin resistance by enhancing oxidative metabolism in skeletal muscle, liver and BAT (Feige et al. 2008). The improved oxidative metabolism in mice was shown to occur through deacetylation of SIRT1 targets genes PGC-1α, FOXO-1 and p53. The gene expression profile study in mice showed that SRT1720 regulates genes involved in mitochondrial biogenesis, metabolic signaling and inflammation (Smith et al. 2009), which further supports a strong metabolic effect of SRT1720. In addition, SRT1720 has been shown to reduce hepatic steatosis in genetic and monosodium
glutamate-induced obese mice due to the reduced expression of genes involved in lipogenesis, in particular SREBPs and its target genes (Yamazaki et al. 2009, Walker et al. 2010). Recently, SRT1720 was reported to improve survival in obese mice and this lifespan extension is accompanied by several health benefits including reduction in liver steatosis, improved insulin sensitivity, suppression of inflammation and apoptosis, and normalization of hepatic gene expression profile (Minor et al. 2011). These results strongly indicate that SRT1720 has protective effects against obesity similar to resveratrol.

To summarize, studies with model organisms have shown that CR has several beneficial effects on health and lifespan. Preliminary data from humans have shown that CR reduces the risk of cardiovascular diseases and diabetes, and mediates similar adaptive responses that occur in lower organisms. The mechanisms underlying the effects of CR are unknown, and recent studies with model systems have shown an important role for highly conserved nutrient sensing pathways; sirtuin, AMPK and mTOR pathways, in mediating the effects. It is also well-established that the insulin/IGF-1 pathway and autophagy are involved in mediating the effects of CR. Compliance to long-term CR is low, and there has been strong effort to find compounds capable of mimicking CR. Accumulating evidence indicates that the SIRT1-activating compounds resveratrol and SRT1720 mimic several effects of CR and attenuate many of the obesity-induced pathologies.
2.3 Dairy products and calcium in obesity

Nutrition has also an important role in the prevention and treatment of obesity. Dairy products are a fundamental part of a healthy diet and an important source of calcium and proteins. Epidemiological studies, clinical trials and animal studies have been shown that a diet high in dairy products supports weight management and facilitates weight loss during CR. Dairy calcium and proteins have been suggested to account for the anti-obesity effects of dairy products.

2.3.1 Potential dairy components affecting obesity

Bovine milk contains approximately 3.5% proteins. Lowering the pH of milk to 4.6 leads to separation of casein and whey protein fractions; casein is coagulated and whey proteins remain soluble (Farrell et al. 2004). The casein to whey ratio ranges during lactation and it varies largely between species. The whey to casein ratio in cow’s milk is on average 20:80.

Caseins
Caseins are the major protein component in bovine milk. Caseins are further divided to four types of molecules, the $\alpha_{\text{s1}}$, $\alpha_{\text{s2}}$, $\beta$, and $\kappa$-caseins in the approximate relative amounts of 4:1:3.5:1.5, respectively. In milk, caseins exist as large aggregates that are called casein micelles. Many of the physical and chemical properties (e.g. white colour, stability in heat) of milk are due to properties of casein micelles (Fox and Brodkorb 2008). Caseins and casein-derived peptides are suggested to have several health properties, including immunomodulatory, anti-oxidative, anti-microbial and osteoprotective properties (for review see Möller et al. 2008). In addition, the anti-hypertensive effects of casein-derived tripeptides isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) have been demonstrated both in animals and humans (for review see Jäkälä and Vapaatalo 2010).

Whey proteins
Whey is the natural by-product of cheese-making process. It is the liquid that remain after removing caseins. Besides whey proteins, that liquid contains minerals, vitamins, lactose and traces of fat. Whey contains five major proteins; $\beta$-lactoglobulin, $\alpha$-lactalbumin, serum albumin, immunoglobulin and proteose-peptone fractions (Farrell et al. 2004). $\beta$-lactoglobulin accounts for approximately 50% and $\alpha$-lactalbumin approximately 25% of the total whey protein in bovine milk. In addition, whey also contains serum albumin, immunoglobulins and protease-peptone fractions (Farrell et al. 2004), and minor amounts of lactoferrin, lactoperoxidase, IGF, $\gamma$-globulins and several other small proteins (Yalcin 2006). Whey proteins have a diverse array of functional properties that affect different biological processes and organ systems, including anti-
inflammatory, immunoregulatory, anti-oxidative and anti-cancer effects (Krissansen 2007). In addition, anti-hypertensive peptides have been derived from whey protein, but the possible clinical benefits of the whey-derived peptides are unclear (for review see Jäkälä and Vapaatalo 2010).

**Calcium**

Bovine milk contains approximately 1.2 g/l of calcium, and dairy products are the most important dietary source of calcium in the Finnish diet (Paturi et al. 2008). In bovine milk, calcium is mainly bound to casein micelles, about one-third of calcium is in the aqueous phase of milk and a small proportion of calcium is also bound to α-lactalbumin (Gaucheron et al. 2005). Calcium is important for the health of bones and teeth. It may help prevent hypertension, decrease colon and breast cancer incidences, and lower the risk of kidney stones (for review see Haug et al. 2007). In addition, the potential of calcium to improve weight control has been studied since the 1980s (see section 2.4.1.1).

### 2.3.2 Dairy products and obesity

**Epidemiological studies**

Several cross-sectional epidemiological studies in adults and children confirm the inverse association between dairy intake and obesity (for review see Dougkas et al. 2011). The largest of those studies was based on the Portuguese Health Interview Survey (n=37 513 adults) which showed significant inverse relationship between BMI and milk consumption (Marques-Vidal et al. 2006). Similar results have been shown in Iranian adults (Mirmiran et al. 2005, Azadbakht and Esmailzadeh 2008) and Italian postmenopausal women (Varenna et al. 2007). However, all studies do not support these findings, and no significant association between dairy intake and obesity was seen in Japanese women (Murakami et al. 2006), US adults (Brooks et al. 2006) or Dutch women (Snijder et al. 2007).

Even though cross-sectional studies mainly support the association between dairy intake and obesity, the results from prospective cohort studies are more controversial. One systematic review of 19 prospective cohort studies has been published recently (Louie et al 2011). Ten of the studies were conducted with children and adolescents, and nine with adults. Together eight studies, three with children (Carruth and Skinner 2001, Moore et al. 2006, Johnson et al. 2007) and five with adults (Pereira et al. 2002, Rosel et al. 2006, Sanchez-Villegas et al. 2006, Halkjaer et al. 2009, Poddar et al. 2009) showed a protective association of dairy intake against weight gain. One study reported significant protective association only in men who were initially overweight (Vergnaud et al. 2008), whereas seven studies reported no effect (Philips et al. 2003, Newby et al. 2004, Striegle-Moore et al. 2006, Tam et al. 2006, Snijder et al. 2008, Fiorito et al. 2009, Huh et al. 2010), and one reported an increased risk (among children) (Berkey et al. 2005). Two studies reported
both increased and decreased risk depending on the type of dairy intake (Drapeau et al. 2004, Rajpathak et al. 2006).

Although the evidence from epidemiological studies is inconclusive, the growing body of evidence suggests that dairy consumption has beneficial effects on the metabolic syndrome (Crichton et al. 2011). Several cross-sectional (Mennen et al. 2000, Yoo et al. 2004, Azadbakht et al. 2005, Liu et al. 2005, Elwood et al. 2007, Ruidavets et al. 2007) and prospective cohort studies (Pereira et al. 2002, Lutsey et al. 2008) have shown that dairy consumption is inversely associated with the prevalence or incidence of the metabolic syndrome. However, some studies failed to see an association between dairy consumption and metabolic syndrome (Snijder et al. 2007, Snijder et al. 2008, Shin et al. 2009), and two studies showed a higher prevalence of metabolic syndrome in subjects with higher dairy intakes (Lawlor et al. 2005, Beydoun et al. 2008).

The type of dairy product used in epidemiological studies has been shown to affect outcomes, and interestingly some studies have shown an inverse association between BMI and high-fat, but not low-fat, dairy intake (Rajpathak et al. 2006, Rosell et al. 2006, Snijder et al. 2007). In some studies higher cheese consumption is associated with higher BMI (Snijder et al. 2007, Beydoun et al. 2008), while milk and yoghurt intake were negatively related to adiposity (Beydoun et al. 2008). In the Bogalusa Heart Study, dairy intake from foods such as pizza, lasagna and cheeseburgers was also included in the analysis (Brooks et al. 2006). This indicates that the type of dairy products strongly varies between cross-sectional studies or it has not been reported at all, which complicates the comparison of the epidemiological studies and can explain why some studies failed to show an inverse association between dairy intake and obesity. In addition, in studies examining the effects of dairy product intake on metabolic syndrome, the differences in metabolic syndrome diagnostic criteria can explain, at least in part, the inconclusive nature of the results.

Clinical trials
The randomized clinical trials have investigated the effect of dairy intake on body weight gain and weight loss. One meta-analysis concerning the effect of dairy consumption on weight and body composition has been published (Abargouei et al. 2012). Overall, the meta-analysis consisting of 14 randomized controlled clinical trials showed that high dairy intake resulted in -0.61 kg (95% confidence interval (CI): -1.29, 0.07, P=0.08) greater reduction in body weight, 0.72 kg (95% CI: -1.29, -0.14. P=0.01) greater reduction in fat mass, 0.58 kg (95% CI: 0.18, 0.99, P<0.01) gain in LBM and 2.19 cm (95% CI: -3.42, -0.96, P<0.001) further reduction in waist circumference (WC) than in the control group. However, there was a significant heterogeneity between studies and therefore studies were categorized into two major groups; those that assessed the effects of high-dairy diets on weight loss (with CR, 500 kcal/day less than the estimated energy requirement) both in intervention and control groups, and those that assessed the effects of high-dairy diets on weight gain (without CR). Ten studies with CR showed that high-dairy intake (additional calcium intake of 550-1000 mg/day via dairy products in intervention group vs. calcium intake of 290-800 mg/day in
control group) resulted in 1.29 kg (95% CI: -1.98, -0.6, P<0.001) greater weight loss, 1.11 kg (95% CI: -1.75, -0.47. P=0.001) greater reduction in body fat mass, 0.72 kg (95% CI: 0.12, 1.32, P=0.02) gain in LBM and 2.43 cm (95% CI: -3.42, -1.44, P<0.001) further reduction in WC compared to controls. However, five studies without CR did not show any significant differences in weight, body fat mass, LBM or WC between subjects with high dairy intake (additional calcium intake of 400-850 mg/day via dairy products) and with low dairy intake (maintained at habitual diet). The meta-analysis demonstrated that increased dairy intake enhances weight and fat loss during CR, but it has no beneficial effects on weight and body composition in situations without CR.

2.4 Dairy components and obesity

2.4.1.1 Dietary calcium and obesity

Epidemiological studies
McCarron and his group (1984) were the first to show that calcium intake was inversely related to body weight. After that, numerous cross-sectional and prospective cohort studies have been published, and several of those studies support the inverse association between calcium intake and body weight both in adults and children (for review see Dougkas et al. 2011). Since dairy is the major dietary source of calcium, studies also subsequently evaluated the role of dairy. Dougkas et al. (2011) conducted a mixed-model regression analysis of the 18 epidemiological studies and created a linear regression equation showing the association between calcium intake and predicted BMI. According to that equation low dietary calcium intake with a 400mg/d dose predicted a BMI of 25.6 kg/m², and high dietary calcium with a 1200mg/d dose predicted a BMI of 24.7 kg/m². In other words, this means that an increase in calcium intake of 800 mg/day is associated with a decrease in BMI of 1.1 kg/m². In addition to BMI, epidemiological studies have also shown that higher calcium intake is associated with lower blood pressure, fasting glucose, insulin and cholesterol levels, insulin resistance (McCarron et al. 1984, Jacqmain et al. 2003, Drouillet et al. 2007, Kruger et al. 2007, dos Santos et al. 2008), and the reduced risk of stroke (Iso et al. 1999, Abbott et al. 1996, Umesawa et al. 2008).

Clinical trials
Even though most of the epidemiological studies show an inverse correlation between calcium intake and BMI, results from clinical trials are more controversial. The meta-analysis including 13 randomized controlled trials showed that high-calcium intake from calcium a supplement source (mean difference -1.79 kg, 95% CI -3.04, -0.55, P=0.05) or dairy product source (mean difference 0.85 kg, 95% CI -4.39, 6.08, P=0.75) did not have significant effect on body weight (Trowman et al. 2006). However, only one of those trials was directed at studying the effects of calcium treatment on body weight (Zemel et al. 2004), while the other studies were investigating bone mass during calcium treatment. Furthermore, one systematic review using 49 randomized trials, of which 38
trials investigated the effect of calcium and/or dairy supplementation on weight gain (without CR) and 11 trials on weight loss (with CR) (Lanou and Barnand 2008). As many as 41 trials had no effect on body weight during weight gain or weight loss. Of the weight gain trials, only one trial showed a lower rate of weight gain (Caan et al. 2008), two trials showed weight gain (Barr et al. 2000, Lau et al. 2001) and one trial showed weight loss (Recker et al. 1996). In contrast, only four trials showed that calcium/dairy supplementation induces weight loss during CR (Zemel et al. 2004, Zemel et al. 2005a, Zemel et al. 2005b).

In most of the clinical trials that showed an effect on body weight, the source of calcium was dairy supplementation (Zemel et al. 2004, Zemel et al. 2005a, Zemel et al. 2005b), which suggests that the source of Ca can affect results. Zemel et al. (2004) reported more effective weight and fat loss during CR in obese subjects who received calcium as dairy products compared to calcium supplements. This is further supported by the other study showing greater fat loss in high-dairy diet compared to calcium supplementation (Zemel et al. 2009). However, the study did not observe significant differences in weight loss. Similarly, in postmenopausal women, high-dairy intake resulted in greater loss of leg fat and a lower increase in the sum of skinfold thickness compared to the high supplemental calcium, even though BMI and weight were unchanged in the study (Manios et al. 2009). In addition, dietary calcium has been shown to be more effective in the prevention of weight re-gain than calcium supplements (Ochner and Lowe 2007).

Animal studies
The anti-obesity effects of dietary calcium has been widely studied in transgenic aP2-agouti mice, which are lean when fed normal chow diets, but are very susceptible to adult-onset diet-induced obesity. aP2-agouti mice gain weight when they are fed a low-calcium diet, and a high-calcium diet suppresses fat accretion and weight gain in animals maintained at identical caloric intakes (Zemel et al. 2000, Sun and Zemel 2006). In addition, high-calcium diets accelerate weight and fat loss during CR more than low-calcium diets (Shi et al. 2001). After CR, aP2-agouti mice fed with high-calcium diets exhibited a 50-85% reduction in weight and fat re-gain (Sun and Zemel 2004a).

Only one study reports a lack of effect of calcium intake on body weight and body fat in rats and mice (Zhang and Tordoff 2004). However, studies in many other animal models, including spontaneously hypertensive rats, Wistar rats and HFD fed C57Bl/6J mice, confirm the observation that increased calcium intake lowers body weight and fat gain (Metz et al. 1988, Papakonstantinou et al. 2003, Parra et al. 2008).

In addition to the effects on body weight and fat content, a high-calcium diet has been shown to decrease adipose tissue intracellular ROS production, and the expression of pro-inflammatory cytokines TNF-α and IL-16 in adipose tissue in aP2-agouti mice (Sun and Zemel 2006, Sun and Zemel 2007a, Zemel and Sun 2008a, Bruckbauer and Zemel 2009). The results indicate that a high-calcium diet suppresses both ROS and inflammatory stress, and both are important contributors to many obesity-associated disorders.
2.4.1.2 Dairy proteins and obesity

Dietary calcium has been estimated to be responsible for approximately 50% of the anti-obesity bioactivity of dairy products, which suggest that dairy products contain also other bioactive components (for review see Zemel 2009). Dairy products are also an important dietary source of high-quality proteins, and accumulating evidence indicates that the intake of proteins may be important during weight loss and weight maintenance (Clifton et al. 2008, Clifton et al. 2009, Westerterp-Plantenga 2009). However, it is unknown whether the type of dietary protein has impact on weight management.

Dairy proteins contain bioactive compounds that may have anti-obesity effects (for review see Krissansen 2007, Möller et al. 2008). Among those bioactive compounds are casein- and whey-derived peptides having angiotensin-converting enzyme (ACE) inhibitor activity (for review see Jäkälä and Vapaatalo 2011). In addition to blood pressure regulation, ACE inhibitor activity is suggested to be associated with weight regulation. The adipose tissue local renin-angiotensin-aldosterone system (RAAS) is linked to the development of obesity and metabolic syndrome (Engeli et al. 2003). Furthermore, angiotensin II is known to up-regulate fatty acid synthase (FAS) expression in adipocytes, and ACE inhibition has been shown to mildly attenuate obesity in hypertensive mice and humans (for review see Zemel et al. 2009).

Accumulating evidence suggests that whey proteins in particular enhance the anti-obesity effects of dairy products. Studies with Wistar rats have shown that whey protein prevents body weight gain more that red meat (Belobrajdic et al. 2004) or casein (Royle et al. 2008). In C57BL/6J mice, a high-calcium whey protein diet inhibited body weight and fat gain, accelerated weight and fat loss during CR and prevented weight and fat re-gain after CR more than a high-calcium casein diet (Pilvi et al. 2007, Pilvi et al. 2009). In addition, comparison between different high-calcium whey protein fraction diets (whey protein isolate, α-lactalbumin, β-lactoglobulin and lactoferrin) showed that α-lactalbumin was the most effective in inducing weight loss and preventing weight re-gain as compared to other whey protein fractions (Pilvi et al. 2009). In addition, a high-calcium whey protein diet has been shown to decrease blood glucose and serum insulin levels more than a casein-based diet during CR (Pilvi et al. 2008a). A high-calcium whey protein diet also beneficially modulates hepatic lipid profile (Pilvi et al. 2008a) and adipose tissue gene expression profile during CR (Pilvi et al. 2008b). Similar beneficial effects have been seen in humans as well and during CR, a whey protein supplement has been shown to enhance weight loss and spare the muscle mass (Frestedt et al. 2008). Clinical trials have also shown that whey protein has blood glucose and/or insulin lowering effects and it may increase muscle protein synthesis (for review see Graf et al. 2011).

The anti-obesity effects of whey protein are suggested to be due to its amino acid composition. Whey contains a high concentration of branched chain amino acids (BCAAs, ~26%) (leucine, isoleucine and valine) (Ha and Zemel 2003). Of the BCAAs, leucine is of particular interest, because...
it has a distinct role in protein metabolism and it is important in the stimulation of muscle protein synthesis (Ha and Zemel 2003). Furthermore, leucine appears to be an important factor in lipid metabolism and energy partitioning between adipocytes and skeletal muscle cells (Sun and Zemel 2007b).

2.4.2 Mechanisms explaining the anti-obesity effects of dairy products

The potential mechanism underlying the effect of high-dairy intake on body weight and body fat is still quite unclear. Currently, the most highly plausible mechanisms refer to dietary calcium and its effects on intracellular calcium and subsequent impact on adipocyte lipid metabolism and fatty acid absorption from the gastrointestinal tract. In addition, whey proteins have been suggested to have satiety and insulinotropic effects, which can enhance the anti-obesity effects of dairy products.

*Calcium and adipocyte lipid metabolism*

Zemel and his group were the first who showed the association between low-calcium intake and fat accumulation (Xue et al. 1998, Zemel et al. 2000). Intracellular calcium has been shown to promote fat storage, which is due to the stimulation of de novo lipogenesis through the regulation of fatty acid synthase (FAS), and the inhibition of lipolysis through the activation of phosphodiesterase 3B (PDE3B) (Zemel 2002). The concentration of intracellular calcium in human adipocytes is regulated by calcium-regulating hormones parathyroid hormone (PTH) and 1,25 dihydroxy-vitamin D3 (1,25(OH)2D3). High-calcium intake decreases the concentration of calcitrophic hormones (PTH and 1,25(OH)2D3) and thereby decreases the concentration of intracellular calcium, which inhibits lipogenesis and stimulates lipolysis leading to decreased lipid storage (Zemel 2003).

In addition, decreased 1,25(OH)2D3 during high-calcium intake has several lipid storage decreasing functions. At first, decreased 1,25(OH)2D3 increases the expression of UCP-2 via the nuclear vitamin D receptor in WAT which leads to improved thermogenesis (Shi et al. 2002). Thereby, high-calcium intake attenuates the decline in thermogenesis which usually occurs during CR. Secondly, the regulation of UCP-2 and intracellular calcium by 1,25(OH)2D3 appears to modulate adipocyte apoptotic death (Sun and Zemel 2004b, Zemel and Sun 2008b). The third possible explanation is that decreased 1,25(OH)2D3 down-regulates 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD) expression and decreases the concentration of glucocorticoid which in turn decreases the size of the fat deposit (Morris and Zemel 2005). Finally, decreased 1,25(OH)2D3 has an impact on human adipocyte differentiation and proliferation via the regulation of ROS and inflammatory cytokines (Sun et al. 2008, Sun and Zemel 2008). Even though evidence from cell-culture and animals studies shows the role of high-calcium intake in adipocyte fat metabolism, recent results from human studies failed to support it (Boon et al. 2005, Boon et al.
2007, Bortoletti et al. 2008) indicating that further studies are required to explore the effect of high-calcium on lipid metabolism in humans.

**Calcium and fat oxidation**
The effect of high-calcium intake on whole body fatty acid oxidation has been examined in randomized clinical trials, and the results from those studies are controversial. Melanson and his group (2005) showed that high-calcium diet during CR increased 24 h whole-body fat oxidation by 28% as compared to a low-calcium diet during CR. Similar results have been seen in other studies (Gunther et al. 2005, Cummings et al. 2006, Teegarden et al. 2008, Zemel et al. 2008), while several studies also fail to support the role of high-calcium consumption in fat oxidation (Boon et al. 2005, Jacobsen et al. 2005, Bortolotti et al. 2008). Although dietary calcium is known to regulate expression of the fat oxidation gene UCP-2 (Shi et al. 2002), the clear mechanism of how dietary calcium regulates fat oxidation is unknown.

**Calcium and fatty acid absorption**
Another possible mechanism that has been suggested as responsible for the effect of calcium on body adiposity is reduced fat absorption from the gastrointestinal tract. Calcium has been shown to increase faecal excretion of fat via the formation of insoluble fatty acid soaps or by the binding of bile acids which weakens the formation of micelles (Denke et al. 1993, Welberg et al. 1994, Shahkhailili et al. 2001, Jacobsen et al. 2005, Bendsen et al. 2008). Recently, meta-analysis of randomized controlled trials examining the impact of both dairy and supplemental calcium on faecal fat excretion has been published (Christensen et al. 2009). It showed that both dairy and supplemental calcium increased faecal fat excretion approximately 2.2 g/day (95% CI 0.63, 1.34, P<0.0001), but there was some heterogeneity (I²=49.5%) among studies indicating some inconsistency in trial outcomes. When only studies using dairy-derived calcium were analyzed, results showed homogeneous outcomes and it was estimated that increasing dairy calcium intake by 1241 mg/day resulted in an increase in faecal fat of 5.2 g/day (95% CI: 1.6, 8.8). Authors also estimated that 5.2 g/day faecal fat excretion is equivalent to 4.7 kcal/day or 1.9 kg body fat or 2.2 kg body weight loss over in one year. In addition to fatty acid absorption, high-calcium intake has been shown to decrease postprandial lipidemia as compared to low-calcium intake, and calcium from dairy products was more effective than supplementary calcium (Lorenzen et al. 2007).
Dairy proteins and satiety

Among the other dairy components, the proteins have the greatest role in appetite control. It is well documented that dietary proteins suppress food intake more than carbohydrates and fats (Anderson and Moore 2004), and diets high in protein content are more satiating than low protein diets (Skov et al. 1999, Weigle et al. 2005). Currently, there is little evidence showing whether the satiety effect of proteins is dependent on the source of protein. Since dairy products are an important source of protein, there has been great interest in investigating the potential of dairy proteins providing satiety signals.

Whey proteins have been shown to be more satiating than casein, soy proteins, turkey or egg proteins (Hall et al. 2003, Anderson et al. 2004, Veldhorst et al. 2009, Pal and Ellis 2010). Some studies, however, have not seen a difference in the satiating effect between whey and casein (Bowen et al. 2006). Whey and casein have different physical properties that contribute to their functionality in food systems. The digestion of whey into peptides and amino acids and then absorption is faster than that of casein and therefore casein and whey proteins are classified as ‘slow’ and ‘fast’ proteins, respectively (for review see Luhovyy et al. 2007). It is assumed that whey and casein have a synergetic effect; whey reduces food intake at 90 min and casein at 150 min after meal consumption. The more satiating effect whey protein is mediated by its effect on the release of satiety hormones. Whey protein ingestion resulted in higher plasma concentration of cholecystokinins (CCKs) and glucagon-like peptide-1 (GLP-1) than casein (Hall et al. 2003). Also, the high concentration of leucine in whey protein is proposed to explain the satiating effect of whey. Leucine enters the brain from blood faster than other amino acids and intracerebroventricular administration of leucine has been shown to reduce food intake which is thought to be due to activation of mTOR in hypothalamus (for review see Potier et al. 2009) (see section 2.2.3.3). Noteworthy is that most of the above mentioned effects of whey are based on high whey protein diets and it is unknown whether similar effects are obtained with normal serving sizes of dairy products which were used in the experimental studies and clinical trials studying the effect of dairy products on body weight.

Dairy proteins and insulinotropic properties

High dairy intake has beneficial effects on metabolic syndrome (Crichton et al. 2011) and, in particular, high dairy intake is linked to a lower risk of type 2 diabetes (for review see Tremblay and Gilbert 2009). Several studies have shown that whey proteins enhance postprandial glycemic response (Nilsson et al. 2004, Frid et al. 2005, Ma et al. 2009, Akhavan et al. 2010). As mentioned above, whey is a rapidly digested protein that promotes a higher concentration of amino acids in plasma after a meal, which can stimulate insulin secretion. The mixture of leucine, isoleucine, valine, lysine and threonine has been shown to induce similar glycemic and insulinemic responses as whey in healthy subjects (Nilsson et al. 2007), suggesting that BCAAs of whey proteins are important for whey protein mediated glycemic response.
In addition, whey protein has been suggested to mediate the insulinotrophic properties through incretin hormones. Incretin hormones, such as insulinotropic polypeptide (GIP) and GLP-1 are gut-derived peptides that potentiate insulin secretion from the islet β-cell in a glucose-dependent manner (Drucker 2006a). Incretin hormones are released after food consumption and they are rapidly degraded by dipeptidyl peptidase IV (DPP-IV). In mice, whey protein has been shown to inhibit DPP-IV and raise GLP-1 concentration resulting in an increased and prolonged insulin response (Gunnarsson et al. 2006). The mechanism for that is unknown, but it has been suggested that dipeptides and tripeptides formed during whey digestion may serve as an endogenous inhibitors of DPP-IV activity in the proximal gut (for review see Drucker 2006b).

* Taken together, the growing body of evidence from epidemiological studies has shown that a diet high in dairy products is inversely associated with BMI and it is related to a lower risk of type 2 diabetes and metabolic syndrome. According to clinical trials high-dairy product intake enhances weight and fat loss during CR. Dairy calcium has been suggested to account for part of the anti-obesity effects of dairy products via increased fat excretion and 1,25(OH)₂D₃-mediated changes in adipocyte lipid metabolism. Importantly, dairy-derived calcium has been shown to be more effective than calcium supplementation, indicating that dairy products also contain other anti-obesity components. Dairy proteins, especially whey proteins, have been shown to prevent weight gain and enhance weight loss during CR. The mechanisms underlying the anti-obesity effects of whey proteins are still unclear, but it has been suggested to be due to their higher satiety and insulinotropic effects. *
3. Aims of study

Weight loss is the primary treatment for obesity and obesity associated diseases. Calorie restriction (CR) with adequate nutrition is the effective method to induce weight loss, but compliance to CR lifestyle is often very low. Better understanding of the mechanism how CR mediates its effects could reveal new targets for anti-obesity drug development. The main aim of the present study was to investigate the molecular and signaling pathways mediating the effects of CR with special emphasis on the sirtuin, AMPK and mTOR pathways. Milk-derived whey proteins have been shown to augment the weight loss effect of CR via a still unknown mechanism. Therefore, the study also aimed to clarify the anti-obesity whey proteins and their mechanism of action.

The specific objectives were the following:

1. To investigate the effects of CR on diet-induced obesity, fatty liver formation, and adipose tissue inflammation. The influences of CR on calorimetry, metabolic performance, and on nutrient sensing pathways in metabolically important tissues were also examined. Finally, the potential of SIRT1 activating compound resveratrol to mimic the beneficial effects of CR was also studied.

2. To compare the adipose tissue cytokine and angiogenesis protein profiles from obese and lean mice and to investigate the influence of CR.

3. To investigate the potential of whey proteins and calcium to modify skeletal muscle gene expression profile during CR.

4. To evaluate the potential of a novel whey protein isolate, rich in lactoperoxidase, lactoferrin, growth factors and immunoglobulins, in the prevention and treatment of diet-induced obesity and fatty liver formation.
4. Materials and methods

4.1 Materials

4.1.1 Experimental animals and animal welfare

All studies were conducted with C57Bl/6J mouse strain, which is a widely used inbred mouse strain for nutritionally-induced obesity. This mouse strain develops obesity and metabolic compliances such as insulin resistance and fatty liver when fed a HFD, and thus serves as a human-like experimental model in obesity research.

6-9 week-old male C57Bl/6J mice were purchased from Charles River Europe (Sulzfeld, Germany) (studies I, II, IV) or from Harlan (Horst, the Netherlands) (study III). The mice were housed 3-5 per cage in a standard experimental animal laboratory (illuminated from 07.00 a.m to 19.00 p.m (06.30-18.30 in study III), 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. Mice had free access to feed and tap water during the experiments. Before the experiments all mice were kept on 1-week acclimatization period when mice were fed with Harlan chow diet (Harlan Tekland 2018; Harlan Holding, Inc., Wilmington, DE, USA).

4.1.2 Study design and dietary interventions

Obesity for mice was induced by feeding the mice with normal-calcium (0.8%, D12492M, Research diet Inc., USA) (studies I, II, IV) or low-calcium high-fat diet (HFD) (0.4%, D05031101M, Research diet Inc.) (study III). 30% calorie restriction (energy intake 70% of ad libitum intake) was used in the studies.

The study designs and dietary interventions are shown in Table 3. In study I mice were fed with high-fat and low-fat diets for 15 weeks (=105 days) and resveratrol (Orchid Chemicals & Pharmaceuticals Ltd., India) was mixed into the HFD with two different doses (2g/food kg and 4g/food kg). The resveratrol dosages were based on the previous study by Lagoug and coworkers (2006).

Studies II-IV consisted of different phases; weight gain phase and calorie restriction (CR) phase, and in study IV a weight re-gain phase was used. During weight gain phase mice were fed with HFD ad libitum for 100 days. After that, body-weight matched mice were divided into study groups and they were moved into the CR phase for 50 days. In studies III and IV, the protein source varied between study groups during the CR phase, the casein in diets was replaced with different combinations of whey proteins (Table 3). Study IV included a weight re-gain phase and after the CR phase mice were fed with same diets ad libitum for 50 days. All diets used in studies
were purchased from Research diet Inc. (New Brunswick, NJ, USA) except for the Harlan chow diet (Harlan Tekland 2018; Harlan Holding, Inc.). The whey protein isolates (WPI) in studies III and IV were different in composition; a novel WPI in the study IV was rich in lactoperoxidase, lactoferrin, immunoglobulins and growth factors, and it has a special amino acid composition.

Table 3. Study groups and dietary interventions used in the thesis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Group</th>
<th>N</th>
<th>Diet characteristics (Fat, protein, Ca(^{2+})(^*))</th>
<th>Dietary protein</th>
<th>Feeding</th>
<th>Total duration of study (days)(#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HFD</td>
<td>18</td>
<td>60%, 18%, 0.8%</td>
<td>casein</td>
<td>AdL</td>
<td>105</td>
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<td>HFD + R2</td>
<td>19</td>
<td>60%, 18%, 0.8%</td>
<td>casein</td>
<td>AdL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HFD + R4</td>
<td>19</td>
<td>60%, 18%, 0.8%</td>
<td>casein</td>
<td>AdL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR</td>
<td>18</td>
<td>60%, 18%, 0.8%</td>
<td>casein</td>
<td>30% CR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LFD</td>
<td>18</td>
<td>10%, 18%, 0.8%</td>
<td>casein</td>
<td>AdL</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Obese</td>
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<td>casein</td>
<td>AdL</td>
<td>150</td>
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<td>casein</td>
<td>30% CR</td>
<td></td>
</tr>
<tr>
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<td>Lean</td>
<td>6</td>
<td>18%, 24%, 1.0%</td>
<td>cannot be separated</td>
<td>AdL</td>
<td></td>
</tr>
<tr>
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<td>Lean CR</td>
<td>6</td>
<td>18%, 24%, 1.0%</td>
<td>cannot be separated</td>
<td>30% CR</td>
<td></td>
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<td>AdL</td>
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<tr>
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<td>18%, 24%, 1.0%</td>
<td>cannot be separated</td>
<td>AdL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CR casein</td>
<td>10</td>
<td>60%, 18%, 0.4%</td>
<td>casein</td>
<td>30% CR</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>60%, 18%, 1.8%</td>
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<td>30% CR</td>
<td></td>
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<tr>
<td></td>
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<tr>
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<td>casein</td>
<td>AdL</td>
<td>200</td>
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<tr>
<td></td>
<td>Casein Control</td>
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<td>casein</td>
<td>30% CR</td>
<td></td>
</tr>
<tr>
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<td>WPI 5%</td>
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<td>60%, 18%, 0.8%</td>
<td>whey 5%/casein 95%</td>
<td>30% CR</td>
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<tr>
<td></td>
<td>WPI 50%</td>
<td>15</td>
<td>60%, 18%, 0.8%</td>
<td>whey 50%/casein 50%</td>
<td>30% CR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WPI 100%</td>
<td>13</td>
<td>60%, 18%, 0.8%</td>
<td>whey 100%/casein 0%</td>
<td>30% CR</td>
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</tbody>
</table>

AdL, ad libitum; CR, calorie restriction; HFD, high-fat diet; LFD, low-fat diet; R2, resveratrol dose 2 g/food kg; R4, resveratrol dose 4g/food kg, WPI, whey protein isolate, * show the amount of energy from fat and proteins and the Ca\(^{2+}\) content of diets, # studies II-IV consisted of different phases; 100 day weight gain phase (HFD AdL) and 50 day calorie restriction (CR) phase, and in study IV a 50 day weight re-gain phase was used.

4.2 Methods

4.2.1 Body weight, body fat percentage and LBM measurements

In study I, the body weight was monitored three times per week by using a standard table scale (Mettler-Toledo, Columbus, USA) and the body fat percentage was analyzed from anesthetized mice magnetic resonance imaging (MRI; Oxford Instrument, Oxford, UK) (Siiskonen et al. 2007).

In other studies (studies II-IV), the body weight was monitored once per week using a standard table scale (Ohaus Scout TM Pro; SP4001, Nänikon, Switzerland), and the body fat percentage and lean body mass (LBM) were analyzed by dual-energy X-ray (DEXA; Lunar PIXIImus, GE Healthcare, Chalfont St. Giles, UK) before and after CR and also after weight re-gain (study IV).
4.2.2 Faecal fat excretion (studies II and IV)

At the end of CR (studies II and IV) and weight re-gain phases (study IV) mice were housed in metabolic cages for 24 h. The faecal samples were collected and the faecal fat content was determined by Schmid-Bondzynski-Ratzlaff method (International standard 1986). The apparent fat digestibility (%) was determined from fat intake and faecal fat content as described previously (Papakonstantinou et al. 2003), using the formula: the apparent fat digestibility (%) = 100 x [(fat intake – faecal fat content)/fat intake].

4.2.3 Oral glucose tolerance test (studies II and IV)

Glucose (glucose dosage 1/kg, given as 20% glucose solution) was given by gavage after 6 h fasting. Blood glucose was determined by a glucometer (Super Glucocard™ II, GT-1630, Arkay Factory Inc., Shiga, Japan) on blood samples taken from the tail vein at time points 0, 15, 30, 60 and 90 min after the gavage. The areas under the curve (AUC) (blood glucose x time) were calculated.

4.2.4 Calorimetry and metabolic performance (study I)

The effects of CR and resveratrol on metabolic performance, physical activity, drinking and feeding behavior were analyzed by housing a group of mice (n=6/group) in a home cage-based monitoring system for laboratory animals (Labmaster TSE System, Bad Homburg, Germany). After a one-week acclimatization period, mice were housed in one-mouse cages during study weeks 12-15. The measuring time was 100 hours (52 h in light phase and 48 h in dark phase). The system consisted of high-precision feeding and drinking sensors, an indirect calorimetry system that allows for determination of O\textsubscript{2} consumption, CO\textsubscript{2} production, respiratory exchange rate and heat production, and infrared light-beams that detected the animals’ physical activity comprising ambulatory, fine and rearing movements.

4.2.5 Tissue sample preparation

Mice were decapitated at the end of the treatment periods. In studies II-IV mice were rendered unconscious with CO\textsubscript{2}/O\textsubscript{2} (95:5% v/v; AGA, Riihimäki, Finland) before decapitation. The blood samples were placed into plastic tubes and the serum was separated by centrifugation at 960 x g at 4°C for 20 min. The subcutaneous, epidymal, abdominal and perirenal fat pads, liver and skeletal muscle (musculus quadriceps femoris) were removed, washed with saline, blotted dry and
weighed. Samples were snap-frozen in liquid nitrogen and stored at -80 °C until assayed. The right tibia length (study IV) was measured by micrometer and it was used for relative organ weight measurements. Liver samples for histology (studies I and IV) and perirenal samples for adipocyte cross sectional area (studies II and IV) measurements were fixed in 10% formaline and embedded in paraffin with routine techniques.

4.2.6 Liver histology (studies I and IV)

Liver sections (4 µm) from the paraffin-embedded liver samples were cut with a microtome. Liver sections were stained with a hematoxylin and eosin (H&E) stain and examined under conventional light microscopy in a “blinded” fashion. The samples were subjected to a semiquantitative histological analysis using the nonalcoholic steatohepatitis (NASH) Clinical Research Network scoring system for NAFLD with slight modifications for mice samples (Kleiner et al. 2005, Tiniakos et al. 2010, Pilvi et al. 2008a).

4.2.7 Adipocyte cross sectional area (studies II and IV)

The fat pads were fixed in 10% formaline and embedded in paraffin with routine techniques. Sections (4 µm) of paraffin-embedded fat samples were cut with a microtome and were mounted on charged glass slides, deparaffinized in xylene and stained. Four (study II, n=6-7/group) or eight (study IV, n=5/group) images were taken from each sample with a microscope camera. The adipocyte cross-sectional area was determined from each image using Leica QWin Standard-software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

4.2.8 Cytokine and angiogenesis arrays (study II)

Proteins from abdominal fats (n=3/group) were isolated by homogenizing the samples with a Bertin Precellys 24 homogenizer (Bertin Technologies, Aix en Provence, France) and ceramic beads (Precellys CK14, Bertin Technologies). Protein analysis was performed using a mouse cytokine array panel A (represents 40 mouse cytokine proteins) and mouse angiogenesis array kits (represents 53 mouse angiogenesis-related proteins) (Proteome Profiler™ antibody arrays, R&D Systems, Inc., MN, USA) according to the protocol of the manufacturer. 750 µg of the total protein from three pooled samples was used for one membrane in which each of the antibodies was spotted in duplicates. Chemiluminescence solution (ECLplus; Amersham Biosciences, GE Healthcare, Little Chalfont, UK) was used for protein detection. The protein expression in the membranes was visualized by FLA-9000 fluorescent image analyzer (Fujifilm, Tokyo, Japan) and the
relative protein expression between study groups was determined by analyzing the pixel densities of spots in each of the arrays.

### 4.2.9 Microarray procedure (study III)

The total RNA from skeletal muscle samples (n=5/group) was isolated by TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) and purified with the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. The concentration and integrity of total RNA were analyzed with a spectrophotometer and 2100 Bionalyzer (Agilent Technologies, Santa Clara CA, USA). The purified RNA (1 µg) was reverse transcribed to cDNA and tagged with biotin with one-cycle target labelling and control reagents (Affymetrix, Santa Clara, CA, USA). The labelled cDNAs were hybridized to Affymetrix GeneChip® Mouse Genome 430 2.0 Arrays, representing over 30,000 mouse transcripts. The arrays were scanned with Affymetrix GeneChip Scanner 3 000.

### 4.2.10 Microarray data processing (study III)

The microarray data were normalized with the RMA reprocessing algorithm and then normalized to the median per chip. The differences between the gene expression in groups were analyzed by Welch type test and Benjamini-Hochberg’s false discovery rate and were used as an adjustment for multiple comparisons. The genes passing the statistical comparison with the corrected p-value 0.05 and exceeding the ±1.2-fold change limit between the compared group and the obese group were then classified as up-regulated and down-regulated in the respective groups. The list of genes with differences in expression between obese vs. lean, and the list with differences in expression between the CR group vs. obese were compared by logical comparison statements to create lists of genes regulated uniquely in the groups. The functional annotation describing the categorical data for gene ontology (GO) and pathway information for the lists of genes were performed with The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 (Dennis et al. 2003).

To detect the putative differences between the CR groups, the expression of the genes on three CR groups was analyzed by using hierarchical clustering. Noise-reduction was applied prior to the clustering by restricting the dataset to contain only the genes which were significantly regulated in at least one comparison between the CR groups vs. obese group. This data for over 3 000 genes were clustered into a hierarchical tree from which the clusters A to R were selected. The KEGG pathways associated with the genes belonging to these created clusters were inspected with DAVID (Dennis et al. 2003).
4.2.11 Quantitative RT-PCR (studies I, III and IV)

Total RNA from perirenal fat (studies I, IV), liver (study I) and skeletal muscle (study III) samples was isolated by TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), treated with DNAse 1 (Deoxyribonuclease 1, Sigma Chemicals Co., St Louis, MO, USA) and 1 µg of RNA was reverse transcribed to cDNA with the reverse transcription enzyme (Im-Prom-II reverse transcription system, Promega, Madison, WI, USA). cDNA samples were amplified using FastStart DNA Master SYBR Green 1 (Roche diagnostics) in the presence of 0.5 µM primers (Table 4). The mRNA expression analysis was performed using Light-Cycler quantitative RT-PCR instrument (Roche Diagnostics, Neuilly-Sur-Seine, France) for detection of adiponectin, aldehyde dehydrogenase family 1 subfamily A7 (Aldh1a7), Cd68, fatty acid synthase (Fasn), leptin, monocyte chemoattractant protein 1 (MCP-1), nuclear respiratory factor 1 (Nrf1), nuclear receptor subfamily 4 group A member 3 (Nr4a3), plasminogen activator-1 (PAI-1), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), stearoyl-coenzyme A desaturase 1 (Scd1), transcription factor A mitochondrial (Tfam), visfatin and 18S. The quantities of the PCR products were quantified with an external standard curve amplified from the purified PCR product. The quantities of the PCR products were quantified with an external standard curve amplified from the purified PCR product. All the values were normalized to 18S mRNA levels.

Table 4. The sequences of qRT-PCR primers used in the thesis.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
</tr>
</thead>
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<tr>
<td>Adiponectin</td>
<td>GTATCGCTCAGCGTTCT</td>
<td>GTCGGTGACGTATCTGC</td>
</tr>
<tr>
<td>Aldh1a7</td>
<td>GTGGTGGACGCGTAAAGA</td>
<td>TTTCGCCCCAGCGACC</td>
</tr>
<tr>
<td>Cd68</td>
<td>CCGAGTCAGTCTACCT</td>
<td>GTGATTGTCTGTCTCGG</td>
</tr>
<tr>
<td>Fasn</td>
<td>AATCCGCACCGGCTAC</td>
<td>ATTACACGCCCCCGAG</td>
</tr>
<tr>
<td>Leptin</td>
<td>AGACCCGGGCAAGAGTG</td>
<td>GCCATAGTGCAAGGTT</td>
</tr>
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<td>MCP-1</td>
<td>CCGAACCATAATGAGATCAG</td>
<td>TCACAGTCGAGTCCAC</td>
</tr>
<tr>
<td>Nrf1</td>
<td>GCACCTGTCGCTCAT</td>
<td>GTTGCACGTGCTGGAT</td>
</tr>
<tr>
<td>Nr4a3</td>
<td>CGCCACCAAATAGGACG</td>
<td>ATCGGTTTCCGGCTGCT</td>
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<tr>
<td>PAI-1</td>
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<td>CCACAACAAAGAGAAAGGA</td>
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<tr>
<td>PGC-1α</td>
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</tr>
<tr>
<td>Scd1</td>
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<td>CTCCGACACACCAGGAG</td>
</tr>
<tr>
<td>Tfam</td>
<td>GGATGCCCAGGCCTCT</td>
<td>CCAACACCTCAGCCAGAT</td>
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<tr>
<td>Visfatin</td>
<td>AGAGTGGCTACCTGGCTACC</td>
<td>CTTTCGCCACGCCGTG</td>
</tr>
<tr>
<td>18S</td>
<td>ACATCCAAGGAGGCAGCAG</td>
<td>TTTTCGCTACTACCTCCCCG</td>
</tr>
</tbody>
</table>
4.2.12 Western blotting (studies I, III and IV)

Proteins from perirenal fat samples were isolated with a cell extraction buffer (Invitrogen, Carlsbad, CA, USA) and complete protease inhibitors (Roche Diagnostics, Neuilly-Sur-Seine, France) (study I). Proteins from the liver (studies I and IV) and muscle samples (studies I and II) were isolated with a lysis buffer (NaCl 136 mM, Na₂HPO₄ 8 mM, KCl 2.7 mM, KH₂PO₄ 1.46 mM, Tween 20 0.001% and complete protease inhibitors (Roche Diagnostics)). In immunoblotting 20 µg of the total proteins were used and protein were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidifluoride membrane (Immobilon-FL, Millipore, Bedford, Massachusetts, USA) and blocked for 2 h with 5% non-fat milk-Tris-buffered saline-0.05% Tween-20 buffer. The membranes were probed with anti-SIR2α (Upstate, Lake Placid, N.Y., USA) at dilution 1:1500, anti-SIRT3 (Abcam, Cambridge, UK) at dilution 1:500, anti-SIRT4 (Abcam) at dilution 1:1000, AMP-activated protein kinase α (AMPKα) (Cell Signaling, Beverly, MA, USA) at dilution 1:1000, phospho-AMPKα (Thr172) (Cell Signaling) at dilution 1:1000, S6 (Cell Signaling) at dilution 1:2000, phospho-S6 (Ser240/244) (Cell Signaling) at dilution 1:2000 and LC3B (Cell Signaling) at dilution 1:500 in a blocking buffer. Alpha-tubulin (Abcam) at dilution 1:2000 was used as a loading control. Horse-radish peroxidise-conjugated antirabbit secondary antibody (Chemicon, Temecula, CA, USA) was used at dilution 1:5000. The localization of horse-radish peroxidise was detected with Amersham ECL Plus (GE Healthcare, Little Chalfont, UK) according to the instructions of manufacturer and visualized by a FLA-9000 fluorescent image analyzer (FUJIFILM Corp, Tokyo, Japan).

4.2.13 Statistical analysis

Data are presented as mean ± SEM. GraphPad Prism, version 4.02 (GraphPad Software, Inc., San Diego, CA, USA) was used for the statistical analyses. Statistically significant differences in mean values were tested by ANOVA followed by the Newman-Keuls multiple comparison test (studies I-III) or the Dunnett’s test (study IV). Analyses in a function of time were done by two-way ANOVA followed by the Bonferroni test (study I). P-values below 0.05 were considered statistically significant.
5. **Results**

5.1 **Body composition and energy intake**

5.1.1 **Body weight**

CR effectively prevented body weight gain (**study I**, p<0.05 CR vs. HFD) and induced weight loss both in obese (**studies II, III**, p<0.05 Obese CR/CR casein vs. Obese) and lean mice (**study II**, p<0.05 Lean CR vs. Lean) compared to the *ad libitum* fed counterparts (Figure 10). Resveratrol both with low- and high-doses was unable to prevent body weight gain compared to HFD group (**study I**) (Figure 10).

High-calcium whey protein diets induced weight loss more than the casein-based diet (**study III**, p<0.05 WPI + Ca and α-lactalbumin + Ca vs. CR casein) (Figure 10). A novel whey protein isolate (WPI) induced weight loss (**study IV**, p<0.05 WPI 5%, 50% and 100% vs. Casein Control) and prevented body weight gain compared to the casein-based diet (**study IV**, p<0.05 WPI 100% vs. Casein control) (Figure 10).

![Figure 10](image-url)

**Figure 10.** Difference in mean body weight after weight gain (**study I**), calorie restriction (CR) (**studies II-IV**) and weight re-gain phases (**study IV**). Differences and comparison between study groups in body weight are calculated using endpoint body weight values from original publications. * p<0.05 vs. HFD/Obese, # p<0.05 vs. CR casein, † p<0.05 vs. Lean (statistical significant differences based on ANOVA Newman-Keuls multiple comparison test (**studies I-III**)), § p<0.05 vs. Casein Control (statistical significant differences based on ANOVA Dunnett’s test (**study IV**)).
5.1.2 Body fat percentage (all studies)

CR prevented fat accumulation (study I, p<0.05 CR vs. HFD) and decreased body fat content both in obese (study II, Obese CR vs. Obese) and lean mice (study II, Lean CR vs. Lean) compared to the ad libitum fed counterparts (Figure 11). Resveratrol treatment with low- and high-doses did not prevent body fat accumulation compared to HFD group (study I) (Figure 11).

The high-calcium whey and α-lactalbumin diets decreased the body fat content more than the casein-based diet during weight loss (study III, p<0.05 WPI + Ca and α-lactalbumin + Ca vs. CR casein) (Figure 11). Compared to the casein-based diet, a novel WPI dose-dependently reduced body fat content during weight loss (study IV, p<0.05 WPI 5%, 50% and 100% vs. Casein Control) and prevented body fat accumulation during the weight re-gain period (study IV, p<0.05 WPI50% and WPI100% vs. Casein Control) (Figure 11).

Figure 11. Difference in mean body fat loss (%) after weight gain (study I), calorie restriction (CR) (studies II-IV) and after weight re-gain phases (study IV). Differences and comparison between study groups in body fat are calculated using endpoint body fat values from original publications. * p<0.05 vs. HFD/Obese, # p<0.05 vs. CR casein, π p<0.05 vs. Lean (statistical significant differences based on ANOVA Newman-Keuls multiple comparison test (studies I, III)), § p<0.05 vs. Casein Control (statistical significant differences based on ANOVA Dunnett’s test (study IV)).
5.1.3  LBM maintenance (studies II-IV)

The impact of CR, whey proteins and calcium on LBM maintenance was studied by comparing the amount of LBM before and after the CR phase. CR in obese (study II, p<0.05 Obese CR vs. Obese) induced an approximately 6%, and in lean mice (study II, p<0.05 Lean CR vs. Lean) an approximately 9%, reduction in LBM maintenance compared to the ad libitum fed obese and lean counterparts (Figure 12). High-calcium whey and α-lactalbumin diets (study III) as well as a novel WPI with different doses (study IV) did not affect LBM maintenance more than casein-based diet (Figure 12).

![Figure 12. Lean body mass maintenance (LBM) (%) after CR phase. Values are calculated by comparing the amount of LBM before and after the CR phase. * p<0.05 vs. Obese, ‡ p<0.05 vs. Lean (statistical significant differences based on ANOVA Newman-Keuls multiple comparison test).](image)

5.1.4  Energy intake (all studies)

In all studies the energy intake of calorie restricted mice was approximately 70% of the ad libitum intake. As compared to HFD group, resveratrol had no significant effect on mice energy intake (59.3 ± 4.0 kcal/mouse/day in the HFD, 57.7 ± 2.6 kJ/mouse/day in the HFD+R2, 56.7 ± 2.0 kJ/mouse/day in the HFD+R4, ANOVA p=0.827) (study I).

In study III, the energy intake during the last two study weeks of the weight gain phase was 55.3 ± 1.3 kJ/mouse/day. This value was used for the calculation of 30% CR, and the energy intake of CR groups on casein, WPI + Ca and α-lactalbumin + Ca diets was approximately 38.7 kJ/mouse/day and no differences were seen between treatment groups.

As compared to the casein-based diet, a novel WPI did not affect energy intake when measured during CR (42.6 ± 0.4 kJ/mouse/day in the casein control, 42.1 ± 0.4 kJ/mouse/day in WPI 5%, 41.8 ± 0.1 kJ/mouse/day in WPI 50%, 43.2 ± 0.5 kJ/mouse/day in WPI 100%, ANOVA p=0.10) or the weight re-gain phase (62.3 ± 3.5 kJ/mouse/day in casein control, 77.7 ± 17.3 kJ/mouse/day in WPI
5%, 65.8 ± 3.6 kJ/mouse/day in WPI 50%, 71.6 ± 5.7 kJ/mouse/day in WPI 100%, ANOVA p=0.69) (study IV).

5.2 Apparent fat digestibility (studies II and IV)

The effect of CR (study II) and a novel WPI (study IV) on apparent fat digestibility was determined from fat intake and faecal fat content.

The apparent fat digestibility of obese mice under CR did not differ from the obese ad libitum fed mice (p>0.05 93.8 ± 2.1% in obese CR vs. 90.7 ± 5.5% in the obese). In lean mice CR significantly increased the apparent fat digestibility compared to the ad libitum fed lean mice (p<0.05 76.5 ± 3.2% in lean CR vs. 65.1 ± 2.5% in lean).

A novel WPI did not influence apparent fat digestibility both in the CR phase (93.8 ± 2.1% in casein, 90.9 ± 1.9% in WPI 5%, 89.7 ± 1.7% in WPI 50% and 90.3 ± 3.4% in WPI 100%, ANOVA p-value 0.61) and in the weight re-gain phase (92.5 ± 1.5% in casein, 93.4 ± 2.3% in WPI 5%, 95.0 ± 1.7% in WPI 50% and 94.6 ± 0.4% in WPI 100%, ANOVA p-value 0.71).

5.3 Oral glucose tolerance test (studies II and IV)

The effects of CR on blood glucose tolerance were tested (study II). The oral glucose tolerance test (OGTT) showed that CR had no effect on blood glucose AUC (blood glucose level x time) in obese (p>0.05 1074.7 ± 22.9 mmol/l x min in obese CR vs. 1259.2 ± 147.9 mmol/l x min in obese) or lean mice (p>0.05 608.8 ± 28.0 mmol/l x min in lean CR vs. 722.7 ± 29.3 mmol/l x min in lean).

The effects of novel WPI on glucose tolerance were also studied (study IV). There was no statistical significant difference in blood glucose among the treatment groups after the CR phase (7.7 ± 0.5 mmol/l in casein, 9.2 ± 1.0 mmol/l in WPI 5%, 8.4 ± 0.6 mmol/l in WPI 50%, 6.6 ± 0.5 mmol/l in WPI 100%, ANOVA p-value 0.115). After the re-gain phase, blood glucose was lower in the WPI 5% and WPI 100% groups compared to the casein control (p<0.05 7.5 ± 0.6 mmol/l in WPI 5%, 6.6 ± 0.2 mmol/l in WPI 100% vs. 10.2 ± 1.1 mmol/l in casein). OGTT showed that blood glucose AUC did not show any significant differences among treatment groups after the CR phase (1074.7 ± 22.9 mmol/l x min in casein, 1094.0 ± 132.5 mmol/l x min in WPI 5%, 922.6 ± 68.3 mmol/l x min in WPI 50%, 1032.6 ± 91.1 mmol/l x min in WPI 100%, ANOVA p-value 0.075) or after the weight re-gain phase (1551.3 ± 114.3 mmol/l x min in casein, 1273.3 ± 129.8 mmol/l x min in WPI 5%, 1067.5 ± 134.8 mmol/l x min in WPI 50%, 1139.5 ± 141.1 mmol/l x min in WPI 100%, ANOVA p-value 0.0913). However, when the AUCs of the same treatment group measured after the CR phase and the re-gain phase were compared, blood glucose AUC in the casein group was increased (p<0.05 casein CR phase vs. casein re-gain phase), whereas in the WPI 100% group the blood glucose AUC were unaltered.
5.4 **Metabolic performance and physical activity (study I)**

The effects of CR and resveratrol on drinking and feeding behavior, metabolic performance, and physical activity were studied by housing a group of mice in a home cage-based monitoring system for laboratory animals *(study I)*.

The food intake in the CR group was approximately 70% of HFD group (p<0.05 2.2 ± 0.1 g/day in CR vs. 3.0 ± 0.2 g/day in HFD) as stated in the study plan. Resveratrol had no effect on food intake as compared to the HFD group (3.0 ± 0.2 g/day in HFD, 2.9 ± 0.1 g/day in HFD+R2 and 2.9 ± 0.1 g/day in HFD+R4, p>0.05). CR had no effect on water intake, but both low- and high-dose resveratrol treatment decreased water intake (2.2 ± 0.1 ml/day in HFD, 2.3 ± 0.1 ml/day in CR, 1.9 ± 0.1 ml/day in HFD+R2, 1.8 ± 0.1 ml/day in HFD+R4, p<0.05 HFD+R2/HFD+R4 vs. CR).

Metabolic performance measured as respiratory exchange ratio (RER), CO₂, O₂ or heat productions were unchanged by CR and resveratrol as compared to the HFD group. CR increased ambulatory movement, total (p<0.05 CR vs. other study groups) and rearing activity (p<0.05 CR vs. HFD+R2/HFD+R4), whereas cumulative fine movement was unchanged by CR. Resveratrol had no significant effect on physical activity.

5.5 **Liver histology (studies I and IV)**

In *(study I)*, mice fed with HFD for 15 weeks showed prominent steatosis. In addition, hepatocyte ballooning indicating the activity of hepatocyte degeneration was also found in mice fed with HFD. Complete absence of steatosis and hepatocyte ballooning was seen in mice under CR. A higher dose of resveratrol tended to ameliorate steatosis and hepatocyte ballooning.

In *(study IV)*, obese mice fed with HFD for 150 days showed prominent steatosis, but merely minimal foci of inflammatory cells and a complete absence of fibrosis was seen. 50 days CR on a novel WPI with different doses (WPI 5%, WPI 50% and WPI 100%) did not show clear improvement in liver morphology. However, after weight re-gain, whey protein fraction with 50% and 100% doses improved liver morphology, and a complete absence of steatosis was seen in the WPI 100% group.

5.6 **Adipocyte cross sectional area (studies II and IV)**

The effects of CR and a novel WPI on adipocyte size (arbitrary units) were measured as a cross-sectional area. The adipocyte size of obese mice under CR was significantly decreased compared to the *ad libitum* fed mice (p<0.05 4303.3 ± 138.1 in obese CR vs. 5207.6 ± 403.0 in obese). The adipocyte size of lean mice under CR was moderately, but not significantly, decreased compared
to the *ad libitum* fed lean mice (p>0.05 922.3 ± 91.3 in lean CR vs. 1567.3 ± 184.4 in lean) (*study II*).

A novel WPI decreased adipocyte size more than the casein-based diet both during the CR phase (p<0.05 3029.3 ± 476.8 in WPI 5%, 2683.2 ± 289.4 in WPI 50% and 2605.1 ± 175.1 in WPI 100% vs. 4947.6 ± 337.5 in casein) and during the weight re-gain phase (p<0.05 3502.9 ± 473.8 in WPI 5%, 4013.4 ± 503.5 in WPI 50% and 2619.8 ± 674.3 in WPI 100% vs. 6616.3 ± 151.4 in casein) (*study IV*).

5.7 **mRNA expression of adipose tissue inflammatory markers and hepatic visfatin (studies I and IV)**

The effects of CR and resveratrol on adipose tissue inflammation were determined by measuring mRNA expression of adiponectin, Cd68, leptin, MCP-1 and PAI-1 (*study I*). CR increased the mRNA expression of anti-inflammatory gene adiponectin (2.8 ± 0.5 in CR vs. 1.0 ± 0.2 in HFD), and decreased mRNA expression of pro-inflammatory genes Cd68 (0.2 ± 0.1 in CR vs. 1.0 ± 0.2 in HFD), leptin (0.4 ± 0.1 in CR vs. 1.0 ± 0.2 in HFD), MCP-1 (0.2 ± 0.1 in CR vs. 1.0 ± 0.2 in HFD) and PAI-1 (0.5 ± 0.1 in CR vs. 1.0 ± 0.2 in HFD) compared to the HFD group (in all p<0.05). Resveratrol with high- and low-doses did not influence the mRNA expression of adiponectin, Cd68, leptin, MCP-1 and PAI-1 in adipose tissue compared to the HFD fed mice. The effects of CR and resveratrol on hepatic visfatin mRNA expression were also studied (*study I*). CR markedly increased hepatic visfatin mRNA expression (p<0.05 1.8 ± 0.1 in CR vs. 1.0 ± 0.1 in HFD), whereas resveratrol had no effect on visfatin mRNA expression.

In *study IV*, the effects of novel WPI on adipose tissue leptin, MCP-1 and PAI-1 mRNA expression were determined. CR per se decreased the mRNA expression of leptin (1.0 ± 0.2 in obese, 0.6 ± 0.3 in casein, 0.4 ± 0.1 in WPI 5%, 0.5 ± 0.2 in WPI 50%, 0.4 ± 0.1 in WPI 100%, p<0.05 WPI 100% vs. Obese) and MCP-1 (p<0.05 0.4 ± 0.2 in casein, 0.4 ± 0.1 in WPI 5%, 0.4 ± 0.2 in WPI 50% and 0.4 ± 0.1 WPI 100% vs. 1.0 ± 0.2 in obese) as compared to obese mice fed *ad libitum*. However, comparison between casein and a novel WPI diet showed no differences between treatment groups. After the weight re-gain phase, there was not any significant difference between study groups.

5.8 **Adipose tissue cytokine and angiogenesis protein profiles (study II)**

The effects of obesity and CR on adipose tissue cytokine and angiogenesis-related protein profiles were analyzed using sensitive high-throughput antibody arrays (*study II*).
**Cytokine protein expression**

Of the 40 analyzed cytokines, the expression of 27 cytokines were more highly expressed in obese than in lean mice. The highly expressed proteins included interleukins IL-1ra, IL-2 and IL-16, chemokines MCP-1, MIG and RANTES, complement component C5a, adhesion molecule sICAM-1 and matrix metalloproteinase inhibitor TIMP-1 (Table 5).

The comparison between calorie restricted and ad libitum fed counterparts revealed that CR in the obese decreased 22 protein and increased 5 protein expressions. In contrast, in lean mice CR increased the expression of 26 proteins. CR both in obese and lean mice increased sICAM-1 and TIMP-1 protein expression (Table 5). Overall, CR showed opposite effect on cytokine profile between obese and lean mice, and several protein expressions including IL-2, MCP-1 and C5a were decreased by CR in obese and increased in lean mice (Table 5).

### Table 5. Adipose tissue cytokine protein expressions in ad libitum and calorie restricted (CR) obese and lean mice.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Obese vs. Lean</th>
<th>Obese CR vs. Obese</th>
<th>Lean CR vs. Lean</th>
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<tbody>
<tr>
<td>IL-1ra</td>
<td>Interleukin</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>IL-16</td>
<td>Interleukin</td>
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<td>↑</td>
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<tr>
<td>MCP-1</td>
<td>Chemokine</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>MIG</td>
<td>Chemokine</td>
<td>↑</td>
<td>↔</td>
</tr>
<tr>
<td>RANTES</td>
<td>Chemokine</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>C5a</td>
<td>Complement component</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>Adhesion molecule</td>
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<td>↑</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>MMP inhibitor</td>
<td>↑</td>
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</tr>
</tbody>
</table>

↓ indicates that protein expression is significantly (p˂0.05) decreased, ↑ indicates that protein expression is significantly (p˂0.05) increased, ↔ indicates that no significant (p˃0.05) difference is seen. Comparisons between study groups have been done with ANOVA followed by Newman-Keuls comparison test using the data from original publication. C5a; complement component 5a, IL-1ra; interleukin-1 receptor antagonist protein, IL-2; interleukin 2, IL-16; interleukin 16, MCP-1; monocyte chemotactic protein-1, MIG; chemokine (C-X-C motif) ligand 9, RANTES; chemokine (C-C motif) ligand 5, sICAM; soluble intercellular adhesion molecule 1, TIMP-1; tissue inhibitor of metalloproteinase 1.

**Angiogenesis-related protein expression**

53 pro- and anti-angiogenesis proteins were analyzed, and of them 17 proteins were expressed at higher levels and 6 proteins at lower levels in obese mice than in lean mice. The protein expressions of cell growth regulators angiogenin, endoglin, endostatin and endothelin-1 as well as angiogenic growth factors IGFBP-3 and leptin were increased in obese mice compared to lean mice (Table 6). In addition, obesity increased the protease MMP-3 and the protease inhibitors PAI-1 and TIMP4 protein expressions in adipose tissue (Table 6). Furthermore, chemokines CXCL16 and platelet factor 4, adhesion molecule DPPIV and coagulation factor III were more highly expressed in obese mice than in lean mice (Table 6).

CR in obese mice decreased 14 protein expressions and increased 6 protein expressions. In lean mice, CR induced major changes in angiogenesis protein profile; 32 protein expressions were increased and 9 protein expressions were decreased. CR increased endoglin, endostatin and
platelet factor 4 expressions, and decreased IGFBP-3, NOV, MMP-9, CXCL16 and osteopontin expressions both in obese and lean mice (Table 6). However, several changes were distinct between obese and lean mice; leptin and TIMP4 expressions were decreased in obese mice and increased in lean mice (Table 6). In addition, CR uniquely in obese mice decreased MMP-3 and PAI-1 expressions, whereas uniquely in lean mice decreased FGF acidic, FGF basic and coagulation factor III and increased angiogenin and DPPIV expressions (Table 6).

Table 6. Adipose tissue cytokine protein expressions in ad libitum and calorie restricted (CR) obese and lean mice.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Obese vs. Lean</th>
<th>Obese CR vs. Obese</th>
<th>Lean CR vs. Lean</th>
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<td>Angiogenin</td>
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<tr>
<td>Endoglin</td>
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<td>Endostatin/Collagen XVII</td>
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<td>Endothelin-1</td>
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<td>FGF acidic</td>
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<td>Osteopontin</td>
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<tr>
<td>Coagulation factor III</td>
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↓ indicates that protein expression is significantly (p<0.05) decreased, ↑ indicates that protein expression is significantly (p<0.05) increased, ↔ indicates that no significant (p>0.05) difference is seen. Comparisons between study groups have been done with ANOVA followed by Newman-Keuls comparison test using the data in original publication. CXCL16; chemokine (C-X-C motif) ligand 16, DPPIV; dipeptidyl peptidase-IV, FGF; fibroblast growth factor, IGFBP-3; insulin-like growth factor-binding protein 3, NOV; nephroblastoma overexpressed, MMP-3/9; matrix metalloproteinase 3/9, PAI-1; plasminogen activator inhibitor-1, TIMP-4; metalloproteinase inhibitor 4.

5.9 Skeletal muscle gene expression (study III)

The potential of whey proteins and calcium to modify skeletal muscle gene expression during CR was investigated using Affymetrix Mouse genome 430 2.0 array representing over 30,000 mouse transcripts (study III). Hierarchical clustering containing over 3000 genes with differences between the CR groups vs. obese groups revealed that 208 and 287 genes were altered by WPI + Ca and α-lactalbumin + Ca diets compared to the casein diet, respectively. 186 genes were common to both the WPI and α-lactalbumin diets, whereas 22 genes were regulated only by the WPI + Ca diet and 101 genes were regulated only by the α-lactalbumin + Ca diet. Several genes from Wnt signaling
were regulated by high-calcium whey protein diets, in particularly by the α-lactalbumin + Ca diet. Wnt signaling is associated with muscle cell differentiation, and modest changes in muscle hypertrophy/atrophy were seen. The expression of atrophy signaling genes Acrv2b and Fbxo32 (also called atrogin-1) were slightly, but >1.2-fold up-regulated by high-calcium whey proteins diets. Correspondingly, the expression hypertrophy signaling genes Eif4ebp1 and Rps6 were >1.2-fold up-regulated during CR irrespective of dietary protein source.

The potential of high-calcium and whey protein to reverse the obesity induced changes were also examined. Of the 2693 genes expressed differently between obese and lean, CR on casein diet reversed partially or completely the expression of 46% (n= 1191), CR on WPI + Ca of 47% (n=1229) and CR on α-lactalbumin + Ca of 51% (n=1322) of the genes. Fatty acid and lipid metabolism was the most enriched gene ontology (GO) term among genes >2-fold up-regulated between obese and lean mice. Quantitative RT-PCR analysis revealed that the mRNA expression of Aldh1a7, Fasn, leptin, Nr4a3 and Scd1 were expressed lower in the WPI + Ca and α-lactalbumin + Ca diet groups than in the casein diet group (in all p<0.05 WPI + Ca and α-lactalbumin + Ca vs. casein), indicating dietary protein source-dependent alterations in muscle lipid and fatty acid metabolism.

5.10 Nutrient sensing pathways (studies I, III, IV and some unpublished data)

5.10.1 Sirtuin pathway

The effects of CR and resveratrol (study I) as well as whey proteins and calcium (studies III-IV) on the sirtuin pathway were studied by analyzing the SIRT1, SIRT3 and SIRT4 in the liver, skeletal muscle and adipose tissue.

Figure 13. Effects of CR and resveratrol on SIRT1 protein level in the liver and skeletal muscle (a), and SIRT4 protein level in the skeletal muscle (b). * p<0.05 vs. HFD groups (statistical significant differences based on ANOVA Newman-Keuls multiple comparison test).
Compared to HFD group, CR increased the SIRT1 protein expression in the liver and skeletal muscle (Figure 13a) and the SIRT4 expression in the skeletal muscle (Figure 13b). CR also increased the SIRT3 protein expression in the liver, skeletal muscle and adipose tissue (Figure 14a).

Resveratrol in a high-dose increased the SIRT1 and SIRT4 protein expressions in skeletal muscle as compared to the HFD group (Figure 13a, b). Resveratrol did not influence the SIRT3 protein expression in any studied tissues (Figure 14a).

High-calcium whey protein diets and (WPI + Ca and α-lactalbumin + Ca) and a novel WPI (WPI5%, WPI50% and WPI100%) did not affect the SIRT1 protein expression in skeletal muscle and the liver, respectively. The SIRT3 protein expression in skeletal muscle was significantly increased by the α-lactalbumin + Ca diet compared to the obese group (Figure 14b). A novel WPI with 50% and 100% dosages increased the SIRT3 protein expression in the liver compared to the casein diet (Figure 14c).

Figure 14. Effects of CR and resveratrol on SIRT3 in the liver, skeletal muscle and adipose tissue (a) as well as effects of whey proteins and calcium on SIRT3 in the liver (b) and skeletal muscle (c). * p<0.05 vs. HFD/Obese (statistical significant differences based on ANOVA Newman-Keuls comparison test), § p<0.05 vs. casein control (statistical significant differences based on ANOVA Dunnett’s test).
5.10.2 Autophagy, AMPK and mTOR pathways

The effect of CR on the hepatic LC3B protein expression, an indicator of autophagy, was analyzed in obese and lean mice (study II, unpublished data). The LC3B ratio (14kDa/16 kDa) was higher in obese mice than in lean mice (Figure 15a). CR in obese decreased the LC3B ratio, but in lean mice the ratio was unchanged as compared to the ad libitum fed counterparts (Figure 15a).

The effects of resveratrol (study I, unpublished data), CR (study II, unpublished data) and novel WPI (study IV) on phosphorylation level of AMPK were studied. There were no significant differences between study groups in the phosphorylation level of AMPK.

CR per se did not affect the hepatic phosphorylation level of S6 (study II, unpublished data). However, novel WPI with all concentrations (5%, 50% and 100%) decreased the phosphorylation of the S6 ribosomal protein in the liver compared to the casein diet, suggesting inhibition of the mTOR pathway (study IV) (Figure 15b).

Figure 15. Effects of CR on LC3B protein (14kDa/16kDa) expression in the liver (a) and a novel whey protein on phosphorylation level of S6 in the liver (b). * p<0.05 vs. Obese (statistical significant differences based on ANOVA Newman-Keuls comparison test), § p<0.05 vs. casein control (statistical significant differences based on ANOVA Dunnett’s test).
6. Discussion

Obesity and obesity-associated health problems are ameliorated by weight loss and the most effective method to induce weight loss is through CR. However, the precise molecular mechanisms by which CR induces health benefits is not yet clear, even though accumulating evidence suggests an important role for nutrient sensing pathways; sirtuins, AMPK and mTOR, as cellular mediators of CR. Epidemiological studies have shown that a diet high in dairy products is inversely associated with BMI, risk of metabolic syndrome and type 2 diabetes. Dairy calcium and proteins, in particular whey proteins, have been suggested to account for the anti-obesity effects of dairy products by promoting weight loss during CR via an unknown mechanism. The study investigated the potential molecular mechanism explaining the effects of CR with special emphasis in the above mentioned nutrient sensing pathways. The study also clarified the anti-obesity effects of whey proteins and their mechanism of action.

6.1 Methodology aspects

Animal model

All studies were conducted with C57BL/6J mice, which is the most widely used diet-induced obesity rodent model (Kanasaki and Koya 2011). These mice are lean and physiologically normal when fed a normal rodent diet, but they develop obesity and hyperinsulinemia, insulin resistance, hypertension and fatty liver when allowed ad libitum access to HFD (Collins et al. 2004). Therefore, HFD fed C57Bl/6J obese mice serve as a very human-like model of obesity and metabolic syndrome as compared to other widely used obese mouse models, such as leptin-deficient ob/ob and leptin-resistant db/db mice.

Several problems linked to obesity research in clinical trials can be avoided by using the HFD fed C57BL/6J mice as an experimental model of obesity. In humans, obesity and obesity-related metabolic disorders develop in the long term. In contrast, C57BL/6J mice develop obesity relatively fast, the increase in body weight can already be noticed after only 2 weeks of HFD feeding, and it becomes apparent after 4 weeks (Wang and Liao 2012). After 16-20 weeks of HFD feeding, mice have usually exhibit a 20-30% increase in body weight when compared to normal diet fed mice. Hyperglycemia develops after 4 weeks of HFD feeding (Collins et al. 2004, Wang and Liao 2012). In addition, the non-compliance to long-term low-calorie diets in clinical trials can be avoided by using the HFD fed C57BL/6J mice as an experimental model for obesity. However, obesity is a multifactorial disease, and the results obtained from animal experiment are only directional and cannot be directly extrapolated to humans.
**Dietary modifications**

CR is a dietary intervention where calorie intake is reduced below usual *ad libitum* intake while adequate intakes of proteins and micronutrients are maintained at sufficient levels to avoid malnutrition. Restricting calories 20-60% of *ad libitum* intake has several beneficial effects on the health of animals (for review see Speakman and Mitchell 2011). In the present study, 30% calorie restriction (CR) was used, which was widely used in previous animal studies and has been shown to be safe and relevant way to induce weight loss.

The anti-obesity effects of different dosages of a novel WPI were investigated. The novel WPI is manufactured from pasteurized whey proteins using ion exchange technology. Its composition differs from normal WPI that has been used in previous studies. The novel WPI is comprised of mainly lactoperoxidase (~50% vs. 0.5% in normal WPI) and lactoferrin (15% vs. ~1% in normal WPI) (Krissansen 2007), and it is rich in immunoglobulins and growth factors. A comparison between casein and normal WPI reveals its high branched chain amino acids (BCAAs) content, whereas the major difference between the novel WPI and casein is related to the levels of cysteine, glutamic acids, methionine and proline.

**Protein and gene expression analysis**

Mouse cytokine and angiogenesis antibody array kits (Proteome Profiler™ antibody arrays) were used to analyze the adipose tissue cytokine and angiogenesis protein profiles of visceral fat. These arrays are sensitive high-throughput tools for simultaneously profiling the relative levels of multiple proteins between samples. Mouse cytokine and angiogenesis array kits allowed for the analysis of the protein expressions of 40 cytokines and chemokines, and 52 angiogenesis-related proteins, respectively. We pooled proteins from three samples together, and used this protein mixture for membranes where each antibody was spotted in duplicates. In the present study, we did not confirm the results in protein or RNA levels by other methods, even though that would be advisable to avoid wrong interpretations.

Microarray genechips were used to analyze skeletal muscle gene expression profiles. Microarray is a fast and quite simple method to generate large amounts of data. The gene expression profiles were analyzed by Mouse Genome 430 2.0 Arrays, which allowed the analysis of 30,000 transcripts simultaneously. The biggest challenge with the microarray, however, is the data analysis. There is no single right method to analyze the data, which exacerbates the comparison of data between different experiments (Cordero et al. 2007). Furthermore, the huge amounts of data complicate the findings of the significant and biologically relevant results. Therefore, important findings must always be confirmed by other methods, and in the present study the significant results were verified with qRT-PCR.
6.2 Calorie restriction in experimental obesity

The present study showed that CR protected against obesity and induced body weight and fat loss. CR also protected against fatty liver formation and ameliorated adipose tissue inflammation. The study also demonstrated that CR was superior in protection against diet-induced obesity and fatty liver formation as compared to oral resveratrol supplementation. The polyphenolic compound resveratrol is a widely used CR-mimetic compound, which effects are believed to be mediated in a SIRT1-dependent manner. In some previous studies, resveratrol has been shown to reduce body weight and adiposity in HFD fed mice (Lagouge et al. 2006) and rats (Aubin et al. 2008, Rivera et al. 2009). In the present study, resveratrol dosages, route of administration, and the treatment period were based on the study by Lagouge and co-workers (2006), but any statistically significant differences in body weight or adiposity were not seen. Only a modest trend toward reduced body weight was seen both with low- and high-doses of resveratrol, suggesting that in this case higher dosages and longer treatment period might be needed for prevention of body weight gain. However, resveratrol was not completely ineffective because with a higher-dose it partially attenuated fatty liver by reducing hepatic steatosis and hepatocyte ballooning. Similar beneficial effects of resveratrol on NAFLD have been seen in previous studies as well, and it has been suggested that it is due to reduced hepatic fatty acid availability and oxidative stress in resveratrol treated animals (Shang et al. 2008, Gómez-Zorita et al. 2012). However, several other studies have also been unable to confirm the beneficial effects of resveratrol on body weight and adiposity (Baur et al. 2006, Rivera et al. 2009, Rocha et al. 2009). These findings and the statement that resveratrol is not a direct activator of SIRT1 (Kaeberlein et al. 2005a, Pacholec et al. 2010) suggest that more specific sirtuin activating compounds are needed to mimic the effects of CR on body composition.

6.3 Molecular and signaling pathways mediating the effects of CR

Effects of CR on adipose tissue cytokine and angiogenesis profiles

Adipose tissue expansion during obesity is characterized by adipocyte hypertrophy (an increase in adipocyte volume), which causes adipocyte dysfunction and leads to changes in adipocyte metabolic and secretion activity (Guilherme et al. 2008, Hajer et al. 2008, Spalding et al. 2008). The present study supported the previous findings, and larger adipocyte size in obese groups associated with higher cytokine protein expression. The most highly up-regulated cytokines were IL-1ra, IL-2, IL-16, MCP-1, MIG, RANTES, C5a and sICAM-1. CR, when performed for obese mice, reduced obesity-induced adipocyte hypertrophy and attenuated adipose tissue cytokine protein expression. Interestingly, in lean mice CR had no effect on adipocyte size, but a much higher cytokine protein expression was seen, indicating that CR might have detrimental effect on adipose
tissue remodeling in lean mice. The distinct effect of CR on cytokine expression between obese and lean mice cannot be explained by different response of obese and lean mice to CR as both the body weight and body fat percentage were decreased by CR to similar extent in obese and lean mice. However, these findings are in line with previous studies showing that CR in lean mice increases serum cytokine levels (Fenton et al. 2009), and ameliorates adipose tissue inflammation in obese mice, in particularly when CR is carried out by restricting the intake of HFD (Wang et al. 2011b).

Adipose tissue is a highly vascularized organ, and therefore expansion of adipose tissue during development of obesity is dependent on the angiogenesis. Some of the anti-angiogenic agents, including TNP-470 and a broad-spectrum of endogenous protein inhibitors, angiostatin and endostatin have been shown to prevent genetically and diet-induced obesity in animals (Rupnick et al. 2002, Bråkenhielm et al. 2004). Therefore, a better understanding of the regulation of angiogenesis process may provide new drug targets for obesity and obesity-related disorders. The present study showed that obesity is associated with marked alteration in the protein expression of cell growth regulators, angiogenic growth factors and proteases, as well as their inhibitors. CR markedly modulated adipose tissue angiogenesis-related protein profiles and the effects of CR were largely distinct between obese and lean mice.

Leptin, a food intake regulator and a potent angiogenic growth factor, was decreased by CR in obese and increased in lean mice. In addition, angiogenic growth factor endoglin and endogenous angiogenesis inhibitor endostatin were up-regulated by CR both in obese and lean mice. In addition to angiogenic growth factors, pericellular proteases have been shown to be important in regulating angiogenesis by participating in extracellular matrix (ECM) remodeling, generating pro- and anti-angiogenic factors from ECM proteins and processing growth factors and receptors (Van Hinsbergh et al. 2006). The proteolytic system consisting of two major component plasminogen activator-plasmin system (fibrinolytic system) and matrix metalloproteinases (MMPs) showed response to CR. MMP-9 expression was decreased by CR in both obese and lean mice, while MMP-3 and PAI-1, an inhibitor of fibrinolytic system, were decreased by CR only in obese mice. MMPs are inhibited by endogenous tissue inhibitors (TIMPs). CR increased TIMP-1 expression both in obese and lean mice, whereas TIMP-4 expression was down-regulated by CR in obese mice and up-regulated in lean mice.

The findings underscore the importance of angiogenesis-related protein and cytokines in adipose tissue remodeling during development of obesity and CR induced weight loss. The findings also support the notion that by modulation of adipose tissue cytokine and angiogenesis-related proteins, it might be possible to influence the development of obesity.
**Effects of CR on nutrient sensing signaling pathways**

The effects of CR on the sirtuin pathway were analyzed. Recent studies strongly suggest that SIRT1 mediates adaptive responses to CR in mammals (Bordone et al. 2007, Boily et al. 2008, Cohen et al. 2009). SIRT1 is suggested to be required for increased physical activity response of CR in mice, as SIRT1-deficient mice do not respond to CR with increased physical activity (Chen et al. 2005a). In accordance with previous studies, the present study also demonstrated higher physical activity in CR mice, and showed increased SIRT1 expression in skeletal muscle and the liver in mice under CR.

Relatively less is known about the other sirtuins role in CR, and in the present study, the effects of CR on mitochondrial sirtuins SIRT3 and SIRT4 expression were investigated. Interestingly, the SIRT4 expression was increased by CR in skeletal muscle. This is in contrast to other studies which have shown that CR decreases the expression of SIRT4 rather than increases it (Haigis et al. 2006). However, although in muscle cells SIRT4 depletion has been shown to improve mitochondrial function (Nasrin et al. 2010), its role in skeletal muscle metabolism is still quite unknown.

The most important finding of the present study was that CR markedly increased SIRT3 protein expression in the liver, skeletal muscle and adipose tissue. SIRT3 has been shown to be responsible for acetylations of several proteins in mitochondria, as the loss of SIRT3, but not other mitochondrial sirtuins SIRT4 and SIRT5, leads to hyperacetylation of mitochondrial proteins (Lombard et al. 2007). Included in those hyperacetylated proteins are several proteins from energy metabolism as well as oxidative stress reducing enzymes and mitochondrial electron transfer components (for review see Huang et al. 2010, He et al. 2012). SIRT3-deficient mice have several metabolic abnormalities, including reduced ATP production, decreased rate of fatty acid oxidation and ketone body production, and fatty liver (Ahn et al. 2008, Hirschey et al. 2010, Shimazu et al. 2010, Kendrick et al. 2011). These findings strongly suggest that the effects of CR are mediated, at least in part, via a SIRT3-dependent manner.

Interestingly, CR increased the mRNA expression of hepatic visfatin (also called NAMPT). Visfatin is the enzyme that converts the NAM to nicotinamide mononucleotide (NMN), which is the first and rate-limiting step in NAD⁺ biosynthesis. Inhibition or down-regulation of visfatin has been shown to lead to reduced SIRT1 activation (Revollo et al. 2004). Recently, it has been demonstrated that HFD feeding and aging reduce visfatin-mediated enzymatic reactions leading to glucose intolerance, whereas treatment with NMN reverses the changes (Yoshino et al. 2011). Higher expressions of hepatic visfatin in calorie restricted mice can thus explain the higher SIRT1 and SIRT3 protein expressions in the liver. Resveratrol, which failed to prevent obesity, induced SIRT1 and SIRT4 expressions in skeletal muscle. However, resveratrol was unable to induce both visfatin and SIRT3 expressions in metabolically important tissues, which can, at least in part, explain its inability to mimic beneficial health effects of CR.

The effects of CR on the AMPK, mTOR and autophagy signaling pathways were also studied. Both the AMPK and mTOR pathways are sensitive to cellular nutritional status (for review see Hardie et al. 2012a, Laplante and Sabatini 2012). However, our study did not support this as we did not notice any significant changes by CR in phosphorylation of AMPK or in phosphorylation of
S6, a main substrate of the mTOR pathway. Interestingly, we demonstrated increased hepatic expressions of autophagic effector protein LC3B-II (14 kDa) in obese mice and the expressions were decreased by CR. This is in contrast to other studies showing that hepatic autophagy is decreased by genetic and HFD-induced obesity (Liu et al. 2009, Yang et al. 2010) and CR is believed to activate autophagy in vivo (for review see Kroemer et al. 2010). However, autophagy is the complex process and over 30 autophagy-related genes (ATGs) have been found from yeast and many of the genes have orthologues in eukaryotes (Xie and Klionsky 2007). Therefore, it would be advisable to investigate how CR influences those other protein expressions to find out which direction CR induces autophagy.

### 6.4 Whey proteins and calcium in experimental obesity

In previous studies, it has been shown that high-calcium whey protein diets prevent weight gain, induce weight loss during CR and prevent weight re-gain after CR more than a casein-based diet (Pilvi et al. 2007, Pilvi et al. 2009). Of the different whey protein fractions, α-lactalbumin has been shown to have the most effective anti-obesity effects (Pilvi et al. 2009). It has also been shown that whey proteins and calcium enhance the blood glucose and insulin lowering effects of CR (Pilvi et al. 2008a), and they have beneficial effects on the liver metabolomic (Pilvi et al. 2008a) and adipose tissue gene expression profiles (Pilvi et al. 2008b). The present study, the novel WPI had unique protein and amino acid composition compared to previously studied whey proteins. The study further underlined the anti-obesity effects of whey proteins as the replacement for casein in the diet with a novel WPI dose-dependently enhanced weight and fat loss during CR and prevented weight and fat re-gain during ad libitum feeding after weight loss. The calcium content was the same in treatment groups and excluded the effects of a high-calcium diet that were used in previous studies. The study revealed that the novel WPI induced weight loss during CR was primarily due to changes in body fat content as the body fat percentage and adipocyte cross sectional area were decreased by a novel WPI, while LBM maintenance was unchanged by a novel WPI. The study also demonstrated that a novel WPI protected against development of fatty liver formation during ad libitum feeding after weight loss. A novel WPI did not influence energy intake or apparent fat digestibility, indicating that the beneficial effects of CR on body weight were not due to changes in satiety or increased faecal fat excretion. Taken into account the high lactoperoxidase, lactoferrin, immunoglobulins and growth factors content and the special amino acid composition of a novel WPI, it suggests that those components might have anti-obesity effects. This is further supported by the previous study showing that enteric-coated lactoferrin reduces visceral adiposity in obese patients (Ono et al. 2010).

In addition to body composition changes, a novel WPI lowered blood glucose levels after the re-gain phase, and when the AUCs from the oral glucose tolerance test after the CR and re-gain phases were analyzed, they showed a tendency towards improved insulin sensitivity. The
insulinotropic effects of whey proteins are suggested to be due to its high-content of BCAAs (Nilsson et al. 2007) or it is regulated through incretin hormones that potentiate insulin secretion from the islet β-cell in a glucose-dependent manner (Gunnarsson et al. 2006). However, the major difference between casein and novel WPI amino acid compositions are not due to BCAAs, suggesting that insulinotropic effects of a novel WPI are mediated through other components.

6.5 Molecular and signaling pathways mediating the effects of whey proteins and calcium

Effects of whey proteins and calcium on skeletal muscle gene expression profile
The effects of high-calcium whey proteins diets on skeletal muscle gene expression profiles were investigated. Microarray results revealed that CR on different dietary regimens reversed approximately 50% of obesity-induced changes in a dietary protein and calcium dependent manner. The qRT-PCR analysis showed that five genes leptin, Scd1, Fasn, Nr4a3 and Aldh1a7 related to skeletal muscle fatty acid and lipid metabolism were decreased by high-calcium whey protein diets as compared to a casein-based diet. Leptin has an important role in whole body energy homeostasis. Leptin improves insulin sensitivity by stimulating fatty acid oxidation and decreasing muscle lipid content in skeletal muscle (Dyck 2009). Obesity is known to induce leptin resistance, which leads to the inability of leptin to suppress food intake and contributes to the development of obesity (ScarpAce and Zhang 2009). In the present study, obesity-induced leptin expression was normalized by high-calcium whey protein diets, which can enhance its anti-obesity effects. Recent studies have shown that Scd1-deficient mice have several metabolic changes that protect mice from diet-induced obesity, insulin resistance and hepatic steatosis (for review see Flowers and Ntambi 2008). Interestingly, the lowest Scd-1 expression was seen in high-calcium α-lactalbumin + Ca, which have been shown to be the most effective whey protein fraction in enhancing weight loss during CR.

As compared to the casein diet, 208 and 287 genes were altered by high-calcium WPI and α-lactalbumin diets, respectively. We noticed that high-calcium whey protein diets, in particular the α-lactalbumin diet, regulated several genes of the Wnt signaling pathway. In skeletal muscle, Wnt signaling improves insulin sensitivity by decreasing intramyocellular lipid depositions through down-regulation of SREBP-1c (sterol-regulatory element binding protein 1c) (Abiola et al. 2009). Both Scd-1 and Fasn are regulated by SREBP-1c (Eberlè et al. 2004), suggesting that Wnt signaling via regulation of SREBP-1c activity might function as a mechanistic link to how high-calcium whey proteins mediate changes in lipid and fatty acid metabolism.

Intense CR can lead to unwanted depletion of skeletal muscle, which may down-regulate metabolic processes, such as protein turnover and basal metabolic rate, exacerbating the weight management after weight loss (Carbone et al. 2012). It has been proposed that whey protein has a role in preservation of muscle mass since it contains a high-proportion of BCAAs (leucine,
isoleucine and valine) important for muscle metabolism and protein synthesis (Ha and Zemel 2003, Zemel 2009). The Wnt signaling pathway has been shown to interact with MyoD, which is important for muscle cell differentiation (Kim et al. 2008a). Therefore, the effects of high-calcium whey protein diets on hypertrophy/atrophy signaling were investigated. Two atrophy signaling genes Fbox32 (also called atrogin-1) and Acvr2b were slightly up-regulated by high-calcium whey protein diets, and two hypertrophy signaling genes Eif4ebp1 and Rsp6 were up-regulated by CR irrespective of dietary protein source. However, it is plausible that the findings were not clinically relevant as much higher Fbox32 expression has been seen in clinically relevant muscle atrophy (Bodine et al. 2001, Gomes et al. 2001), and any significant difference in LBM maintenance were not seen between the study groups.

**Effects of whey proteins and calcium on nutrient sensing signaling pathways**

Bieganowski and Brenner (2004) discovered nicotinamide riboside (NR), a NAD$^+$ precursor, from whey protein fraction of cow’s milk, leading to the hypothesis that whey proteins might regulate the NAD$^+$-dependent sirtuin pathway. Recently, NR has been shown to increase the NAD$^+$ level in mammalian cells and mouse tissues and activate SIRT1 and SIRT3, resulting in enhanced oxidative metabolism and protection against HFD-induced metabolic abnormalities (Canto et al. 2012). In the present study, neither high-calcium whey protein diets (WPI and α-lactalbumin) nor a novel WPI with any dosages influenced the SIRT1 protein expression. The SIRT3 protein expression was increased in skeletal muscle by CR, especially with the high-calcium α-lactalbumin diet. After the CR phase, a novel WPI increased the hepatic SIRT3 protein expression more than the casein-based diet. However, during the re-gain phase a novel WPI did not influence the SIRT3 protein expression indicating that CR is a necessity for an increased SIRT3 expression by a novel WPI. These results strongly suggest that anti-obesity effects of whey proteins and calcium are, at least in part, mediated by the mitochondrial SIRT3 pathway. The effects of whey proteins and calcium on cellular energy status sensitive pathways AMPK and mTOR were also investigated. A novel WPI did not influence on AMPK phosphorylation. S6 phosphorylation was decreased by a novel WPI, indicating inhibition of the mTOR pathway. The high calcium WPI and α-lactalbumin diets did not affect S6 phosphorylation.

### 6.6 Clinical relevance and future aspects

The aim in the obesity treatment is to improve health, and to prevent and alleviate obesity associated co-morbidities which can be achieved by at least 5% permanent weight loss (Aikuisten lihavuus, Käypä hoito –suositus, 2011, Tsigos et al. 2008). In the present study, a 30% decrease in calorie intake below *ad libitum* intake induced weight and fat loss and the weight loss effects of CR were enhanced by whey proteins and calcium. Even thought results cannot be directly
extrapolated to humans, the study verifies the anti-obesity effects of whey proteins and suggests that increasing the amount of whey proteins in diets would be advisable during weight loss.

However, the compliance to long-term low-calorie diet is very often poor in clinical trials and many fail in weight loss and weight management. Since SIRT1 is thought to mediate the effects of CR, several SIRT1 activating compounds have been developed (Howitz et al. 2003, Milne et al. 2007). In this study the SIRT1 activator resveratrol was used and it did not protect against obesity or metabolic disorders. Currently, more potent SIRT1-activating compounds are developed. Multiple clinical studies have been initiated with three selective SIRT1 activators SRT2104, SRT2379 and SRT3025 to find out those compounds potential in inflammatory, metabolic and cardiovascular diseases (ClinicalTrials 2012). The present study also suggested that mitochondrial SIRT3 is an important mediator of CR, suggesting that it would be advisable to investigate the SIRT3 pathway potential in obesity treatment and prevention more closely.

The adipose tissue cytokine and angiogenesis profiles showed great response to changes in body weight. Recent studies suggest that anti-angiogenic agents might have a role in the treatment of obesity and metabolic disorders (for review see Cao 2010). Early clinical studies with the anti-angiogenic drug bevacizumab have shown promising signs in the prevention of progression of diabetic retinopathy and diabetic maculopathy, both the complications related to type 2 diabetes (Steinbrook 2006). However, the safety of the anti-angiogenic drugs in treatment of obesity should be evaluated carefully as angiogenesis has several important physiological functions, including the healing of wounds.
7. **Summary and conclusions**

The present study aimed to investigate the molecular mechanisms and signaling pathways mediating the beneficial effects of calorie restriction (CR) on experimental obesity. The study also aimed to clarify the cellular mechanisms by which whey proteins enhance CR.

The main findings and conclusions of the present study are as follows:

1. CR protected against diet-induced obesity and fatty liver formation as well as ameliorated adipose tissue inflammation, and showed superior protection against diet-induced obesity as compared to the SIRT1 activating compound resveratrol. CR induced the sirtuin pathway and increased the SIRT3 protein expression in metabolically important tissues, suggesting an important role for sirtuins as mediators of CR.

2. Obesity induced cytokine and angiogenesis protein expression in adipose tissue and these changes in protein profiles were largely ameliorated by CR. In contrast, in lean mice, CR increased the expression of several cytokines and angiogenesis-related proteins. The results underscore the importance of adipose tissue cytokine and angiogenesis-related proteins in adipose tissue remodeling and the development of obesity.

3. High-calcium WPI and α-lactalbumin diets enhanced the anti-obesity effects of CR compared to casein. Both WPI and α-lactalbumin modified skeletal muscle gene expression profile, in particular, the genes associated with the Wnt signaling pathway and skeletal muscle lipid and fatty acid metabolism. These findings can explain, at least in part, the greater anti-obesity effects of whey proteins and calcium compared to casein.

4. A novel WPI, rich in lactoperoxidase, lactoferrin, growth factors and immunoglobulins, protected against fatty liver and exerted superior anti-obesity effects compared to casein. The beneficial effects of WPI were dose-dependent and mediated, at least in part, via activation of the sirtuin pathway.
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10. Original publications