PhD Thesis
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The dietary composition and source of macronutrients determine obesity development

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PREFACE

This PhD project started in 2010 as a collaboration between the Department of Biology, University of Copenhagen and National Institute of Nutrition and Seafood Research (NIFES). The work presented here has been performed at both institutions by supervision of Prof. Karsten Kristiansen and Associated Prof. Lise Madsen.
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ABSTRACT

The drastic worldwide increase in obesity during the last decades is accompanied with several different health disorders. The underlying mechanisms for this escalation is not clear, but certain alterations in the dietary macronutrient composition are suggested to be of importance. In addition to an elevation in energy intake, these alterations include increased consumption of refined carbohydrates and a relative decrease in protein consumption. The relative intake of dietary fat has not increased during the last decades, but the proportion of vegetable oils has increased at the expense of saturated fat and marine oils.

To further investigate the importance of the macronutrient composition on obesity development, we have performed a series of mice experiments. Our results demonstrate that both the amount and source of macronutrients influence obesity development and related disorders. The anti-obesity and insulin-sensitizing effect of marine n-3 polyunsaturated fatty acids (PUFAs) are well described in rodents. In line with this, we show that replacing marine oil with soybean oil in salmon feed, translates into a lower content of n-3 PUFAs in salmon fillets leading to aggravated insulin resistance in mice fed salmon in a Western diet. However, a high dietary content of sucrose or other high glycemic index carbohydrates attenuate the anti-obesogenic effect of n-3 PUFAs. When casein is used as the protein source, a high protein:carbohydrate ratio prevents high fat diet induced obesity, this is observed in studies where the dietary fat is enriched with either marine n-3 PUFAs or vegetable n-6 PUFAs. The protein:carbohydrate ratio furthermore influence on the accumulation of POPs in adipose tissue. However, we also demonstrate that the protein source determines the potential of high protein diets to attenuate obesity development. The gut microbiota is less affected by alterations in protein:carbohydrate ratio and adiposity, but is altered in response to an elevated fat intake.

The macronutrient composition is able to affect obesity development through direct influence on energy consuming metabolic processes, such as gluconeogenesis, amino acid degradation, urea synthesis and nonshivering thermogenesis.
**SAMMENDRAG**

Den drastisk globale økningen i fedme de siste tiårene medfører flere ulike helseforstyrrelser. De underliggende mekanismene for denne økningen er uklare, men visse endringer i kostens sammensetning av makronæringsstoffer er foreslått å være viktige. I tillegg til et økt energiinntak, omfatter disse endringene et økt inntak av raffinerte karbohydrater og en relativ reduksjon i proteininntak. Det relative inntaket av kostfett har ikke økt det siste tiåret, men andelen av vegetabilske oljer har økt på bekostning av mettet fett og marine oljer.

For å videre undersøke betydningen av makronæringsstoffsammensetningen på fedmeutvikling har vi utført flere dyreforsøk ved bruk av gnagere. Våre resultater viser at både type makronæringsstoffer og deres sammensetning påvirker fedmeutvikling og relaterte forstyrrelser. Effekten av marine omega-3 fettsyrer på å hemme fedme og forbedre insulinsensitivitet hos gnagere er veldokumentert i litteraturen. Relatert til dette viser vi at ved å erstatte marine oljer med soyaolje i før til laks, reduseres innholdet av omega-3 fettsyrer i laksefileten og medfører ytterligere redusert insulinsensitivitet i gnagere gitt laksefilet i en vestlig diet. Derimot forhinder en høy protein:karbohydrat ratio fedmeutvikling på høy fett diet, dette er observert i studier hvor fettkilden er beriket med enten marine omega-3 fettsyrer eller vegetabilske omega-6 fettsyrer. Videre viser vi også at protein:karbohydrat ration påvirker akkumulering av persistente organiske miljøgifter i fettvev. I tillegg ser vi at proteinkilden påvirker potensialet av høy protein dieter til å hemme fedmeutvikling. Tarmfloraen er mindre påvirket av en endring i protein:karbohydrat ratio og fedme, men er endret i respons til et høyt fettinntak.

Sammensetningen av makronæringsstoffer påvirker fedmeutvikling via direkte endringer i energikrevende metabolske prosesser, som glukoneogenese, aminosyredegradering, ureasyntese og varmeproduksjon i brune fettceller.
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**ABBREVIATIONS**

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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched chained amino acid</td>
</tr>
<tr>
<td>BCKA</td>
<td>Branched chain keto acid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichloro-diphenyl-trichloroethane</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>GI</td>
<td>Glycemic index</td>
</tr>
<tr>
<td>GL</td>
<td>Glycemic load</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyl</td>
</tr>
<tr>
<td>POP</td>
<td>Persistent organic pollutant</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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1.0 INTRODUCTION

1.1 The global obesity epidemic

Today, obesity is considered one of the main public health problems worldwide and is often referred to as the global obesity epidemic. In 2003, the World Health Organization (WHO) called out attention to the public that “if immediate action is not taken, millions will suffer from an array of health disorders” (WHO, 2003). Obesity is defined as “abnormal or excessive fat accumulation that may impair health”, and is normally classified by body mass index (BMI) (kg/m²), with BMI from 25-30 defined as overweight and above 30 as obese (WHO, 2014).

Historically, the problems related to body weight gain and adiposity have been dramatically changed. In retrospect, a struggle to survive on a minimal amount of food was the reality. Globally, this has dramatically changed, today obesity is an epidemic problem worldwide and among the leading risks of death (WHO, 2014). In the year 2000, the number of overweight people exceeded the number of underweight people for the first time (Gardner and Halweil, 2000). Today, overweight-related co-morbidities cause more deaths than underweight in areas where 65% of the population live (WHO, 2014).

While obesity has only been considered as a problem during the last decades, the link between an impaired health and the state of obesity was recognized already in the 1930’s. An increase in obesity was, however, first observed in 1960 (Caballero, 2007). The worldwide prevalence of obesity has been steadily increasing ever since, with a number of over 1.4 billion adults being overweight today (2008), including 10% defined as obese (WHO, 2014). One of the earlier solutions to health problems caused by malnutrition, consisted of supplementing the diet with sugar and fat to increase energy intake (Caballero, 2007). Ironically, refined sugars and vegetable oils continued to be important for the increase in energy intake, and are today among the dietary factors that are considered responsible for the obesity epidemic (Blasbalg et al., 2011; Malik et al., 2013; WHO, 2002).

1.1.1 Factors contributing to the obesity epidemic

A positive energy balance is the fundamental ground rule that has to be present for body weight gain and adipose tissue mass expansion. However, factors contributing to the obesity epidemic constitute a long list of complex interactions and controversial opinions (Caballero, 2007). The factors linked to obesity development involve the interaction of genetics, the environment and a psychological aspect (Kopelman, 2000). However, a positive energy balance is necessary to promote body weight gain and this has been linked to the altered life style related to the dietary
INTRODUCTION

composition and physical activity (Blasbalg et al., 2011; Caballero, 2007; Malik et al., 2013; WHO, 2002).

The view on the state of obesity has shifted from the judgmental individual perspective of gluttony, to a more global focus on external contributors (Caballero, 2007; WHO, 2003). A report from a WHO consultation concluded that the fundamental causes of the obesity epidemic are a sedentary lifestyles combined with energy-dense high fat diets. This was reported to be a result of the alterations in todays society from the traditional living and towards the increased urbanization and industrialization present today (WHO, 2000). The changes of environmental factors, like automation, physical inactivity, an unhealthy and persuasive food marketing and the increased dependence on already prepared food, are listed as contributors to the global obesity epidemic in todays society (Caballero, 2007; WHO, 2002).

WHO has listed dietary factors as a major contributor, together with physical inactivity, influencing the unbalanced energy equation in obesity. However, this represents a factor possible to modify. One of the main methods applied for treatment of obesity is dietary management (WHO, 2000), and the advice to the general population is to limit the energy intake from fat and sugar and increase the consumption of fruit and vegetables (WHO, 2014). Thus, dietary intake of energy is demonstrated to be increased in the US population together with the highest prevalence of obesity in the world. A great part of this increase in energy intake has been assigned to the consumption of sweetened beverages (Caballero, 2007). In the last century, a dramatic increase in the intake of dietary oils, especially soybean oil, were reported in the US, leading to higher consumption of linoleic acid (LA), linked to obesity (Alvheim et al., 2012; Blasbalg et al., 2011). Other important sources suggested to increase the energy intake is the low cost of energy-dense foods, prepared meals and a restricted dietary eating pattern (Caballero, 2007; WHO, 2002). A nutritional change with higher consumption of animal fat and protein, refined grains and sugar is reported, which are considered as dietary alterations contributing to an increase in energy intake (Malik et al., 2013).

Since research started focusing on obesity, several factors with potential impact on obesity development and related metabolic disorders have been discovered. The gut microbiota has recently gained attention and is associated with changes in obesity (Ley et al., 2005; Ridaura et al., 2013; Turnbaugh et al., 2006; Turnbaugh et al., 2009) and diet (David et al., 2014; Hildebrandt et al., 2009; Ley et al., 2008; Muegge et al., 2011; Wu et al., 2011). In relation to changes in environmental factors in todays society, the dietary exposure of environmental pollutants are associated with obesity, mainly supported by epidemiological studies correlating
plasma levels of persistent organic pollutants (POPs) with obesity (Dhooge et al., 2010; Lee et al., 2014; Ronn et al., 2011; Roos et al., 2013).

While our genes have an impact on the susceptibility for obesity development (Kopelman, 2000), the evidence of epigenetics being involved has gained more attention recently (Campion et al., 2010). Epigenetics has been defined as heritable changes in gene expression that is not caused by alterations in DNA sequences (Christensen and Marsit, 2011). The impact of nutrition and environmental factors on epigenetics are suggested and further linked to influence obesity development (Campion et al., 2010). Thus, the discovery of potentially contributing obesogenic factors provide new approaches to evaluate obesity and counteract the obesity epidemic.

1.1.2 Obesity-related metabolic disorders

Obesity is stated as a “major risk for serious diet-related non-communicable diseases, including metabolic diseases like diabetes, cardiovascular disease, hypertension, stroke and certain forms of cancer” (WHO, 2003). Today, obesity is among the leading risks for global death and its obesity-related diseases are associated with health consequences that reduce the overall quality of life (WHO, 2003). The metabolic disturbances, including elevated plasma levels of insulin, insulin resistance and hyperlipidemia, are normally evident in the obese state (Kopelman, 2000).

Lifespan

Today, obesity is considered one of the main risks for death worldwide (WHO 2003), emphasizing the impact of obesity on lifespan. While our genes are of great importance when estimating life expectancy, caloric restriction is the main environmental variable found to extend longevity (Bluher, 2008). The increase in lifespan correlates with a reduction in adipose tissue mass in different organisms (Picard and Guarente, 2005), either by caloric restriction (Weindruch, 1996; Weindruch et al., 1986) or mutations in the pathway of insulin/insulin-like growth factor-1 (IGF-1) signaling through genetically modified animals (Bluher, 2008; Bluher et al., 2003; Katic and Kahn, 2005).

Insulin resistance, glucose intolerance and dyslipidemia

An increase in levels of plasma insulin is normally present in fasted obese individuals, together with an elevated response in both plasma insulin and glucose to an oral glucose load, accompanied with peripheral insulin resistance. When the pancreatic insulin secreting β-cells
are unable to compensate for the reduced insulin sensitivity, hyperglycemia occurs and type 2 diabetes is evident with β-cell failure (Kahn et al., 2006). Impaired secretion and action of insulin are linked to several metabolic disturbances, including reduced insulin-stimulated glucose uptake, uncontrolled hepatic glucose production and dyslipidemia (Muoio and Newgard, 2008).

Obesity is associated with an excess of dietary nutrients, leading to production of toxic by-products from metabolism, further linked to insulin resistance (Muoio and Newgard, 2008). An increase in non-esterified fatty acids (NEFAs) in plasma are associated with insulin resistance (Kahn et al., 2006) and are found in obese and type 2 diabetic subjects (Boden, 1997; Reaven et al., 1988; Roden et al., 1996). The relationship between increased levels of NEFA and insulin resistance is associated with an increase in intracellular levels of the fatty acid metabolites diacylglycerol (DAG), fatty acyl-CoA and ceramides, which possibly suppress insulin-signaling (Huffman et al., 2009; Shulman, 2000; Tai et al., 2010). Further, elevated blood levels of branched chain amino acids (BCAAs) are also related to obesity and insulin resistance (Newgard, 2012; Newgard et al., 2009).

Adipose tissue is an important regulator of lipid and glucose homeostasis. Metabolic disturbances linked to a dysfunctional adipose tissue in obesity lead to deranged lipid and glucose metabolism, impaired storage of triglycerides, disturbed secretion of cytokines and an increase in lipolysis. Further complications related to the impairments of adipocyte functions are increased blood levels of fatty acids and peripheral accumulation of fatty acids (Guilherme et al., 2008). As the storage capacity of adipocytes may reach its limit in obesity, this results in fat accumulation in other tissues like the liver, skeletal muscle and heart (Muoio and Newgard, 2008). The importance of a functional adipose tissue is further proved by the fact that the absence of white adipose tissue results in severe insulin resistance in mice (Reitman and Gavrilova, 2000) and the fat-specific disruption of GLUT4 leads to an impaired glucose tolerance and insulin resistance (Abel et al., 2001).

**Low-grade inflammation**

Inflammation has also been considered as a key feature of obesity and type 2 diabetes. Obesity, insulin resistance and type 2 diabetes are related to chronic inflammation with an activation of signaling pathways involved in inflammation, an abnormal cytokine production and elevated levels of acute-phase reactants (Hotamisligil, 2006). Enlarged adipocytes are related to increased secretion of monocyte chemoattractant protein-1 (MCP-1), resulting in macrophage
infiltration in the adipose tissue and increased levels of inflammatory components such as tumor necrosis factor-α (TNF-α) (Weisberg et al., 2003). Thus, an increased infiltration of macrophages is found in adipose tissue of obese animals and humans (Hotamisligil, 2006; Weisberg et al., 2003) and genetic deletion of MCP-1 (Kanda et al., 2006) and TNF-α (Uysal et al., 1997) has been proven to protect from high fat diet-induced insulin resistance.

1.2 Dietary macronutrients in diet-induced obesity development

During evolution, there has been a dramatic dietary change. The Western diets today have in particular changed the glycemic load, fatty acid composition and macronutrient composition, among other nutrients, compared to our ancestors diet 10 000 years ago (Cordain et al., 2005; Simopoulos, 2002), illustrated in Figure 1. The changes in dietary composition the past decade are suggested to have happened rapidly in the context of evolution and considered responsible for promoting chronic diseases, like obesity, diabetes, different forms of cancer, atherosclerosis and hypertension (Simopoulos, 2002). The increased prevalence of obesity happened simultaneously with great dietary changes, emphasizing the importance of investigating how these new dietary trends and inclusion of more processed and highly refined food products impact on our health and contribute to obesity development.

A Western diet consists on average of about 50 % energy from carbohydrate, 35 % from fat and 15 % from protein. One health advice is to reduce the fat intake to 30%, but in comparison to the hunter-gatherers dietary composition, the intake of carbohydrates has been elevated at the expense of proteins. Modulating the ratio of protein to carbohydrate has demonstrated a potential effect in prevention of obesity in animals (Freudenberg et al., 2013; Ma et al., 2011; Madsen et al., 2008; Morens et al., 2005; Pichon et al., 2006).
Figure 1: The diagram illustrates the dietary changes in some of the main nutrients in the hunter-gatherer society compared to the Western diet today. The y-axis displays the amount of the different nutrients in the diet, graded into low, moderate and high. The diagram is based on information from (Simopoulos, 2002).

1.2.1 Dietary proteins

The ability of high protein diets to reduce body weight and fat mass has been related to the high amino nitrogen content, stimulating satiety and reducing food intake, partly by increased water intake (Freudenberg et al., 2013; Petzke et al., 2014). A high protein intake has also been demonstrated to induce a higher thermic effect, representing an increase in energy expenditure after ingestion, compared to a low protein intake. The high thermic effect of proteins is possibly due to their metabolic processing, where the synthesis of proteins are especially energy demanding due to high ATP cost of peptide bond synthesis, urea production and gluconeogenesis (Halton and Hu, 2004; Veldhorst et al., 2009; Westerterp-Plantenga et al., 2012; Westerterp, 2004). In agreement with this, increasing the protein intake from 10 energy% to 25 energy% at the expense of dietary fat was demonstrated to increase 24h energy expenditure and increase the sleeping metabolic rate, together with an elevated satiety in human subjects (Hochstenbach-Waelen et al., 2009). High protein diets are also suggested to be
beneficial in terms of improving blood lipid profiles and insulin sensitivity, in addition to preventing loss of muscle mass when losing weight (Westerterp-Plantenga et al., 2012).

Comparisons of different protein sources have been demonstrated to impact obesity development. Inclusion of fish protein hydrolysate from saithe in the diet, rich in taurine and glycine, led to elevated levels of bile acids in the plasma and reduced abdominal obesity, circulating triacylglycerols (TAGs) and hepatic lipid accumulation, compared to proteins from soy or casein in rats (Liaset et al., 2009). Fish protein hydrolysate from salmon also prevents high-fat diet induced obesity, compared to casein, together with an improved lipid metabolism and increased expression of genes related to nonshivering thermogenesis (Liaset et al., 2011). Intake of proteins from cod and soy have a positive impact on both glucose metabolism and insulin sensitivity in high sucrose fed rats compared to casein (Lavigne et al., 2000). Further, scallop was demonstrated to be the most beneficial protein source in protection from high fat high sucrose induced obesity and dyslipidemia, when compared with proteins from casein, chicken, cod and crab in mice. Among all the different protein sources, the dietary content of taurine and glycine were inversely correlated with adipose tissue mass and plasma lipids (Tastesen et al., 2014). The reduced expansion of adipose tissue mass and accumulation of hepatic lipids in mice fed high fat diets, were evident with proteins derived from whey compared to casein, which were linked to elevated levels of tricarboxylic acid cycle metabolites in the urine, preventing lipogenesis and lipid accumulation (Lillefosse et al., 2014).

The dietary form of proteins can also affect the outcome of diet-induced obesity development in mice. The intake of hydrolyzed casein prevented obesity development and reduced plasma insulin and glucose, compared to intact casein, due to an induced expression of genes involved in fatty acid oxidation and browning of white adipose tissue (Lillefosse et al., 2013). Overall, the effects of proteins on obesity and related metabolic alterations seem to be dependent on not just the proportion of proteins in relation to carbohydrates, but also on the source and form of the dietary proteins. The increased popularity of high protein diets in weight loss management further emphasizes the importance of elucidating the impact of different protein sources and also the long-term effects in regards to safety and efficiency by a high protein intake on obesity.

Lifespan is indicated to be impaired by a high protein intake and improved when proteins are replaced with carbohydrate. This study only evaluated diets with proteins derived from casein and methionine, but indicates that high protein intake is not beneficial in regards to longevity in mice (Solon-Biet et al., 2014). Another study demonstrated an increase in lifespan in mice fed a high carbohydrate low fat diet compared to mice fed high fat diets. The reduction in
lifespan due to a high fat intake was more prominent when the mice also had a high intake of carbohydrates, and improved when the high intake of carbohydrates was replaced with proteins (Keipert et al., 2011). An epidemiological study on US men and women in the age 50-65 years reported an increase in overall mortality in persons with a high protein intake, however, this was abolished or reduced in subjects who consumed plant-derived proteins, accentuating the importance of protein source. Negative effects of a low protein intake were linked to subjects above age 66, suggesting that a high protein intake is beneficial for health and lifespan in older but not middle-aged subjects (Levine et al., 2014). Earlier studies have linked the reduction in adipose tissue mass to increase in lifespan, by caloric restriction, insulin/IGF-1 signaling or genetic manipulation (Bluher, 2008; Bluher et al., 2003; Katic and Kahn, 2005; Picard and Guarente, 2005; Weindruch, 1996; Weindruch et al., 1986). Thus, the latter suggests that the dietary effect on adipose tissue/obesity mass may be crucial for longevity.

Branched chain amino acids (BCAAs) in obesity

The impact of elevated levels of BCAAs in obesity and insulin resistance is a matter of debate, presenting contradictory results. Elevated blood levels of BCAAs, branched chain keto acids (BCKAs) or carnitine esters derived from BCAA catabolism are reported in obesity, insulin resistance and type 2 diabetes (Adams, 2011; Newgard et al., 2009). In contrast, disruption of the enzyme catalyzing the first step in the peripheral BCAA metabolism in mice leads to elevated levels of plasma BCAAs and prevention of diet-induced obesity, in addition to increasing energy expenditure and improving glucose tolerance and insulin sensitivity (She et al., 2007). Further, intake of leucine or BCAAs is reported to reduce body weight and adipose tissue, in addition to be beneficial for glucose metabolism (Bianchi et al., 2005; Layman and Walker, 2006; Zhang et al., 2007). However, rats given BCAAs with a high fat diet developed insulin resistance despite being lean (Newgard et al., 2009). The interaction of BCAAs with an excess of dietary fat is suggested to induce insulin resistance, as a high fat diet together with BCAAs induce insulin resistance (Newgard, 2012; Newgard et al., 2009). A reduction in the catabolism of BCAAs in white adipose tissue (especially omental/visceral fat) have been linked to metabolic unhealthy obese humans and rodents (Lackey et al., 2013).

1.2.2 Nonshivering thermogenesis

While white adipocytes function as a lipid storage, the brown adipose tissue (BAT) is able to burn off fat by the production of heat, contributing as a potential target in prevention of obesity (Bartelt and Heeren, 2014). In genetic models of obesity, there are reported an atrophy of BAT and a reduction in uncoupling protein 1 (UCP1) content (Trayhurn, 1984), and the lack of brown
adipose tissue has been demonstrated to induce obesity development (Lowell et al., 1993). However, the absence of UCP1 in mice did not lead to obesity development (Enerback et al., 1997), but the transgenic expression of Ucp in white adipose tissue reduced body weight and adipose tissue mass in a strain of genetically obese mice (Kopecky et al., 1995).

Cold exposure is the main stimulus for activation of brown adipose tissue. Animals exposed to cold, compared to thermoneutrality (28-30°C), can have up to four-fold higher metabolism due to nonshivering thermogenesis (Cannon and Nedergaard, 2004). The increase in thermogenesis of mice housed at room temperature (20-22°C) versus thermoneutrality leads to an increase in energy expenditure, resulting in a need to eat 60% more food to maintain body weight compared to mice at thermoneutrality (Peirce et al., 2014). A reduction in sympathetic activity, the main signal for BAT stimulation, induces apoptosis of BAT (Briscini et al., 1998; Lindquist and Rehnmark, 1998) and is related to obesity (Nisoli et al., 2000). TNF-α is also related to apoptosis of brown adipocytes (Nisoli et al., 1997) and could potentially be a link between obesity and reduced BAT activity, as obese subjects have elevated levels of TNF-α (Hotamisligil et al., 1995; Hotamisligil et al., 1993). In addition to potential beneficial effects on weight reduction, BAT has proved to be important in glucose and lipid clearance (Arbeeny et al., 1995). Mice with insulin receptor knockout in BAT have reduced insulin secretion and an impaired glucose tolerance (Guerra et al., 2001).

More recently, adipocytes have been divided into white, beige/brite and brown adipocytes. The thermogenic beige/brite adipocytes are found within the white adipose tissue (WAT) and express UCP1 (Peirce et al., 2014). The recruitment of beige adipocytes is demonstrated after cold exposure (Bartelt and Heeren, 2014) and is found to derive directly from white adipocytes, however, the conversion of beige to white adipocytes has also been demonstrated with adaptation to warm temperatures (Rosenwald et al., 2013). This recruitment of beige adipocytes or so-called WAT “browning” is also found to prevent obesity (Ma et al., 2011; Madsen et al., 2008; Schulz et al., 2013).

1.2.3 Dietary protein:carbohydrate ratio

The protein:carbohydrate ratio is able to modulate the outcome of high fat diets irrespective of whether the fat source is based on n-3 or n-6 polyunsaturated fatty acids (PUFAs). Increasing the protein content at the expense of carbohydrates prevents obesity development and inhibit the induction of inflammation and lipogenesis in mice fed a high fat diet (Ma et al., 2011; Madsen et al., 2008). The increase in protein:sucrose ratio reduces blood insulin levels and elevate the glucagon/insulin ratio and cAMP signaling, further inducing the expression of
cAMP responsive genes and stimulating nonshivering thermogenesis (Madsen et al., 2008). The energy consuming processes, like gluconeogenesis, amino acid degradation and urea synthesis, are induced by high intake of proteins at the expense of sucrose (Ma et al., 2011; Madsen et al., 2008). Thus, feed efficiency is reduced in response to high protein intake, and mice fed a high protein diet would need to eat six times more calories compared to mice with high carbohydrate intake to achieve the same body weight gain on a high fat diet (Madsen et al., 2008). In summary, the adipogenic potential of a high fat diet is induced by including high amounts of sucrose in the diet, while prevented if proteins are added instead.

1.2.4 Dietary glycemic index

The impact of protein:sucrose ratio on obesity development is suggested, to partly, be a result of the different response on stimulating insulin secretion. As various carbohydrate sources have different glycemic index (GI) and modulate the rise in plasma glucose and insulin after intake, this can potentially affect obesity development. The use of low-GI diets is suggested as a tool for reducing adiposity based on promising results of low-GI diets tested in both humans and animals (Brand-Miller et al., 2002; Pawlak et al., 2004; Solomon et al., 2013; Solomon et al., 2010). A study of healthy humans did not reveal any difference in either glucose or insulin response or appetite between the intake of low- and high-GI foods (Alfenas and Matter 2005 Diabetes Care). However, the glycemic response is dependent on both the quality given by the glycemic index and the quantity of carbohydrates, where both factors are only taken into account when calculating the glycemic load (GL) (Foster-Powell et al., 2002). Comparison of the glycemic and insulinemic response to a wide variety of isoenergetic portions of foods demonstrated, as expected, a high correlation between blood glucose and insulin response. Further, the calculated insulin response correlated positively with the total content of carbohydrates and sugar, and negatively with the energy per cent of fat and protein. Foods which are typically found in a Western diet, induced greater insulin responses than the more traditional diets with less refined foods (Holt et al., 1997). A better completion and maintenance of weight loss among adults are also found due to reduction in glycemic index and a modest increase in dietary protein content (Larsen et al., 2010). The importance of the insulin response to different diets or dietary components is further emphasized by results from animal studies, which indicate a causal relationship of insulin in promoting diet-induced obesity development (Ma et al., 2011; Madsen et al., 2008; Mehran et al., 2012). The high intake of sucrose leads to high circulating levels of insulin and promote high fat diet-induced obesity in mice (Madsen et al., 2008), and attenuate the beneficial outcome of fish oil on obesity and inflammation (Ma et
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al., 2011). This outlines the potential impact of high GI carbohydrates in the diet to induce obesogenic effects, especially combined with a high dietary fat intake.

**Insulin and impact on diet-induced obesity development**

A profound anabolic effect of insulin in its responsive tissues, including muscle, adipose tissue and liver, is evident through stimulated cell growth and differentiation, lipogenesis, synthesis of glycogen and protein and the inhibition of hepatic glucose production (Saltiel and Kahn, 2001). While hyperinsulinemia is a common feature seen in obese insulin resistant individuals, it is commonly viewed as a compensatory response to the reduced insulin sensitivity. However, new findings link the role of insulin as a causal factor in the development of obesity (Bluher et al., 2002; Mehran et al., 2012).

Insulin signaling in adipose tissue has been demonstrated to be essential in obesity development and its related metabolic disturbances, where mice with fat-specific disruption of the insulin receptor gene display low fat mass and protection from glucose intolerance (Bluher et al., 2002). The prevention of hyperinsulinemia in transgenic mice with pancreas-specific $\text{Ins}1^{+/-}:\text{Ins}2^{-/-}$, completely protects the mice from diet-induced obesity. This is related to induction of UCP1 in white adipose tissue and increased energy expenditure. In addition, the $\text{Ins}1^{+/-}:\text{Ins}2^{-/-}$ phenotype also demonstrates protection from an excess of circulating free fatty acids and hepatic lipid accumulation. This study provides evidence for hyperinsulinemia as a causal factor for diet-induced obesity (Mehran et al., 2012), which also is demonstrated by dietary alterations in earlier studies (Ma et al., 2011; Madsen et al., 2008). Thus, reasonable evidence consists to suggest that reducing hyperinsulinemia by nutritional or pharmalogical modulation could be an effective way to reduce obesity development.

**1.3 Dietary fat**

The dietary intake of fat has often been considered one of the main contributors to the increase in obesity (Astrup et al., 2000; Bray and Popkin, 1998; Hariri and Thibault, 2010; Hill et al., 2000). As fat is the most energy-dense macronutrient, it has been suggested that diets with high levels of fat lead to an increase in the overall energy intake. Thus, limiting the intake of dietary fat has been suggested to reduce weight gain and is still one of the main advice from WHO for a healthy diet (WHO, 2014). However, conflicting results are reported from the impact of dietary fat intake on obesity. It is stated that there is no conclusive evidence to attribute the dietary fat intake for obesity more than the other macronutrients, at least not from epidemiological studies (Seidell, 1998). The focus on reducing dietary fat has also been
described as a serious mistake in the attempt to curb the obesity epidemic (Willett, 2002). This misinterpretation of the role of dietary fat is linked to the decline in dietary fat intake in the US during the last decades concomitant with the dramatic increase in obesity (Austin et al., 2011). The increased focus of the fat source, and not only the amount of fat, is becoming more evident as the fat has been suggested to be of great importance to health (Simopoulos, 2002). The intake of vegetable oils has increased as a result of the oil-seed processing industry and the change to grain feeding of cattle and livestock, which further contribute to a higher dietary intake of n-6 PUFAs at the expense of n-3 PUFAs. The change in dietary fat composition has resulted in an increased dietary n-6/n-3 PUFA ratio, due to a higher intake of n-6 PUFAs (Blasbalg et al., 2011). The dietary n-3/n-6 ratio is estimated to have been approximately 2-3:1 in the hunter-gatherer society, today the ratio is 10:1 in the US diet (Cordain et al., 2005). Fish derived raw materials are a limited resource and need to be replaced in the fish farming industry, the use of vegetable oils are considered suitable replacements (Turchini et al., 2009). However, this also affects the fatty acid composition of the fish fillets, reducing the amount of n-3 PUFAs (Liland et al., 2013; Torstensen et al., 2005).

The reported changes in the industry of oil-seed processing, animal- and marine farming, change the fatty acid composition of various foods, and constitute an important factor which impacts on our health. The balance of n-6 and n-3 PUFAs has been demonstrated to be of importance according to impact on health and chronic diseases (Simopoulos, 2002), where n-3 PUFAs are reported to reduce the risk for cardiovascular disease and prevent or reduce many inflammatory and autoimmune diseases (Kris-Etherton et al., 2002; Simopoulos, 2002). The present increase in n-6/n-3 ratio leads to an elevation in eicosanoids derived from arachidonic acid (AA), with physiological effects promoting a more pro-inflammatory state, linked to chronic conditions like obesity, autoimmune disease, diabetes, cancer and cardiovascular disease (Simopoulos, 2002). An elevation in the dietary LA intake from 1 to 8 E% is reported in the US, this elevation is demonstrated to increase obesity development in mice on a high fat diet. The obesity promoting effect of increased dietary LA intake is associated with endocannabinoid hyperactivity, as LA is the precursor of arachidonic acid (AA) which constitute the backbone of some endocannabinoids. However, inclusion of 1 E% eicosapentaenoic acid (EPA)/docosapentaenoic acid (DHA) reversed the effect of 8 E% LA and reduced adiposity and feed efficiency (Alvheim et al., 2012).

Numerous animal studies demonstrate beneficial effects of the marine n-3 PUFAs on obesity development and related metabolic complications. The replacement of different vegetable oils
or saturated fatty acids with marine n-3 PUFAs prevents high fat diet-induced obesity (Rokling-Andersen et al., 2009; Ruzickova et al., 2004; Samane et al., 2009) insulin resistance (Storlien et al., 1987), adipocyte hypertrophy and hepatic lipid accumulation (Huber et al., 2007). An improved glucose (Samane et al., 2009) and lipid metabolism (Rokling-Andersen et al., 2009) are also reported upon intake of n-3 PUFAs (Ruzickova et al., 2004), as well as reduced inflammation in adipose tissue (Todoric et al., 2006). The beneficial outcome from intake of marine PUFAs is associated with an induction of mitochondrial fatty acid oxidation (Froyland et al., 1997; Madsen et al., 1999), together with a reduction in lipogenesis in adipose tissue (Flachs et al., 2005). The administration of marine PUFAs (EPA and DHA) in the form of phospholipids, compared to triglycerides, increases the dietary bioavailability of EPA and DHA and demonstrates more beneficial effects on obesity related metabolic disturbances in mice (Rossmeisl et al., 2012).

While animal studies support the anti-obesogenic effect of marine n-3 PUFAs, human evidence is inconclusive regarding the effects of marine n-3 PUFAs on obesity development (Flachs et al., 2014). However, this is suggested to be caused by differences in the background diet, such as alterations in the macronutrient- and fatty acid composition, and possibly the exposure of persistent organic pollutants (POPs) (Madsen and Kristiansen, 2012). High levels of sucrose in the background diet abrogate the anti-adipogenic and anti-inflammatory effect of fish oil in mice (Ma et al., 2011). The impact of other carbohydrate sources to influence the effect of n-3 PUFAs is unknown, but needs to be further evaluated as sucrose attenuated beneficial effects of fish oil. Thus, other high GI carbohydrates prevalent in our diet today could also have a possible deteriorating effect on the beneficial n-3 PUFAs.

1.3.1 Persistent organic pollutants (POPs)
During the last decade, there has been increased focus on the possible negative health effects of environmental pollution. The lipophilic and highly persistent organic pollutants (POPs) are especially important in regards to exposure from dietary intake, as they bio-accumulate along the food chain and are further stored in the adipose tissue of the consumer. Thus, fat-rich foods of animal origin (Boada et al., 2014), including seafood, oils/fats and dairy products contribute as dietary sources for POP exposure (Perello et al., 2012). A change from animal-based diets to more plant-based diets has been suggested as one way to reduce the exposure of environmental POPs, as animal-derived food sources generally contain more POPs (Boada et al., 2014; Lee et al., 2014).
Several studies demonstrate a link between obesity and insulin resistance to the exposure of POPs, such as the polychlorinated biphenyls (PCBs) and dichloro-diphenyl-trichloroethanes (DDTs) (Arsenescu et al., 2008; Grun and Blumberg, 2007; La Merrill et al., 2014; Newbold, 2010; Ruzzin et al., 2010; Wahlang et al., 2013). A correlation between the plasma levels of different POP congeners and obesity is reported (Dhooge et al., 2010; Lee et al., 2012; Ronn et al., 2011; Roos et al., 2013). However, evidence for a direct causal relationship between exposure of POPs to obesity is scarce, and whether the elevated levels of POPs actually are involved in a direct impact on the development of obesity or rather reflect the obese state, needs to be elucidated. As adipose tissue functions as a storage site for the accumulation of the lipophilic POPs, this could possibly be a safe way of storage to protect other tissues. However, alterations in adipose tissue could further lead to a change in the pharmacodynamics of POPs and constitute an endogenous exposure route. The discrepancy between many studies, according to negative and positive correlations between different POP congeners and obesity and/or insulin resistance, has been suggested to possibly result from a reported inverted U shape relationship. Thus, depending on the POPs dose of exposure the association with obesity and/or diabetes could be positive, inverse or zero (Lee et al., 2014).

The total body burden of POPs is found to be higher (2-3 times) in obese compared to lean humans, although this is not evident through POP concentrations in adipose tissue due to a diluting effect of more adipose tissue mass. Drastic weight loss through surgery leads to a reduced total body burden of POPs and to elevated POP levels in blood, linked to repressed response in improvement of lipid values and hepatic toxicity markers (Kim et al., 2011). It is unknown whether POPs are distributed equally across all adipose tissue depots and lipids in blood. A small study in humans indicated a complicated distribution pattern of POPs in different adipose depots, where the level of POPs in either serum or subcutaneous fat not necessarily indicated the level in other adipose tissues (Yu et al., 2011).

The half-life of different POP congeners is reported to vary, but a nearly linear relationship is demonstrated between the half-life of POPs and body fat (Milbrath et al., 2009). Pharmacokinetic balance studies by injections of PCB153 in rats have demonstrated that accumulation and excretion of this compound is dependent on whether the adipose tissue mass is expanding, constant or decreasing (Jondorf et al., 1983; Muhlebach and Bickel, 1981; Wyss et al., 1986). The expansion of adipose tissue mass was associated with an irreversible storage of a major part of the PCB injected (Muhlebach and Bickel, 1981), and a constant adipose tissue mass was related to a lower percentage of PCB stored and a higher fecal excretion (Wyss et al.,
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1986). However, a reduction in adipose mass led to an even greater percentage excreted through feces, and also a redistribution to the skin (Jondorf et al., 1983).

The metabolism, detoxification and elimination of xenobiotics involve a range of different drug-metabolizing enzymes, functioning as a defense against toxic compounds, with highest expression in liver and intestine. Thus, alterations in the drug-metabolizing enzymes can potentially lead to changes in absorption, distribution, metabolism and excretion of xenobiotics (Wei et al., 2012). Diet-induced changes in the drug-metabolizing enzymes could also be an alternative way that lead to alterations in the pharmacokinetics of xenobiotics. Dietary components like protein, fat, carbohydrate and also total energy intake have been reported to be involved in food-drug interactions (Harris et al., 2003). The human gut microbiota possesses microorganisms which are able to impact the metabolism of xenobiotics (Maurice et al., 2013). Metabolic function analyses of the human microbiome have also revealed an enrichment in metabolism of xenobiotics (Gill et al., 2006). The presence of different hydrocarbons in the gut are suggested to impact the gut microbiota (Lee et al., 2014), as the presence of a type of bacteria, methanogenic archaea, is linked to body weight gain (Samuel and Gordon, 2006) and also associated with higher body levels of POPs (Lee et al., 2011). A direct causal link between obesity and POP exposure is not established, and it is not obvious whether the link between obesity and POP levels are due to increased accumulation because of adipose tissue expansion and/or a direct result of the POPs.

1.3.2 Gut microbiota

A characteristic of the gut microbiota in obese and lean humans demonstrate an association of lower bacterial richness in persons with higher adiposity, insulin resistance, dyslipidemia and inflammation, relating the gut microbiota diversity with a metabolic risk profile (Le Chatelier et al., 2013). The human microbiota is also reported to possess a lower proportion of Bacteriodetes in obese compared to lean persons, which is increased upon low-calorie induced weight loss. In agreement with this, genetically obese mice (ob/ob) also have a reduced ratio of Bacteriodetes to Firmicutes compared to lean mice (Braak and Smilauer, 2012). The change in the relative abundance of Bacteriodetes and Firmicutes is linked to an alteration in metabolism of the gut microbiota, with an increase in dietary energy harvest associated with obesity. Thus, the capacity of increased energy harvest due to changes in microbiota composition, is possible to transfer into germ-free mice and induce adiposity (Turnbaugh et al., 2006). Mice receiving fecal microbiota from obese mice increased the adipose tissue mass, compared to mice receiving fecal microbiota from lean mice. Further, cohousing mice with microbiota from obese
mice together with mice having microbiota from lean mice, leads to prevention of obesity development. This is associated with metabolic features, related to an invasion of Bacteriodetes from the lean-derived microbiota into the obese-derived microbiota (Ridaura et al., 2013). The transplantation of microbiota from obese mice fed Western diet demonstrate an increase in adiposity in the new host, which has been proven also in mice with a human derived microbial community. The Western-diet induced change in the humanized mouse microbiota revealed an increase in classes of bacteria within the Firmicutes phylum, and a decrease in member of the Bacteroidetes (Turnbaugh et al., 2009).

The dietary impact on human health has been reported to partly be a result of alterations in the composition of gut microbiota/microbiome. The bacterial composition or “enterotype” determined by sequencing were strongly associated with the long-term dietary intake, with a divergence between gut profile of humans with a dietary intake of protein and animal fat compared to carbohydrates (Wu et al., 2011). The enterotypes have been divided into three main variants dominated by Bacteriodes, Prevotella and Ruminococcus (Arumugam et al., 2011). The Bacteriodes enterotype have been associated with animal protein, variety of amino acids and saturated fats. A short-term dietary intervention study revealed a rapid, but only modest change, in microbiota composition in comparison to a long-term diet intake, which strongly was associated with enterotype clustering (Wu et al., 2011).

Diet-induced adaptation of the gut microbiota is found to be similar across different mammalian species, including humans. This divergence in the microbiota induced by diet is suggested to be a result of a divergence in microbiota related to their functions, like enzymatic breakdown of components in the diet. Many of the enzymes of the gut microbiome in carnivores are involved in amino acid metabolism compared to the herbivores (Muegge et al., 2011). The bacterial diversity is altered across carnivores, omnivores and herbivores, with an increased bacterial diversity from the carnivores to omnivores and further to herbivores (Ley et al., 2008). The microbiota profile of short-term dietary intake (4 days) composed of only animal products, revealed a change in microbiota, with an increased abundance of bile-tolerant microorganisms and decreased level of Firmicutes compared to the intake of a pure plant-based diet. The animal-based diet was also related to a higher intake of fat and protein, and a reduction in fiber intake (E%) (David et al., 2014). High fat diet-induced changes in microbiota were detected in wild-type mice developing obesity and in RELMβ KO mice that stayed lean, which indicates high fat diet-induced changes in microbiota independent of obesity development (Hildebrandt et al.,
2009). The exact impact of the gut microbiota in diet-induced obesity is not completely established and it is difficult to isolate the impact of diet from that of obesity per se.

1.4 Objectives
The primary aim of this PhD study was to evaluate how the dietary composition of macronutrients influences obesity development and related metabolic disturbances in mice, with focus on both the source and amount of different macronutrients. This was more specifically aimed to determine:

- The influence of different carbohydrate sources on the reported anti-obesity and anti-inflammatory effects of n-3 PUFAs.
- How the dietary intake of salmon fillets, fed different vegetable oils, affect obesity development and insulin resistance in mice.
- How the dietary macronutrient composition affects accumulation of POPs in adipose tissue and the association with obesity development.
- The long-term impact of high fat diets with different protein:carbohydrate ratio on gut microbiota and obesity development.
- The effect of different protein sources on obesity development in high fat high protein diets.
2.0 LIST OF ARTICLES


3.0 SUMMARY OF RESULTS

PAPER I: High-glycemic index carbohydrates abrogate the anti-obesity effect of fish oil in mice

- The macronutrient composition of the diet modulates the effects of fish oil in mice.
- Fish oil combined with sucrose, glucose, or high-GI starch promotes obesity and abrogate the reported anti-inflammatory actions of fish oil. More specifically demonstrated by:
  - Increasing amounts of sucrose in the diet dose-dependently increase energy efficiency and white adipose tissue (WAT) mass, further linked to:
    - High dietary levels of sucrose repress expression of Ucp1 in WAT, reduce the expression of genes involved in gluconeogenesis and increase expression of Fasn involved in lipogenesis in liver.
  - Replacing sucrose or glucose with fructose reduces obesity development, and further:
    - Reduces feed efficiency and levels of plasma glucose and insulin in fed state.
    - Induces expression of Ucp1 and other brown marker genes in inguinal WAT.
    - Increases hepatic expression of genes involved in gluconeogenesis and lipogenesis, and reduces expression of genes related to fatty oxidation.
    - Reduces expression of genes related to inflammation in WAT.
  - High GI starch intake induces higher WAT mass and plasma insulin compared to low GI starch.
    - High GI starch induces expression of lipogenic genes in liver, inflammatory marker genes in WAT and represses expression of Ucp1 in inguinal WAT
  - Glybenclamide administrated to induce insulin secretion leads to:
    - No effects on obesity development.
    - Improved glucose tolerance, increased expression of Pparg in WAT, reduced hepatic expression of genes related to gluconeogenesis, amino acid degradation and ureagenesis
  - Nifedipine included in the diet to reduce insulin secretion leads to:
    - Reduced plasma insulin in the fed state, WAT mass and adipocyte diameter in epididymal WAT, glucose tolerance and improved insulin sensitivity.
In conclusion, the results imply that glycemic control of insulin secretion modulates metabolic effects of fish oil by demonstrating that high-GI carbohydrates attenuate the anti-obesity effects of fish oil.

PAPER II: Intake of Farmed Atlantic Salmon Fed Soybean Oil Increases Insulin Resistance and Hepatic Lipid Accumulation in Mice

Replacement of fish oil with vegetable oils, rapeseed oil, olive oil or soybean oil, in feed to Atlantic Salmon leads to:
- Reduced n-3/n-6 polyunsaturated fatty acid ratio and level of persistent organic pollutants (POPs) in the salmon fillets and in the tissue of mice fed Western diets including the salmon fillets.
- Aggravated insulin resistance and increased hepatic lipid accumulation in mice fed Western diets with fillets from salmon fed soybean oil, rich in linoleic acid (LA).
- No difference in body weight gain or adipose tissue mass.

In conclusion, the results indicate that the content of linoleic acid in vegetable oils for salmon feed may be a matter of concern that warrants further investigation.

PAPER III: Macronutrient composition determines total accumulation of persistent organic pollutants from dietary exposure in adipose tissue of mice

The dietary composition of macronutrients modulates the accumulation of several persistent organic pollutants (POPs) in adipose tissue of mice.

A high protein:sucrose ratio in mice fed high fat diets leads to:
- Reduced total accumulation of POPs from dietary exposure in the adipose tissue of mice.
- Enhanced hepatic expression of genes involved in metabolism and elimination of xenobiotics and protection from obesity development.

In conclusion, the results suggest that the macronutrient composition is an important factor regulating the body burden of POPs.
PAPER IV: Effect of a long-term high protein diet on lifespan, obesity development and gut microbiota in mice

- A long-term high dietary protein:sucrose ratio in mice leads to:
  - Attenuated life-long high fat diet-induced weight gain, adipose tissue expansion, hepatic lipid accumulation, mortality and initially also expression of inflammatory markers.
  - Partly prevention in the over-time changes in global gene-expression in liver and adipose induced by a high fat high sucrose diet.
- The long-term dietary fat intake is more important than the protein:sucrose ratio in alterations of gut microbiota composition, and there is also a marked alteration in the microbiota during time.
- In conclusion, the results suggest that a long-term high protein intake have sustained life-long beneficial effects on high fat diet-induced obesity development, related metabolic disturbances and reduction of lifespan. While, dietary fat rather than adiposity appear to be a major driver shaping the gut microbiota.

PAPER V: The protein source determines the potential of high protein diets to attenuate obesity development

- The source and amount of protein influence development of obesity and insulin resistance in mice.
- A high protein:sucrose ratio attenuates obesity development, independent if the protein source is derived from casein or pork.
- Different protein sources have great impact on the outcome of obesity development in mice fed high fat and high protein diets, demonstrated by:
  - A high proportion of casein prevents obesity development, reduced glucose tolerance and insulin resistance.
  - Mice fed a high proportion of cod, beef, chicken or pork protein gained a substantial amount of adipose tissue, became glucose intolerant and mice fed beef, chicken and pork were also insulin resistant.
- The alterations observed in obesity development due to different protein sources could, at least partly, be a result of the impact on the brown adipose tissue, demonstrated by:
  - Maintenance of a brown phenotype in the classical interscapular brown adipose depot, evident in the mice fed casein.
SUMMARY OF RESULTS

- Mice fed pork had morphological transformation of the interscapular brown adipocytes, displayed as larger intracellular fat droplets and low UCP1 immunoreactivity.

➢ In conclusion, the results indicate that casein is not representative for all high protein diets, due to great differences on obesity development between high fat and high protein diets based on casein compared to proteins from animal source.
4.0 DISCUSSION OF RESULTS

4.1 The impact of the dietary fat level on obesity development

High fat intake is often suggested as the main dietary contributor in the worldwide obesity epidemic (Astrup et al., 2000; Bray and Popkin, 1998; Hariri and Thibault, 2010; Hill et al., 2000). However, the health impact of dietary fat has been a matter of debate and contradictory evidence has been reported (Austin et al., 2011; Seidell, 1998; Willett, 2002). The focus and advice aimed to reduce dietary fat intake have, by some, actually been referred to as a great misinterpretation and mistake (Willett, 2002). We have investigated how a high dietary fat intake modulates obesity development in combination with other macronutrients. Overall, our results demonstrate that a high fat intake combined with high levels of sucrose leads to weight gain in mice compared to a low fat diet (Paper I-V). The obesity promoting effect of a high fat high sucrose diet is demonstrated for diets based on either fish oil (Paper I), corn oil (Paper III-V) or Western diets combined with a mixture of fat derived from salmon and milk fat (Paper II). The obesogenic potential of high dietary levels of fat and sucrose is further evident by an increased feed efficiency compared to low fat diets, demonstrated in all the animal trials (Paper I-V). A high fat intake also leads to marked alterations in obesity development due to the dietary background levels of carbohydrates and/or proteins (Paper I and V).

4.2 The effect of marine n-3 PUFAs is dependent on other dietary macronutrients

The literature on human studies is inconclusive regarding the beneficial effects of n-3 PUFAs on obesity and related metabolic disturbances. However, the effect of n-3 PUFAs to reduce plasma triglycerides is generally evident in most studies with human subjects (Flachs et al., 2014). The impact of marine derived fatty acids on obesity seems to be highly dependent on the composition of other nutrients, such as the dietary level of sucrose (Ma et al., 2011) and n-6 PUFAs (Alvheim et al., 2012), suggested to be part of the explanation for the various outcomes of human studies (Madsen and Kristiansen, 2012).

Despite inconclusive effects in humans, several animal studies support a beneficial effect of marine n-3 PUFAs related to obesity, insulin resistance, dyslipidemia and inflammation (Huber et al., 2007; Rokling-Andersen et al., 2009; Ruzickova et al., 2004; Samane et al., 2009; Storlien et al., 1987; Todoric et al., 2006). We demonstrate here that the effect of fish oil is dependent on the level of other nutrients in the diet (Paper I and II). Using fish oil in the diet combined with high GI carbohydrates such as sucrose, glucose or high GI starch induce elevated circulating levels of insulin and promote obesity development (Paper I). The inhibitory effect
DISCUSSION OF RESULTS

of dietary n-3 PUFAs on lipogenesis (Flachs et al., 2005) and inflammation (Todoric et al., 2006) is abrogated by a concomitant intake of high GI carbohydrates (Paper I). The inclusion of high GI carbohydrates leads to an increase in lipogenesis and adipose tissue inflammation (Paper I). Thus, increasing amounts of high GI carbohydrates abolish the beneficial impact of fish oil. Although, fish oil still presents a positive effect on inhibition of hepatic lipid accumulation despite high intake of GI carbohydrates (Paper I-III), in accordance with earlier results (Ma et al., 2011).

In addition to being affected by the intake of carbohydrates, the outcome n-3 PUFA intake is also demonstrated to be dependent on the n-3/n-6 PUFA ratio (Alvheim et al., 2012). Our results demonstrate that the n-3/n-6 PUFA ratio in the fish fillets also can be modulated through the fish feed, and further impact the consumer (Paper II). Replacement of fish oil with soybean oil in the salmon feed, reduced the n-3/n-6 PUFA ratio and increased especially linoleic acid (LA) levels, in the salmon fillet used in the mouse diets (Paper II). The change in fatty acid composition was reflected in the red blood cells in the mice and associated with reduced insulin sensitivity.

The results from animal studies are generally more conclusive than human studies, regarding beneficial effects of n-3 PUFAs on obesity (Flachs et al., 2014). The significant impact of other dietary nutrients to influence the effect of n-3 PUFAs seems to be a possible explanation for conflicting results, as stated earlier (Madsen and Kristiansen, 2012). Further, the dietary form of n-3 PUFAs can modulate the bioavailability and affect the outcome on obesity (Rossmeisl et al., 2012). In animal trials, all ingested nutrients are known and the environmental conditions highly controlled. However, this is not obtainable to the same degree in human trials and could lead to diverging results obtained between animals and humans. Another suggested reason for contradictory effects in rodents, compared to humans are the differences in peroxisome proliferator-activated receptor α (PPARα) signaling (Flachs et al., 2014; Rakhshandehroo et al., 2009). In rodents, PPARα activation stimulates peroxisomal β-oxidation promoting an anti-obesity effect of n-3 PUFAs (Fiamoncini et al., 2013), whereas in humans, PPARα does not stimulate formation of peroxisomes to the same degree as in rodents (Cheung et al., 2004; Delarue et al., 2004).

4.3 High fat high sucrose induced obesity development

High dietary levels of fat and sucrose induce obesity and related metabolic disturbances in mice, independent of the dietary n-3/n-6 PUFA ratio (Paper I-V). A high dietary level of fat combined with a low protein:carbohydrate ratio, leads to stimulation of lipogenesis and inhibition of
catabolic and energy consuming processes, including amino acid degradation, urea synthesis and gluconeogenesis (Paper I and IV). High dietary levels of sucrose also induce obesity-related low-grade inflammation in white adipose tissue of mice fed diets enriched in either n-3 or n-6 PUFAs (Paper I and IV). Further, the plasma lipid profile is negatively affected by high dietary levels of sucrose and fat, displayed by an increase in plasma cholesterol levels in mice after 3 and 18 months of feeding (Paper IV). However, a deteriorated plasma lipid profile is only demonstrated for diets enriched with n-6 PUFAs (Paper IV), while sucrose intake combined with fish oil do not display any significant impact on plasma lipids in mice compared to a low fat intake (Paper I). Further, the marine n-3 PUFAs prevent hepatic lipid accumulation despite a high sucrose intake (Paper II), but a high fat diet enriched in n-6 PUFAs and sucrose promotes accumulation of lipids in liver (Paper III and IV). Increased lipid accumulation in liver is related to reduced insulin sensitivity (Muoio and Newgard, 2008), and both features are present in mice fed high fat diets with n-6 PUFAs and high levels of sucrose (Paper II and III).

Overall, the combination of high dietary levels of fat and sucrose induces obesity development and related metabolic disturbances, including increased inflammation in adipose tissue and alterations in metabolic processes, independent of the fat source. In accordance with earlier studies (Ma et al., 2011), the high fat high sucrose induced accumulation of lipids in liver and plasma, together with reduced insulin sensitivity, are only demonstrated when the dietary fat is enriched with n-6 PUFAs, prevented by marine n-3 PUFAs (Paper II-IV).

4.3.1 Persistent organic pollutants (POPs)
Dietary fat intake leads to the exposure of persistent, fat-soluble pollutants. The highly persistent organic pollutants (POPs) bio-accumulate through the food chain and into fat-rich foods from marine and animal sources, further contributing as a source of dietary exposure. POPs have recently been reported as a factor contributing to obesity development and insulin resistance (Arsenescu et al., 2008; Grun and Blumberg, 2007; Lee et al., 2014; Newbold, 2010; Ruzzin et al., 2010; Wahlang et al., 2013). However, our results do not demonstrate any induction of obesity caused by dietary POP exposure in mice (Paper III). The total amount of POPs accumulating in the adipose tissue is positively related to the adipose tissue mass (Paper III), supporting the correlation reported between increased levels of POPs and obesity (Dhooge et al., 2010; Lee et al., 2012; Ronn et al., 2011; Roos et al., 2013). However, obesity developed in accordance with changes in the dietary macronutrients and was not further affected by the inclusion of POPs. Higher accumulation of POPs, may be promoted by an expanding adipose tissue mass, or by the change in macronutrients. High intake of dietary proteins reduced POP
accumulation and adiposity, but also increased expression of genes related to xenobiotic metabolism and excretion (Paper III).

4.4 High protein intake

High fat high sucrose induced obesity can be prevented by replacing sucrose with proteins (Paper I, III, IV and V). A dose-dependent increase in adiposity was found when sucrose replaced the level of proteins in a high fat diet (Paper I), which also demonstrates that increased dietary levels of protein dose-dependently reduces the obesogenic potential of a high fat diet (Paper I). A long-term intake of high protein diets, with casein, displays a sustained reduction in body weight and adipose tissue mass in mice fed high fat diets (Paper IV). In summary, replacing high levels of sucrose with proteins can reverse the adipogenic potential of a high fat diet, at least when the protein source is casein. While intake of sucrose leads to an induction in lipogenesis (Paper I and IV), replacing sucrose with proteins prevents an increase in lipogenesis (Paper I and IV). Further, intake of proteins at the expense of sucrose up-regulate energy consuming processes, including gluconeogenesis, amino acid degradation and urea synthesis, in mice fed high fat diets (Paper I and IV), in agreement with previous studies (Ma et al., 2011; Madsen et al., 2008).

4.4.1 Different protein sources

Casein is a widely used protein source in animal studies, but other relevant protein sources present in a human diet deserve attention. This is especially important, as high protein diets are increasingly popular in weight management. Proteins from animal sources are commonly present in our diet and are therefore of particular importance to evaluate. We demonstrate that different protein sources modulate obesity development (Paper V). High fat high protein diets based on animal proteins, beef, chicken or pork, were highly obesogenic and induced significantly higher adipose tissue mass also compared to a casein based high fat diet with sucrose. Overall, our results demonstrate that the adipogenic potential of a high fat diet is not only determined by the protein:sucrose ratio but also dependent on the protein source.

Proteins derived from casein are especially high in branched chain amino acids (BCAAs), leucine, isoleucine and valine (Paper V). Earlier studies have demonstrated a reduction in obesity by the intake of leucine or BCAAs in general (Bianchi et al., 2005; Layman and Walker, 2006; Zhang et al., 2007), however, a more recent study demonstrated an induction of insulin resistance by the intake of BCAAs with a high fat diet (Newgard et al., 2009). Further, several studies demonstrate elevated plasma levels of BCAAs related to obesity and insulin resistance (Adams, 2011; Newgard et al., 2009). Our results demonstrate prevention from both high fat
diet induced obesity development and insulin resistance in response to a high intake of casein, the protein source investigated with the highest BCAA level (Paper V). Overall, we did not find any elevations in plasma levels of BCAAs in associated with either obesity or insulin resistance.

4.4.2 Nonshivering thermogenesis

Alterations in the dietary composition modulated the expression of genes involved in nonshivering thermogenesis, indicating a dietary impact on the activity of nonshivering thermogenesis (Paper I, IV and V). Increased levels of high GI carbohydrates suppress gene expression of Ucp1 and other brown adipose tissue (BAT) markers in the white adipose tissue (WAT) of mice (Paper I), while replacement of carbohydrates with casein proteins restores “browning” of WAT (Paper I). Of note, the long-term effect of a high protein intake on WAT “browning” is unclear (Paper IV).

We did not detect any differences in gene expression of BAT markers in white adipose tissue of mice fed high fat diets together with high levels of protein from different sources. However, these mice were fasted for 4 hours, and this could have contributed to repressed expression of genes related to nonshivering thermogenesis (Paper V), as the activity of nonshivering thermogenesis is reported to be inhibited during fasting (Cannon and Nedergaard, 2004). The relative mRNA expression does not necessarily always correlate with the protein levels due to rapid feed-back regulations, and a time-delay is reported to account for an acute decrease in mRNA UCP1 levels that is not associated with an equal change in protein level (Jacobsson et al., 1994; Nedergaard et al., 2001; Puigserver et al., 1992). Further evaluating UCP1 in the brown adipose tissue by the use of immunohistochemistry, revealed significantly higher UCP1 expression in BAT of mice fed high fat diets with high intake of proteins. This was more pronounced in mice fed proteins from casein compared to pork (Paper V). Thus, the changes of UCP1 in BAT were positively associated with reduced adiposity, which could indicate a protection from obesity development due to a more active BAT. The potential impact of dietary proteins to induce nonshivering thermogenesis in BAT, might be an additional way of high protein diets to reduce feed efficiency and contribute to weight reduction. However, the effect of high dietary levels of protein to activate BAT was also dependent on the protein source (Paper V). High levels of sucrose and/or the use of pork as protein source led to reduced levels of UCP1 in BAT. Further histological examination also revealed morphological alterations in the brown adipose tissue, displaying large intracellular fat droplets, indicating an inactivated and more “white-like” BAT. This suggests that while a high protein intake from casein
maintains a more active BAT, a high sucrose intake and/or proteins derived from pork lead to a “whitening” of BAT.

4.5 The impact of dietary fat on gut microbiota and lifespan

Recently, the gut microbiota has been related to changes in diet and obesity, but the impact on gut microbiota is difficult to distinguish from effects of diet or obesity per se. Our results aimed to investigate the long-term effects of high fat diets with a different protein:sucrose ratio on the microbiota profile. While evidence from the literature links a change in the gut microbiota to both obesity and dietary alterations (David et al., 2014; Le Chatelier et al., 2013; Ley et al., 2008; Muegge et al., 2011; Turnbaugh et al., 2006; Wu et al., 2011), our study revealed the greatest change in microbiota due to dietary alterations and not obesity development (Paper IV). The most clear changes were evident as a result of increased levels of dietary fat, but less affected by the protein:sucrose ratio and thus the obese state. This is in agreement with an earlier published study, demonstrating that high fat diet induced changes in microbiota was independent of obesity development (Hildebrandt et al., 2009).

Lifespan is negatively correlated with an increase in adipose tissue mass (Picard and Guarente, 2005), demonstrated by caloric restriction (Weindruch, 1996; Weindruch et al., 1986) or mutations in the pathway of insulin signaling (Bluher, 2008; Bluher et al., 2003; Katic and Kahn, 2005). In regards to the impact of macronutrient composition, the replacement of a high protein intake with carbohydrates was reported to be beneficial (Solon-Biet et al., 2014). However, another study displayed reduced longevity in response to a high fat intake, but less pronounced in combination with a high protein intake compared to a high carbohydrate intake (Keipert et al., 2011). Our results are in agreement with this last study, demonstrating a reduction in longevity upon high fat feeding compared to a low fat diet (Paper IV). The reduced longevity upon high fat feeding was only evident when combined with a high sucrose intake, but not high protein intake. Thus, our results indicate a reduction in lifespan related to increased obesity/fat mass by alterations in the macronutrient composition.

4.6 The global obesity epidemic related to alterations in dietary intake

The global obesity epidemic has been associated with dietary and lifestyle changes, characterized by increased consumption of animal fat and proteins, refined grains, added sugar and a more sedentary lifestyle (Malik et al., 2013). With an overall view on the dietary macronutrients, a meta-analysis of intervention trials led to the conclusion that diets with a low content of fat and sugar-rich beverages, and rather high content of carbohydrates, fiber, grains and protein, were optimal in regards to prevention of obesity, diabetes and cardiovascular
DISCUSSION OF RESULTS

disease (Astrup, 2005). With the aim of weight loss in already obese persons, a carbohydrate-
restricted diet was more efficient compared to a calorie- and fat-restricted diet. This was also
accompanied with improved insulin sensitivity and plasma triglyceride levels (Samaha et al.,
2003). Paper I-V demonstrate that inclusion of sugar or other high GI carbohydrates promote
obesity in a high fat diet, irrespective of fat source. The replacement of high GI carbohydrates
with proteins from casein prevents high fat diet induced obesity development, demonstrated in
Paper I, III, IV and V. Further, the source of proteins is also decisive for the impact of high fat
diet induced obesity (Paper V).

The increased consumption of animal derived food products is not sustainable during a long-
term perspective. Over the last 50 years, the consumption of meat, dairy and fish have increased,
leading to great impact and loss of terrestrial and marine biodiversity (Westhoek et al., 2011).
Results from Paper II highlight challenges related to the replacement of fish feed ingredients
for a sustainable fish production, and further emphasize that we also should be aware on the
effect of this replacement. However, while the use of soybean oil in the fish feed may lead to
adverse outcomes on the consumer, the use of rapeseed oil could potentially be a sustainable
and preferable replacement for fish derived raw materials in the future salmon production.

The consumption of foods from animal sources have been increased with three to four times
between 1989 and 2000 (Malik et al., 2013; Popkin, 2006). Alterations aimed to reduce the
environmental pressure and the reduction in the consumption of animal products in Europe is
highlighted to be of importance (Westhoek et al., 2011). In addition to the environmental
benefits, there are also possible health gains into reducing the consumption of animal products
(Malik et al., 2013; Westhoek et al., 2011). Paper V demonstrates that the intake of animal
derived proteins, especially chicken and pork, are more adipogenic than other protein sources
such as casein in mice (Paper V). This indicates a potential obesity promoting effect of animal
derived proteins, in accordance with the increased prevalence of obesity related to the intake of
animal derived food products (Malik et al., 2013).

Moreover, the dietary intake of foods from animal origin also constitutes an increased risk for
exposure of POPs (Boada et al., 2014), and the estimated intake of POPs from dietary sources
have been linked to higher incidence of obesity (Donat-Vargas et al., 2014). It is difficult to
determine whether POPs are causing obesity or if nutrients in the dietary food sources are
involved. Blood levels of POPs correlate with obesity and related diseases in many studies
(Codru et al., 2007; Dhooge et al., 2010; Lee et al., 2006; Lee et al., 2012; Ronn et al., 2011;
Roos et al., 2013; Taylor et al., 2013), but the causative relationship between body burden of
POPs and obesity is unknown. Higher intake of animal fat and proteins are dietary factors directly related to the increasing obesity burden worldwide (Malik et al., 2013). Moreover, a higher exposure of POPs are evident with the intake of animal derived food sources (Boada et al., 2014), and increased half-life of POPs is reported with higher body fat (Milbrath et al., 2009). Overall, a close link between the body burden of POPs and obesity is anticipated. Thus, the intake of an obesogenic, animal derived diet, leading to expansion of adipose tissue mass and higher accumulation of POPs, could possibly be a causal explanation for the link between elevated levels of POPs and obesity.
5.0 CONCLUSIONS

The results from Paper I-V demonstrate that the dietary composition and source of macronutrients determine the development of obesity and related metabolic disturbances, including insulin resistance, dyslipidemia, hepatic lipid accumulation and inflammation in mice, illustrated in Figure 2.

- A low protein:sucrose ratio attenuates high fat diet induced obesity development, which further is modulated by the protein source.
- The effect of marine n-3 PUFAs is dependent on the dietary level of other nutrients in the background diet, including n-6 PUFAs, carbohydrates and proteins.
- The dietary macronutrient composition impact lifespan, gut microbiota and determines the accumulation of POPs in adipose tissue of mice from dietary exposure.
- The dietary macronutrients possibly modulate obesity and related diseases through impact on metabolic processes including lipogenesis, gluconeogenesis, amino acid degradation and activity of brown adipose tissue and/or “browning” of white adipose tissue.

*Figure 2: The figure summarizes the obesity related disturbances (blue boxes) influenced by changes in the dietary macronutrient composition (grey boxes) demonstrated in Paper I-V.*
6.0 FUTURE PERSPECTIVES

In light of the results gained in Paper I-V, new questions are also raised. The composition of macronutrients demonstrate a great impact on obesity and related metabolic disturbances, but many answers still remain to be elucidated.

We have demonstrated how different macronutrients modulate obesity in mice, in regards to both the source and composition. The long-term intake of high fat diets with altered levels of protein:sucrose ratio is determined, however this was only performed by the use of casein as the protein source. We further demonstrate how different protein sources modulate obesity, although only during short term feeding. Thus, the effect of long-term intake of high fat diet could possibly also be dependent on the protein source and not only the protein:sucrose ratio. This emphasizes the importance of evaluating how a long-term intake of different protein sources impact obesity development, gut microbiota and also lifespan.

The diet constitute a complex matrix, including the known and preferable nutrients, but also unknown and undesirable substances. This further highlights the potential, but still fairly unknown, interactions between nutrients and other undesirable compounds like persistent organic pollutants, which possibly can provide valuable knowledge related to obesity and associated diseases.

The gut microbiota is a rather new field of study in association with diet-induced obesity development. We demonstrate that the dietary level of fat is an important determinant in alterations of the gut microbiota, but the impact of other dietary substances such as persistent organic pollutants are not determined. The possible impact of dietary POP exposure on gut microbiota, and/or the possible impact of the gut microbiota on the breakdown and accumulation of POPs could be of value to determine.

The use of animal trials to evaluate the effect of different nutrients provide a valuable tool to study how nutrients affect different tissues under a controlled environment. The use of animals can provide valuable knowledge on the outcome and further mechanisms of different treatments, but the results and findings always need to be verified in humans before any conclusions regarding the impact in humans are drawn. Thus, our results from animal trials remain to be validated in humans, but indicate potentially important effects of the dietary composition in obesity and related disturbances.
7.0 REFERENCES


REFERENCES


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8.0 APPENDIX


High-glycemic index carbohydrates abrogate the antiobesity effect of fish oil in mice

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Fish oil rich in n-3 polyunsaturated fatty acids is known to attenuate diet-induced obesity and adipose tissue inflammation in rodents. Here we aimed to investigate whether different carbohydrate sources modulated the antiobesity effects of fish oil. By feeding C57BL/6J mice isocaloric high-fat diets enriched with fish oil for 6 wk, we show that increasing amounts of sucrose in the diets dose-dependently increased energy efficiency and white adipose tissue (WAT) mass. Mice receiving fructose had about 50% less WAT mass than mice fed a high fish oil diet supplemented with either glucose or sucrose, indicating that the glucose moiety of sucrose was responsible for the obesity-promoting effect of sucrose. To investigate whether the obesogenic effect of sucrose and glucose was related to stimulation of insulin secretion, we combined fish oil with high and low glycemic index (GI) starches. Mice receiving the fish oil diet containing the low-GI starch had significantly less WAT than mice fed high-GI starch. Moreover, inhibition of insulin secretion by administration of nifedipine significantly reduced WAT mass in mice fed a high-fish oil diet in combination with sucrose. In conclusion, our data indicate that glycemic control of insulin secretion and the reported anti-inflammatory actions of fish oil are abrogated. In mice fed a fish oil-enriched diet in combination with sucrose had markedly higher feed efficiency and required less than 50% of the calories to achieve the same weight gain as mice fed a fish oil-enriched diet in combination with protein.

A major difference between proteins and sucrose is the ability of sucrose to elicit a rise in blood glucose and stimulate insulin secretion. Insulin is a powerful anabolic hormone that stimulates adipocyte differentiation and adipose tissue expansion (41), and activation of insulin signaling is crucial for the development of obesity (9). Moreover, increased insulin signaling by transgenic expression of insulin receptor substrate-1 is sufficient to induce obesity (52). Thus, it is possible that increased insulin signaling and glucose uptake in adipose tissue in sucrose-fed mice may override the anti-inflammatory and antiobesity effects of fish oil.

The glucose moiety of sucrose is responsible for the rise in blood insulin upon intake of sucrose because fructose, unlike glucose, is unable to stimulate insulin secretion (16). This in part relates to the very low levels of Slc2a5 [solute carrier family 2 (facilitated glucose transporter), member 5, GLUT5] in pancreatic β-cells (64). Furthermore, fructose does not stimulate the release of gastric inhibitory peptide, which stimulates insulin secretion indirectly (27, 67). Thus, the ability of sucrose to counteract the beneficial effects of fish oil seems to relate to a glucose-dependent stimulation of insulin secretion. The fructose moiety of sucrose may further modulate the effect of fish oil on the development of obesity. Thus, the increased consumption of fructose over the past decades has been linked to development of metabolic disorders (59), and fructose is routinely used to induce glucose intolerance in rats (70).

Because different types of starch differ in their ability to increase postprandial blood glucose and insulin secretion, different types of starch may also modulate the effect of fish oil. The glycemic index (GI) is a measurement of the ability of different types of carbohydrate-based foods to raise blood glucose levels within 2 h (34). The interest in low-GI diets as a tool in weight management is increasing. Although reviews and meta-analyses conclude that such diets may be effective, their efficiency in terms of lasting weight reduction is still a matter of debate (3, 5, 20, 38, 68). Different types of starches with different GI are known to induce different responses in plasma glucose and insulin in rodents (57). However, it is unknown whether different types of starches modulate the effects of fish oil-enriched diets. Here, we have performed systematic analyses to investigate the influence of different

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The mice were fed for 4–8 wk as indicated. In separate experiments, mice were treated with nifeidine (N7634; Sigma-Aldrich, St. Louis, MO) or glybenclamide (G0639; Sigma-Aldrich). Nifeidine was included in the diets at a dose (1 g/kg) that was demonstrated earlier to reduce plasma insulin levels in agouti mice (37). The sulfonylurea glybenclamide was used as an insulin secretagogue and was administered daily by intraperitoneal (ip) injection at a dose of 2 μg/g body wt. Control mice received placebo by daily ip injection. The mice were fed the experimental diets and treated with nifeidine and/or glybenclamide after 1 wk of acclimatization, with free access to a standard low-fat chow diet. Mice fed nifeidine-containing diets or treated with glybenclamide were pair-fed and were terminated after 4 wk of treatments. In experiments where glybenclamide or nifeidine was included, experimental feeding time was reduced to 4 wk to limit possible adverse effects resulting from increased or decreased insulin secretion/signaling.

At the end of all experiments, mice were euthanized in the fed or fasted state by cardiac puncture under anesthesia with isoflurane (Iso-ba-vet, Schering-Plough, Denmark) using the Univentor 400 Analgesia Unit (Univentor), and plasma was prepared from blood. Liver, tibialis anterior muscle, and adipose tissues were dissected out, weighted, freeze-clamped, and frozen at −80°C. The epididymal white adipose tissue (eWAT) depots were selected as representative visceral depots, whereas inguinal brown adipose tissue (iBAT) was taken from the anterior subcutaneous interscapular region (12). Brown interscapular brown adipose tissue (iBAT) was taken from the anterior subcutaneous interscapular region (12). All mice experiments were approved by National Animal Health Authorities (Denmark and Norway). Adverse events were not observed.

**Glucose and insulin tolerance tests.** In glucose tolerance tests (GTT), mice were fed-deprived for 6 h before injection of 2 g/kg glucose in saline. In insulin tolerance tests (ITT), mice were fed-deprived for 4 h before ip injection of 0.5 U/kg human recombinant insulin (Actrapid; Novo Nordisk, Bagsværd, Denmark) in saline. Blood was collected from the lateral tail vein at indicated time points, and blood glucose was measured with a Bayer Contour glucometer (Bayer).

**Energy digestibility and indirect calorimetry.** Male C57BL/6J BomTac mice (n = 5 for each group) were adapted to experimental diets ad libitum for 4 days. Feces, urine, and feed residues were collected during a 24-h period before respiration measurements. The crude protein (N × 6.25) content in feed, feces, and urine was determined.
using the Tecator-Kjeltec system 1026 (Foss Tecator, Höganäs, Sweden). The gross energy content of feed and feces was determined using an adiabatic bomb calorimeter (System C700; IKA Analysetechnic, Heitersheim, Germany). An open-air circuit system (Oxymax; Columbus Instruments, Columbus, OH) consisting of two respiration chambers was used to measure 22-h respiratory gaseous exchange.

Plasma analyses. Insulin, glucose (44), and lipid metabolites (39) were measured in plasma, as described previously. IL-6 (no. KNC0061; Invitrogen), TNFα (no. KMC3011; Invitrogen), adiponectin (no. EZMADP-60K; Millipore), and leptin (RD291001200R; BioVendor, Brno, Czech Republic) were measured using ELISA kits.

Tissue lipid analyses. Lipid classes (39) and fatty acid composition (2) were analyzed as described previously.

Histology. Sections of adipose tissue were fixed, dehydrated, embedded in paraffin blocks, cut into 3-μm-thick sections, and stained with eosin and hematoxylin, as described previously (4). Sections were visually examined using an Olympus BX 51 binocular microscope (Olympus, Tokyo, Japan) fitted with an Olympus DP50 3.0 camera.

Quantitative reverse transcriptase PCR. RNA purification and quantitative reverse transcriptase PCR (qRT-PCR) were performed as described earlier (45). Primers for qRT-PCR were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA) and are presented in Table 3.

Statistical analyses. All data represent means ± SE or means + SE. All data sets were tested for homogeneity of variances using Levene’s test. Data were then analyzed using ANOVA post hoc pairwise comparisons. Homogenous data sets were analyzed using a Tukey HSD test. Nonhomogenous data sets from qRT-PCR analyses were log-transformed and retested for homogeneity of variances using Levene’s test. Data sets that remained nonhomogenous were analyzed using the Kruskal-Wallis test. The use of a Tukey HSD test following Levene’s test. Data sets that remained nonhomogenous were analyzed using an ad hoc parametric test specifically mentioned in the figure and/or table legends. A value of P < 0.05 was considered statistically significant. The Statistica 9.0 software (StatSoft, Tulsa, OK) was used for statistical analyses.

RESULTS

Sucrose dose-dependently counteracts the obesity-protective effect of fish oil. To investigate whether sucrose dose-dependently counteracted the obesity-protective effect of fish oil, male C57BL/6J mice were fed isocaloric high-fat diets with different sucrose/protein ratios (Table 1) for 6 wk. Earlier studies have demonstrated that a low sucrose/protein ratio slightly reduced feed intake in mice (40). To secure equal energy intake, all groups were pair fed. In this particular case, all groups received the same amount of feed as that consumed by the mice receiving the lowest sucrose/protein ratio. Body weight gain and white adipose tissue (WAT) mass increased in parallel with the increase in the sucrose/protein ratio in the feed (Fig. 1, A, B, and E). The weights of iBAT, liver, and tibialis anterior muscle were not influenced by the different feeds (not shown). We have demonstrated earlier that increasing the amount of sucrose in the diet increased the respiratory exchange ratio (RER) but did not significantly reduce O2 consumption (40). However, weight loss during 24-h feed deprivation was reduced by increasing the amount of sucrose in the feed (Fig. 1C). Because total energy intake was kept equal, energy efficiency increased dose dependently in response to the increased amount of dietary sucrose (Fig. 1D).

Expression of inflammatory markers such as Serpine1 [serine (or cysteine) peptidase inhibitor, clade E, member 1] or plasminogen activator inhibitor-1 (PAI-1), Ccl2 [CCL2 chemokine (C–C motif) ligand 2, MCP1], and Cd68 (CD68 antigen) was increased in both eWAT and iWAT in the obese mice, suggesting that sucrose attenuated the anti-inflammatory effect of fish oil (Fig. 1F). Expression of Emr1 (epidermal growth factor-like module containing, mucin-like, hormone receptor-like sequence 1, F4/80) was increased significantly in iWAT only (Fig. 1F). The expression of Pparg [peroxisome proliferator-activated receptor-γ (PPARγ)], Srebf1 [sterol regulatory element-binding transcription factor 1c (SREBP1c)], and Fasn (fatty acid synthase) did not change significantly (Fig. 1F).

In iBAT, Ucp1 (uncoupling protein 1) expression was similar in all groups (Fig. 1G), but the expression of Ucp1 was significantly lower in iWAT in mice fed the high amount of sucrose compared with mice fed the low amount of sucrose (Fig. 1F). This indicates that adipocytes in iWAT from mice receiving a low amount of sucrose and a high amount of protein had a more brownish phenotype.

Sucrose dose-dependently reduced expression of Ppargc1a (peroxisome proliferator-activated receptor-γ, coactivator 1α), Pck1 (phosphoenolpyruvate carboxykinase 1, cytosolic), and Agrp (alanyl-glyoxylate aminotransferase) in the liver (Fig. 1H). Expression of the lipogenic gene Fasn was increased, whereas expression of enzymes involved in fatty acid oxidation such as Acox1 (acyl-CoA oxidase 1, palmitoyl) and Cpt1a (carnitine palmitoyltransferase 1a) was unchanged when the sucrose/protein ratio was increased (Fig. 1H).

The glucose moiety of sucrose is responsible for the obesity-promoting effect of sucrose. To investigate whether the obesity-promoting effect of sucrose fed in combination with fish oil depended on the glucose or fructose moiety of sucrose, we prepared diets where fish oil was combined with sucrose, glucose, or fructose (Table 2). Male C57BL/6J mice were fed isocaloric high-fat diets with different carbohydrate sources ad libitum. After 8 wk, mice receiving fish oil in combination with fructose had gained less weight than mice receiving fish oil in combination with sucrose or glucose (Fig. 2A). Energy intake was not significantly different (Fig. 2B), and hence, energy efficiency was significantly lower in mice fed the fructose-supplemented diets (Fig. 2B). Calculation of digestibility demonstrated a minor but not significantly reduced digestibility of protein and fat in fructose-fed mice (Fig. 2C). Indirect calorimetric measurements were performed in a second set of mice. These measurements revealed that both O2 consumption and CO2 production were similar in all groups in both the fasted and the fed state (Fig. 2D). Still, the mice receiving fructose had ∼50% less white adipose tissue mass, iWAT, pWAT, and eWAT than mice receiving sucrose or glucose combined with a tendency toward a slight decrease in the weight of the tibialis anterior muscle (Fig. 2E).

We have previously provided evidence that the different obesogenic effect of corn oil fed in combination with either protein or sucrose is related to the effect of the macronutrient composition on hormonal status (44). Because sucrose and glucose, unlike fructose, stimulate insulin secretion, we measured plasma levels of glucose and insulin in the fed state. As expected, plasma glucose and insulin were lower in fructose-fed mice (Fig. 2F). Similar to mice fed a high-fat diet in combination with proteins, expression of Ppargc1a and Pck1 in the liver was increased (Table 4). However, unlike mice fed a high-protein diet, expression of genes involved in amino
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*Continued*
Acaca, acetyl-CoA carboxylase-1α; Acosl, acetyl-CoA oxidase 1; Agxt, alanine-glyoxylate aminotransferase; Ccl2, CCL2 chemokine (C-C motif) ligand 2; Cplt1a and Cplt2, carnitine palmitoyltransferase 1a and 2, respectively; Crem, cAMP-responsive element modulator; Cyt COXII, cytochrome c oxidase subunit II; Dio2, deiodinase, iodothyronine, type II; Emr1, epidermal growth factor-like module containing, mucin-like, hormone receptor-like sequence 1; Fasn, fatty acid synthase; Got1, glutamate oxaloacetate transaminase 1, soluble; Gpi, glutamic pyruvic transaminase, soluble; Hmgcs2, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2; Lep, leptin; Lpl, lipoprotein lipase; Pck1, phosphoenolpyruvate carboxykinase 1, cytosolic; Ppargc1α and Ppargc1β, peroxisome proliferator-activated receptor-γ coactivator 1α and 2, respectively; Scd1, stearoyl-CoA desaturase 1; Slc27a1, solute carrier family 27, member 1; Srebfl, sterol regulatory element-binding transcription factor 1; Tbp, TATA-box binding protein; Ucp1, uncoupling protein 1. *National Center for Biotechnology Information Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene).

Table 3.—Continued

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Acid degradation, such as Pck1 and Agxt, was not increased in fructose-fed mice (Table 4). Plasma levels of 2-hydroxybutyrate, a marker for fatty acid β-oxidation, were similar in all groups (Fig. 2F), but the expression of genes involved in hepatic fatty acid oxidation, such as Acosl and Cplt1a, was reduced in livers from fructose-fed mice (Table 4). Hepatic expression of lipogenic genes was higher in fructose-fed mice (Table 4). Importantly, however, excess lipid accumulation was not seen in liver or tibialis anterior muscle (Fig. 3A).

Expression of genes involved in lipid uptake and triacylglycerol uptake was not significantly different in WAT (Fig. 3C). The finding that Ucp1 expression was strongly induced in iWAT (Fig. 3C) but not iBAT (Fig. 3B) in fructose-fed mice indicates that energy, as observed in protein-fed mice, may be dissipated in the form of heat. Indeed, a higher expression of markers for brown adipocytes such as Pparg1a and Cyt COXII (cytochrome c oxidase subunit II) suggests a higher number of brown-like adipocytes in iWAT in fructose-fed mice (Fig. 3C).

Circulating levels of leptin were positively correlated with adipose tissue mass and are known to stimulate the production of TNFα and IL-6 (56). As expected, expression of Lep (leptin) decreased in WAT, and circulating levels of leptin were lower in mice fed fructose than in mice fed sucrose (Fig. 3, C and D). However, neither expression nor circulating levels of TNFα and IL-6 were significantly different between the two groups of mice (Fig. 3, C and D). Surprisingly, circulating levels of adiponectin, an anti-inflammatory adipokine inversely correlated with the state of obesity (56), were also lower in fructose- than in sucrose-fed mice (Fig. 3D). WAT expressions of Ccl2 involved in monocyte recruitment and macrophage marker genes Cd68 and Emr1 were significantly higher in mice fed fish oil in combination with glucose or sucrose than fructose (Fig. 3C). Thus, the glucose moiety of sucrose appears to be responsible for the ability of sucrose to attenuate the anti-inflammatory effect of fish oil.

Because fructose feeding is frequently used to induce glucose intolerance in rats (32, 70) and infiltrating macrophages are causally linked to the development of glucose intolerance (10, 28), a GTT was performed in mice fed the sucrose-, glucose-, and fructose-based diets. Of note, during GTT, blood glucose levels reached higher concentrations in glucose compared with fructose-fed mice, and the area under the curve was significantly higher in glucose than in both sucrose- and fructose-fed mice, although fasting glucose levels in the glucose-fed mice did not differ from that of fructose-fed mice (Fig. 3, E and F).

High-GI starch increases the obesogenic potential of fish oil. To further investigate whether other types of carbohydrates, such as starch with different capacity to stimulate insulin secretion, are able to modulate the antiobesogenic potential of fish oil, we prepared isocaloric diets (Table 2) where fish oil was combined with high or low GI starches (100% amylpectin vs. 60% amylose/40% amylpectin) previously demonstrated to induce the expected differences in postprandial blood glucose levels in a meal tolerance test when combined with a high-fat diet (13, 57). Male C57BL/6J mice were fed the isocaloric high-fat diets with different carbohydrate sources ad
libitum for 8 wk. In agreement with earlier findings (57, 65), where mice were fed high- and low-GI starches in low-fat diets, body weights were similar, but adipose tissue mass was lower in mice receiving fish oil in combination with low-GI starches (Fig. 4, A and C). Energy intake was also similar, and no significant difference in energy efficiency was found (Fig. 4D). However, mice fed fish oil in combination with the low-GI starch had significantly lower levels of plasma insulin and less WAT (Fig. 4, B and C). The reduced adiposity observed in mice receiving fish oil in combination with the low-GI starch was not due to decreased digestibility, because the digestibility of the diet containing the low-GI starch was slightly higher than the digestibility of the diet containing the high-GI starch (Fig. 4D). In the fed state, mice fed fish oil in combination with the low-GI starch consumed more O₂ and produced more CO₂ than mice fed the high-GI starch (Fig. 4F). RER was not significantly different (Fig. 4F), and expression of genes involved in fatty acid oxidation [Acox1 and Hmgcs2 (3-hydroxy-3-methylglutaryl-coenzyme A synthase 2)], gluconeogenesis (Pck1), and amino acid degradation and urea synthesis [Agxt, Got1 (glutamate oxaloacetate transaminase 1, soluble) and Cps1 (carbamoyl-phosphate synthetase 1)] in the liver was similar in the two groups of mice (Table 5). However, expression of the lipogenic genes Scd1 (stearyl-coenzyme A desaturase 1) and Fasn was reduced in mice fed low-GI starch (Table 5). Expression of Ucp1 was similar in iBAT, but expression in iWAT in mice fed the low-GI starch...
Expression of genes involved in lipid uptake and triacylglycerol syntheses was not changed significantly (Fig. 4, G).

As expected, expression of Lep (leptin) increased in WAT, and circulating levels of leptin were higher in mice fed the high-GI starch than in mice fed the low-GI starch (Fig. 4, G and H). Expression levels of Il6 was higher in eWAT in mice fed high-GI starch than in mice fed low-GI starch (Fig. 4G), but circulating levels of IL-6 were similar (Fig. 4H). Neither expression levels nor circulating levels of TNFs were significantly different in the two groups of mice (Fig. 4, G and H). Moreover, both expression and circulating levels of the anti-inflammatory adipokine adiponectin were similar (Fig. 4, G and H). However, WAT expressions of the macrophage marker genes Cdo8 and Emr1 were significantly higher in mice fed fish oil in combination with high-GI starch than fructose low-GI starch (Fig. 3C), suggesting that infiltration of macrophages is related to the state of obesity. Altogether, results from the experiments combining fish oil with sucrose, glucose, or fructose as well as high- or low-GI starches suggested that stimulation of insulin secretion decreased the antiobesogenic effect of a diet enriched with dietary fish oil.

The obesity-promoting effect of sucrose in combination with fish oil is associated with increased insulin secretion. To investigate whether increased insulin secretion is able to...
increase the antiobesogenic effect of fish oil when the carbohydrate load is low, we fed male C57BL/6J mice the high-fish oil 13% sucrose diet (Table 1) or a standard low-fat diet (Table 2) ad libitum. The sulfonyleurea glybenclamide was used as an insulin secretagogue (21, 74). Body weight gain (Fig. 5A) and energy efficiency (not shown) were not affected by glybenclamide, but the amount of white adipose tissue mass tended to increase, albeit not significantly (Fig. 5B). Moreover, the mean average diameter of the adipocytes (Fig. 5B) was not increased by glybenclamide in either eWAT (52 ± 10 vs. 45 ± 12 μm) or iWAT (35 ± 6 vs. 38 ± 4 μm). However, expression of Pparγ1a was increased significantly in both eWAT and iWAT (Fig. 5C). Because insulin levels in the fed state tended only to increase, a GTT was performed to validate whether the dose of glybenclamide used was sufficient to increase insulin levels. After 6-h feed deprivation, fasting glucose levels were similar, but as expected, glucose tolerance was improved by nifedipine treatment relative to untreated fish oil and sucrose-enriched diet had lower adipose tissue mass. The adipocytes had a normal appearance, but nifedipine attenuated the increased average diameter of the adipocytes in eWAT induced by the fish oil diet supplemented with sucrose (Fig. 6C). When nifedipine was added to a standard low fat diet, no effect on adipose tissue mass was observed (not shown). In line with unchanged energy efficiency, we did not detect any increased expression of Ucp1 in iWAT in nifedipine-treated mice (Fig. 6D). Nifedipine did not alter plasma levels of free fatty acids, triacylglycerol, glycerol, or 2-hydroxybutyrate in the fed or fasted state (Fig. 7A). Moreover, nifedipine did not increase hepatic expression of Pck1 or genes involved in lipogenesis or amino acid degradation (Fig. 7B). Thus, inhibition of insulin secretion was able to attenuate the obesogenic effect of sucrose in combination with fish oil but did not reduce energy efficiency. Blood glucose levels after 6-h feed deprivation was normalized by nifedipine, but nifedipine did not improve the reduced glucose tolerance observed after feeding fish oil in combination with sucrose (Fig. 6E). However, the ITT indicated that insulin sensitivity was improved (Fig. 6F). Together, our data indicate that inhibition of insulin secretion attenuated the adipogenic effect of sucrose in combination with fish oil. However, inhibition of insulin secretion was insufficient to reduce energy efficiency.

**DISCUSSION**

We have described previously that sucrose counteracted the anti-inflammatory effect of fish oil in adipose tissue and increased obesity development in mice (40). Here, we show that the glucose moiety of sucrose was responsible for this effect, and this effect of sucrose can be mimicked by high-GI starch. We further show that a high-fat diet combined with high-GI carbohydrates actually promoted obesity even if the diet contained 18 weight% fish oil. Thus, the background diet critically influenced the ability of fish oil to curb obesity development.

Several lines of evidence have suggested that increased insulin secretion plays a crucial role in the obesity-promoting effect of high-fat diets containing sucrose, glucose, or high-GI starch. Dietary sucrose via the glucose moiety, as well as high-GI starch, stimulates secretion of insulin from pancreatic β-cells, and inhibition of insulin secretion by administration of nifedipine attenuated the adipogenic effect of fish oil in combination with sucrose. Insulin is an important driver for adipocyte differentiation (41). Mice lacking insulin receptors in adipose tissue (FIRKO mice), are protected against obesity and remain glucose tolerant on a high-fat diet (9), and white adipose tissue mass is reduced dramatically in Irs1+/−/Irs2Δ/Δ double-knockout mice (48). Furthermore, weight gain is a well-recognized side effect of type 2 diabetic drugs that increase insulin sensitivity (25, 49). However, an increase in insulin secretion alone was insufficient to promote obesity.
development because mice receiving glybenclamide in combination with proteins and fish oil did not become obese. This finding is in keeping with the observation that a high-fat diet is unable to increase adipose tissue mass in the absence of carbohydrates (47, 50). Together, these observations suggest that hyperinsulinemia is a contributing factor to the development of obesity, and reducing hyperinsulinemia would possibly counteract obesity development.

The obesity-promoting effect of increased insulin secretion is supported further by our finding that glucose, but not...
Fig. 4. High-glycemic index (GI) starch increases the adipogenic potential of fish oil. Male C57BL/6J mice (n = 6) were fed isocaloric high-fat diets with different carbohydrate sources ad libitum for 8 wk. A: energy efficiency was calculated as body weight gain divided by feed energy intake. B: fed state glucose and insulin levels were measured in plasma. C: adipose tissue weights were recorded. D: digestibility of energy, N, OM, and fat was calculated. F: indirect calorimetry measurements were performed in separate sets of mice (n = 5). Relative gene expression was measured in iBAT (E) and eWAT and iWAT (G) using qRT-PCR and normalized to Tbp. H: circulating levels of adiponectin, leptin, TNFα, and IL-6 were measured in plasma. Data are presented as means ± SE. Data sets were analyzed using Tukey HSD test except for the following: Serpinel1 in eWAT and Ucp1 in iWAT were analyzed using Tukey HSD test after log transformation of data (P = 0.052). Different small letters denote significant differences (P < 0.05). Statistics were performed separately for each individual tissue.
fructose, is the obesity-promoting moiety of sucrose. Unlike sucrose and glucose, fructose does not stimulate insulin secretion from pancreatic β-cells (70), and mice receiving fish oil in combination with fructose had less adipose tissue mass than mice receiving fish oil in combination with glucose or sucrose. However, because the mass of adipose tissues in sucrose-fed mice was as high as in glucose-fed mice, it is not likely that fructose is able to counteract the obesity-promoting effect of glucose. Mice receiving fructose in combination with fish oil were also less glucose intolerant than mice fed fish oil in combination with sucrose or glucose. Fructose is commonly used to induce glucose intolerance in rats, and some mice strains also develop metabolic syndrome in response to high-fructose feeding (53). However, in C57BL/6J mice neither hyperinsulinemia nor hyperglycemia developed in response to high-fructose feeding (53).

Our finding that inclusion of sucrose abolished the anti-obesity effect of fish oil seems to be at variance with a recent study from Sato et al. (63), because these authors demonstrated that inclusion of 5 g/kg of the (n-3) PUFA EPA into a high-fat/high-sucrose diet reduced body weight gain in mice. The reason for this discrepancy is not clear, but differences in the dietary compositions may account for the different results. Although the amount of n-3 PUFA used in this study, 6 g/kg n-3 fatty acids, is comparable with the 5 g/kg EPA used by Sato et al. (63), they used purified EPA ethyl ester, whereas the n-3 PUFAs used in our study comprised a mixture (32 ± 3 and 18 ± 3 g/kg EPA and DHA, respectively) in the form of triacylglycerols. This is a significant difference, because it is well established that both the metabolism and the metabolic effects of EPA supplementation differ from those of DHA, although the underlying molecular mechanisms remain to be fully elucidated (7, 15, 26, 29, 43, 46). Furthermore, because the kinetics of absorption of fatty acids from triacylglycerols and ethyl esters in the gut differs (51), we cannot exclude that this difference may contribute to the observed differences between the two studies. It should also be mentioned that the main fat source in the diets used in our study is corn oil, which is rich in n-6 fatty acids, whereas Sato et al. (63) used milk fat containing >60% saturated fat. Moreover, the highest dose of sucrose used in our study was higher than that used by Sato et al. (63). Expression of Ucp1 in iWAT decreased dose-dependently with increasing concentrations of sucrose (Fig. 1F), which would suggest that expression of Ucp1 in iWAT in our experiments was lower than in the experiments of Sato et al. (63). This becomes of particular importance because our experiments, in contrast to those of Sato et al. (63), were conducted at thermoneutrality, where lower or complete lack of Ucp1 expression, particularly in white adipose tissues, promotes adipose tissue mass expansion and obesity, which is not observed at temperatures below thermoneutrality (11, 22, 42).

Obviously, increased adipose tissue mass is related to energy intake. However, macronutrient composition can influence energy efficiency in such a way that mice consuming the same amount of calories end up with quite different amounts of adipose tissue. Thus, increasing the amount of sucrose in the feed from 13 to 43% led to approximately fivefold higher energy efficiency. In mice fed increasing amounts of protein at the expense of sucrose, we observed a dose-dependent increase in Ucp1 expression in iWAT, suggesting increased uncoupled respiration and dissipation of energy in form of heat. We and others have demonstrated recently that cyclooxygenase-depen-
dent induction of Ucp1 expression in WAT counteracts diet-induced obesity (42, 71). Additionally, we observed a dose-dependent increase in Pparγc1a and Pck1 expression in response to a reduced intake of dietary sucrose, suggesting increased hepatic gluconeogenesis (23). Moreover, reduced sucrose combined with increased protein content in the feed would increase the energy cost associated with amino acid catabolism. Thus, UCP1-dependent uncoupled respiration in iWAT in combination with increased energy cost from gluconeogenesis and ureagenesis may account for the reduced energy efficiency that was observed when sucrose levels in the diet were low.

Obesity is associated with a state of chronic low-grade inflammation, and following the onset of obesity, secretion of proinflammatory adipokines is increased. The anti-inflammatory effect of fish oil in adipose tissue is well described (33, 36, 55, 69). Thus, it was remarkable that mice receiving fish oil in combination with sucrose, glucose, or high-GI starch had increased expression of macrophage marker genes in adipose tissue. In agreement with the positive correlation between adipose tissue mass and circu-
loration levels of leptin, mice fed fish oil in combination with sucrose, glucose, or high-GI starch had increased expression of macrophage marker genes in adipose tissue. In agreement with the positive correlation between adipose tissue mass and circu-

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Values represent means ± SE; n = 6/diet. Different letters indicate statistical difference, P < 0.05.
state than subcutaneous adipose tissue, which was reviewed recently by Blüher (8). However, a few recent reports have demonstrated that subcutaneous adipose tissues compared with visceral adipose tissue in certain circumstances may express similar or higher levels of inflammatory markers (19, 72), and our observations add to this list.

To summarize, we demonstrate that the background diet exerts a crucial influence on the ability of fish oil to protect against obesity development and adipose tissue inflammation. Thus, it cannot be excluded that several additional beneficial effects of fish oil intake might be diminished or completely abrogated by a simultaneous intake of high-GI carbohydrates. If similar effects are found in humans, this is of great concern because the intake of refined sugars from sources such as soft drinks has increased dramatically during the last several decades (14). Moreover, n-3 supplements are often taken in combination with morning meals containing high-GI carbohydrates such as cereal, bread, and orange juice. Thus, comprehensive studies of the interaction between dietary macronutrients and fish oil in humans seem warranted.

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**Fig. 5.** Increased insulin secretion by glybenclamide is insufficient to increase the obesogenic effect of fish oil when the carbohydrate intake is low. Male C57BL/6J mice (n = 6) were fed a standard low-fat diet or high-fat/low-carbohydrate diet for 4 wk. The mice were injected daily with glybenclamide or placebo. A: body weight development in pair-fed mice is shown as relative increase. B: the weights of eWAT and iWAT were recorded, and sections were stained with hematoxylin and eosin. Scale bars, 50 μm. C: relative gene expression was measured in eWAT and iWAT (C) and in liver by qRT-PCR and normalized to Tbp (D). In a separate set of mice (n = 10), fasting blood glucose levels were determined (E), and an intraperitoneal glucose tolerance test was performed (F). Data are presented as means ± SE or means + SE. Different small letters denote significant differences (P < 0.05). Statistics were performed separately for each individual tissue.
Fig. 6. The obesity-promoting effect of sucrose in combination with fish oil is diminished by inhibition of insulin secretion by nifedipine. Male C57BL/6J mice \((n = 6)\) were fed a standard low-fat diet or high-fat/high-carbohydrate diet with or without nifedipine supplementation for 4 wk. A: body weight development in pair-fed mice is shown as relative increase. B: fed state levels of insulin and glucose were measured in plasma. C: the weights of eWAT and iWAT were recorded; sections were stained with hematoxylin and eosin (scale bars, 50 \(\mu\)m), and the average diameter of 200 cells was calculated. D: relative gene expression was measured in eWAT and iWAT by qRT-PCR and normalized to \(Tbp\). Intraperitoneal glucose tolerance test \((E)\) and insulin glucose tests \((F)\) were performed in a separate set of mice \((n = 10)\). Data are presented as means \(\pm\) SE or means + SE. Different small letters denote significant differences \((P < 0.05)\). Statistics were performed separately for each individual tissue and each time point.
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DISCLOSURES
The authors have no conflicting interests, financial or otherwise.

AUTHOR CONTRIBUTIONS
REFERENCES


Intake of Farmed Atlantic Salmon Fed Soybean Oil Increases Insulin Resistance and Hepatic Lipid Accumulation in Mice

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Abstract

Background: To ensure sustainable aquaculture, fish derived raw materials are replaced by vegetable ingredients. Fatty acid composition and contaminant status of farmed Atlantic salmon (Salmo salar L.) are affected by the use of plant ingredients and a spillover effect on consumers is thus expected. Here we aimed to compare the effects of intake of Atlantic salmon fed fish oil (FO) with intake of Atlantic salmon fed a high proportion of vegetable oils (VOs) on development of insulin resistance and obesity in mice.

Methodology/principal findings: Atlantic salmon were fed diets where FO was partly (80%) replaced with three different VOs; rapeseed oil (RO), olive oil (OO) or soy bean oil (SO). Fillets from Atlantic salmon were subsequently used to prepare Western diets (WD) for a mouse feeding trial. Partial replacement of FO with VOs reduced the levels of polychlorinated biphenyls (PCB) and dichloro-diphenyl-tricloooethanes (DDT) with more than 50% in salmon fillets, in WDs containing the fillets, and in white adipose tissue from mice consuming the WDs. Replacement with VOs, SO in particular, lowered the n–3 polyunsaturated fatty acid (PUFA) content and increased n–6 PUFA levels in the salmon fillets, in the prepared WDs, and in red blood cells collected from mice consuming the WDs. Replacing FO with VO did not influence obesity development in the mice, but replacement of FO with RO improved glucose tolerance. Compared with WD-FO fed mice, feeding mice WD-SO containing lower PCB and DDT levels but high levels of linoleic acid (LA), exaggerated insulin resistance and increased accumulation of fat in the liver.

Conclusion/Significance: Replacement of FO with VOs in aqua feed for farmed salmon had markedly different spillover effects on metabolism in mice. Our results suggest that the content of LA in VOs may be a matter of concern that warrants further investigation.

Introduction

The ability of marine n–3 polyunsaturated fatty acids (PUFAs) to protect against the development of cardiovascular disease is well documented [1–3]. Thus, increasing the dietary intake of n–3 PUFAs is currently recommended by several health authorities. During the recent years, there has been increasing focus on the possible ability of n–3 PUFAs to protect against other life-style diseases such as obesity and type 2 diabetes. Although human studies are inconclusive [4], rodent feeding trials demonstrate that marine n–3 PUFAs are able to protect against development of obesity [5–10], and insulin resistance [9,11–15]. Fish and seafood, in particular fatty fish such as Atlantic salmon (Salmo salar L.), are rich in n–3 PUFAs. Thus, both health authorities and consumers have accepted farmed Atlantic salmon as part of a healthy diet. Farmed Atlantic salmon have traditionally been fed diets with high levels of fish meal and fish oil (FO), and a single meal of 200 g Atlantic salmon, providing 4 g of n–3 PUFAs, would be sufficient to cover more than the weekly recommended intake of n–3 PUFAs [16]. However, due to the pressure on wild fish stocks, and hence limited access and variable prices of fish meal and FO for the rapidly growing global aquaculture industry [17], efforts are channelled towards development of aqua feed that rely less on fish meal and FO [18,19].
Vegetable oils (VOs) are presently recognized as suitable alternatives in aqua feed [20], and consequently, the nutrient composition in the Atlantic salmon fillets will change [16]. Although the Atlantic salmon have the ability to elongate and desaturate fatty acids [21,22], Atlantic salmon fed vegetable feed have lower content of marine n–3 PUFA content in aqua feed, Atlantic salmon fillets and plasma in patients with coronary heart disease (CHD) consuming the fillets [23]. Documentation related to the health beneficial effects of fish has focused on the content of marine n–3 PUFA 2ω6 ratio 1.75 0.63 0.54 0.32 0.13 0.29

Table 1. Composition of the experimental mice diets (g/kg).

<table>
<thead>
<tr>
<th>Components added (g/kg)</th>
<th>WD-FO</th>
<th>WD-RO</th>
<th>WD-OO</th>
<th>WD-SO</th>
<th>LF</th>
<th>WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>97</td>
<td>97</td>
<td>97</td>
<td>197</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>Protein from salmon</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Milk fat, anhydrous</td>
<td>138</td>
<td>131</td>
<td>129</td>
<td>135</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Fat from salmon</td>
<td>62</td>
<td>69</td>
<td>71</td>
<td>65</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Corn oil</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>38</td>
<td>46</td>
<td>41</td>
<td>41</td>
<td>50</td>
<td>–</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>539</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>340</td>
<td>340</td>
<td>340</td>
<td>90</td>
<td>340</td>
<td></td>
</tr>
</tbody>
</table>

All diets were supplemented with 40 mg/kg ethoxyquin, 2g/kg Choline bitartrate, 4g/kg calcium carbonate, 10g/kg Vitamin mix V1001, 35g/kg Mineral mix, 3g/kg L-Methionine and 50g/kg cellulose. Cholesterol levels in WDs were balanced to a final concentration of 1.5 g/kg.

Table 2. Fatty acid composition of the experimental mice diets.

<table>
<thead>
<tr>
<th>Fatty acid (mg/g)</th>
<th>WD-FO</th>
<th>WD-RO</th>
<th>WD-OO</th>
<th>WD-SO</th>
<th>LF</th>
<th>WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum SFA</td>
<td>93.2</td>
<td>78.8</td>
<td>84.2</td>
<td>85.2</td>
<td>7.1</td>
<td>103.5</td>
</tr>
<tr>
<td>Sum MUFA</td>
<td>58.2</td>
<td>67.1</td>
<td>73.4</td>
<td>55.4</td>
<td>7.6</td>
<td>50.8</td>
</tr>
<tr>
<td>LA, 18:2=6</td>
<td>9.6</td>
<td>15.6</td>
<td>15.3</td>
<td>25.8</td>
<td>8.0</td>
<td>8.1</td>
</tr>
<tr>
<td>AA, 20:4=6</td>
<td>&lt;0.1</td>
<td>0.3</td>
<td>0.3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Sum n–6</td>
<td>10.5</td>
<td>17.3</td>
<td>17.0</td>
<td>28.5</td>
<td>8.1</td>
<td>8.3</td>
</tr>
<tr>
<td>ALA, 18:3–3</td>
<td>1.8</td>
<td>3.9</td>
<td>2.7</td>
<td>2.7</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>EPA, 20:5–3</td>
<td>4.1</td>
<td>1.3</td>
<td>1.2</td>
<td>1.2</td>
<td>&lt;0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>DHA, 22:6–3</td>
<td>6.2</td>
<td>2.6</td>
<td>2.5</td>
<td>2.3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Sum n–3</td>
<td>18.4</td>
<td>10.9</td>
<td>9.2</td>
<td>9.2</td>
<td>1.1</td>
<td>2.5</td>
</tr>
<tr>
<td>n–3/n–6 ratio</td>
<td>1.75</td>
<td>0.63</td>
<td>0.54</td>
<td>0.32</td>
<td>0.13</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Data represent mean of duplicate measurements. Sum n–3 and sum n–6 include additional fatty acids not indicated in the table.

Materials and Methods

Ethical Statement

Animal handling and experiments were performed in accordance with the guidelines of the Norwegian State Board of Biological Experiments with Living Animals [Norwegian approval identification nr, FOTS Id: 3196]. No adverse events were observed.

Production of Atlantic Salmon

The salmon feeding trial was carried out at Skreting ARC, Fish Trials Station, Stavanger, Norway. A total of 600 Atlantic salmon (Salmo salar L.), mean weight 815±28 g, were randomly distributed in 12 tanks. The salmon were divided into four experimental groups and fed different experimental diets for 28 weeks as described earlier [22]. The fat source in the control diet was 100% FO, whereas 80% of the FO was replaced by rapeseed oil (RO), olive oil (OO), or soy bean oil (SO), in the experimental diets. These oils were chosen based on their different n–6 PUFA purified salmon oil attenuated development of insulin resistance and obesity in Wistar rats, whereas intake of crude salmon oil containing POPs exaggerated obesity development [32]. Moreover, we have recently shown that chronic consumption of Atlantic salmon with a high level of POPs caused insulin resistance and obesity in mice [33]. When the levels of POPs in the salmon were reduced, development of insulin resistance and obesity in the mice decreased concomitantly [33].

Since both the nutrient and contaminant status of Atlantic salmon are affected by the use of plant-based feed ingredients, a spillover effect on consumer health might be expected. In the present study we compared the development of insulin resistance and obesity in mice fed a western type diet containing either Atlantic salmon fed FO or Atlantic salmon where the FO in the feed was replaced by a high proportion of VOs. Our results indicate that the altered fatty acid composition, the increased content of linoleic acid (LA) in particular, rather than the altered content of POPs, in fillets from Atlantic salmon, influenced development of insulin resistance and hepatic accumulation of lipids in mice.
content [22]. In all diets 70% of fish meal was replaced with plant proteins. The complete dietary compositions and analyses of the diets and fillets have been reported earlier [22]. Upon sampling, salmon fillets were freeze dried, homogenized and analyzed for fat and protein content.

**Mouse Diets**

All diets formulations were based on the standard Western diet (WD), D12079B Research Diets, Inc. NJ, USA prepared by Ssniff Spezialdiäten (Soest, Germany). We replaced 50% of the protein source in the standard Western diet with proteins from salmon fed FO, RO, OO or SO. Since the fat content differed in salmon fed the different diets, milk fat was used to achieve isocaloric diets. The major fatty acid source in the milk fat used was 16:0, 18:1, 14:0 and 18:0, representing, 29, 26, 10 and 11%, respectively. Composition of the mice diets are shown in Table 1. As references, two groups of mice were fed either a casein based standard WD (Ssniff S8672), or a regular low fat diet (LF), D12450B (Ssniff EF R/M Control, Germany). Complete fatty acid composition of the WD and LF diet is available at http://www.sniff.com/documents/10_catalogue_ef_engl_1.pdf.

**Animals**

48 male C57BL/6J BomTac mice were obtained from Taconic (Eby, Denmark) at 8 weeks of age. The mice were maintained in a controlled environment with an artificial 12-h-light/dark cycle at thermoneutrality (30°C). Before the start of the feeding experiment animals were allowed to acclimatize to a regular LF diet for 5 d. After acclimatization, the mice were housed individually and randomly assigned to the experimental diets.

The animals were fed the experimental diets described in Table 1 for 10 weeks ad libitum. Throughout the experiment, all mice were weighed once a week and feed intake was assessed every Monday, Wednesday and Friday.

**Insulin and Glucose Tolerance Tests (ITT and GTT)**

ITT and GTT were performed on conscious mice after 7 and 8 weeks of feeding, respectively. ITT was performed in fed state by intraperitoneal (i.p.) injection of insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark, 0.75 U/kg body weight), GTT was performed in 6 h fasted mice by i.p. injection of glucose (2.0 mg/g body weight). Blood was collected from the lateral tail vein and glucose levels were measured at different time-points using a glucometer (Ascensia Contour, Bayer Healthcare, Oslo, Norway). For the GTT, incremental area under the curve (AUC) was calculated as blood glucose concentrations above baseline levels.

**Plasma and Tissue Sampling**

At the end of the feeding period, overnight fasted mice were sacrificed by cardiac puncture under Isoflurane anesthesia (Isoba-vet, Schering-Plough, Denmark). Liver, muscles, and adipose tissue depots were quickly dissected out, weighed, snap-frozen in liquid nitrogen and stored at −80°C for further analyses. Blood was collected in tubes containing 10 µl heparin (10 mg/ml Sigma-Aldrich, United Kingdom), and plasma was prepared by centrifugation. Red blood cells (RBCs) and plasma were stored in aliquots at −80°C for further use.

**Plasma Analyses**

A commercial ELISA kit was used in accordance with the manufacturer’s instruction for determination of plasma insulin (Mouse Insulin ultrasensitive ELISA, DRG, Marburg, Germany). Plasma glucose was determined using an automated analyzer (Maximat PII, Multi-purpose diagnostic analyzer system, Montpellier, France).

**Lipid Analyses**

Total fat content in the diets was measured gravimetrically after acidic hydrolysis and petroleum ether extraction. Tissue lipids were extracted from liver and muscle samples with chloroform: methanol, 2:1 (v/v). Lipid classes were analyzed using an automated High Performance Thin Layer Chromatography (HPTLC) system (Camaq, Switzerland) and separated on HPTLC plates coated with silica gel 60 F [34]. Fatty acid composition of total lipids in red blood cells was analyzed on a capillary gas chromatograph with flame ionization detector (Perkin Elmer, USA) [35].

**Energy in Feces and Diets**

Energy content was determined in a bomb calorimeter following the manufacturer’s instruction (Parr Instruments, Moline, IL, USA).

**Contaminant Analyses**

The levels of 7 polychlorinated biphenyls (7 PCBs) and dichlorodiphenyl-trichloroethanes (DDTs) in salmon fillets, mouse feed, liver and adipose tissue from mice were analyzed as described earlier [22,25], but the spillover effects of such changes for the composition of both nutrients and POPs in Atlantic salmon were exchanged with vegetable proteins and 80% of the FO were exchanged with VO. The VOs used were rape seed oil (RO), olive oil (OO) and soybean oil (SO), chosen based on their different content of n-3 PUFAs [22]. Compositions of the salmon diets are listed in Liland et al. 2012 [22]. To investigate the potential spillover implications of such changes in the aqua feed for the consumers, we prepared standard WDs containing these salmon fillets, replacing 50% of the protein source (casein) with protein from salmon (Table 1). The fat content was slightly different in

**Results**

Replacing Fish Oil with Vegetable Oils in Fish Feed Reduces the n−3/n−6 Ratio in Atlantic Salmon Fillets and in the Red Blood Cells from Mice Consuming the Salmon Fillets

It is well known that replacing FO with VO in fish feed changes the composition of both nutrients and POPs in Atlantic salmon fillets [22,25], but the spillover effects of such changes for the consumers are not thoroughly examined. To investigate this, Atlantic salmon were fed diets in which 70% of marine proteins were exchanged with vegetable proteins and 80% of the FO were exchanged with VO. The VO’s used were rape seed oil (RO), olive oil (OO) and soybean oil (SO), chosen based on their different content of n-6 PUFAs [22]. Compositions of the salmon diets are listed in Liland et al. 2012 [22]. To investigate the potential spillover implications of such changes in the aqua feed for the consumers, we prepared standard WDs containing these salmon fillets, replacing 50% of the protein source (casein) with protein from salmon (Table 1). The fat content was slightly different in
Figure 1. Composition of salmon aqua feed changes n-3/n-6 PUFA ratio in mice consuming the salmon fillets. Fish oil (FO) in aqua feed was partly replaced with rapeseed oil (RO), olive oil (OO) or soy bean oil (SO) and fed Atlantic salmon. The salmon fillets were used in Western diets (WDs) fed male C57BL/6J mice (n = 8/diet) for 10 weeks. Fatty acid composition was measured and the n-3/n-6 PUFA ratio calculated in (A) Atlantic salmon fillets (B) the WDs containing the fillets and (C) red blood cells (RBC) collected from mice consuming the WDs and reference diets. Data represent mean of duplicate measurements in A and B and mean±SEM (n = 5) in C. Asterisk(s) indicates significant different from FO-WD.

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Compared with the WD-FO diet all the WD-VOs significantly reflected the ratio in the salmon fillets and mouse diets (Fig. 1). The omega-3 indices were reduced 18 and 21% in WD-RO and WD-OO fed mice, respectively (Table 3). However, all mice fed diets containing salmon had an omega-3 index higher than 8% of the vegetable n-3 fatty acids, hereafter collectively referred to as WD-VOs, had significantly lower, but comparable levels of the marine n-3 fatty acids (PUFAs) lower in RBCs in mice fed WD-VOs compared with WD-FO (Table 2). Of note, the levels of LA were comparable in WD-RO and WD-OO, but particularly high in WD-SO (Table 2). Furthermore, the levels of the vegetable n-3 PUFA, α-linolenic acid (ALA) was higher in the WD-RO than the other WD-VOs (Table 2). Thus, the n-3/n-6 ratio varied between the WD-VOs. The n-3/n-6 ratio was lower in WD-OO than in WD-RO, and the lowest n-3/n-6 ratio was measured in the WD-SO (Table 2).

To investigate possible spillover effects for consumers, C57BL/6J mice were fed these diets for 10 weeks and fatty acid composition was measured in the red blood cells (RBC). As references, two groups of mice were fed either a standard casein based WD or a regular low fat (LF) diet. As predicted, the combined levels of n-6 PUFAs were higher and the levels of n-3 PUFAs lower in RBCs in mice fed WD-VOs compared with WD-FO (Table 3). Thus, the n-3/n-6 ratio in RBCs in the mice reflected the ratio in the salmon fillets and mouse diets (Fig. 1). Compared with the WD-FO diet all the WD-VOs significantly reduced the omega-3 index - with over 30% reduction in mice fed the WD-SO diet compared to mice fed the WD-FO diet (Table 3). The omega-3 indices were reduced 18 and 21% in WD-RO and WD-OO fed mice, respectively (Table 3). However, all mice fed diets containing salmon had an omega-3 index higher than 8% [2], indicating that intake of the WD-VO diets still might exert a cardio-protective effect. For comparison, the reference diets resulted in an omega-3 index below the recommended limit of 8%.

Replacing Fish Oil with Vegetable Oils in Fish Feed Reduces the Levels of PCBs and DDTs in Atlantic Salmon Fillets and Accumulation of these in Adipose Tissue from Mice Consuming the Salmon Fillets When FO was replaced with VO in salmon feed, the levels of PCBs and DDTs were reduced by more than 50% in the salmon fillets (Fig. 2A). PCBs and DDTs are lipid soluble contaminants that accumulate in adipose tissue in rats and mice [32,33]. Thus, the contaminant levels in adipose tissue, but not in liver of the mice mirrored the levels in the salmon fillets and the mouse diets (Fig. 2). Together, changes in the salmon feed were translated into altered fatty acid composition in RBCs and levels of POPs in adipose tissue in mice consuming the salmon fillets.

The Nutrient- and Contaminant Changes in Salmon Fillets do not Influence Obesity Development in Mice We have previously demonstrated that chronic consumption of commercially available Atlantic salmon with a high level of POPs caused obesity in obesity prone C57BL/6J mice [33]. When the levels of POPs in the salmon were reduced, obesity development in the mice were slightly, but significantly, decreased. Here we demonstrate that although the accumulation of both PCBs and DDTs in adipose tissue was significantly lower in mice fed the various WD-VOs than in mice fed WD-FO, (Fig. 2C) body weight gain (Table 4) and adipose tissue masses (Table 5) were comparable. As expected, mice fed the LF diet, consumed significantly less energy and gained less weight than WD-FO fed mice. However, energy intake and apparent fat digestibility in the WD-VOs fed mice were comparable to those of mice fed the WD-FO (Table 4). Thus, neither reduced levels of PCBs and DDTs nor lower n-3/n-6 ratio in the WD-VOs had any effect on the body weight or obesity development (Table 4 and 5).

Table 3. Fatty acid composition in RBCs from mice after consuming the experimental diets for 10 weeks.

<table>
<thead>
<tr>
<th></th>
<th>WD-FO</th>
<th>WD-RO</th>
<th>WD-OO</th>
<th>WD-SO</th>
<th>LF</th>
<th>WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum FA (mg/g)</td>
<td>3.1±0.2</td>
<td>3.1±0.2</td>
<td>3.2±0.1</td>
<td>3.2±0.2</td>
<td>3.1±0.1</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>Sum SFA (mg/g)</td>
<td>1.26±0.06</td>
<td>1.14±0.01*</td>
<td>1.18±0.02</td>
<td>1.19±0.01</td>
<td>1.18±0.01</td>
<td>1.13±0.02*</td>
</tr>
<tr>
<td>Sum MUFA (mg/g)</td>
<td>0.58±0.03</td>
<td>0.59±0.01</td>
<td>0.66±0.01*</td>
<td>0.51±0.02</td>
<td>0.61±0.02</td>
<td>0.70±0.02***</td>
</tr>
<tr>
<td>LA, 18:2n-6 (mg/g)</td>
<td>0.23±0.02</td>
<td>0.32±0.01***</td>
<td>0.32±0.02**</td>
<td>0.37±0.01***</td>
<td>0.29±0.02**</td>
<td>0.26±0.01</td>
</tr>
<tr>
<td>AA, 20:4n-6 (mg/g)</td>
<td>0.22±0.01</td>
<td>0.25±0.01</td>
<td>0.27±0.01**</td>
<td>0.32±0.01***</td>
<td>0.56±0.02***</td>
<td>0.51±0.01***</td>
</tr>
<tr>
<td>Sum n-6 (mg/g)</td>
<td>0.49±0.03</td>
<td>0.65±0.02***</td>
<td>0.69±0.01***</td>
<td>0.82±0.02***</td>
<td>0.97±0.01***</td>
<td>0.91±0.02***</td>
</tr>
<tr>
<td>EPA, 20:5n-3 (mg/g)</td>
<td>0.21±0.01</td>
<td>0.15±0.01***</td>
<td>0.13±0.01***</td>
<td>0.09±0.01***</td>
<td>0.01±0.00***</td>
<td>0.02±0.00***</td>
</tr>
<tr>
<td>DHA, 22:6n-3 (mg/g)</td>
<td>0.40±0.02</td>
<td>0.34±0.00**</td>
<td>0.35±0.00*</td>
<td>0.34±0.01**</td>
<td>0.23±0.01***</td>
<td>0.22±0.01***</td>
</tr>
<tr>
<td>Sum n-3 (mg/g)</td>
<td>0.73±0.04</td>
<td>0.59±0.01***</td>
<td>0.58±0.01***</td>
<td>0.51±0.01***</td>
<td>0.28±0.01***</td>
<td>0.31±0.01***</td>
</tr>
<tr>
<td>Calculated n-6 HUFA (%)</td>
<td>26.6±0.2</td>
<td>36.1±0.5***</td>
<td>39.4±0.6***</td>
<td>47.5±0.5***</td>
<td>71±1***</td>
<td>680±6.8***</td>
</tr>
<tr>
<td>Calculated n-3 index (%)</td>
<td>19.4±0.1</td>
<td>15.9±0.2***</td>
<td>15.2±0.2***</td>
<td>13.3±0.3***</td>
<td>7.7±0.1**</td>
<td>7.8±0.2***</td>
</tr>
</tbody>
</table>

The n-3 index: EPA+DHA content of erythrocytes expressed as a percent of total fatty acids in RBCs. Sum n-3 and sum n-6 include additional fatty acids not indicated in the table.

Data are presented as mean ± SEM (n = 5). Asterisk(s) indicates significant different from WD-FO.

*p<0.05, **p<0.01, ***p<0.005.

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid, FA, fatty acids; HUFA, highly unsaturated fatty acids (HUIA, ≥20 carbons and ≥3 carbon-carbon double bonds), LA, linoleic acid; MUFA, monounsaturated fatty acids; RBCs, red blood cells; SFA, saturated fatty acids.

doi:10.1371/journal.pone.0053094.t003
Composition of salmon aqua feed changes accumulation of POPs in mice consuming the salmon fillets.

Fish oil (FO) in aqua feed was partly replaced with rapeseed oil (RO), olive oil (OO) or soy bean oil (SO) and fed Atlantic salmon. The salmon fillets were used in Western diets (WDs) fed male C57BL/6J mice (n = 8/diet) for 10 weeks. Concentrations of 7PCBs and DDTs were measured in (A) Atlantic salmon fillets (B) the WDs containing the fillets and (C) epididymal white adipose tissue (eWAT) and (D) liver collected from mice consuming the WDs and a low fat reference diet. Data represent mean of duplicate measurements in A and B. Tissues from two animals were pooled to achieve sufficient material for POP analyzes, and data in C and D thus represent mean+SEM (n = 4). Asterisk(s) indicates significant different from WD-FO. doi:10.1371/journal.pone.0053094.g002
The Fatty Acid Composition in Salmon Fillets Influences Development of Insulin Resistance and Hepatic Lipid Accumulation in Mice

Given the earlier findings that POPs appear to exacerbate insulin resistance [32,33], whereas n−3 PUFAs have been demonstrated to ameliorate development of diet-induced insulin resistance [9,11–15], we measured fasting levels of glucose and insulin, and performed glucose and insulin tolerance tests.

Overnight fasting plasma glucose levels were comparable in all groups (Fig. 3A). Compared with intake of WD-FO, intake of WD-VOs did not affect plasma insulin levels (Fig. 3B). The glucose tolerance in mice fed the WD-FO was comparable to the glucose tolerance in mice fed the standard WD, but lower than in mice fed the LF diet (Fig. 3C and E). WD-RO had a significantly decreased incremental area under the curve (AUC) compared with WD-FO (Fig. 3E) indicating an improved glucose tolerance. During the insulin tolerance test, AUC in WD-RO fed mice was not significantly lower than in mice fed WD-SO, but significantly lower (p<0.05) than in WD-FO fed mice (Fig. 3D). Mice receiving WD-RO had significantly higher blood glucose levels at 15 and 45 min after insulin injection than mice fed the WD-FO (Fig. 3D). LF fed mice had lower plasma glucose 30 and 45 min after injection of glucose (Fig. 3D). The difference was confirmed by a repeated-measures ANOVA test during the ITT revealing that the glucose response over time was significantly higher after in WD-RO fed mice as compared to WD-FO fed mice (p<0.05), whereas the glucose response over time in LF fed mice was significantly lower (p<0.01). Furthermore, mice receiving WD-RO had significantly larger AUC during ITT than mice fed the WD-FO (Fig. 3F). These results suggest that insulin sensitivity in mice fed WD-RO was decreased.

The reduced insulin sensitivity in WD-SO fed mice was not associated with increased adipose tissue mass. However, as not only increased adipose tissue mass, but also ectopic deposition of lipids have been suggested to contribute to the development of insulin resistance [11,36–38] we quantified lipids in liver and the tibials anterior muscle. We did not observe any significant changes in muscle lipid content (not shown). Hepatic levels of PL and free fatty acids were similar in all groups but mice fed the WD-SO had significantly higher levels of total lipids, free cholesterol, steryl ester (SE) and triacylglycerol (TAG) in the liver than mice fed the WD-FO (Fig. 4). A similar tendency was seen in mice fed the WD-OO, but due to large individual differences this did not reach statistical significance. Given the ability of n−3 PUFAs to protect against diet-induced accumulation of fat in liver [39,40], and the indication of increased accumulation of lipids with increasing amount of n−6 in the diet, our data suggest that fatty acid composition in the salmon fillets appears to play a more important role than the levels of 7 PCBs and DDTs.

Discussion

The acceptance of Atlantic salmon as part of a healthy diet has to a large extent been based on its high content of marine n−3 PUFAs. However, the traditional use of fish-derived raw materials such as fish meal and FO in aquaculture is not sustainable due to the pressure on wild fish stocks. Thus, VOAs are presently recognized as suitable alternatives in aqua feed [20]. We recently

Table 4. Body weight, energy intake, energy efficiency and apparent fat digestibility in mice fed the different diets for 10 weeks.

<table>
<thead>
<tr>
<th></th>
<th>WD-FO</th>
<th>WD-RO</th>
<th>WD-OO</th>
<th>WD-SO</th>
<th>LF</th>
<th>WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial BW (g)</td>
<td>25.4±.5</td>
<td>25.3±.4</td>
<td>25.4±.5</td>
<td>25.5±.4</td>
<td>25.4±.6</td>
<td>25.4±.5</td>
</tr>
<tr>
<td>BW gain (g)</td>
<td>18.6±.6</td>
<td>16.7±.9</td>
<td>17.0±1.0</td>
<td>18.5±1.1</td>
<td>8.2±.7***</td>
<td>15.3±.7*</td>
</tr>
<tr>
<td>Total energy intake (kJ)</td>
<td>4125±140</td>
<td>4016±127</td>
<td>4113±231</td>
<td>4032±195</td>
<td>3423±133*</td>
<td>3840±104</td>
</tr>
<tr>
<td>Energy efficiency (g/MJ)</td>
<td>4.5±.2</td>
<td>4.2±.3</td>
<td>4.2±.4</td>
<td>4.6±.2</td>
<td>2.4±.2**</td>
<td>4.0±.2</td>
</tr>
<tr>
<td>Fat digestibility (%)</td>
<td>97.7±.3</td>
<td>98.1±.3</td>
<td>98.5±.1</td>
<td>98.6±.1</td>
<td>96.7±.1</td>
<td>96.2±.6**</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM (n=8). Asterisk(s) indicates significant different from WD-FO.

* p<0.05,
** p<0.01,
*** p<0.005.

Abbreviations: BW, body weight.

doi:10.1371/journal.pone.0053094.t004

Table 5. Organ weights (g) in the mice fed the experimental diets for 10 weeks.

<table>
<thead>
<tr>
<th></th>
<th>WD-FO</th>
<th>WD-RO</th>
<th>WD-OO</th>
<th>WD-SO</th>
<th>LF</th>
<th>WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.35±.06</td>
<td>1.33±.03</td>
<td>1.54±.10</td>
<td>1.59±.14</td>
<td>1.11±.04</td>
<td>1.47±.05</td>
</tr>
<tr>
<td>eWAT</td>
<td>1.98±.05</td>
<td>1.97±.08</td>
<td>1.87±.16</td>
<td>1.96±.08</td>
<td>0.70±.08*</td>
<td>1.79±.08</td>
</tr>
<tr>
<td>iBAT</td>
<td>0.07±.02</td>
<td>0.68±.05</td>
<td>0.65±.09</td>
<td>0.71±.05</td>
<td>0.25±.03*</td>
<td>0.63±.05</td>
</tr>
<tr>
<td>Muscle (Tibialis)</td>
<td>0.102±.006</td>
<td>0.085±.006</td>
<td>0.089±.008</td>
<td>0.100±.006</td>
<td>0.080±.007</td>
<td>0.084±.005</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM (n=8). Asterisk(s) indicates significant different from WD-FO.

* p<0.05,
** p<0.01,
*** p<0.005.

Abbreviations; eWAT, epididymal white adipose tissue; iBAT, interscapular brown adipose tissue; iWAT, inguinal white adipose tissue.

doi:10.1371/journal.pone.0053094.t005
demonstrated that a net production of marine proteins, but not marine n-3 PUFAs, is achievable when 70% of the marine proteins and 80% of the marine oils are replaced by vegetable alternatives [22]. Here we demonstrate that replacing FO with RO, OO or SO, decreased the n-3/n-6 ratio in salmon fillets by 79, 76 and 92%, respectively, with a concomitant reduction in

Figure 3. Fatty acid composition in salmon fillets influences development of insulin resistance in mice. Male C57BL/6J mice (n = 8/diet) were fed WD-FO, WD-RO, WD-OO and WD-SO for 10 weeks. As references, two groups of mice received regular WD or LF diet. Plasma glucose (A) and insulin (B) were measured after overnight fasting. An intraperitoneal glucose tolerance test (GTT) was performed after 7 weeks of feeding (C) and an intraperitoneal insulin tolerance test (ITT) was performed after 8 weeks of feeding (D). Area under the curve (AUC) was calculated from the glucose tolerance (baseline was set to fasted blood glucose levels) (E) and insulin tolerance test (F). Data are presented as means±SEM (n = 8). *represents significant different from WD-FO (P<0.05). **represents significant different from WD-FO (P<0.01). ***represents significant different from WD-FO (P<0.005).
doi:10.1371/journal.pone.0053094.g003

Figure 4. Fatty acid composition in salmon fillets influences development of hepatic lipid accumulation in mice. Male C57BL/6J mice (n = 8/diet) were fed WD-FO, WD-RO, WD-OO and WD-SO for 10 weeks. As references, two groups of mice received standard WD or LF diet. Lipids were extracted from liver and (A) total lipids; (B) triacylglycerol (TAG); (C) free cholesterol, and (D) steryl ester (SE) were quantified. Data are presented as means±SEM (n = 8). Asterisk(s) indicates significant different from WD-FO.
doi:10.1371/journal.pone.0053094.g004
marine n−3 PUFAs in RBCs collected from mice fed WDs containing the salmon fillets. The omega-3 PUFA content in RBC membranes reflects the levels in cardiae membranes, and an omega-3 index below 4% is considered a risk factor for cardiovascular disease [2]. The omega-3 index was lower in mice fed the WD-VOs, WD-SO in particular, than in mice fed the WD-FO, however, the omega-3 index remained above 8% in all mice fed WDs containing salmon. Thus, the cardio-protective effects of salmon ingestion appeared to be maintained in diets based on VO fed salmon.

The ability of marine n−3 PUFAs to protect against development of obesity in rodents is well documented [3–10], whereas n−6 PUFAs, LA in particular, have been associated with increased weight gain [10,41–44]. We recently demonstrated that elevation of dietary LA from 1 to 8% increased endocannabinoid tone and obesity development in mice, an effect that was prevented by adding 1% EPA and DHA to the 8% LA diets [10]. Furthermore, excessive dietary LA from SO in salmon feed increased the endocannabinoid tone, both in Atlantic salmon and in mice fed high fat diets containing the salmon fillets. The increased endocannabinoid tone in the mice was accompanied by increased weight gain [24]. Here, the WD-SO contained more n−6 PUFAs, mainly LA, than the WD-FO and the other WD-VOs. Thus, the calculated n−6 HUFA content in WD-SO fed mice was significantly higher than in WD-FO and the other WD-VO fed mice. However, obesity development and weight gain were not influenced by replacing FO with VO in the present study. Compared to WD-FO fed mice, glucose tolerance was comparable in mice fed WD-OO and WD-SO, and actually improved in WD-VO fed mice. Despite similar body weight, adipose tissue mass and glucose tolerance, WD-SO fed mice became more insulin resistant and accumulated more lipids in the liver than WD-FO fed mice. Thus, in this study insulin resistance was accompanied by ectopic fat accumulation rather than obesity. Although a causal relationship is not fully established, hepatic insulin resistance is frequently accompanied by excessive deposition of fat in the liver [37]. The sequence of events leading to insulin resistance is not elucidated, but it was recently demonstrated that development of diet-induced hepatic insulin resistance precedes development of peripheral insulin resistance [45]. The finding that WD-SO fed mice had higher accumulation of hepatic TAG and were more insulin resistant than WD-FO fed mice might thus be related to the fatty acid composition of the diets. A number of studies have demonstrated the ability of marine n−3 PUFAs to prevent diet-induced accumulation of hepatic TAG [2,46,47]. Of note, the levels of the marine omega-3 fatty acids, EPA and DHA, were comparable in all WD-VOs. However, the level of LA was higher in WD-SO than in WD-OO and WD-RO. This might be of great importance for the omega-3 index, as the conversion of LA to arachidonic acid (AA), allows competition between AA and the n−6 PUFAs, EPA and DHA, for incorporation into membrane phospholipids (PL) [10]. In the study by Alvheim et al, 100% replacement of FO with SO in salmon feed led to increased LA and decreased EPA and DHA levels in salmon liver and fillets [24]. The mice fed SO salmon had higher AA and lower EPA and DHA levels in the PLs. Glucose tolerance and insulin resistance were not measured in the study by Alvheim et al., but the lower levels of EPA and DHA were accompanied with a 2.5-fold increased accumulation of hepatic TAG in mice fed SO salmon. Although a cause relationship is not demonstrated, it is possible that the high levels of n−6 PUFAs, LA in particular, in the WD-SO attenuated the ability of marine n−3 PUFAs to reduce hepatic TAG, and thereby contributed to the augmented insulin resistance in WD-SO fed mice.

In the study by Alvheim et al., the Atlantic salmon were fed refined and purified FO and SO. However, the oils commonly used in the aquaculture industry contain POPs, and the use of vegetable oils in aquafeed will introduce different POPs in addition to changing the nutrient composition of salmon fillet. This is an important aspect, as the POPs present in commercially available farmed salmon are transferred to mice consuming the salmon, and these POPs may contribute to development of obesity and insulin resistance [32,33]. Chronic long term consumption of commercially available Atlantic salmon with current levels of POPs caused obesity and insulin resistance in mice [33], and the accumulation of POPs in adipose tissue was similar to levels in mice fed the WD-FO. When the levels of POPs in the salmon were reduced, obesity development and insulin resistance were slightly, but significantly decreased [33]. This study, however, neither obesity nor insulin resistance correlated with accumulation of POPs in adipose tissue. Since obesity in general is often associated with insulin resistance, we cannot exclude the possibility that insulin resistance in the earlier studies [32,33], at least in part, simply reflected the degree of obesity. Laboratory rodents are normally fed a diet where casein is used as the protein source. In a previous salmon study, we replaced 100% of the casein with salmon proteins [33]. Mice receiving salmon as the sole protein source gained significantly more adipose tissue mass than mice receiving casein. The increased adiposity was accompanied with increased digestibility of fat. Thus, in order to limit the difference in fat digestibility, we exchanged only 50% of the casein with proteins from salmon in the present study. The difference in fat digestibility between WD and WD-FO was still significant with higher digestibility in the WD-FO. All the WD-VOs had the same fat digestibility as WD-FO, suggesting that the uptake of fat from the different WDs was comparable.

In the present experiment insulin resistance was more pronounced in mice fed the WD-SO than in mice fed WD-FO although the degree of adiposity was similar in all WD groups. Of note, the levels of PCBs and DDTs reported to accumulate in adipose tissue [33] were comparable to the levels found after 10 weeks feeding with WD-FO in the present study, whereas mice fed the WD-SO diet had lower accumulation of PCBs and DDTs than reported for mice fed Atlantic salmon with reduced POP levels [33]. Obviously, the levels of POPs in adipose tissue are not the sole determining factor of insulin resistance. We cannot exclude the possibility that POPs contributed to the development of insulin resistance, but our results suggest that other factors, such as fatty acid composition in the diets, are of importance.

In view of the limited access to marine fish meal and fish oil for the rapidly growing aquaculture industry, a global contributor to food security, the use of alternative feed ingredients is inevitable. Exchanging FO with VO in aquafeed reduced the accumulation of POPs in adipose tissue by more than 50% in mice consuming the salmon. Importantly, in relation to replacement of fish oil with vegetable oil in future feed for salmon farming, our data clearly demonstrate that VOVs have markedly different spillover effects on metabolism in mice, and that the content of LA may be a matter of concern. This raises the important question as to whether similar effects will be observed in humans consuming Atlantic salmon fed VOVs. SO in particular, and in this context our study suggests that RO and/or OO represent a better choice than SO to replace FO.
References


Macronutrient composition determines total accumulation of persistent organic pollutants from dietary exposure in adipose tissue of mice

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Running title: Accumulation of persistent organic pollutants

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ABSTRACT

Background: Accumulation of persistent organic pollutants (POPs) has been linked to adipose tissue expansion, and therefore, different nutrients may modulate accumulation of POPs.

Objective: To investigate the influence of dietary composition on POP accumulation, and the possible link between accumulation of POPs in adipose tissue and obesity development.

Methods: We fed mice fish oil-based high fat diets during a long-term feeding trial. Further, we performed dose-response experiments using four POPs found in marine sources, PCB-153, PCB-138, PCB-118, and pp’-DDE as single congeners or as mixtures in combination with different diets. We measured accumulation of POPs in adipose tissue and liver, determined obesity development, glucose tolerance, insulin sensitivity, lifespan and hepatic expression of genes involved in metabolism of xenobiotics.

Results: Compared with mice fed diets with a low protein:sucrose ratio, mice fed diets with a high protein:sucrose ratio had significantly lower total burden of POPs in adipose tissue, were protected from obesity development and had enhanced hepatic expression of genes involved in metabolism and elimination of xenobiotics. Exposure to POPs, either as single compounds or mixtures, had no effect on obesity development, glucose tolerance or insulin sensitivity.

Conclusion: The dietary composition of macronutrients modulates adipose tissue accumulation of several POPs. This finding adds a new dimension to the concept of a POP cocktail, which should also be considered in the context of co-ingested macronutrients. Our results further indicate that alterations in macronutrient composition might be an important and additional way of reducing total body burden of POPs.
Lipid-soluble persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) and dichloro-diphenyl-trichloroethanes (DDTs), bio-accumulate through the food web and are stored in human adipose tissue. During the last decade increasing attention has been paid to the possible relationship between POP exposure and the current obesity epidemic (Grun and Blumberg 2007; Newbold 2010; Ruzzin et al. 2012). Although their use was banned in most countries several decades ago, DDTs and PCBs are still found at considerable levels in human adipose tissue due to their earlier widespread use and persistency (Brauner et al. 2012; Malarvannan et al. 2013; Wang et al. 2010). Studies have reported an association between obesity and plasma levels of certain PCBs and pesticides (Dhooge et al. 2010; Lee et al. 2012; Ronn et al. 2011; Roos et al. 2013). Thus, concerns about the possible connection between exposure of environmental contaminants and the epidemics of obesity as well as the type 2 diabetes have been raised (Lee et al. 2014). However, a causal relationship between POP exposure and obesity development has not yet been demonstrated, and inverse relationships between obesity and plasma levels of POPs, in particular highly chlorinated PCBs, are also reported (Dirinck et al. 2011; Lee et al. 2012; Nawrot et al. 2002; Ronn et al. 2011; Roos et al. 2013). Blood levels of POPs per se are not reliable predictors of POP exposure, whereas age and level of serum lipids are more strongly correlated with POP levels in serum (Dirinck et al. 2011; Laden et al. 1999; Lee et al. 2006; Nawrot et al. 2002).

A direct causal link between POP exposure and obesity in humans is difficult to establish, but repeated injections of PCB-153 (Wahlang et al. 2013) and PCB-77 (Arsenescu et al. 2008) are reported to exaggerate obesity in mice. We have previously observed POPs of marine origin accumulate in adipose tissue concomitant with obesity development in rats fed a high fat diet with crude salmon oil (Ruzzin et al. 2010), and in mice fed farmed Atlantic salmon (Ibrahim et
These studies indicated a relationship between dietary POP exposure and development of obesity. However, mice fed high levels of POPs from whale meat were leaner than control casein fed mice, despite a high accumulation of POPs in adipose tissue (Ibrahim et al. 2012). In a recent study we reduced the levels of PCBs and DDTs by 50% in salmon filets by partial replacement of fish oil with vegetable oils in the aquatic feed (Midtbo et al. 2013). The salmon was used for mice feed, and despite a two-fold difference in PCB and DDT concentrations in adipose tissue of salmon fed mice, obesity development was not affected (Midtbo et al. 2013). Importantly, however, in the experiments with mice fed salmon (Ibrahim et al. 2011) or whale (Ibrahim et al. 2012), the diets containing different levels of POPs also had altered nutrient composition. Thus, it is not obvious whether the observed differences in obesity development in these studies are nutrient and/or POP load dependent.

Nutrients and POPs may interact in a complex manner (Hennig et al. 2012). For example, the ability of n-3 polyunsaturated fatty acids (PUFAs) to attenuate the action of PCBs in different cell systems is well documented (Hennig et al. 2012). Such interactions may be of importance as fatty fish and fish oil are sources of both marine n-3 PUFAs and POPs. Generally, the amounts of marine n-3 PUFAs and PCBs increase concomitant with the lipid level in seafood. Although suggested (Ruzzin et al. 2010), it is not known, if the anti-obesogenic effect of n-3 PUFAs is attenuated by POPs. Importantly, however, dietary constituents, such as linoleic acid (Alvheim et al. 2012), sucrose (Ibrahim et al. 2011) and other high glycemic index carbohydrates (Hao et al. 2012) interact with n-3 PUFAs and attenuate their anti-obesogenic effect. Thus, different nutrients may interact with POPs, as well as with each other in a complex manner.
Accumulation of the highly chlorinated and nearly non-metabolizable PCB-153, in adipose tissue of rats depends on whether adipose tissue mass is expanding or decreasing (Jondorf et al. 1983; Muhlebach and Bickel 1981; Wyss et al. 1986). Given that nutrients influence obesity development, the dietary composition may also modulate POPs accumulation. To investigate the potential influence of dietary composition on POP accumulation, and the potential link between accumulation of POPs in adipose tissue and obesity development, a series of animal feeding trials were performed. In several studies age exhibits the strongest correlation with tissue accumulation of POPs. Therefore, we measured accumulation of POPs in adipose tissue in mice fed fish oil-enriched diets with different macronutrient compositions for 6 and 18 months and determined the effect on metabolic parameters and lifespan. We further selected four of the most abundant POPs in adipose tissue (PCB-118, PCB-138, PCB-153 and pp’-DDE) for dose-response studies and measured their deposition in adipose tissue in order to examine the correlation between accumulation in adipose tissue with obesity development in mice. Finally, we administrated the selected four POPs in diets with different fatty acid composition and different protein:sucrose ratios to investigate possible interactions between POPs and feed composition.

Materials and methods

Animals. All animal handling and experimental protocols were approved and performed in accordance with the guidelines of Norwegian Animal Research Authority (NARA). Female C57BL/6J BomTac mice 3 weeks of age were housed four mice per cage at room temperature (22°C) with a 12-h light/dark cycle. The animals had free access to water and were fed their respective diets (Table 1) ad libitum. In all the experiments, the mice were weighed once per week, fresh water provided twice per week and food changed and the intake recorded three times per week. In all the experimental diets with POPs added, the selected amount of POP congeners were first dissolved in dimethyl sulfoxide (DMSO), next dissolved in part of the oil
for the diet and finally added together with rest of the oil to the respective diets. The amount of DMSO did not exceed 0.9 g/kg feed. DMSO was added to the reference diets in a similar concentration to the POPs containing diets. The amount of POPs added to the different diets is shown in Table 2 and 3.

**Determination of POP levels.** Persistent organic pollutants (POPs) were measured in the feed of the long term fed mice. The analysis included a selection of congeners within the group of polychlorinated biphenyls (PCBs), organochlorine pesticides, dioxins (PCDDs), furans (PCDFs) and brominated diphenylethers (PBDEs) as shown in Supplemental Material, Table S1. The levels of POPs were measured according to methods previously described (Berntssen et al. 2010). A selection of relevant POPs was measured in the adipose tissue of long term fed mice (See Supplemental Material, Table S2). In all animal trials, a selected group of relevant POPs was measured in the adipose tissue and liver of mice, as indicated for each experiment in the results.

**Body composition of mice.** Whole body composition of fat mass, lean mass and free water mass was determined in live mice by a non-invasive scan using the Bruker’s minispec LF50 Body Composition Analyzer mq 7.5 (Bruker Optik GmbH), which uses a time domain nuclear magnetic resonance (TD-NMR) system as previously described (Halldorsdottir et al. 2009).

**Glucose-, insulin- and pyruvate tolerance test.** Glucose tolerance test (GTT) was performed in 6h fasted mice by oral gavage of 3 mg glucose/g lean body mass, and in 16h fasted mice by oral gavage of 1.5 mg glucose/g total body mass. Insulin tolerance test (ITT) was performed by injection of 1 U insulin/kg lean body mass (Actrapid, Denmark) in fed mice. Pyruvate tolerance test (PTT) was performed by injection of 3 mg sodium pyruvate (Sigma-Aldrich)/g lean body mass in 6h fasted mice. During all tests, blood was collected from the lateral tail vein at the
indicated time points and blood glucose measured using a glucometer (Ascensia Contour, Bayer, Norway).

**Tissue sampling.** Mice under 4% isofluran anaesthesia (Isoba vet, Schering-Plough, Denmark) were sacrificed by cardiac puncture. Blood samples were collected in tubes containing the anticoagulant heparin (2U/µL), centrifuged (5 minutes at 2400g) to separate plasma and stored at -80°C for further analyses. Organs were immediately dissected out, weighed, snap-frozen in liquid nitrogen and stored at -80°C.

**Plasma measurements.** Insulin levels in plasma were determined using Insulin (Mouse) ELISA kit (DRG Diagnostics, Germany), and plasma glucose was measured by a glucose assay kit (BioVision, USA). The quantitative insulin-sensitivity check index (QUICKI) was calculated using the measured plasma levels of insulin and glucose according to the formula: 1/(log(fasting insulin µU/ml) + log(fasting glucose mg/dl)) (Chen et al. 2005).

**Lipid analysis.** The composition of lipid classes in liver was quantified using high-performance thin-layer chromatography (HPTLC) according to method previously described (Torstensen et al. 2011).

**Quantitative reverse transcriptase PCR.** Total RNA was purified from frozen liver samples, cDNA synthesized and qRT-PCR run as previously described (Lillefosse et al. 2013). Gene-specific primers for qRT-PCR analyses were designed using Primer Express 2.0 (Applied Biosystems) (Primer sequences are available on request). The most stable housekeeping gene as indicated in the results was determined using geNorm and further used to normalize the gene expression level of target genes.

**Statistical analysis.** All data are presented as mean ± standard error of the mean (SEM). The variance of all data sets were tested for homogeneity by Levene’s test, and log-transformed if not homogenous. Statistical differences between several groups were determined by ANOVA.
using unequal LSD post hoc test, and Dunnet’s post hoc test when only compared to one control group. A factorial ANOVA was used when indicated, with the exposure of POPs and dietary macronutrient composition as categorical predictors with Unequal N HSD post hoc test. Repeated measures ANOVA were performed on growth curves. Data with P<0.05 is indicated as significant different. Statistics were performed using Statistica 12 (StatSoft). For glucose-, insulin- and pyruvate tolerance tests the area under the curve was determined by GraphPad Prism 6 (GraphPad Software, Inc.), this software was also used for comparison of survival curves.

Results

Long-term accumulation of POPs. The protein:sucrose ratio determines the obesogenic effect of a fish oil-enriched diet (Hao et al. 2012; Ma et al. 2011), and further, the accumulation of POPs has been reported to depend on whether adipose tissue mass is increasing, stable or decreasing (Jondorf et al. 1983; Muhlebach et al. 1991; Wyss et al. 1986). Therefore, we fed mice fish oil-based high fat diets (Table 1) with high sucrose (FOS) or high protein (FOP) content during a long-term feeding trial. As expected, the level of POPs were higher in the fish oil based diets compared to the low fat reference diet (LF), with comparable levels of POPs in the FOS and FOP diets (Figure 1A and Supplemental Material, Table S1). As expected, we observed an increase in the accumulation of POPs in adipose tissue of the fish oil fed mice from 6 to 18 months (Figure 1C and Supplemental Material, Table S2). Noteworthy, the protein:sucrose ratio of the high fat diets affected the total accumulation of POPs in adipose tissue, where a high protein intake led to reduced accumulation of POPs in adipose tissue (Figure 1C). Thus, long-term accumulation of POPs from fish oil intake is determined by the protein:sucrose ratio. The protein:sucrose ratio also defined body weight and adipose tissue mass gain after 6 and 18 months, where a high protein intake attenuated the obesity development demonstrated in the FOS fed mice (Figure 1B and D). Of note, despite
significantly greater adipose tissue mass in FOS fed mice compared with FOP fed mice, lifespan as determined by 50% survival seemed unaffected by the protein:sucrose ratio, whereas lifespan of both groups was reduced relative to mice fed the LF diet (See Supplemental Material, Figure S1).

**Dose-dependent accumulation of POPs in adipose tissue.** Four abundant POPs found in marine sources (PCB-138, -153, -118 and pp’-DDE) reported to be associated with obesity and/or type 2 diabetes (Dhooge et al. 2010; Lee et al. 2012; Lee et al. 2011a; Lee et al. 2010; Ruzzin et al. 2010; Taylor et al. 2013) were selected, and given separately or as a mixture at different doses (Table 2) in a fish oil-based high fat and high sucrose diet (FOS) (Table 1). The mice fed single POPs or a mixture of POPs exhibited a dose-dependent accumulation of the POPs in adipose tissue (Figure 2A and B). However, despite a wide range of accumulation of POPs in adipose tissue, no effect of either the single congeners or the POP mixtures was observed with respect to body weight gain or fat mass (Figure 2C-F). Further, no differences were observed in feed efficiency, glucose tolerance or insulin sensitivity estimated by the quantitative insulin sensitivity check (QUICKI) (See Supplemental Material, Figure S2). Thus, dose-dependent accumulation of POPs in adipose tissue does not affect obesity development or glucose tolerance.

**Total accumulation of POPs in adipose tissue.** To further evaluate the impact of macronutrient composition on adipose tissue accumulation of POPs as a possible contributing factor in obesity development, we included a low-dose POPs mixture, with POP levels comparable to those found in marine sources. As fish oil previously has been demonstrated to protect against obesity development and insulin resistance (Flachs et al. 2014; Madsen et al. 2005), and corn oil constitutes an abundant fat source in western diets today, we used three different corn oil based diets. High fat diets with high content of sucrose (COS) or protein (COP) and a low fat diet (LF) (Table 1) were given with and without a low-dose POP mixture of the four selected.
POPs (PCB-138, -153, -118 and pp’-DDE) (Table 3). The POPs accumulated in adipose tissue of the mice in response to intake of the POP mixture (Figure 3A). However, the total accumulation of POPs was dependent on the macronutrient composition, and could not be explained by the total intake of POPs. Despite a higher intake of POPs in the mice fed COP+POPs as compared to the LF+POPs fed mice (P=0.034) (not shown), the COP+POPs fed group deposited less POPs in the adipose tissue. As expected, intake of the different diets elicited significant alterations in body mass and fat mass (Figure 3B-C). The COS fed mice had increased body mass, fat mass and adipose tissue burden of POPs compared to the COP fed mice. In contrast, the COP fed mice had the lowest body mass, fat mass and adipose burden of POPs compared to COS- and LF fed mice. However, within each diet, inclusion of POPs did not alter body or fat mass in mice. Accordingly, total burden of POPs in adipose tissue is determined by the macronutrient composition, but has no impact on obesity development.

Glucose tolerance and insulin sensitivity. Insulin sensitivity, glucose- and pyruvate tolerance were measured. Thus, insulin sensitivity, estimated by QUICKI and insulin tolerance test, was reduced in the COS fed mice as compared to the LF- and COP fed mice. However, inclusion of the POP mixture in the diets had no impact on insulin sensitivity (Figure 4A-B). Furthermore, in keeping with previous observations (Ma et al. 2011), intake of the COP diet reduced glucose- and pyruvate- tolerance possibly related to a higher rate of gluconeogenesis and thereby increased hepatic glucose output in the fed state (Fig 4C-D). Neither glucose nor pyruvate tolerance was affected by intake of the POP mixture. Thus, in the present study, insulin sensitivity, glucose tolerance and gluconeogenesis did not change with intake of POPs, but were highly dependent on the dietary macronutrient composition.

Hepatic accumulation of POPs, lipid metabolism and detoxification. The liver is a central organ in lipid metabolism and the main site for detoxification. We observed a dose-dependent accumulation of POPs in the livers of mice according to the exposure to POPs, both as single
congeners and as a mixture (Figure 5A and Supplemental Material, Figure S3). However, the hepatic accumulation of POPs did not affect levels of liver triacylglycerols (TAGs) and total lipids in mice (Figure 5B and See Supplemental, Material Figure S3). Rather, intake of diets with different macronutrient ratios had great impact on hepatic lipid composition and POP accumulation, where a high protein intake (COP) reduced hepatic accumulation of TAGs (Figure 5B) and POPs (with the exception of the PCB-138 congener) (Figure 5A). In addition, the ratio between accumulated POPs and the amount of TAGs in liver was similar in mice fed the LF and COS diets, but we observed an increase in the level of PCBs relative to liver TAGs in response to COP feeding (Figure 5C). The increase in level of hepatic PCBs relative to TAGs indicates that other mechanisms than a change in lipid composition could be involved in the deposition of hepatic PCBs in the COP fed mice.

To further investigate possible mechanisms underlying the alterations in accumulation of POPs in mice in relation to intake of diets with different dietary macronutrient composition, we determined hepatic expression of genes involved in metabolism of xenobiotics, including modification (phase I), conjugation (phase II) and excretion (phase III) of xenobiotics. Interestingly, we observed that intake of the COP diet in itself led to increased expression of six genes involved in metabolism of xenobiotics, comprising genes involved in phase I (Cyp1a2, Cyp4a14), phase II (Sult1a1, Gsto1, Gstt2) (Figure 6) and phase III (Abcb1b) of xenobiotic metabolism (Figure 7A). In line with altered expression of genes encoding enzymes involved in detoxification and excretion, we also observed changes in the hepatic concentration of phosphatidyl choline (PC), and in the ratio of phosphatidyl ethanolamine (PE) to PC (Figure 7C-D) which could indicate an increase in bile excretion upon high protein feeding. The POP mixture per se had minor effect on gene expression, but slight induction of Cyp4a14 mRNA and Gstt2 mRNA in liver in response to an exposure to pp’-DDE and PCB-153 was observed (See Supplemental, Material Figure S4). Together these findings are in keeping with the
observation that only minor alterations in gene expression were detected in response to
exposure to the individual POP congeners in the dose-response trials. Thus, inclusion of POPs
in the concentrations used in our experiments only slightly induced expression of a subset of
genes involved in clearance of xenobiotics. However, dietary macronutrient composition
appeared to be a more powerful driver of expression of genes involved in metabolism and
elimination of xenobiotics. This may, at least in part, explain the lower accumulation of POPs
in the COP fed mice.

**DISCUSSION**

In the present study we fed mice fish oil-based high fat diets with high sucrose (FOS) or high
protein (FOP) content during a long-term feeding trial, and further we performed extensive
dose-response experiments using four POPs found in marine sources. In keeping with previous
observations we found a striking age-dependent accumulation of POPs in adipose tissue. Of
note, mice fed the diet with high protein:sucrose ratio accumulated less POPs in adipose tissue
than mice fed diets with low protein:sucrose ratio. The relative abundance of the individual
PCBs found in adipose tissue of mice fed fish oil for 18 months in this study was similar to
their relative abundance in human adipose tissue (Wang et al. 2010). Moreover, the
concentrations of the most abundant PCBs found in the adipose tissue from the mice fed fish
oil in the present study were within the ranges measured in human adipose tissue in Europe
(Malarvannan et al. 2013; Wingfors et al. 2000) and USA (Stellman et al. 1998), but higher
than those reported in Asia (Wang et al. 2010). In our study the concentration of the primary
metabolite of the pesticide DDT, p,p’-DDE, was lower in fish oil fed mice than in adipose tissue
from humans (Malarvannan et al. 2013; Poon et al. 2005; Stellman et al. 1998). Thus, the
concentration found in adipose tissues in the present study can be considered relevant in the
context of normal levels accumulating in humans.
We selected the two most abundant di-ortho-PCBs, PCB-153 and PCB-138 and the most
abundant mono-ortho PCB, PCB-118, as well as the pesticide metabolite pp’-DDE for further
studies, and as non-monotonic dose-response curves are observed for many toxicants (Lee et
al. 2007; Lee et al. 2010; Taylor et al. 2013), we performed several dose-response trials
including a wide range of doses given to mice as single congeners or as mixtures.

We demonstrate that PCB-118, PCB-138, PCB-153 and pp’-DDE accumulated in adipose
tissue in a dose-dependent manner. However, neither in mice fed the fish oil-based diets nor in
mice fed diets supplemented with the pure selected PCBs and pp’-DDE did we observe effects
on obesity development, glucose tolerance or insulin sensitivity irrespective of doses or whether
the POPs were given as single compounds or in mixtures. The accumulation of POPs in the
dose response experiments ranged from concentrations similar to those found in human adipose
tissue or up to 1000-fold higher (Malarvannan et al. 2013; Poon et al. 2005; Stellman et al.
1998; Wang et al. 2010; Wingfors et al. 2000). In comparison with earlier rodent experiments,
where obesity was reported to be affected (Ibrahim et al. 2011; Ruzzin et al. 2010), the
accumulation of POPs in adipose tissues was similar or up to more than 100-fold higher. The
relationship between intake of POPs and development of obesity and type-2 diabetes is
currently controversial and vigorously debated (Lee et al. 2014) and conflicting results have
also been observed in animal studies (Ibrahim et al. 2012; Ibrahim et al. 2011; Midtbo et al.
2013; Ruzzin et al. 2010). Thus, the present study do not support the notion that accumulation
of POPs accentuates obesity development and metabolic disorders associated with obesity.

However, we cannot exclude the possibility that one or more of the POP congeners and/or POPs
metabolites present in seafood and other food matrices not investigated here could have
changed the outcome on obesity development.
We observed that the total burden of POPs in adipose tissue in mice fed diets with a high protein:sucrose ratio was significantly lower than that of mice fed diets with a low protein:sucrose ratio. This phenomenon may be directly related to the well described ability of high protein diets to attenuate obesity development (Ma et al. 2011; Madsen et al. 2008). This interpretation would also be in line with the earlier observation that accumulation of PCBs, at least the highly chlorinated and nearly non-metabolizable PCB-153, in adipose tissue of rats is depending on whether adipose tissue mass is increasing or decreasing (Jondorf et al. 1983; Muhlebach and Bickel 1981; Wyss et al. 1986). When rats were fed an energy-restricted diet that led to a reduction in adipose tissue mass, less than 0.6 per cent of the injected PCB-153 accumulated in adipose tissue (Jondorf et al. 1983). Furthermore, when adipose tissue mass was kept constant only 38 per cent of the PCB given to the rats were present in adipose tissue after 40 weeks (Wyss et al. 1986). By contrast, rats with an expanding adipose tissue mass exhibited an irreversible storage of 75 per cent of the PCB-153 given to the rats in adipose tissue (Muhlebach and Bickel 1981). Together, these results suggest that the accumulation of POPs in adipose tissue increases when adipose tissue expands. In agreement with this, a nearly linear relationship between body fat and the half-life of POPs has been reported in a wide range of different studies (Milbrath et al. 2009). Thus, the positive correlation between POP levels and obesity reported in several studies (Kim et al. 2011; Lee et al. 2012; Uemura et al. 2009) may not necessarily indicate that POP exposure is a direct causal factor in obesity development, but suggest that a higher body burden of POPs may reflect a continuously expanding adipose tissue mass.

The non-monotonic dose response effects of POPs, demonstrated in several studies (Lee et al. 2007; Lee et al. 2011b, 2010; Taylor et al. 2013), have been suggested to provide a possible explanation for the discrepancies between many studies regarding the relation between POP exposure and obesity development. The present study covers only a limited selection of POPs
found in the environment and a wide range of other POPs might potentially influence obesity
development and insulin resistance. On the other hand, in this study we have used POPs
introduced under condition with control of the background diet, an important factor that is
unaccounted for in many studies. The selected and purified POPs used here are present in many
dietary products today, and thus, relevant in the context of human exposure. However, the
present study provides no evidence that exposure to and accumulation of these POPs affected
either obesity development, glucose homeostasis or insulin sensitivity despite evaluation of a
wide range of POP doses. Importantly, the accumulation of selected POPs was dependent on
the composition of macronutrients in the background diet. A reduction in body burden of POPs
was demonstrated in relation to high dietary protein intake, this was demonstrated for several
of the POPs present in fish oil from the long term fed mice, and also for the selected POPs
evaluated further (PCB-118, -153, -138 and pp'-DDE). It is conceivable that accumulation of
compounds with similar structure (physicochemical properties) would be reduced in response
to a high protein diet.

We demonstrate that intake of POPs in a diet with high protein:sucrose ratio caused lower tissue
accumulation of POPs than intake of POPs in a diet with low protein:sucrose ratio. Reduced
tissue deposition of POPs could either be due to decreased POP absorption from the intestine,
or it could be caused by a higher elimination from the body. Our hepatic gene expression data
supported the latter, as genes involved in phase I-III of the detoxifying machinery were induced
in mice fed a diet with high protein:sucrose ratio diets, irrespective of whether POPs were
included in the diet or not. The present study was not designed to investigate biliary excretion
of POPs. However, based on our data it is tempting to speculate whether differences in
elimination of POPs through bile could contribute to the observed differences in tissue POPs
accumulation. Excretion of POPs through bile would be dependent on bile flow, which is
determined by both a bile acid-dependent fraction and a bile acid-independent fraction (Esteller
We have previously shown that plasma bile acid concentration, likely to reflect hepatic excretion and intestinal reabsorption, can be regulated by the dietary protein source in rats (Liaset et al. 2011; Liaset et al. 2009). Moreover, hepatic glutathione concentration, important for generation of bile acid-independent bile flow (Ballatori and Truong 1992; Esteller 2008), was also regulated by the dietary protein source in rats (Liaset et al. 2011). Thus, as different protein sources can regulate factors important for bile flow, it is not unlikely that dietary protein level can modulate bile flow, and hence, the elimination of undesirable substances such as POPs from the body. Whether or not this was the case in the current study is not known but should be elucidated in future studies.

The possible negative effects of environmental pollutants on human health are matters of great concern considering that many of these chemicals are highly resistant to degradation, bio-accumulate in the food web, and constitute compounds present in all animal products. Accumulating evidence pointing to possible negative health effects linked to exposure of a wide range of different POPs emphasizes the need to reduce the exposure to POPs. If the dietary composition of macronutrients has impact on the detoxification and excretion of several POPs, this could potentially be an important and additional way of reducing body burden of POPs and thus reduce the possible detrimental effects of exposure over time.
REFERENCES


Jondorf WR, Wyss PA, Muhlebach S, Bickel MH. 1983. Disposition of 2,2',4,4',5,5'-hexachlorobiphenyl (6-CB) in rats with decreasing adipose tissue mass. II. Effects of restricting food intake before and after 6-CB administration. Drug Metab Dispos 11(6): 597-601.


## Table 1. Dietary macronutrient composition of the mouse feed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Low fat (g/kg)</th>
<th>Fish oil (g/kg)</th>
<th>Corn oil (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose</td>
<td>Protein</td>
<td>Sucrose</td>
</tr>
<tr>
<td>Protein (Casein)</td>
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<td>540</td>
<td>200</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>669</td>
<td>149</td>
<td>489</td>
</tr>
<tr>
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<td>90</td>
<td>90</td>
<td>430</td>
</tr>
<tr>
<td>Starch</td>
<td>529</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Fat</td>
<td>70</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Corn oil</td>
<td>70</td>
<td>70</td>
<td>250</td>
</tr>
<tr>
<td>Fish oil</td>
<td>-</td>
<td>180</td>
<td>180</td>
</tr>
<tr>
<td>Energy (kcal/g)</td>
<td>4.5</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 2. The concentration of each POP congener in the POPs mixtures added to the fish oil and sucrose diets.

<table>
<thead>
<tr>
<th>Group</th>
<th>PCB-138</th>
<th>PCB-153</th>
<th>pp'-DDE</th>
<th>PCB-118</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOS + low POPs mix (µg/kg)</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>FOS + high POPs mix (µg/kg)</td>
<td>500</td>
<td>500</td>
<td>500</td>
<td>50</td>
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</tbody>
</table>
Table 3. The concentration of each POP congener in the POPs mixture added to the low fat-, corn oil and sucrose- and corn oil and protein diets.

<table>
<thead>
<tr>
<th>Group</th>
<th>PCB-138</th>
<th>PCB-153</th>
<th>pp’-DDE</th>
<th>PCB-118</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS and COP + POPs mix (µg/kg)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>LF + POPs mix (µg/kg)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>0.4</td>
</tr>
</tbody>
</table>
**FIGURE LEGENDS**

**Figure 1.** POP levels measured in mouse feed (A) white adipose tissue mass (n=9-10) (B), levels of POPs measured in gonadal white adipose tissue (gWAT) (n=1-4) (C), and body weight gain (n=9-10) (D) (mean ± SEM) of mice fed low fat- (LF), fish oil and sucrose- (FOS) and fish oil and protein (FOP) diets for 6 and 18 months. Detection limit (DL) <0.3 µg/kg (<0.2 µg/kg for PCB118).

*p<0.05 compared with LF and #p<0.05 compared with FOS at same time points, by one- way ANOVA using Unequal N HSD post hoc test.

**Figure 2.** POP levels (ng) measured in gonadal white adipose tissue (gWAT) (n=2-6) (A and B), body weight gain (C and D) and white adipose tissue mass (n=11-16) (E and F) from mice fed fish oil and sucrose (FOS) diets spiked with the denoted POPs congeners as shown in the figure and in Table 2 for the POPs mixtures after 12-14 weeks of feeding. All data are presented as mean ± SEM.

*p<0.05 compared with FOS as control group by one-way ANOVA using Dunnet’s post hoc test.

**Figure 3.** POP levels (ng) measured in gonadal white adipose tissue (gWAT) (n=2-4) (A), body composition (B) and body weight development (n=16) (C) from mice fed low fat- (LF), corn oil and sucrose- (COS) and corn oil an protein (COP) diets both with and without added POPs mixture as shown in Table 3. All data are presented as mean ± SEM.

*p<0.05 compared to LF, #p<0.05 compared to COS by factorial ANOVA using Unequal N HSD post hoc test with dietary macronutrient composition and dietary POPs as categorical predictors.

*Diet: p<0.05 between all diets by repeated measures ANOVA.
Figure 4. Estimated QUICKI from measured 16h fasted plasma glucose and insulin at sampling after 16 weeks of feeding (A), the area under the curve (AUC) of insulin tolerance test (ITT) in fed animals performed in week 11 (B), AUC of oral glucose tolerance test (OGTT) in 6h fasted animals dosed by lean body mass (3 mg/g) in week 12 (C) and AUC of pyruvate tolerance test (PTT) as measured in change of blood glucose (mmol/L) in 6h fasted animals in week 13 performed in mice fed low fat- (LF), corn oil and sucrose- (COS) and corn oil an protein (COP) diets both with and without added POPs mixture as shown in Table 3. All data are presented as mean ± SEM.

*p<0.05 compared to LF, #p<0.05 compared to COS by factorial ANOVA using Unequal N HSD post hoc test with dietary macronutrient composition and dietary POPs as categorical predictors.

Figure 5. Hepatic levels of POPs (ng) (A), triacylglycerol concentration (mg/g) (B) and amount of POPs (ng) per TAGs (g) (n=4) (C) from mice fed low fat- (LF), corn oil and sucrose- (COS) and corn oil and protein (COP) diets both with and without added POPs mixture as shown in Table 3. All data are presented as mean ± SEM.

*p<0.05 compared to LF, #p<0.05 compared to COS by factorial ANOVA using Unequal N HSD post hoc test, with dietary macronutrients and dietary POPs as categorical predictors.

Figure 6. Hepatic expression of genes involved in xenobiotic metabolism; Cyp1a2 (A) and Cyp4a14 (B) in phase I, and Sult1a1 (C), Gstol (D) and Gstt2 (E) (n=6-7) in phase II from mice fed low fat- (LF), corn oil and sucrose- (COS) and corn oil and protein (COP) diets both with and without added POPs mixture as shown in Table 3. Expression of genes are displayed relative to TATA box binding protein (TBP). All data are presented as mean ± SEM.
*p<0.05 compared to LF, #p<0.05 compared to COS by factorial ANOVA using Unequal N HSD post hoc test, with dietary macronutrients and dietary POPs as categorical predictors.

Figure 7. Hepatic expression of genes involved in bile acid transport and phase III of xenobiotic metabolism; *Abcb1b* (A) and *Abcc2* (B) displayed as relative to TATA box binding protein (TBP) (n=6-7), the measured level of phosphatidyl choline (mg) in liver (C) and the hepatic ratio of phosphatidyl ethanolamine (PE) per phosphatidyl choline (PC) (D) (n=4). All data are presented as mean ± SEM.

* p<0.05 compared to LF, # p<0.05 compared to COS, †POPs effect p<0.05 by factorial ANOVA using Unequal N HSD post hoc test, with dietary macronutrients and dietary POPs as categorical predictors.
Figure 1

A. POPs in feed (μg/kg)

- pp'-DDE
- pp'-DDD
- PCB-101
- PCB-138
- PCB-153
- PCB-118

< DL

B. White adipose tissue (g)

- 6 LF
- 18 LF
- 6 FOS
- 18 FOS
- 6 FOP
- 18 FOP

C. POPs in gWAT (ng)

- 6 LF
- 18 LF
- 6 FOS
- 18 FOS
- 6 FOP
- 18 FOP

D. Body weight gain (g)

- 6 LF
- 18 LF
- 6 FOS
- 18 FOS
- 6 FOP
- 18 FOP
Figure 2

**A** POPs in gWAT (ng)

- PCB-138
- PCB-153
- PCB-118
- pp'-DDE

**B** POPs in gWAT (ng)

- PCB-138
- PCB-153
- PCB-118
- pp'-DDE

**C** Body weight gain (g)

- PCB-138
- PCB-153
- PCB-118
- pp'-DDE

**D** Body weight gain (g)

- PCB-138
- PCB-153
- PCB-118
- pp'-DDE

**E** White adipose tissue (g)

- PCB-138
- PCB-153
- PCB-118
- pp'-DDE

**F** White adipose tissue (g)

- PCB-138
- PCB-153
- PCB-118
- pp'-DDE

Legend:
- LF - 0
- FOS - 0
- FOS - 10 µg/kg
- FOS - 100 µg/kg
- FOS - 1000 µg/kg
- FOS - 10000 µg/kg
- FOS - low POPs mix
- FOS - high POPs mix
Figure 3

**POPs in gWAT (ng)**

- **A**
  - PCB-138
  - PCB-153
  - PCB-118
  - pp’-DDE

**Body composition (g)**

- **B**
  - Body weight
  - Lean mass
  - Fat mass

**Body weight development (g)**

- **C**

* LF
* LF + POPs mix
* COS
* COS + POPs mix
* COP
* COP + POPs mix

* Diet
Figure 4

**QUICKI**

- A

**AUC of ITT (mmol/Lxh)**

- B

**AUC of OGTT (mmol/Lxh)**

- C

**AUC of PTT**

(delta blood glucose from t0 x 90min)

- D

Legend:
- LF
- LF + POPs mix
- COS
- COS + POPs mix
- COP
- COP + POPs mix

* Indicates significant difference from LF.
# Indicates significant difference from LF + POPs mix.
Figure 5

**A**

POPs in liver (ng)

**B**

Triacylglycerols in liver (mg/g)

**C**

Ratio POPs/TAGs (ng/g) in liver

Legend:
- LF
- LF + POPs mix
- COS
- COS + POPs mix
- COP
- COP + POPs mix
Macronutrient composition determines total accumulation of persistent organic pollutants from dietary exposure in adipose tissue of mice

Lene Secher Myrmel, Even Fjære, Lisa K. Midtbø, Annette Bernhard, Rasmus K. Petersen, Si B. Sonne, Alicja Mortensen, Qin Hao, Trond Brattelid, Bjørn Liaset, Karsten Kristiansen and Lise Madsen

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Supplemental Material, Figure S3
Supplemental Material, Figure S4
Supplemental Material, Table S1. Persistent organic pollutants measured in the mouse feed given to long-term fed mice.

<table>
<thead>
<tr>
<th>Persistent organic pollutants (weight/g diet)</th>
<th>Low energy</th>
<th>High fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sucrose</td>
</tr>
<tr>
<td>Polychlorinated biphenyls (PCBs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-ortho-substituted PCBs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCB-77 (pg/g)</td>
<td>&lt;LOQ</td>
<td>2.7</td>
</tr>
<tr>
<td>PCB-81 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-126 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-169 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>Mono-ortho-substituted PCBs</td>
<td>&lt;LOQ</td>
<td>46</td>
</tr>
<tr>
<td>PCB-105 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-114 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-118 (pg/g)</td>
<td>&lt;200</td>
<td>200</td>
</tr>
<tr>
<td>PCB-123 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-156 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-157 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-167 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-189 (pg/g)</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
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<tr>
<td>Di-ortho-substituted PCBs/Non-dioxin like PCBs</td>
<td>&lt;0.30</td>
<td>0.77</td>
</tr>
<tr>
<td>PCB-153 (ng/g)</td>
<td>&lt;0.30</td>
<td>0.60</td>
</tr>
<tr>
<td>PCB-138 (ng/g)</td>
<td>&lt;0.30</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>PCB-180 (ng/g)</td>
<td>&lt;0.30</td>
<td>&lt;0.30</td>
</tr>
<tr>
<td>PCB-101 (ng/g)</td>
<td>&lt;0.30</td>
<td>0.47</td>
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<tr>
<td>PCB-28 (pg/g)</td>
<td>&lt;0.30</td>
<td>&lt;0.30</td>
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<tr>
<td>PCB-52 (pg/g)</td>
<td>&lt;0.30</td>
<td>&lt;0.30</td>
</tr>
<tr>
<td>Sum PCB 7 (ng/g)</td>
<td>-</td>
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<tr>
<td>Organochlorine pesticides</td>
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<tr>
<td>Alpha-endosulfane (ng/g)</td>
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<tr>
<td>Beta-endosulfane (ng/g)</td>
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<tr>
<td>Cis-nonachlor (ng/g)</td>
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<td>Dieldrin (ng/g)</td>
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<td>Isodrin (ng/g)</td>
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<td>Endosulfane-Sulfate (ng/g)</td>
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<td>&lt;LOQ</td>
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<td>Alpha-hexachlorocyclohexane (ng/g)</td>
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**Polychlorinated dibenzo-p-dioxins (PCDDs)**  
2,3,7,8-Tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) (pg/g)  
1,2,3,7,8-Pentachlorodibenzo-dioxin (1,2,3,7,8-PCDD) (pg/g)  
1,2,3,4,7,8-Hexachlorodibenzo-dioxin (1,2,3,4,7,8-HxCDD) (pg/g)  
1,2,3,6,7,8-Hexachlorodibenzo-dioxin (1,2,3,6,7,8-HxCDD) (pg/g)  
1,2,3,7,8,9-Hexachlorodibenzo-dioxin (1,2,3,7,8,9-HxCDD) (pg/g)  
1,2,3,4,6,7,8-Heptachlorodibenzo-dioxin (1,2,3,4,6,7,8-HpCDD) (pg/g)  
Octachlorodibenzo-dioxin (OCDD) (pg/g)  

**Polychlorinated dibenzofurans (PCDFs)**  
2,3,7,8-Tetrachlorodibenzofuran (2,3,7,8-TCDF) (pg/g)  
2,3,4,7,8-Pentachlorodibenzofuran (2,3,4,7,8-PCDF) (pg/g)  
1,2,3,4,7,8-Hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) (pg/g)  
1,2,3,6,7,8-Hexachlorodibenzofuran (1,2,3,6,7,8-HxCDF) (pg/g)  
1,2,3,7,8,9-Hexachlorodibenzofuran (1,2,3,7,8,9-HxCDF) (pg/g)  
2,3,4,6,7,8-Hexachlorodibenzofuran (2,3,4,6,7,8-HxCDF) (pg/g)  
1,2,3,4,6,7,8-Heptachlorodibenzofuran (1,2,3,4,6,7,8-HpCDF) (pg/g)  
1,2,3,4,7,8,9-Heptachlorodibenzofuran (1,2,3,4,7,8,9-HpCDF) (pg/g)  
Octachlorodibenzofuran (OCDF) (pg/g)  

**Polybrominated diphenyl ethers (PBDEs)**  
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Polybrominated diphenyl ether 138 (PBDE 138) (ng/g)  
Polybrominated diphenyl ether 153 (PBDE 153) (ng/g)  
Polybrominated diphenyl ether 154 (PBDE 154) (ng/g)  
Polybrominated diphenyl ether 183 (PBDE 183) (ng/g)  
Polybrominated diphenyl ether 28 (PBDE 28) (ng/g)  
Polybrominated diphenyl ether 66 (PBDE 66) (ng/g)  
Polybrominated diphenyl ether 99 (PBDE 99) (ng/g)
Supplemental Material, Table S2. Persistent organic pollutants measured in the gonadal white adipose tissue of the long-term fed mice.

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<tr>
<td>Sum DDT</td>
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Figure S1. Longevity demonstrated as per cent mice alive until 50 per cent survival of mice fed low fat- (LF), fish oil and sucrose- (FOS) and fish oil and protein (FOP) diets.

*Survival: p<0.05 compared with LF by comparison of survival curves using Log rank (Mantel-Cox) test.
**Supplemental Material, Figure S2**

**Figure S2.** Feed efficiency (n=3) (A and D), area under the curve (AUC) of oral glucose tolerance test (OGTT) in 16h fasted animals dosed by total body mass (1.5 mg/g) (n=8-10) (B and E) and estimated QUICKI from 16h fasted plasma at termination (n=5-6) (C and F) from mice fed fish oil and sucrose (FOS) diets spiked with the denoted POP congeners as shown in the figure for the single POP congeners and in Table 2 for the POP mixtures after 11-14 weeks of feeding. All data are presented as mean ± SEM. *p<0.05 compared with FOS as control group by one-way ANOVA using Dunnet’s post hoc test.
**Figure S3.** Hepatic levels of POPs (ng) (A and C) and concentration (mg/g) of sum lipids in liver (n=3) (B and D) from mice fed fish oil and sucrose (FOS) diets spiked with the denoted POP congeners as shown in the figure and in Table 2 for the POP mixtures after 12-14 weeks of feeding. All data are presented as mean ± SEM.

*p*<0.05 compared with FOS as control group by one-way ANOVA using Dunnet’s post hoc test.
Supplementary figure 4

**Cyp1a2**

**Gsto1**

**Cyp4a14**

**Gst2**

**Sult1a1**

**Abcb1b**

[Legend for colors and conditions]
Figure S4. Hepatic expression of genes involved in xenobiotic metabolism; *Cyp1a2* (A) and *Cyp4a14* (B) in phase I, and *Sult1a1* (C), *Gsto1* (D) and *Gstt2* (E) (n=6-7) in phase II and *Abcb1b* (F) in phase III from mice fed fish oil and sucrose (FOS) diets spiked with the denoted POP congeners as shown in the figure (n=2-4). Expression of genes are displayed relative to calnexin. All data are presented as mean ± SEM.

* p<0.05 compared with FOS as control group by one-way ANOVA using Dunnet’s post hoc test.
Effect of a long-term high protein diet on lifespan, obesity development and gut microbiota in mice

Pia Kiilerich¹, Lene Secher Myrmel¹,², Even Fjære¹,², Qin Hao¹, Floor Hugenholtz³, Si Brask Sonne¹, Murriel Derrien³, Lone Møller Pedersen¹, Rasmus Koefoed Pedersen¹, Alicja Mortensen⁴, Tine Rask Licht⁴, Maria Unni Rømer⁵, Ulla Vogel⁶, Linn Jeanette Waagbø², Qiang Feng⁷, Liang Xiao⁷, Chuan Liu⁷, Bjørn Liaset², Michiel Kleerebezem³, Jun Wang¹,⁷,⁸,⁹,¹⁰, Lise Madsen¹,²,⁷* and Karsten Kristiansen¹,⁷*

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Summary

Female C57BL/6J mice were fed a regular low fat diet or high fat diets with high or low protein:sucrose ratio during their entire lifespan to examine the long-term effect on metabolism and gut microbiota. A high dietary protein:sucrose ratio attenuated life-long high fat diet-induced weight gain and adipose tissue expansion, and initially also expression of inflammatory markers. Three distinct phases of weight gain were observed in all groups, with the largest difference in feed efficiency observed during the second and longest phase (week 10-65). In mice fed the diet with high protein:sucrose ratio, this coincided with increased Ucp1 expression in inguinal adipose tissue. A high dietary protein:sucrose ratio attenuated high fat diet-induced hepatic lipid accumulation. Digital gene expression analyses demonstrated that the protein:sucrose ratio modulated global gene-expression in liver and adipose tissue and indicated that a high protein diet partly prevented the over-time changes in gene expression and adiposity induced by high fat high sucrose diets. Using Mouse Intestinal Tract Chip analysis of fecal bacterial DNA we detected marked effects on the composition of the gut microbiota over time. Dietary fat content, and not the protein:sucrose ratio, was the major driver of gut microbiota composition. Accordingly, dietary fat rather than adiposity appeared to shape the gut microbiota. Our findings support the notion that decreased longevity in response to high fat high sucrose feeding is linked to the high rate of weight gain early in life observed in mice fed a high fat diet with a low protein:sucrose ratio.
Introduction

Intake of high fat diets have been associated with the development of obesity and several metabolic dysfunctions, including insulin resistance, hepatic steatosis, hyperlipidemia and low grade systemic inflammation (Samuel & Shulman 2012). Accumulating evidence indicate that the gut microbiota contributes to the development of diet-induced obesity (Backhed et al. 2004; Turnbaugh et al. 2006; Turnbaugh et al. 2008; Ridaura et al. 2013; David et al. 2014), and additionally influences the development of metabolic dysfunctions associated with obesity (Le Chatelier et al. 2013). Thus, the microbiota may determine uptake of nutrients and augment dietary energy utilization (Turnbaugh et al. 2006), and furthermore the gut microbiota represents an important link to systemic low grade inflammation and the on-set of type 2 diabetes and metabolic dysfunctions (Cani et al. 2008; Qin et al. 2012).

Current Western diets are generally characterized by a high content of dietary fat and carbohydrates. Energy dense high fat diets may promote obesity by increasing the probability of a positive energy balance. Diets with more than 30% energy originating from fat promote obesity in both humans and mice, and in rodents high fat diet-induced obesity is a frequently used model to study obesity and related metabolic disorders (Hariri & Thibault 2010). However, such models do not allow to distinguish whether observed metabolic dysfunction results from the obese state or from the high fat feeding per se. During the last decades, high fat high protein diets, such as the Atkins diet, have increased in popularity as a tool for weight loss, but safety issues for such diets are currently debated (Noto et al. 2013). It is evident from rodent studies that obesity is prevented if the increase in dietary fat is accompanied with a high protein:sucrose ratio (Morens et al. 2005; Pichon et al. 2006; Madsen et al. 2008; Ma et al. 2011; Hao et al. 2012; Freudenberg et al. 2013). This may be due to higher satiety and diet-induced thermogenesis when the protein content in the diet is increased, as well as to an increase in the energy required to utilize amino acids for gluconeogenesis and a concomitant increased ureagenesis (Madsen & Kristiansen 2010). Conversely, reducing the fat content from 40 to 30 energy% is sufficient to counteract insulin resistance (Harris & Kor 1992). Together with the finding that glucose intolerance and insulin resistance are detectable within the first week of high fat feeding, these findings suggest that development of insulin resistance and glucose intolerance may be directly related to the dietary fat content (Turner et al. 2013).

It is generally accepted that obesity reduces longevity (Bluher 2008) and is associated with increased all-cause mortality in humans (Flegal et al. 2013). It is also well documented that
energy restriction increases whereas high fat feeding reduces life-span in rodents, but in most experiments it has been difficult to separate the beneficial effects of caloric restriction *per se* from that of leanness (Bluher 2008). However, the finding that longevity is increased in feed restricted *ob/ob* mice, despite high levels of obesity suggested that longevity is related to feed intake rather than the state of obesity (Harrison *et al.* 1984). On the other hand, the increased longevity in fat specific insulin receptor knockout mice that are protected against diet- and age-induced obesity, despite normal feed-intake, suggested that the reduced fat mass and thereby the possibly reduced burden of obesity related disorders may be of importance (Bluher 2008). Moreover, increasing the protein:sucrose ratio increased the longevity in high fat diet fed mice, and conversely decreased longevity in response to a diet with a low protein:sucrose ratio was linked to a high rate of weight gain early in life (Keipert *et al.* 2011). Still, median life-span in high fat high protein fed mice was reduced compared with low fat fed mice and these findings underscore the importance of macronutrient composition. Interestingly, a recent article using a complex geometric framework for nutrition concluded that the ratio of macronutrients, and not caloric intake was determining various metabolic parameters as well as longevity (Solon-Biet *et al.* 2014).

Reduced biodiversity and compromised stability of the intestinal microbiota have been reported in elderly humans (Tiihonen *et al.* 2010), and modulation of the gut microbiota has been suggested as a modality for longevity extension (Ottaviani *et al.* 2011). Dietary composition (Turnbaugh *et al.* 2008; Hildebrandt *et al.* 2009; Turnbaugh *et al.* 2009; David *et al.* 2014) and the state of obesity (Ley *et al.* 2005) are also important factors determining the microbial diversity in the gut and recent studies indicate that obesity correlates with decreased microbial gene richness (Le Chatelier *et al.* 2013). The linkage between diet, obesity and gut microbiota is, however, not elucidated. On one hand, the findings that conventionalization of germ-free mice with microbiota from both diet-induced (Turnbaugh *et al.* 2008) and genetically obese mice results in recapitulation of the original phenotype (Backhed *et al.* 2004), suggest that the changes are not directly linked to the dietary composition but rather the state of obesity. Further, when body weights were stabilized by reducing the levels of carbohydrates or fat, conventionalization of germ-free mice with microbiota from these mice did not induce obesity in the recipients (Turnbaugh *et al.* 2008). However, high fat feeding studies using obesity resistant RELMβ KO mice suggested that dietary factors are more relevant than obesity *per se* (Hildebrandt *et al.* 2009) and switching from a low fat diet to a high fat diet resulted in changes in the microbiota composition within a single day (Turnbaugh *et al.* 2009). It has been reported
that high-protein diet-induced weight loss in humans was accompanied with changes in the microbiota associated with a potential detrimental metabolic profile in the gut (Russell et al. 2011). It remains to be established if a high protein:sucrose ratio in a high fat diet modulates the gut microbiota in mice.

In this study we aimed to further investigate the long-term effect of high fat diets with low and high protein:sucrose ratios on lifespan, gut microbiota and the development of obesity and related dysfunctions in mice. This approach would also enable us to distinguish whether the observed effects on the gut microbiota reflected the obese state or the high fat feeding per se.
Results

*A high dietary protein:sucrose ratio attenuates high fat diet-induced weight gain and mortality.*

The mice were fed the experimental diets *ad libitum* from three weeks of age and onwards (Table 1) until 50% of the mice in each experimental group had died. This time point was recorded as 50% survival time and all the remaining mice were terminated. In agreement with earlier studies (Madsen *et al.* 2008; Keipert *et al.* 2011; Ma *et al.* 2011), a high protein:sucrose ratio strongly attenuated high-fat diet-induced weight gain (Fig. 1A), but high fat high protein (HFP) fed mice still gained more weight than mice fed the low fat reference (REF) diet. Mice receiving the high fat diet supplemented with sucrose (HFS) were significantly heavier than the reference group already at week 10, whereas the difference between the reference and HFP was significant from week 53. The body weight (BW) gain curves revealed 3 distinct growth phases during the experiment; Phase 1 (week 0-8), Phase 2 (week 10-65) and Phase 3 (week 70-95). Linear fit of each curve in the 3 phases revealed significant differences in growth rates between the diets and phases (Fig. 1A and Supplementary Figure 1). Growth rates for HFS fed mice were higher than HFP fed mice in phase 1 and 2. Overall, growth rates declined significantly with time for all diets (p<0.0001).

Weekly and cumulative energy intakes were significantly higher in both HF groups compared to the low fat reference group (p<0.0001 for all comparisons, Fig. 1B and Supplementary Figure 2). Moreover, weekly and cumulative energy intakes were significantly higher in the HFS group compared to the HFP group (p<0.0039 and p<0.0001, respectively). Feed efficiencies, calculated as Meal energy consumed per gram body weight, were comparable in HFS and HFP fed mice in Phase 1. In Phase 2, the feed efficiency in HFS fed mice, but not HFP fed mice was significantly higher than in the reference group (Fig. 1C). In Phase 3, HFS and HFP fed mice lost body mass.

The 50% survival time was significantly shorter for mice fed the HFS diet compared to the REF group, while over time survival of mice in the HFP group did not differ significantly from either the REF or the HFS group (Fig. 1D). Of note, between 65% and 80% survival, HFP fed mice seemed to have better survival than the HFS fed mice. All deceased animals were subjected to an autopsy, but there was no systematic cause of death in either groups. Thus, in agreement with Keipert *et al.* (2011), a high protein:sucrose ratio attenuated high fat diet-induced weight...
gain in Phase 1 and 2, and furthermore, 50% survival time in mice fed HFP was not statistically different from REF fed mice.

In agreement with previous 3 months studies in male mice (Madsen et al. 2008; Ma et al. 2011; Hao et al. 2012), intake of a diet with high protein:sucrose ratio did not protect the mice against high fat diet-induced glucose intolerance at 3 months (Fig. 2A). Both groups of high fat fed mice displayed an impaired glucose clearance compared to mice receiving the REF diet with the HFP fed mice exhibiting the highest level of glucose intolerance. At 18 months we observed no difference in glucose clearance between the groups (Fig. 2B). A similar finding of a transient impaired glucose tolerance in high fat fed mice has been reported previously (Keipert et al. 2011).

**A high dietary protein:sucrose ratio attenuates high fat diet-induced adipose tissue expansion and expression of inflammatory markers.**

As expected, white adipose tissue (WAT) mass (iWAT, gWAT and rWAT) increased with time in all groups (Fig. 3). Mice fed the diet with a high protein:sucrose ratio were in part protected against high fat diet-induced obesity. Thus, HFS, but not HFP fed mice, had significantly higher WAT mass than the REF group. iBAT mass was significantly lower in HFP than in HFS fed mice, but after 18 months of feeding this difference was no longer significant (Fig. 3). We observed no significant changes in the relative weights of liver, heart, and muscle comparing the HFS and the HFP fed mice, but the relative weight of the kidneys in HFP fed mice was significantly increased (Supplementary figure 3).

To further compare changes over time in response to the low fat diet and the two high fat diets, RT-qPCR analyses of selected genes were performed. An increase in expression of marker genes for browning of white fat, *Ucp1*, *Ppargc1a*, and *Dio2*, was observed in HFP mice compared to HFS mice in the second, and longest growth phase (Fig. 4A) suggesting an increase in energy expenditure in iWAT. The lack of *Ucp1* induction in iWAT in the HFP fed mice during the first growth phase is noteworthy, as we previously observed an increase in *Ucp1* expression in iWAT in HFP fed male mice in the same period (Madsen et al. 2008), suggesting that the response to HFP feeding may exhibit gender differences. The lack of *Ucp1* induction and thus a diminished capacity for uncoupled respiration in the female mice during the first growth phase is also reflected in an equal feed efficiency in the HFP and HFS fed mice in the first phase of growth. This is in contrast to the reduced feed efficiency observed in the HFP fed mice in the second growth phase (Fig. 1C). Reflecting the lower feed efficiency and increased
capacity for uncoupled respiration in WAT of the HFP fed mice, the relative mass of WAT was significantly lower in the HFP fed mice compared with HFS fed mice at 6 months and 18 months, and a similar tendency was observed comparing iWAT and rWAT (Fig. 3). Compared to the REF group, expression of Ucp1 in iWAT in both HFS and HFP fed mice was significantly higher at 6 months with the highest level observed in the HFP fed mice. The same was observed for Ppargc1a and Dio2. However, at 18 months, the REF fed mice exhibited the highest expression of Ucp1, Ppargc1a and Dio2, and the differences in expression between HFS and HFP fed mice were no longer observed.

High fat feeding and adipose tissue expansion are associated with low-grade inflammation in adipose tissue. Thus, we measured mRNA expression of selected markers for inflammation and macrophage infiltration in iWAT (Fig. 4B). HFS feeding led to increased expression of mRNA encoding inflammatory markers, Ccl2 (chemokine (C-C motif) ligand 2) and Serpinel (serine (or cysteine) peptidase inhibitor, clade E, member 1), but not in macrophage infiltration markers, Cd68 and Emr1 (EGF-like module containing mucin-like, hormone receptor-like sequence 1), at 3 and 6 months. This increase was attenuated in mice fed the diet with high protein:sucrose ratio. However, after 18 months of feeding expression levels were similar in all groups, except that expression of Cd68 and Emr1 mRNA was elevated in the HFP fed mice compared with HFS and REF fed mice.

A high dietary protein:sucrose ratio attenuates high fat diet-induced hepatic lipid accumulation.

Obesity and high fat feeding, in particular, are associated with hepatic steatosis. Mice fed a diet with high protein:sucrose ratio were protected against high fat diet-induced accumulation of triacylglycerol in the liver for at least 6 months (Fig. 5A). Hepatic levels of free cholesterol did not differ significantly between HFP and HFS fed mice. By contrast hepatic levels of steryl esters were significantly lower in HFP fed mice compared to HFS fed mice after both 3 and 6 months of feeding. After 18 months of feeding, the hepatic lipid levels were not significantly different; however, plasma cholesterol levels were still significantly lower in HFP fed mice (Fig. 5B).

To investigate if hepatic lipid accumulation was associated with changes in expression of genes involved in fatty acid synthesis and oxidation, RT-qPCR analyses were performed. We observed dramatic changes in the levels of mRNA encoding key enzymes in fatty acid synthesis over time and in response to the different diets (Fig. 6A). After 3 months of feeding, the levels
of mRNAs encoding FAS and SCD1 were high in LF and HFS fed mice, whereas much lower levels were observed in HFP fed mice. However, the differences in Fasn expression were less pronounced with time, and at 18 months there were no significant differences comparing the low fat with the two high fat groups. By 18 months, the level of Scd1 mRNA was significantly higher in the low fat fed mice compared to the HF fed mice (Fig. 6A). Expression of Srebf1, another key gene involved in lipogenesis, exhibited no clear difference between the three feeding groups as determined by mRNA levels, but similar to Fasn and Scd1 expression decreased markedly over time. Surprisingly, levels of Acc1(Acaca) mRNA increased significantly over time. Overall, the lower expression of lipogenic genes in HFP fed compared to HFS fed mice at 3 months, suggesting a lower level of de novo hepatic fatty acid synthesis, was largely lost during the second and third growth phase. However, still the HFP animals remained less obese than the HFS animal throughout the study.

By 3 months of feeding the levels of mRNAs encoding enzymes involved in fatty acid oxidation (ACO, CPT-1 and MCAD) and ketogenesis (HMGCS2) were similar in LF and HF fed mice. By 6 and 18 months of feeding, expression of Acox1, Cpt1a and Hmgcs2 mRNAs had declined, whereas levels of Acadm mRNA remained high. Yet, by 6 months, expression of the genes involved in fatty acid oxidation, Acox1 and Cpt1a, were up-regulated in the HF mice compared to LF fed mice (Fig. 6B). A sustained increase in mRNA encoding enzymes involved in amino acid degradation (GOT1, CPS1, AGXT and GPT-1) and gluconeogenesis (PEPCK) was observed throughout the experiment in HFP mice (Fig. 6C-D) indicative of an increased energy expenditure in HFP animals compared to HFS animals. This is in keeping with the decreased feed efficiency in HFP fed in the second growth phase, and in agreement with the lower body weight and fat pad mass in the HFP animals. Thus, the protein:sucrose ratio strongly affected gene expression during the first phase of feeding, but the difference between the HFS and HFP fed mice diminished over time.

_The protein:sucrose ratio modulates global gene expression in liver and adipose tissue._

To further investigate the influence of the protein:sucrose ratio and length of treatment, digital gene expression (DGE) analyses were performed for liver and two metabolically different adipose tissue depots, iWAT and gWAT.

PCA plots show clear separations between the samples due to time (6 vs. 18 months) more than the protein:sucrose ratio in all 3 tissues (Fig. 7A). Relatively few genes shared between HFS and HFP were differentially regulated with time in the liver, while 361 genes were differentially
regulated between 6 and 18 months in liver of HFP fed mice. The HFS diet caused many more genes to be differentially regulated between 6 and 18 months in iWAT (663+150) and gWAT (1014+199) compared to HFP (iWAT (154+150) and gWAT (269+199)), indicating that HFP feeding in part prevented the over time changes in gene expression that are taking place in WAT of mice fed a HFS diet (Fig. 7B).

The HFS diet increased the expression of genes involved in fatty acid synthesis in the liver and storage of fat in gWAT compared to mice fed the HFP diet after 6 months feeding as determined by the DGE analysis (Supplementary Figure 4A). Furthermore, expression of genes involved in lipid metabolism and fatty acid oxidation was increased in the liver and gWAT, respectively, along with an inhibition of lipolysis in gWAT. Compared to HFP feeding, HFS feeding increased expression of proinflammatory genes in iWAT, especially Pai-1/Serpine1, along with an increase in expression of genes encoding adipokines after 6 months (Supplementary figure 4B). This suggests that the combination of high intake of sucrose and fat accentuated a state of local inflammation in white adipose tissues, possibly related to adipocyte hypertrophy, in addition to systemic inflammation.

**The dietary fat content is a major driver of gut microbiota composition.**

To investigate the temporal influence of the protein:sucrose ratio on the bacterial composition in the gut, bacterial DNA from feces was analyzed using a Mouse Intestinal Tract Chip (MITChip). The MITChip analysis showed a distinct microbiota profile at the probe level for the high fat diets, clearly separated from the low fat reference (Fig. 8A), whereas the microbial diversity did not show any significant differences (Supplementary Figure 5A). In mice fed the REF or the HFS diet we observed a trend towards an over time increase in the relative abundance of Bacteroidetes with a corresponding decline in the relative abundance of Firmicutes. In the HFP fed mice this increase in the relative abundance of Bacteroidetes and the decrease in the relative abundance of Firmicutes became significant (Fig. 8B).

To further examine the microbial changes, genus level data were analyzed by principal response curves (PRC) and redundancy analysis (RDA). PRC analysis showed that the microbial composition in animals fed either of the HF diets deviated from animals that received the low fat REF diet (Supplementary figure 5B). The total variation explained by all explanatory variables is 38.8%. Of this variation, 29.6%, 24.2% and 46.4% could be attributed to the different diets, time, and differences between replicates, respectively. The diet as explanatory variable had a significant influence on the microbiota composition during the different time
points in the first and second PRC (p<0.05, Monte Carlo tests). The genus-like/bacterial groups depicted in Supplementary Figure 5B display a positive weight decrease in abundance in mice fed either of the high fat diets. Bacteria belonging to the class of Clostridia (Coprobacillus, C. ramnosum), Mollicutes (Allobaculum, Acholeplasma and Solobacterium moorei) and the order of Lactobacilliales (L. salivarius, L. Delbrueckii, L. paracasei, L. Acidophilus and L. plantarum) decreased in abundance in HF fed mice, whereas bacteria belonging to the class of Gammaproteobacteria (E. coli, Vibrio et rel., Pasteurella and Labrys methylaminiphilus) and the Fusobacteria phylum (Fusobacterium) as well as Clostridium difficile and Akkermansia muciniphila increased in abundance in HF fed mice.

To identify bacteria for which abundances differed over time with respect to the protein:sucrose ratio, a PRC analysis was performed on the HFS and HFP groups only (Supplementary Figure 5C). This latter PRC explains 8.9% of the total variation and the axes were not significant. The main drivers in the difference between HFS and HFP over time were Sutterella wadsorthia et rel., Akkermansia muciniphila and Olsenalla et rel. that were found at higher abundances, and unclassified Porphyromonadaceae and Clostridium cluster XVIa together with Labrys methylaminiphilus and Fibrobacter succinogenes, which were found at lower abundances when protein content was high. Supplementary Table 1 summarizes the relative abundances of the genus-like groups shown in the PRCs.

To confirm the importance of the fat content in the diet over time, RDA plots were made for each time point (Supplementary Figure 6A). For all three time points, the low fat REF diet contributed significantly to explain the variation in the microbiota composition (p<0.05, Monte Carlo permutation tests). The genus-like groups that correlate at all three time points with the high fat diets are two Clostridialis (Clostridium leptum et rel. and Anaerovorax et rel.) and two Lactobacillales, Lactobacillus spp (Lactobacillus delbrueckii et rel. and Lactobacillus plantarum et rel.)

The RDA plots of the diets (Fig. 9), where only time and weight are the explanatory variables, show clear separation of the different time-points indicating time-dependent differences in the microbial composition for all three diets. Interestingly, the bacterial species driving the differences between time-points exhibited a time-dependent coincidence amongst the diets. No clear overlap of common bacterial taxons were seen at 3 months (Fig. 9A), while Desulfovibrionales (Desulfovibrio), Coriobacteriales (Olsenella) and Verrucomicrobia (Akkermansia) are mutually driving differences in microbiome composition between 6 months
bacteria belonging to the order Clostridiales (C. sphenoides, R. obeum, R. Callidus, C. leptum, C. herbivorans, Anaerotruncus and E. hallii) seem to be major determinants of microbiome composition at 16 months, suggesting a concurrence of the microbiota with age regardless of diet. Together, these three RDA plots clearly demonstrate that the drivers of the microbial composition in the high fat diet fed mice at all time-points were quite different from those identified in low-fat fed mice.

Interestingly, the specific differences between the HFS and HFP animals seem to vanish with time, as evidenced by a good separation between HFS and HFP at 3 months that gradually disappeared from 3-6 and 6-16 months (Supplementary Figure 6A and 6B). There were no common species correlating with one of the diets on all three time points. Together these data demonstrate that dietary fat is a major factor determining the composition of the gut microbiota.

**Discussion**

Rodent models of high fat diet-induced obesity have provided considerable insight into obesity-related metabolic disorders (Hill et al. 2000). However, in this setting it is difficult to distinguish obesity related effects from effects elicited by high fat feeding per se. In this study we took advantage of the notion that obesity is prevented if the increase in dietary fat is accompanied with an increased protein:sucrose ratio (Morens et al. 2005; Pichon et al. 2006; Madsen et al. 2008; Ma et al. 2011; Hao et al. 2012; Freudenberg et al. 2013). Although such high fat high protein diets have increased in popularity, the long-term safety remains an issue (Noto et al. 2013). Therefore, we fed mice high fat diets with both a high and low protein:sucrose ratio during their entire lifespan.

Compared to the low fat REF group, 50% survival time was significantly shorter for mice fed the HFS diet, but not for mice fed the HFP. Thus, in agreement with Keipert et al (2011), increasing the protein:sucrose ratio, at least in part, protects mice against high fat diet-induced reduced lifespan. The initial increased caloric intake during the first 10 weeks observed in mice fed the HFS diet has previously been seen in male mice in some (Voigt et al. 2013), but not all studies (Madsen et al. 2008; Ma et al. 2011). The higher weekly and cumulative energy intake in the HFS group compared to the HFP group may in part be related to an increased food intake, particularly in week 5 and 6, where extra feed had to be placed in 4 out of 6 cages in the HFS group. Still, similar to our observation of the early increased caloric intake and expansion of adipose tissue and shortened life span of HFS fed mice, Keipert et al (2011) concluded that the
harmful effects of high fat diets on longevity were linked to the early and rapid obesity development. The conclusion that a diet with a high protein:sucrose ratio supported longevity is apparently at variance with the conclusion of a recent study, reporting that longevity and health were improved when protein was replaced by carbohydrates (Solon-Biet et al. 2014). However, in that study a complex matrix of macronutrients was used, and the conclusions on the effects of protein versus carbohydrates were based on diets with greatly different fat content, and thus, these results cannot be directly compared with our studies using a fixed content of fat only varying the ratio between carbohydrates and protein.

Unlike shorter experiments using male mice (Madsen et al. 2008; Ma et al. 2011; Hao et al. 2012), mice fed the HF diet supplemented with protein gained more weight than mice fed the low fat reference diet. Still, after 18 months of feeding the masses of the white adipose tissue depots in HFP fed mice, were not significantly higher than in low fat fed mice. Interestingly, the body weight gain curves revealed three distinct growth phases during the experiment; Phase 1 (0-8 weeks of feeding), Phase 2 (10-65 weeks of feeding) and Phase 3 (70-95 weeks of feeding). Overall, the increased body weight in both high fat fed groups in Phase 1 can be explained by the increase in feed intake in this period in parallel with equal feed conversion rates between the groups. On the other hand, while the feed intake during Phase 2 seems to be equal among the groups, the curves representing the cumulative energy intake are parallel and higher feed efficiency in the HFS group could serve to explain the continued significantly higher increase in body weight in this group compared to the HFP group.

Female mice are largely protected against the metabolic syndrome, despite obesity and increased serum TAG and cholesterol, when fed a high fat diet (Pettersson et al. 2012). However, similar to our earlier finding in male mice, high fat feeding in combination with high amounts of sucrose led to impaired glucose tolerance (Ma et al. 2011; Hao et al. 2012). Increasing the protein:sucrose ratio did not protect the mice against high fat diet-induced glucose intolerance when mice were fed for 3 months, despite protection against obesity. Supporting this, expression of Pepck, the main regulator of gluconeogenesis and hepatic glucose output in diabetes was increased in HFP fed mice. In agreement with a similar report of a transient impaired glucose tolerance in high fat fed mice (Keipert et al. 2011), we observed no difference in glucose clearance between the groups after 18 months.

Increasing the protein:sucrose ratio, protected the mice against high fat diet-induced accumulation of triacylglycerol in the liver for at least 6 months. Moreover, the high
protein:sucrose ratio alleviated the initial high fat diet-induced expression of inflammatory markers in adipose tissue. In line with an earlier report that female mice were protected against high fat diet-induced macrophage infiltration (Pettersson et al. 2012), we did not detect increased expression of macrophage selective markers in the first two growth phase. However, after 18 months of feeding, expression of the inflammatory markers, Cd68 and Emr1, in adipose tissue was surprisingly higher in HFP fed mice than in HFS and REF fed mice. In agreement with our earlier short term experiments in male mice (Madsen et al. 2008; Ma et al. 2011; Hao et al. 2012), RT-qPCR analyses of liver mRNA indicated that a high protein:sucrose ratio led to increased amino acid metabolism and ureagenesis, as well as reduced lipid synthesis during the first months of feeding. Of note, however, these changes were not sustained throughout the study, and the analysis of global gene expression also demonstrated that the number of differentially regulated genes in the HFS and HFP fed mice decreased over time.

Although a number of studies have identified the state of obesity as an important factor affecting the gut microbial composition (Ley et al. 2005), the linkage between diet, obesity and gut microbiota remains to be elucidated. Increasing the protein:sucrose ratio in the HF diet did not lead to prominent changes in the gut flora. Only minor differences between mice fed HFP and HFS were observed and these gradually disappeared after three months. Although we cannot rule out that the early differences are of more importance than those persisting, or the possibility that observed differences, such as higher abundance of Sutterella wadsorthia et rel., and Akkermansia muciniphila and lower amounts of unclassified Porphyromonadaceae in HFP fed mice compared to HFS mice play a role in obesity development, our study strongly suggests that the fat content of the diet is the most prominent factor determining the gut microbiota. For instance, we observed that high fat feeding with both low and high protein:sucrose ratio was accompanied by lower abundance of Akkermansia muciniphila, a bacterium earlier associated with leanness and inversely correlated with body weight (Everard et al. 2013). Furthermore, we observed a higher Firmicutes to Bacteroidetes ratio associated with an increased capacity for energy harvest by the gut microbiota in both high fat diet groups despite different rates of obesity development and feed efficiency. Thus, our study is in line with the suggestion that the early notion indicating that obesity is associated with a high ratio of Firmicutes to Bacteroidetes is not universal (Duncan et al. 2008). However, the age-dependent decrease in Firmicutes and increase in Bacteroidetes in all feeding groups are in keeping with the changes observed in elderly people (Claesson et al. 2012) which were associated with a decrease in energy harvest from the food and also reflected in lower feed efficiency in all groups with aging. Together, our
MITChip analyses support the notion that the amount of fat in the diet, and not obesity *per se* is the driving force determining the composition of the gut microbiota.

In humans, weight loss induced by a protein-rich diet has previously been shown to be accompanied by a reduction in *Roseburia/Eubacterium rectale* and associated with a potentially detrimental metabolic profile in the gut (Russell *et al.* 2011). In the present study, increasing the protein content in the diet did not affect levels of *Roseburia/Eubacterium rectale*. However, the abundance of *Roseburia inulinivorans* was reduced when mice were fed high fat diets and this is in line with previous rodent studies (Neyrinck *et al.* 2012). The abundance of this bacterium has been shown to be reduced in type 2 diabetic humans (Qin *et al.* 2012). *Roseburia inulinivorans*, which convert a variety of dietary oligosaccharides ranging from inulin to sucrose, but also starch, into butyrate (Scott *et al.* 2011), has been demonstrated to elicit a protective effect against development of obesity and insulin resistance in mice (Gao *et al.*, 2009), but butyrate production, on the other hand was also recently reported to increase the incidence of colorectal cancer (Belcheva *et al.* 2014).

Together, our findings support the notion that decreased longevity in response to high fat feeding is linked to the high rate of weight gain early in life observed in mice fed a diet with a low protein:sucrose ratio. Increasing the protein:sucrose ratio in the diet protects against high fat diet-induced obesity, as well as hepatic lipid accumulation. Accordingly, we observed the greatest diet-dependent differences in gene expression during the first phase of growth, week 1-8. Over time these differences diminish, emphasizing the importance of the early growth phase. We detected marked effects on the composition of the gut microbiota over time, and fat rather than adiposity or protein:sucrose seemed to be a major driver shaping the gut microbiota.
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Author Contributions

UV, LM and KK designed the experiment. PK, LSM, EF, QH, FH, SBS, RKP, CC, AM, TRL, MUR, QH, MK, LJW, QF, LX, CL and BL performed the experiments and analyzed data. PK, FH, LM and KK wrote the manuscript; all authors read, commented and approved the final version of the manuscript.

Experimental Procedures

Animals. 150 female C57BL/6 mice (3 weeks of age, SPF, Taconic Europe) were divided into 3 experimental groups with 50 mice in each with an overall equal body weight mean for all the groups. Within in each group mice were randomly divided into 10 cages with 5 animals in each. Mice were kept at 55±5% humidity, 22±1°C, air change ratio 8-10 times/hour in a 12:12 light:dark cycle.

Mice were fed *ad libitum* with a low fat diet or a corn-oil based high-fat diet (25%) enriched with either protein or sucrose (43%) (Table 1) for their entire lifespan. Body weight, feed and water intake were recorded once a week throughout the experiment. Animals dying during the experiment were autopsied and internal organs examined for tumors and other anomalies. Clinically sick animals were euthanized and autopsied when their condition was estimated to cause death within 24 hours or when it was deemed unethical to keep the animal alive. For each feeding group the experiment was terminated when 50% of the animals were dead.

10 mice from each group were sacrificed 3, 6 and 18 months after the start of the experiment by cardiac puncture after anesthesia. Animals were weighed; blood was collected and separated into plasma and RBC before freezing at -80°C. Internal organs, liver, heart, kidney, tibialis anterior, interscapular brown adipose tissue (iBAT), inguinal white adipose tissue (iWAT), gonadal white adipose tissue (gWAT), retroperitoneal white adipose tissue (rWAT)) were weighed and stored at -80°C until further analyses. Feces were collected from the cages during 2 days at 3, 6 and 16 months after the start of the experiment.
The experiment was approved by the Animal Experiment Inspectorate in Denmark and was conducted in compliance with the European convention for the protection of vertebrate animals used for experiments and other scientific purposes (Council of Europe, no. 123, Strasbourg, France, 1985).

**Oral glucose tolerance test.** Glucose (1.5 g/kg body weight) was administered orally in overnight fasted mice. Blood was collected from the tail vein of conscious animals and blood glucose was measured at baseline and at the indicated time points using a glucometer (Ascensia Contour, Bayer, Norway).

**RNA extraction and qRT-PCR.** RNA was extracted from liver, iWAT and gWAT using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized in duplicates using TaqMan Reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) containing Multiscribe Reverse Transcriptase. Gene expression was determined in 384-well reaction plates by RT-qPCR using LightCycler 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland). Expression of target genes was normalized to TATA box-binding protein (TBP). Primer sequences are available upon request.

**Tissue lipid extraction and lipid class analysis.** Total lipid was extracted from liver samples with chloroform:methanol, 2:1 (v/v) and quantified on a Camaq HPTLC system and separated on HPTCL silica gel as previously described (Ma et al. 2011).

**Plasma insulin.** Insulin (mouse) ELISA-kit (DRG Instruments, GmbH, Germany) was used to measure insulin levels in plasma.

**Plasma lipids.** Plasma triacylglycerol and cholesterol were determined using conventional enzymatic kits (DIALAB, Austria) and a MaxMat PL II (MAXMAT S.A., Montpellier, France).

**Digital gene expression profiling (DGEP) and data analysis.** Tag library preparation from liver, iWAT and gWAT was performed using Illumina NlaIII Gene expression sample preparation kit and sequenced using Ilumina Genome Analyzer II system (BGI-Shenzhen, P.R. China) according to the manufacturer’s recommendations. Image analysis, base calling and extraction of tags were performed using Illumina pipeline. The tag entities of DGEP libraries were mapped to mRNA reference using bwa (Li & Durbin 2009). The matched tags were filtered by the match position to NlaIII recognition CATG sites by custom perl scripts. Bioconductor package edgeR (Robinson et al. 2010) was used to perform statistical analysis.
for determine the significantly different genes between diets and time points in different tissues. GO annotation was performed using bioconductor package GOstats (Falcon & Gentleman 2007).

**MITChip analysis.** The microbial community in the intestinal samples were analysed using the Mouse Intestinal Tract Chip (MITChip). This phylogenetic microarray was designed using criteria of the Human Intestinal Tract Chip (HITChip) developed by Rajilic-Stojanovic et al., (2009). The MITChip consists of 3,580 different oligonucleotides specific for the mouse intestinal microbiota (Geurts et al. 2011). The array targets the V1 and V6 regions of 16S rRNA genes of bacteria. The 16S rRNA genes were amplified from twenty nanogram of intestinal metagenomic DNA with the primers T7prom-Bact-27-F and Uni-1492-R (Table 2). These PCR products were transcribed, labelled with Cy3 and Cy5 dyes and fragmented as described elsewhere (Rajilic-Stojanovic et al. 2009). Finally the samples were hybridized on the arrays at 62.5°C for 16 hours in a rotation oven (Agilent Technologies, Amstelveen, The Netherlands). After washing and scanning of the slides, data was extracted with the Agilent Feature Extraction software, version 9.1. The data were normalized and analyzed using a set of R-based scripts in combination with a custom-designed relational database, which operates under the MySQL database management system.

The RPA signal intensities were taken to analyze microbiota profiles at different levels of taxonomic resolution as indicated in the text (Lahti et al. 2011). Phylum level data was used to calculate the Bacteroidetes and Firmicutes levels, and differences were tested with the Student’s t-test. To determine correlation of genus-like level microbial groups detected on the MITChip with a specific diet, redundancy analysis (RDA) and Principal response curve (PRC) analysis as implemented in Canoco for Windows 5 was used (Braak & Smilauer 2012). RDA is a linear method of canonical ordination, which linearly combines explanatory variables on the ordination axis. PRC combines the amounts of genus-like groups, after logarithmic transformation, to a new single variable. The bacterial groups with large deviations weigh high in this combination while groups that have equal amounts in the control diet and the high fat diets have zero weight. The quality of the PRC graph is expressed by the ratio of the variance explained by the PRC axis, and the variance of all deviations across time. The Monte Carlo Permutation test was used to assess the significance of the variation in the RDA’s and PRC’s. Probe level data was used to calculate the Shannon diversity with an in-house R-script.
Statistics. All data are presented as means ± standard error of the mean. All statistical analyses were carried out in GraphPad Prism, except Digital Gene Expression Profiling and MITChip analysis (see their respective paragraphs for details). One-way, two-way and repeated measures ANOVA were followed by a Bonferroni adjusted Fisher’s Least Significant Difference (LSD) test where appropriate, taking into account the total number of pairwise comparisons. In all cases a significance level of $\alpha = 0.05$ was used.
References


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Figure legends

**Figure 1:** Body weight gain (A), weekly energy intake (B), feed efficiency (C), and survival (D) of female mice on a high-fat diet supplemented with either 43 energy% sucrose (HFS) (red) or 43 energy% protein (HFP) (blue) compared to a low fat reference diet (REF) (black). (A) A two-way ANOVA was applied to detect overall effects of diet on body weight (p<0.0001 for all comparisons) and time (p<0.0001 for all diets). Post-hoc Bonferroni adjusted pair-wise comparisons were used to detect weekly differences between diets (p<0.05). (B) Weekly energy intake in the three experimental groups during the lifespan of the animals. Two-way ANOVA was applied to detect overall effect of diet. p<0.0001 between REF-HFS and REF-HFP and p<0.0111 between HFS-HFP. (C) Feed efficiencies were calculated as the ratio between the amount of BW gained (in grams) divided by the amount of kcal ingested on average in the three growth phases. One-way ANOVA was used to detect overall effect of diet (p<0.0365 in the second growth phase) within one growth phase followed by post-hoc Bonferroni corrected pairwise comparisons (p<0.05). Uppercase and italic letters denote significant difference within the second and third growth phase, respectively. (D) Kaplan-Meier survival curves were analyzed using a log-rank test showing significant difference in survival only between REF and HFS (p=0.0148).

**Figure 2.** Oral glucose tolerance test (OGTT), area under the curve (AUC) for OGTT and fasting blood (16h) glucose after 3 (A) and 18 months (B) of feeding REF, HFS and HFP diets. The OGTT and AUC panels were analyzed using a repeated measures two-way ANOVA and one-way ANOVA, respectively, followed by Bonferroni corrected pair-wise comparisons, n=8.

**Figure 3.** Fat pad weights of iWAT (A), gWAT (B), rWAT (C) and iBAT (D) after 3, 6 and 18 months of REF, HFP or HFS feeding as percentage of total body weight. Lower-case, upper-case and italic letters denote significant differences within the 3, 6 and 18 months samples, respectively. Values with no letters in common are significantly different as determined by one-way ANOVA followed by Bonferroni corrected pair-wise comparisons, p<0.05, n=9-10.

**Figure 4.** RT-qPCR analysis of gene expression in inguinal white adipose tissue during long-term LF-REF, HFS and HFP feeding. Genetic markers for browning of white fat (Ucp1, Ppargc1a, and Dio2) (A) and macrophage infiltration (Cd68 and Emr1) (B) and inflammation (Ccl2 and Serpine1) (C) in iWAT. Significant differences between diets were detected using a
one-way ANOVA followed by Bonferroni-corrected pairwise comparisons (p<0.05). Lowercase, uppercase and italic letters denote significant difference between diets within the 3, 6 and 18 month time-point, respectively. n=4-8

**Figure 5.** Lipids in liver (A) and plasma (B). Significant differences between diets were detected using a one-way ANOVA followed by Bonferroni-corrected pairwise comparisons (p<0.05). Lowercase, uppercase and italic letters denote significant difference between diets within the 3, 6 and 18 month time-point, respectively. n=4-8.

**Figure 6.** qRT-PCR analysis of gene expression in liver during long-term REF, HFS and HFP feeding. Liver markers for lipogenesis (FAS (Fasn), ACC1 (Acaca), SCD1 (Scd1), and SREBP-1c (Srebf1)) (A), Fatty acid oxidation (ACO (Acox1), CPT-1 (Cpt1a), MCAD (Acadm)), and ketogenesis (HMGCS2 (Hmgcs2)) (B), Amino acid degradation (GOT1 (Got1), CPS1 (Cps1), AGXT (Agxt) (C), and GPT-1 (Gpt)) and gluconeogenesis (PGC1α (Ppargca), and PEPCK (Pck1) (D). Significant differences between diets were detected using a one-way ANOVA followed by Bonferroni-corrected pairwise comparisons (p<0.05). Lowercase, uppercase and italic letters denote significant difference between diets within the 3, 6 and 18 month time-point, respectively. n=4-8.

**Figure 7.** Digital gene expression profiling show differential regulation with time and diet. PCA plots showing distinct clustering of samples mostly dependent on time (A) and with corresponding Venn diagrams showing the degree of overlapping gene expression between the diets (FDR<=5%) (B). Differentially expressed genes at 6 months in gWAT, iWAT and liver (FDR<=5%).

**Figure 8.** MITChip-based analysis of fecal microbiota. (A) Pearson distance-based clustering of microbiota profiles obtained by 16S rRNA gene-targeted MITChip analysis. The clustering was done using log10 transformed probe level data. (B) Firmicutes and Bacteroidetes relative abundances. Data was taken from the RDP values of the phylum level. * indicates significant differences compared with the 3 months value (p<0.05, Student-t test).

**Figure 9.** Redundancy analysis, RDA, of microbiota composition over time. Redundancy analysis, RDA of microbiota composition observed in the individual diets REF (A), HFS (B) and HFP (C). The explanatory variables are the weight of the animals and the time points. These variables explain 39.1% (A); 45.3% (B) and 47.9% (C) of total variation. The relative
abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best fitting 15 genus-like groups are shown in the plots.

Supplementary figure 1. Linear fit of growth curves for the three diets (A). Significant effects of time (p<0.0001 for all comparisons) and diet (p<0.0001 for all comparisons in the first and second growth phase, p<0.0129 for difference between REF-HFS in the third growth phase) on growth rates determined by linear fit of the BW curves were detected using a two-way ANOVA followed by Bonferroni adjusted pair-wise comparisons (p<0.05) (B).

Supplementary figure 2. Cumulative energy intake in the three experimental groups during the life span of the animals. Two-way ANOVA was applied to detect overall effect of diet. p<0.0001 for all comparisons.

Supplementary figure 3. Organ weights of liver (A), heart (B), kidneys (C) and tibialis (D) at 3, 6 and 18 months of feeding depicted as percentage of total body weight. Lower-case, upper-case and italic letters denote significant differences within the 3, 6 and 18 months samples, respectively. Values with no letters in common are significantly different as determined by one-way ANOVA followed by Bonferroni corrected pair-wise comparisons, p<0.05, n=9-10.

Supplementary figure 4. Heatmap of digital gene expression profiles in gWAT, iWAT and liver according to sampling time-point and diet (A) and according to diet at 6 months (B). As the color gradient goes from white over orange to deep red the larger the fold change in expression of the particular gene between HFS and HFP, where red denotes higher expression in HFS than HFP, n=3.

Supplementary figure 5. (A) Principal Response Curve (PRC) analysis summarizing the differences in microbiota composition between the high fat diets and the REF diet over time. Time accounts for 24.2% of the total variation and is displayed on the horizontal axis. The diets explain 29.4% of the total variation, where 55.8% of the variance is displayed on the first PRC (upper panel) and 21.1% in the second PRC (lower panel). The diet as explanatory variable had a significant influence on the microbiota composition during the different time points in the first and second PRC (p<0.05, Monte Carlo permutation tests). The matching bar on the right side of both curves represents the genus-like group weights. These groups can be interpreted as the main drivers of the differences between different feeding groups that fall
over 0 on the response curve, while those on the negative scale follow the opposite pattern. Only genus-like groups with weights below -1 and above +1 are shown (B) Principal Response Curve, PRC, where the explanatory variables are the diets HFS and HFP, with the species weights for the genus level data set, indicating the effects of the dietary treatments on the genus groups. Only genus-like groups with weights below -1 and above +1 are shown. The explanatory variables explain 8.9% of total variation. The first PRC shows 65.8% of this variation, but was not significant.

**Supplementary figure 6.** (A) Redundancy analysis, RDA, of microbiota composition at month 3 (upper panel), month 6 (middle panel) and month 16 (lower panel). Animal weight and the different diets (REF, HFS, HFP) were included as explanatory variables. These variables explain 42.6% (upper panel); 45.9% (middle panel) and 44.4% (lower panel) of total variation. The relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best fitting 15 genus groups are shown in the plots. (B) Redundancy analysis, RDA, of microbiota composition observed at month 3 (upper panel), month 6 (middle panel) and month 16 (lower panel). The explanatory variables are the weight of the animals, and the HFP and HFS diets. These variables explain 19.7% (upper panel), 25.5% (middle panel) and 26.6% (lower panel) of total variation. The relative abundances of the genus-like groups of the MITChip were used as species input for the RDA plots. The best fitting 15 genus groups are shown in the plots.

**Supplementary table 1.** Relative abundances of the genus-like groups shown in the PRCs.
Table 1: Diet composition.

<table>
<thead>
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<th>Protein (g/kg)</th>
<th>High corn oil</th>
<th>Low fat</th>
<th>High sucrose</th>
<th>High protein</th>
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<tr>
<td>Casein</td>
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<tr>
<td>L-Cysteine</td>
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<tr>
<td>Carbohydrate (g/kg)</td>
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Table 2. Primers (from Rajilic-Stojanovic et al., 2009)

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Figure 1

A.  Body weight (g) over weeks.

B.  Energy intake (kcal/g BW/week) over weeks.

C.  Feed efficiency (g/Mcal) in different weeks.

D.  % survival over weeks.
Figure 2

A  3 months

- REF
- HFS
- HFP

OGTT, area under the curve

Fasting blood glucose

B  18 months

OGTT, area under the curve

Fasting blood glucose
Figure 3
Figure 4

A  Markers for browning

Ucp1

Ppargca

Dio2

B  Markers for inflammation

Cd68

Emr1

Ccl2

Serpine1
Figure 5

A

Liver TAG (mg/g)

Liver cholesterol (mg/g)

Liver sterol ester (mg/g)

Plasma TAG (mmol/L)

Plasma cholesterol (mmol/L)

REF

HFS

HFP

Legend:
**Figure 6**

**A** Lipogenesis

**Fasn**

- REF
- HFS
- HFP

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**Acaca**

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**Scd1**

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**Srebfl**

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<tr>
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**B** Fatty acid oxidation

**Acox1**

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**Cpt1a**

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**Acadm**

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**Hmgcs2**

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<tr>
<td>B</td>
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Figure 6

C  Amino acid degradation

Got1

Cps1

Agxt

Gpt

D  Gluconeogenesis

Pck1

Ppargc1a
Figure 9

A  RDA plot 3, 6, 16 months
REF

B  RDA plot 3, 6, 16 months
HFS
Supplementary Figure 1

A

B

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<th>Phase 1 (1-8 weeks)</th>
<th>Phase 2 (10-65 weeks)</th>
<th>Phase 3 (70-95 weeks)</th>
<th>Overall effect of time</th>
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<td>Slope</td>
<td>SEM</td>
<td>R²</td>
<td>Slope</td>
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<td>HFS</td>
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<td>HFP</td>
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Supplementary Figure 2

Cumulative energy intake (kcal/g BW) vs. weeks

0 10 20 30 40 50 60 70 80 90 100

REF
HFS
HFP
Supplementary Figure 3

A

Liver weight (% of BW)

REF
HFS
HFP

3 months 6 months 18 months

B

Heart weight (% of BW)

3 months 6 months 18 months

C

Kidney weight (% of BW)

3 months 6 months 18 months

D

Tibialis weight (% of BW)

3 months 6 months 18 months

Supplementary Figure 3
Supplementary Figure 4A and B:
Heatmap of digital gene expression profiles
<table>
<thead>
<tr>
<th>gWAT</th>
<th>6mon</th>
<th>18mon</th>
<th>iWAT</th>
<th>6mon</th>
<th>18mon</th>
<th>Liver</th>
<th>6mon</th>
<th>18mon</th>
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</table>

- Negative regulation of cellular component movement
- Negative regulation of locomotion
- Response to inorganic substance
- Cell-cell signaling
- Fat cell differentiation
- Positive regulation of multicellular organismal process
- System process
- Polysaccharide metabolic process
- Hormone metabolic process
- Behavior
- Regulation of hormone levels
- Blood circulation
- Positive regulation of growth
- Circulatory system process
- Regulation of intracellular protein kinase cascade
- Humoral immune response
- Cell differentiation
- Response to oxygen-containing compound
- Cellular developmental process
- Cellular component movement
- Negative regulation of multicellular organismal process
- Protein tetramerization
- Intracellular protein kinase cascade
- Muscle adaptation
- Oxidation-reduction process
- Muscle system process
- Single-organism metabolic process
- Lipid metabolic process
- Muscle organ development
- Carboxylic acid metabolic process
- Ion transport
- Organic acid metabolic process
- Lipid biosynthetic process
- Muscle structure development
- Glycolysis
- Regulation of lipid metabolic process
- Anion transport
- Chemical homeostasis
- Small molecule biosynthetic process
- Lipid localization
- Single-organism biosynthetic process
- Steroid metabolic process
- Organic anion transport
- Sodium ion transport
- Lipid transport
- Sodium ion transmembrane transport
- Tissue development
- Cellular aldehyde metabolic process
- Immune system process
- Defense response
- Inflammatory response
- Response to wounding
- Response to stimulus
- Regulation of immune system process
- Response to external stimulus
- Response to chemical stimulus
- Leukocyte migration
- Cellular response to chemical stimulus
- Response to other organism
- Multi-organism process
- Response to biotic stimulus
- Regulation of response to external stimulus
- Multicellular organismal process
- Multicellular organismal process
- Single-organism process
- Locomotion
- Response to organic substance
- Regulation of response to stimulus
- Cellular response to stimulus
- Localization of cell
- Anatomical structure development
- Organ development
- Cell adhesion
- Hematopoietic or lymphoid organ development
- Biological adhesion
- Signaling
- Single organism signaling
- Blood vessel morphogenesis
- Developmental process
- Cell communication
- Negative regulation of immune system process
- Homeostatic process
- Negative regulation of response to stimulus
- Death
- Biological regulation
- Regulation of behavior
- Acute inflammatory response
- Regulation of acute inflammatory response
- Anatomical structure morphogenesis
- Anatomical structure formation involved in morphogenesis
- Positive regulation of cell proliferation
- Apoptotic process
- Regulation of cell shape
- Response to organic nitrogen
- Complement activation
- Protein activation cascade
- Response to metal ion
- Regulation of molecular function
- Enzyme linked receptor protein signaling pathway
- Positive regulation of lipid metabolic process
- Amin metabolite process
- Retinoid metabolic process
- Isoprenoid metabolic process
- Regulation of molecular function
Supplementary Figure 5

A

PRC plots, REF, HFS, HFP
PRC plot, HFS, HFP
Supplementary Figure 6A

RDA plot, REF, HFS, HFP
3 months

RDA plot, REF, HFS, HFP
6 months
RDA plot, REF, HFS, HFP
16 months

Clostridium leptum et rel.
Olsenella et rel.
Lactobacillus debriecii et rel.
Lactobacillus gasseri et rel.
Vibrio et rel.
Lactobacillus plantarum et rel.
Phasobacter saccharogenes et rel.
Olsenella et rel.
Bryantella et rel.

Weight

Month 16

Unclassified Clostridiales XIVa
- close to Anaerostipes caccae
Ruminococcus obeum et rel.
Rikenella et rel.
Turicibacter et rel.
Supplementary Figure 6B

RDA plot, HFS, HFP
3 months

RDA plot, HFS, HFP
6 months
RDA plot, HFS, HFP
16 months
<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
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<th>6 months</th>
<th>16 months</th>
<th>3 months</th>
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<td>0.01±0.01</td>
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<td>3.8±0.56</td>
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<td>3.8±0.56</td>
<td>3.4±0.48</td>
<td>3.6±0.52</td>
<td>3.8±0.56</td>
<td>3.4±0.48</td>
<td>3.6±0.52</td>
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The protein source determines the potential of high protein diets to attenuate obesity development.

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Running head: Protein sources and obesity
ABSTRACT

The notion that the obesogenic potential of high fat diets in rodents is efficiently attenuated by increasing the protein:carbohydrate ratio is largely based on studies where casein or whey are used as protein sources. To evaluate to what extent protein source might modulate the effect of high protein diets, we fed mice high fat diets with a high protein:carbohydrate ratio using six different protein sources. We observed striking differences in weight gain and accretion of adipose mass. Whereas a high proportion of casein prevented obesity, mice fed a high proportion of soy, cod, beef, chicken or pork protein, gained a substantial amount of adipose tissue and became insulin resistant and glucose intolerant. Using a factorial design, where casein and pork filets were chosen as protein sources, we demonstrated that both protein source and amount influenced feed efficiency as well as development of obesity and insulin resistance. We observed a remarkable difference in response to both protein amount and source in the morphological appearance and UCP1 immunoreactivity of adipocytes from interscapular brown adipose tissue. Our data indicate that maintenance of a brown phenotype in the adipocytes in the interscapular region by a high proportion of casein may protect against obesity development. By contrast, adipocytes in the interscapular adipose depot in mice fed pork filets exhibited a clear morphological transformation, acquiring larger fat droplets and displaying less UCP1 immunoreactivity. We conclude that diets with high protein:carbohydrate ratio where casein is used as protein source are not representative for all high protein diets.
INTRODUCTION

High protein diets have become increasingly popular as a mean to prevent obesity development and lose weight. The efficiency and safety of high protein diets, in particular when combined with a high fat intake, however, are vigorously debated (1, 2). In rodents, it is evident that obesity development is prevented if a high fat diet is accompanied with an increased protein:carbohydrate ratio (3-10). Worth noting, however, in rodent experiments where high fat diet-induced obesity has been prevented by increasing the protein:carbohydrate ratio, casein or whey was used as protein a source.

A major consequence of increasing the protein:carbohydrate ratio derives from the attenuated rise in blood glucose and insulin secretion. The important role of insulin secretion and action in adipose tissue in development of diet-induced obesity is underscored by the findings that both pancreas-specific Ins1+/−:Ins2−/− mice (11) and fat-specific insulin receptor knockout (FIRKO) mice are protected against diet-induced obesity (12). This is in line with our previous study demonstrating that the glycemic index of the carbohydrate component of the feed determines the obesogenic effect of high fat diets (9).

There are several additional mechanisms by which a high protein:carbohydrate ratio may prevent high fat diet-induced obesity. A high intake of protein is known to have high satiating effect, thereby reducing energy intake (13), but importantly, pair-feeding experiments by us and others have demonstrated that high protein diets also reduce feed efficiency and obesity development, independently of feed intake (4, 7, 9). The reduced energy efficiency may relate to the higher thermic effect of proteins (20-30%) compared with carbohydrates (5-10%) (14). Furthermore, high protein intake is associated with augmented energy expenditure due to an increase in ATP-consuming processes such as protein degradation and re-synthesis as well as synthesis of urea. Moreover, if the dietary carbohydrate level is low, ATP is required for gluconeogenesis (15, 16).

Mitochondrial ATP synthesis may be impaired by the uncoupling protein 1 (UCP1) expressed in brown or brite (brown-in-white) adipocytes, also known as beige adipocytes, that can be found together with white adipocytes in several fat depots (17). As UCP1 allows energy to be dissipated in the form of heat, its expression is positively correlated with metabolic inefficiency and UCP1 expression is induced by cold exposure and overfeeding (18). The number of UCP1-
expressing adipocytes in different adipose depots varies between mouse strains and may account for their different propensity for diet-induced obesity (19, 20). A more brown phenotype of typically white adipose tissue with a concomitant resistance to diet induced obesity can be obtained by transgenic expression of UCP1 itself (21), as well as by modulation of several key molecules involved in brown adipocyte differentiation (22). Given the high capacity of activated brown adipocytes to take up glucose, browning of adipose tissue has received interest as a strategy to improve glucose homeostasis (23-26). Browning of white adipocytes may occur by both pharmacological and nutritional agents (27). We have earlier observed increased expression of Ucp1 in inguinal white, but not in interscapular brown adipose tissue in mice fed high fat diets by increasing the protein:carbohydrate ratio (7, 9). However, in rats it has been reported that increasing the protein:carbohydrate ratio in a low fat diet led to increased expression of Ucp1 in interscapular brown fat (28). Still, it is not yet known if the protein:carbohydrate ratio can modulate the number of UCP1-expressing cells.

Different type of proteins may influence both adipose tissue mass and function in various ways, and few rodent studies have demonstrated that diets with standard levels of different types of proteins differ in their ability to stimulate Ucp1 expression and accordingly, display different obesogenic potential (29-31). Still, the general notion that an increased intake of dietary protein attenuates obesity development in rodents is more or less exclusively based on studies where casein or whey are used as the protein source. Casein and whey have a high content of branched chain amino acids (BCAAs), valine, leucine and isoleucine. In particular, leucine is recognized as a nutrient signal proposed to mediate, at least in part, the effect of high protein diets on metabolism (10, 32, 33). Of note, the chronic elevated levels of BCAAs in mice with disrupted mitochondrial branched chain aminotransferase were associated with increased energy expenditure. However, the lean phenotype in these mice was accompanied by insulin resistance (33). Moreover, metabolic profiling identified elevated BCAAs as a signature related to obesity and insulin resistance in humans (34). Given the relative high amounts of BCAAs in casein and whey, a high dietary protein:carbohydrate ratio using these protein sources may not be representative for high protein diets in general. Thus, in this study we aimed to evaluate the development of obesity and insulin resistance in rodents fed high fat diets with a high protein:carbohydrate ratio using different protein sources.
MATERIALS AND METHODS

Ethical statement
The animal experiments were performed in accordance with the guidelines of the Norwegian Animal Research Authority (NARA) (Norwegian approval identification FOTS id.nr 3750). No adverse effects were observed.

Mouse diets
The macronutrient composition of the diets is presented in Tables 1-4. A low fat reference diet (RD) and regular high fat/high sucrose (HF/HS) diet, both with casein as protein source, were used as reference diets. In the experimental diets, part of the carbohydrate was exchanged with protein to prepare high fat/high protein (HF/HP) diets using either casein (Sigma, batch nr. BCBC3986V and 080M0006), soy powder (Ssniff Spezialdiäten, Soest, Germany), cod fillet powder (Seagarden AS), beef tenderloin (H. Bragstad A/S, Bergen), chicken breast fillet (Prior, Norway) or pork sirloin (H. Bragstas A/S, Bergen) as protein sources. Beef, chicken and pork filets were freeze dried and pulverized. The protein sources were analyzed for crude protein (N*6.25) and total fat content as earlier described (35) in order to balance the diets with respect to total protein and fat content. The diets were mixed using a Crypto Peerless EF20 blender, kept at -20 °C, and analyzed for gross energy, fat, protein and amino acid composition as described (35).

Animals
The data in this paper are based on the results from three separate animal studies using male C57BL/6J Bomtac mice (Taconic), 8 weeks of age. In experiment 1 and 2, the mice were kept at thermoneutrality (28-30°C) with a 12 h light/dark cycle in single cages. The mice were assigned into experimental groups (n=9) by body weight and body composition determined by nuclear magnetic resonance (Minispec mq 7.5, NMR analyser, Bruker, Germany) after five days acclimatization. The mice were weighed once a week and fed ad libitum three times a week. Body composition was determined after 8 weeks of feeding. After 11 weeks of feeding the mice were fasted for 4 h before they were sacrificed by cardiac puncture under Isoflurane anesthesia (Isoba-vet, Schering-Plough, Denmark). Blood was collected in tubes containing EDTA (Medinor AS, Oslo, Norway), centrifuged at 2500 g in 4°C for 5 min. Plasma was stored at -80°C before further analysis. Liver, muscle and adipose tissue were dissected out, weighed, snap-frozen in liquid nitrogen and stored at -80°C until further analyses. A portion of each
adipose depot was fixed for histology. See histology section for further details. A third cohort (experiment 3) of C57BL/6J mice was used for indirect calorimetry measurements (see below).

**Insulin and glucose tolerance tests (ITT and GTT)**

After 9 and 10 weeks, respectively, of receiving the experimental diets, GTT and ITT were performed on mice in the conscious state in experiment 1 and 2. Prior to the GTT the mice were fasted for 6 h and then received an intraperitoneal (i.p) injection of 2 mg glucose/g body weight. Blood was collected from the lateral tail vein and glucose levels were measured using a glucometer (Ascensia Contour, Bayer Healthcare, Oslo, Norway) before and 15, 30, 60 and 120 min after glucose injection. Additionally, 20 µl of blood were collected at time point 0 and 15 min to measure plasma insulin in experiment 2. Before ITT the mice had free access to feed, but they were deprived of feed during the test. They received an i.p injection of 0.75 U insulin (Actrapid, Denmark)/kg body weight and blood was collected and glucose measured before as well as after 15, 30, 45 and 60 min.

**Feed efficiency and apparent digestibility**

Feed efficiency was calculated as body mass gain per energy intake (g/MJ). As GTT and ITT may influence on feed intake, data prior to testing (first 8 weeks) were used. After six weeks of feeding the mice were placed in cages with paper lining for the purpose of collecting feces for one week. Feed intake was monitored and feces left behind in cages were collected, weighted and frozen at −80°C. The content of total fat and nitrogen in diets and feces was analyzed as described earlier (35). Based on feces measurements and feed intake, apparent digestibility of fat and nitrogen was calculated as follows: 100 × (intake (mg) - fecal output (mg))/(intake (mg)).

**Indirect calorimetric measurements**

In experiment 3, VO$_2$ and VCO$_2$ were measured in open-circuit indirect calorimetry cages as described previously (36) using CaloCages (Phenomaster, TSE Systems), equipped with infrared light-beam frames (ActiMot2). The mice were placed in the metabolic cages and fed a low fat RD for 72 h. Gas exchange and beam breaks, as a proxy for activity, were recorded during the last 48 h. The mice (n=4) were subsequently fed the experimental diets for another 72 h and gas exchange and activity were recorded. Diet-induced changes for each individual mouse were calculated. Based on two consecutive light (06.00-17.30h) and dark (18.00-05.30h) phases respiratory exchange ratio (RER) was calculated from VO$_2$ and VCO$_2$ and spontaneous
locomotor activity was defined as total counts of light-beam breaks. Energy expenditure (EE) was calculated as; 16.3 kJ/L × L VO\(_2\) + 4.6 kJ/L × L VCO\(_2\).

**Plasma analyses**

Plasma insulin was determined using a commercial ELISA kit in accordance with the manufacturer’s instructions (Mouse insulin ELISA, DRG). MaxMat PL II analyzer (MAXMAT S.A.) and conventional kits were used to measure 4-hydroxybutyrate and non-esterified fatty acids. Free amino acids and urea in plasma were measured on the Biochrom 30+ instrument (Cambridge, UK), as previously described (31).

**qRT-PCR**

Total RNA was extracted from mouse adipose tissue using the Trizol reagent (Invitrogen). RNA quantity was assessed with the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) and RNA quality was tested on a random selection of samples with BioAnalyzer – RNA 6000 Nano (Agilent Technologies). Reverse transcription and real time quantitative PCR were then performed as described elsewhere (36). The mRNA expression was normalized to that of the housekeeping gene TATA box binding protein (Tbp).

**Western blotting**

Western blotting was preformed essentially as described earlier (37). Mouse adipose tissue was lysed in the lysis buffer described previously (37) with a Precellys 24 homogenizer (Bertin Technologies). Antibodies used were against glyceraldehyde 3-phosphate dehydrogenase (Ab8245, Abcam) and UCP1 (Ab10983, Abcam).

**Histology**

Sections of different adipose depots were fixed in 4 % formaldehyde in 0.1 mol/L phosphate buffer (PB) overnight, dehydrated, embedded and stained with eosin and hematoxylin (38). Adipocyte cell size was determined and immunohistological detection of UCP1-positive cells was performed by an avidin-biotin peroxidase method as described earlier (39). The anti UCP1-antibody was kindly provided by Prof. Jan Kopecky.
**Statistical analyses**

All data are presented as mean ± SEM. Figure preparation as well as some of the statistical analysis were performed using Graph Pad Prism version 6 (GraphPad Software Inc.). The data from the first experiment was analyzed using 1-way ANOVA analyses followed by Tukey’s multiple comparison, and group means were considered statistically different at P < 0.05. Growth, GTT and ITT were analyzed by repeated measurements ANOVA followed by Tukey’s post hoc test. In experiment 2 and 3 we used a factorial ANOVA design with protein:carbohydrate ratio and protein source as categorical predictors (Statistica 9.0, StatSoft Inc).
RESULTS

High fat diets with a high proportion of cod, beef, chicken and pork are obesogenic.

In order to investigate if a high protein:carbohydrate ratio is able to attenuate high fat diet-induced obesity when other protein sources than casein are used, we prepared diets where casein was replaced with soy, cod, beef, chicken and pork (Table 1). A casein-based low fat RD as well as a high fat high sucrose (HF/HS) diet with casein were used as reference diets. Exchanging casein with other protein sources led to a reduced level of BCAAs (Table 2). Exchanging casein with cod or terrestrial animal protein also led to a reduced level of phenylalanine. As expected, the soy-based diets contained less methionine and lysine than diets with animal proteins (Table 2), but the sulphur-amino acid requirement (Nutrient Requirements of Laboratory Animals, Forth Revised Edition, 1995) was met. Exchanging casein also changed the composition of dispensable amino acids as the amount of arginine and cysteine increased and the amounts of glutamine, proline and tyrosine were reduced (Table 2).

As expected, the mice fed the high fat diets with a high proportion of casein did not gain more body and fat mass than mice fed the low fat RD, whereas mice fed a high fat diet with a high proportion of sucrose gained significantly more body and fat mass (Fig. 1A-B). Of note, only mice fed a high proportion of casein protein had significantly lower fat mass than mice fed the HF/HS reference diet (Fig. 1B). Fat masses in mice fed a high proportion of soy, cod and beef were comparable to those fed HF/HS (Fig. 1B). Strikingly, compared with mice fed the HF/HS reference diet, mice fed diets with high content of proteins from chicken and pork had significantly more fat mass (Fig. 1B), also verified by dissection of different adipose tissue depots (Supplementary Fig. 1). Compared with mice fed the low fat RD and mice fed a high proportion of casein or soy, the pork and chicken fed mice also had reduced lean masses (Fig. 1C).

Replacement of casein with cod, but not any other protein sources, led to a reduced feed intake (Fig. 1D). Consequently, compared with casein, feed efficiency was higher with all other protein sources tested, with the exception of soy (Fig. 1E). Based on intake and excretion, the apparent digestibility of fat in the cod containing diet was lower than in the casein based diet (Fig. 1F), and the apparent nitrogen digestibility of soy and beef protein was lower than that of casein (Fig. 1G). However, these differences cannot explain the different obesogenic potential of the various high protein diets.
Fasting blood glucose and insulin as well as glucose tolerance and insulin sensitivity were comparable in mice fed the high fat diet with a high content of casein and the low fat reference diet (Fig. 2A-F). Replacement of casein with other protein sources led to a significantly lower glucose tolerance (Fig. 2C-D). Fasting insulin levels were higher in mice fed chicken and pork and insulin sensitivity was significantly reduced in mice fed diets with a high content of beef, chicken or pork (Fig. 2B, E-F). Furthermore, compared with mice fed a diet with a high proportion of casein, insulin sensitivity tended (P=0.062) to be reduced in mice fed diets with high amount of soy (Fig. 2E-F).

**Protein source and protein:carbohydrate ratio modulate the obesogenic effect of high fat diets.** To further analyse the influence of protein intake and source, we performed a second experiment using a factorial design with protein:carbohydrate ratio and protein source as categorical predictors. As mice fed casein and pork represented the extremes in experiment 1, these were chosen as protein sources. A casein-based HF/HS diet was used as a reference. The dietary compositions are presented in Table 3. As expected, body weight gain and adipose tissue mass in mice fed a high proportion of casein were comparable to those mice fed the reference diet (Fig. 3A-B). Both protein level and source influenced on body weight gain and adipose tissue mass (Fig. 3A-B), but lean masses were comparable in this experiment (Fig. 3C). Casein fed mice were less obese than pork fed mice, but increasing the protein:carbohydrate ratio attenuated obesity development independently of the protein source (Fig. 3B). As feed intake was similar in all four groups, calculation of feed efficiency mirrored body weight gain (Fig. 3D). Feed efficiency was, however, not directly linked to digestibility, as both fat and nitrogen digestibility were reduced with a low protein:carbohydrate ratio (Fig. 3D-F). However, independent of protein amount, fat digestibility was higher in pork than casein fed mice (Fig. 3E).

Plasma levels of urea and hydroxybutyrate were higher in mice fed casein than in mice fed pork, suggesting that catabolism of both protein and fat were higher in casein than pork fed mice (Fig. 3G-H). Moreover, plasma levels of free fatty acids (FFA) were higher in casein than in pork fed mice, suggesting increased lipolysis (Fig. 3I). The protein:carbohydrate ratio did not modulate plasma levels of FFA or hydroxybutyrate, but as expected, plasma levels of urea were higher in mice fed a high proportion of proteins.
We utilized indirect calorimetry to evaluate the apparent differences in metabolism. As body mass and body composition are strong determinants for both O₂-utilization and CO₂-production (40), a new set of mice was used in this experiment. The mice were placed in metabolic cages and fed a low fat reference diet for 72 h. Gas exchange and beam breaks, as a proxy for activity, were recorded during the last 48 h. The mice were subsequently fed the experimental diets, gas exchange and activity were recorded, and diet-induced changes for each mouse were calculated. The source of protein, but not the amount of protein, influenced the activity of the mice. Interestingly, when mice were fed diets containing pork, their activity was not significantly different during the light period (Fig. 4A), but significantly reduced during the dark period (Fig. 4B). The type of protein did, however, not affect energy expenditure (Fig. 4C-D). It is well documented that high fat diets increase EE and reduce RER, but unexpectedly, a low proportion of proteins relative to sucrose led to a stronger diet-induced EE during the light- (Fig. 4C), but not the dark period (Fig. 4D). Still, the reduction in RER was more pronounced when the proportion of proteins was high (Fig. 4E-F), indicating a lower utilization of carbohydrates.

*Protein source and protein:carbohydrate ratio modulate glucose homeostasis in mice fed high fat diets.*

Although only mice fed a high proportion of casein were protected against reduced insulin sensitivity (Fig. 5A-B) and glucose tolerance (Fig. 5C-D), both protein:carbohydrate ratio and the protein source were able to alter these parameters. We also measured insulin levels before and 15 min after glucose injection during the GTT (Fig. 5E-F). At both time points, the insulin levels reflected the state of obesity as insulin levels were higher in pork than casein fed mice, and higher when the dietary protein:carbohydrate level was low.

When dietary fat intake is high, BCAAs are suggested to contribute to development of insulin resistance (34). Compared with pork protein, the levels of BCAAs in casein are high (Table 4). Analyses of the amino acid profile in plasma collected from mice that were feed deprived for 4 h revealed higher levels of circulating BCAAs in casein compared with pork fed mice (Table 5). Since BCAA metabolism in adipose tissue is able to modulate the circulating BCAA levels (41), we measured the expression of the two first enzymes required for BCAA oxidation, branched chain aminotransferase (*Bcat2*) and the branched chain ketoacid dehydrogenase complex (*BCKDHC*) subunits, *Bckdha* and *Dbt*, as well as *Dld* (a subunit of BCKDHC that is shared with pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes), in inguinal white (iWAT) and interscapular brown adipose tissue (iBAT). In iBAT, expression of
Bcat2 was higher in mice fed a high protein:carbohydrate ratio, but expression of these genes was not significantly affected by the type of protein (Fig. 6A). However, in iWAT, expression of Dbt and Dld were higher in mice fed pork than in mice fed casein, independent of protein amount (Fig. 6B).

**Protein source and protein:carbohydrate ratio influence on iBAT histology.**

To investigate whether an increased protein:carbohydrate ratio lead to an increased amount of brite adipocytes in iWAT, histological examinations were performed. Increasing the protein:carbohydrate ratio led to smaller adipocytes in iWAT (Fig. 7A). Furthermore, the adipocytes in iWAT from casein fed mice were smaller than adipocytes from pork fed mice (Fig. 7A). Expression levels of marker genes for brown/brite adipocytes were not significantly altered by either the type or amount of dietary protein (Fig. 7B). UCP1 was not detectable by neither immunohistochemistry nor Western blotting in iWAT (not shown).

Histological examination of iBAT revealed that only adipocytes from mice fed a high proportion of casein, maintained a classic brown phenotype (Fig. 8A). Mice fed a low protein:carbohydrate ratio, in particular those fed pork, had large “white-like” adipocytes with a single large lipid droplet in the iBAT depot (Fig. 8A). With the exception of Cpt1b, expression levels of brown adipocyte marker genes were not significantly altered by either the type or amount of dietary protein in iBAT (Fig. 8B). Moreover, immunohistochemical analyses demonstrated that a higher proportion of adipocytes in casein compared to pork fed mice stained positive for UCP1 (Fig. 8C). This was verified by Western blotting (Fig. 8D). Thus, our data indicate that maintenance of a brown phenotype of adipocytes in the interscapular region by a high proportion of casein may protect against obesity development.
DISCUSSION

It is well described that high protein diets are able to attenuate high fat diet-induced obesity. However, in these studies casein or whey is used as protein sources (3-10). Thus, in this study we aimed to evaluate the development of obesity in rodents fed high fat diets with a high protein content using different protein sources.

We report striking divergence between different protein sources in relation to obesity development. Whereas a high proportion of casein attenuated obesity, mice fed a high proportion of cod, beef, chicken or pork accumulated higher amounts of adipose tissue, became insulin resistant and glucose intolerant. The observed differences in obesity development were not related to energy intake. Thus, we observed large differences in feed efficiency. However, feed efficiency appeared not to be directly related to neither fat nor nitrogen digestibility. Using a factorial design with protein:carbohydrate ratio and protein source as categorical predictors, where casein and pork protein were chosen as protein sources, we demonstrated that both protein source and amount influenced on the development of obesity and insulin resistance. However, different mechanisms may underlie the observed effects.

We utilized indirect calorimetry to evaluate energy metabolism. As both body mass and composition are strong determinants for the gas exchange (40), we subjected the mice to indirect calorimetric measurements before onset of obesity at the transition from a low fat regular reference diet to the experimental diets. Of note, we observed no significant difference in EE that could explain the difference in feed efficiency or adiposity. Changing to high fat diets led to an expected reduction in RER, in particular during the dark phase. The reduction in RER was more pronounced when the proportion of proteins was high, indicating a lower utilization of carbohydrates. The higher plasma levels of urea in mice fed diets with a high proportion of proteins for 11 weeks reflected dietary substrate availability, i.e. increasing the protein:carbohydrate ratio in high fat diets led to a higher utilization of amino acids at the expense of carbohydrates. The loss of energy in form of ATP used in syntheses of urea and by the required conversion of amino acids to glucose (15, 16), may contribute to the reduced feed efficiency in mice fed diets with high protein:carbohydrate ratios.

The protein source did not modulate RER, but plasma levels of urea and hydroxybutyrate were higher in mice fed casein than pork, indicating that catabolism of both protein and fat were higher in casein than pork fed mice. Interestingly, the source of protein, but not the amount,
altered the activity of the mice. When mice were fed diets containing pork, their activity tended to be reduced during the light period and their activity was significantly reduced during the dark period. In line with this observation, we have previously reported an inverse correlation between locomotor activity and development of diet-induced obesity, without being able to detect differences in EE (36). Lower locomotor activity in mice fed protein from pork may very well over time impact on feed efficiency and obesity development.

Energy may also be lost by conversion to heat by uncoupling of mitochondria in brown adipocytes. An increased number of UCP1 expressing adipocytes protects against diet induced obesity (22), whereas UCP1 ablation augments obesity in mice exempt from thermal stress (42). White and brown adipocytes are found together in both visceral and subcutaneous fat depots forming a plastic organ (17). Exposure to thermoneutrality, aging and obesity leads to a “whitening” of the adipose organ (43). However, we have earlier demonstrated that a high proportion of casein in the diet led to increased expression of Ucp1 in inguinal adipose tissue (7-9). Here we confirm that the proportion of dietary protein influenced the size of the adipocytes in the inguinal depot (8). Fasting is known to down regulate Ucp1 expression (44), and the four h feed deprivation before collection of tissue in this experiment may account for the lack of significant differences in Ucp1 expression. Furthermore, we were unable to detect UCP1 with either immunohistochemistry or Western blotting in iWAT.

Of note, a lower abundance of UCP1 immunoreactive cells with a more brown-like phenotype was observed in the interscapular region collected from mice fed diets with a low content of proteins. In particular, a large proportion of the adipocytes in iBAT from the obese mice fed pork protein in combination with a low protein:carbohydrate ratio was unilocular and not UCP1-immunoreactive. By contrast, a large proportion of adipocytes from the lean mice fed the HF/HP diet based on casein were multilocular and stained positive for UCP1. This observation was supported by Western blotting. Thus, maintenance of a brown phenotype of adipocytes in the interscapular region by a diet with a high content of casein may protect against obesity development.

The protective effect of intake of high amounts of casein and whey (10) on both obesity development and insulin resistance may relate to the high content of BCAAs in the diets, as leucine partially mimics the effect of high protein diets (32, 45). In our studies, the reduced glucose tolerance and insulin sensitivity in pork fed mice may be directly related to the state of
obesity. However, both amino acid composition and amino acid metabolism may directly influence glucose homeostasis. On one hand, leucine may directly interact in the insulin signaling pathway, and may furthermore increase the recycling of glucose via the glucose-alanine-cycle (32, 45). However, metabolic profiling has identified elevated BCAAs as a metabolite signature related to obesity and insulin resistance in humans (34), and chronic elevated levels of BCAAs in lean mice with disrupted mitochondrial branched chain aminotransferase are accompanied by insulin resistance (33). In accordance with the higher content of BCAAs in casein than pork, mice fed casein had higher levels of circulating BCAAs.

We conclude that diets with high protein:carbohydrate ratio where casein is used as protein source may not be representative for high protein diets. These observations are in line with rodent studies demonstrating that diets with standard levels of different types of proteins have different obesogenic potential as well as different effect on insulin sensitivity (29-31, 35, 46, 47). Given the popularity of high protein diets, this warrants further investigations in humans.
ACKNOWLEDGMENTS
We thank Dr. Pavel Flachs and Prof. Jan Kopecky for kindly providing the UCP1 antibody used for immunohistochemistry and the staff at NIFES for technical assistance and animal care. We would like to specifically acknowledge the early contribution of Vigdis Misje Hagen to this project.

GRANTS
This project was supported by the European Union FP7 project DIABAT (HEALTH-F2-2011-278373) to J.B.H., K.K. and L.M. This work was also, in part, supported by The Norwegian Seafood Research Fund (FINS 900842), the Norwegian Research Council (200515/ I30), the SHARE Cross Faculty Ph.D. Initiative of the University of Copenhagen and NIFES.

DISCLOSURES
The authors have no conflicting interests, financial or otherwise.

AUTHOR CONTRIBUTIONS
FIGURE LEGENDS:

FIGURE 1. **Effect of high fat high protein diets with different protein sources on body mass, body composition and digestibility.** Male C57BL/6J mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) using casein as the protein source or high fat diets with a high protein:carbohydrate ratio (HF/HP) using different protein sources for 11 weeks. Mice fed a low fat regular diet (RD) were used as a reference. Body mass development was recorded (A) and fat mass (B) and lean mass (C) determined after 8 weeks of feeding. Energy intake (D) and feed efficiency (E) were calculated based on data collected during the first 8 weeks of feeding. Apparent fat (F) and nitrogen (G) digestibility were calculated based a feed intake and feces collection during the 6th week of feeding. Data represent mean ± SEM (n=9). Different small letters denote significant differences between the groups (p<0.05).

FIGURE 2. **Effect of high fat high protein diets with different protein sources on glucose homeostasis.** Male C57BL/6J mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) using casein as the protein source or high fat diets with a high protein:carbohydrate ratio (HF/HP) using different protein sources for 11 weeks. Fasting 4 h glucose (mmol/L) (A) and insulin (pmol/L) levels (B) were measured in plasma at termination. A glucose tolerance test (GTT) was performed (C) and area under the curve (AUC) was calculated (D) after 9 weeks of feeding. An insulin tolerance test (ITT) was performed (E) and AUC was calculated (F) after 10 weeks of feeding. Data represent mean ± SEM (n=9). Different small letters denote significant differences between the groups (p<0.05).

FIGURE 3. **Effect of high fat diets with high and low protein:carbohydrate ratios on body mass gain, body composition and digestibility.** Male C57BL/6J mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before blood collection and termination. Body mass development was recorded and body weight (BW) gain calculated (A). Fat mass (B) and lean mass (C) were determined after 8 weeks of feeding. Feed efficiency (D) was calculated based on data collected during the first 8 weeks of feeding. Apparent fat (E) and nitrogen (F) digestibility were calculated based on feed intake and feces collection during the 6th week of feeding. Plasma levels of urea (G), hydroxybutyrate (H) and free fatty acids (FFA) (I) were measured in plasma collected at termination. Data represent
mean ± SEM (n=9). Significant differences (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with *.

FIGURE 4. **Effect of high fat diets with high and low protein:carbohydrate ratios on activity, energy expenditure (EE) and respiratory exchange ratio (RER).** Male C57BL/6J mice were fed a low fat reference diet. After three days the diets were switched to high fat diets with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources. Diet induced changes in activity (A and B), EE (C and D) and RER (E and F) for each mouse were calculated. Data represent mean ± SEM (n=4). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with *.

FIGURE 5. **Effect of high fat diets with high and low protein:carbohydrate ratios on glucose homeostasis.** Male C57BL/6J mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before blood collection and termination. An insulin tolerance test (ITT) was performed (A) and AUC was calculated (B) after 10 weeks of feeding. A glucose tolerance test (GTT) was performed on animals fasted for 6 h (C) and area under the curve (AUC) was calculated (D) after 9 weeks of feeding. Blood was collected and serum prepared for insulin measurements before (E) and 15 min after (F) glucose administration. Data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with *.

FIGURE 6. **Effect of high fat diets with high and low protein:carbohydrate ratios on expression of genes involved in branched chain amino acid transamination in adipose tissue.** Male C57BL/6J mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4 h before termination. RNA was purified from interscapular brown (iBAT) and inguinal white (iWAT) adipose tissue. cDNA was synthesized and expression of branched chain aminotransferase (Bcat2) and the branched chain ketoacid dehydrogenase complex subunits, Bekdha, Dbt and Dld were measured in iBAT (A) and iWAT (B). Expression levels were normalized to TATA-box binding protein (Tbp). Data represent
mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with *

FIGURE 7. Effect of high fat diets with high and low protein:carbohydrate ratios on histological appearance and gene expression in inguinal white adipose tissue (iWAT). Male C57BL/6J mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before termination. Sections of iWAT (n=4) were stained with eosin and hematoxylin, scalebar = 100µm (A) and expressions of uncoupling protein 1 (Ucp1), deiodinase, iodothyronine, type II (Dio2), peroxisome proliferator-activated receptor-γ coactivator 1α (Ppargc1a) and cell death-inducing DFFA-like effector (Cidea) were measured and expression levels were normalized to TATA-box binding protein (Tbp) (B). Gene expression data represent mean ± SEM (n=9). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with *

FIGURE 8. Effect of high fat diets with high and low protein:carbohydrate ratios on histological appearance, protein and gene expression in interscapular brown adipose tissue (iBAT). Male C57BL/6J mice were fed a high fat diet with a low protein:carbohydrate ratio (HF/HS) or a high protein:carbohydrate ratio (HF/HP) using casein or pork as protein sources for 11 weeks. The mice were feed-deprived 4h before termination. Sections of iBAT (n=4) were stained with eosin and hematoxylin, scalebar = 100µm (A) and expressions of uncoupling protein 1 (Ucp1), deiodinase, iodothyronine, type II (Dio2), peroxisome proliferator-activated receptor-γ coactivator 1α (Ppargc1a), cell death-inducing DFFA-like effector (Cidea), carnitine palmitoyltransferase 1B (Cpt1B) and Leptin were measured and expression levels were normalized to TATA-box binding protein (Tbp) (B). Gene expression data represent mean ± SEM (n=9). Sections of iBAT (n=4) were immunohistologically stained with an UCP1-antibody and the area quantified, scalebar = 200µm (C). UCP1 detected by Western-blotting (n=3) (D). Significant difference (p<0.05) between the protein sources are presented with different letters and differences between high and low protein:carbohydrate ratio with *.
Table 1. Compositions of the experimental diets used in experiment 1.

<table>
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<tr>
<th>Component (g/kg)</th>
<th>HF/HS RD</th>
<th>Casein</th>
<th>Soy</th>
<th>Cod</th>
<th>Beef</th>
<th>Chicken</th>
<th>Pork</th>
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**Analyzed**

| Fat (g/kg) | 71 | 252 | 246 | 258 | 246 | 238 | 247 | 270 |
| Crude protein § (g/kg) | 192 | 180 | 360 | 350 | 360 | 380 | 380 | 370 |
| Energy kJ/g | 18 | 23  | 24  | 24  | 23  | 24  | 24  | 24  |

Composition of the regular reference diet (RD), the high fat high sucrose reference diet (HF/HS) and the experimental high fat high protein diets (HF/HP) with different protein sources. Analyzed values represent mean of triplicate measurements.

* The calculated contribution of fat present in the protein sources. † AIN93G. ‡ AIN93VX NCR95 compliant. § N*6.25
Table 2. *Amino acid compositions of the experimental diets used in experiment 1*

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<th>Casein</th>
<th>Soy</th>
<th>Cod</th>
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The values represent mean of triplicate measurements. Σ BCAAs represent the sum of branched chain amino acids (Leu, Ile and Val).
Table 3. Compositions of the experimental diets used in experiment 2 and 3.

<table>
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<tr>
<th>Component (g/kg)</th>
<th>Casein</th>
<th>Pork</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RD</td>
<td>HF/HS</td>
</tr>
<tr>
<td>Casein</td>
<td>207</td>
<td>207</td>
</tr>
<tr>
<td>Pork</td>
<td></td>
<td>237</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>440</td>
</tr>
<tr>
<td>Fat from protein powder*</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Corn oil</td>
<td>68.9</td>
<td>248.9</td>
</tr>
<tr>
<td>Dextrin</td>
<td>532.5</td>
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</tr>
<tr>
<td>L-Cysteine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>50</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
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<td>0.01</td>
</tr>
<tr>
<td>Mineral mix †</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix ‡</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
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</table>

**Analyzed**

<table>
<thead>
<tr>
<th>Component</th>
<th>Casein</th>
<th>Pork</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (g/kg)</td>
<td>68</td>
<td>234</td>
</tr>
<tr>
<td>Crude protein § (g/kg)</td>
<td>171</td>
<td>171</td>
</tr>
<tr>
<td>Energy kJ/g</td>
<td>18</td>
<td>22</td>
</tr>
</tbody>
</table>

Compositions of the regular reference diet (RD), the high fat high sucrose (HF/HS) and the high fat high protein (HF/HP) using casein and pork as protein sources. Analyzed values represents mean of triplicate measurements.

* The calculated contribution of fat present in the protein sources. †AIN93G. ‡AIN93VX NCR95 compliant. § N*6.25
Table 4. Amino acid compositions of the experimental diets used in experiment 2 and 3

<table>
<thead>
<tr>
<th></th>
<th>Casein</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RD</td>
<td>HF/HS</td>
<td>HF/HP</td>
<td>HF/HS</td>
</tr>
<tr>
<td><strong>AA (mg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Indispensable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>4.9</td>
<td>4.9</td>
<td>10.5</td>
<td>7.4</td>
<td>15.0</td>
</tr>
<tr>
<td>Ile</td>
<td>9.3</td>
<td>9.0</td>
<td>19.2</td>
<td>8.9</td>
<td>18.2</td>
</tr>
<tr>
<td>Leu</td>
<td>17.5</td>
<td>17.0</td>
<td>35.8</td>
<td>16.0</td>
<td>32.1</td>
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<tr>
<td>Lys</td>
<td>16.0</td>
<td>15.4</td>
<td>31.1</td>
<td>19.4</td>
<td>39.2</td>
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<tr>
<td>Met</td>
<td>5.9</td>
<td>5.8</td>
<td>11.5</td>
<td>6.5</td>
<td>11.9</td>
</tr>
<tr>
<td>Phe</td>
<td>8.5</td>
<td>8.7</td>
<td>18.8</td>
<td>6.8</td>
<td>13.7</td>
</tr>
<tr>
<td>Thr</td>
<td>7.6</td>
<td>7.4</td>
<td>15.3</td>
<td>8.8</td>
<td>17.5</td>
</tr>
<tr>
<td>Trp</td>
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<td>1.9</td>
<td>4.3</td>
<td>1.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Val</td>
<td>11.9</td>
<td>11.6</td>
<td>24.6</td>
<td>9.3</td>
<td>19.2</td>
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<tr>
<td>Dispensable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>5.7</td>
<td>5.5</td>
<td>11.4</td>
<td>11.5</td>
<td>23.0</td>
</tr>
<tr>
<td>Arg</td>
<td>5.4</td>
<td>5.4</td>
<td>11.9</td>
<td>11.1</td>
<td>22.9</td>
</tr>
<tr>
<td>Asx</td>
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<td>27.2</td>
<td>19.9</td>
<td>39.5</td>
</tr>
<tr>
<td>Cys</td>
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<td>4.1</td>
<td>4.2</td>
<td>5.1</td>
<td>7.2</td>
</tr>
<tr>
<td>Glx</td>
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<td>41.1</td>
<td>82.6</td>
<td>31.0</td>
<td>61.6</td>
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<td>Gly</td>
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<td>3.1</td>
<td>6.6</td>
<td>7.9</td>
<td>15.9</td>
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<td>≤ 0.3</td>
<td>≤ 0.3</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Pro</td>
<td>19.9</td>
<td>19.3</td>
<td>41.0</td>
<td>6.9</td>
<td>13.9</td>
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<tr>
<td>Ser</td>
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<td>8.0</td>
<td>15.9</td>
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<td>6.9</td>
<td>6.9</td>
<td>17.3</td>
<td>4.8</td>
<td>11.8</td>
</tr>
<tr>
<td>Tau</td>
<td>≤ 0.3</td>
<td>≤ 0.3</td>
<td>≤ 0.3</td>
<td>0.4</td>
<td>0.8</td>
</tr>
<tr>
<td>Σ BCAAs</td>
<td>38.7</td>
<td>37.6</td>
<td>79.6</td>
<td>34.2</td>
<td>69.6</td>
</tr>
</tbody>
</table>

The values represent mean of triplicate measurements. Σ BCAAs represent the sum of branched chain amino acids (Leu, Ile and Val).
Table 5. Plasma concentrations of amino acids in mice fed high fat high sucrose (HF/HS) or high fat high protein (HF/HP) diets with casein or pork as the protein source.

<table>
<thead>
<tr>
<th>AA (µmol/100 ml plasma)</th>
<th>Casein</th>
<th>Pork</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HF/HS</td>
<td>HF/HP</td>
<td>HS vs HP</td>
</tr>
<tr>
<td>Indispensable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>6.7 ± 0.3</td>
<td>5.8 ± 0.2</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>Ile</td>
<td>11.0 ± 0.6</td>
<td>11.4 ± 0.5</td>
<td>8.3 ± 0.5</td>
</tr>
<tr>
<td>Leu</td>
<td>18.0 ± 0.9</td>
<td>19.1 ± 0.9</td>
<td>14.4 ± 0.7</td>
</tr>
<tr>
<td>Lys</td>
<td>25.6 ± 0.8</td>
<td>21 ± 1</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Met</td>
<td>3.8 ± 0.2</td>
<td>3.9 ± 0.3</td>
<td>3.5 ± 0.2</td>
</tr>
<tr>
<td>Phe</td>
<td>6.3 ± 0.2</td>
<td>6.1 ± 0.3</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Thr</td>
<td>18.0 ± 0.9</td>
<td>18 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Trp</td>
<td>8.7 ± 0.6</td>
<td>8.1 ± 0.5</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>Val</td>
<td>23 ± 1</td>
<td>24 ± 1</td>
<td>17.9 ± 0.9</td>
</tr>
<tr>
<td>Dispensable</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>38 ± 1</td>
<td>37 ± 2</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>Arg</td>
<td>5.5 ± 0.4</td>
<td>5.1 ± 0.2</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td>Asp</td>
<td>6.9 ± 0.3</td>
<td>6.6 ± 0.3</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Glu</td>
<td>5.2 ± 0.2</td>
<td>4.5 ± 0.3</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>Gly</td>
<td>25.4 ± 0.9</td>
<td>31 ± 1</td>
<td>21.9 ± 0.9</td>
</tr>
<tr>
<td>Pro</td>
<td>8.9 ± 0.4</td>
<td>8.3 ± 0.5</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>Ser</td>
<td>12.5 ± 0.4</td>
<td>11.6 ± 0.6</td>
<td>11.1 ± 0.6</td>
</tr>
<tr>
<td>Tyr</td>
<td>7.9 ± 0.4</td>
<td>8.0 ± 0.4</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>Tau</td>
<td>52 ± 2</td>
<td>49 ± 3</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Σ BCAAs</td>
<td>52.2 ± 2.5</td>
<td>54 ± 2.3</td>
<td>40.5 ± 2.1</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM (n=8). Σ BCAAs represent the sum of branched chain amino acids (Leu, Ile and Val).
REFERENCES


35. Tastesen HS, Keenan AH, Madsen L, Kristiansen K, and Liaset B. Scallop protein with endogenous high taurine and glycine content prevents high-fat, high-sucrose-induced obesity and improves plasma lipid profile in male C57BL/6J mice. Amino Acids 46: 1659-1671, 2014.


**Figure 1**

**A** BW development (g)

**B** Fat mass (g)

**C** Lean mass (g)

**D** Energy intake (MJ)

**E** Feed efficiency (g/MJ)

**F** Apparent fat digestibility (%)

**G** Apparent nitrogen digestibility (%)
**Figure 2**

**A**  
Fasting (4h) glucose (mmol/L)

**B**  
Fasting (4h) insulin (pmol/L)

**C**  
Blood glucose during GTT (mM)

**D**  
AUC GTT (mmol/Lxh)

**E**  
Blood glucose during ITT (mM)

**F**  
AUC of ITT (mmol/Lxh)

---

- RD
- HF/HP Casein
- HF/HP Cod
- HF/HP Chicken
- HF/HS Casein
- HF/HP Soy
- HF/HP Beef
- HF/HP Pork
Figure 3

A  BW gain (g)  
B  Fat mass (g)  
C  Lean mass (g)  
D  Feed efficiency (g/MJ)  
E  Apparent fat digestibility (%)  
F  Apparent nitrogen digestibility (%)  
G  Plasma urea (mmol/L)  
H  Plasma OH-butyrate (mmol/L)  
I  Plasma FFA/NEFA (mmol/L)  

RD  [ ]  HF/HS Casein  [ ]  HF/HP Casein  [ ]  HF/HS Pork  [ ]  HF/HP Pork

Figure 3
**Figure 4**

- **A** Change in activity during light phase (beam breaks)
- **B** Change in activity during dark phase (beam breaks)
- **C** Change in EE during light phase (kJ/h*kg BW)
- **D** Change in EE during dark phase (kJ/h*kg BW)
- **E** Change in RER during light phase
- **F** Change in RER during dark phase

Legend:
- H/F HS Casein
- H/F HP Casein
- H/F HS Pork
- H/F HP Pork
Figure 5

A. Blood glucose during ITT (mM)
B. AUC of ITT (mmol/Lxh)
C. Blood glucose during GTT (mM)
D. AUC of GTT (mmol/Lxh)
E. Fasting (6hrs) plasma insulin (pmol/L)
F. Insulin (15 min) during GTT (pmol/L)
Figure 6

Gene expression iBAT (relative expression)

A

Bcat2

Bckdha

Dbt

Did

Gene expression iWAT (relative expression)

B

Bcat2

Bckdha

Dbt

Did
Figure 7

A

**Gene expression in WAT (relative expression)**

### Casein

<table>
<thead>
<tr>
<th>High sucrose</th>
<th>High Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="high_sucrose_casein" alt="Image" /></td>
<td><img src="high_protein_casein" alt="Image" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>High sucrose</th>
<th>High Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="high_sucrose_pork" alt="Image" /></td>
<td><img src="high_protein_pork" alt="Image" /></td>
</tr>
</tbody>
</table>

- **HF/HS Casein**
- **HF/HP Casein**
- **HF/HS Pork**
- **HF/HP Pork**

### Mean diameter (μM)

- **a**
- **b**

B

**Gene expression in WAT (relative expression)**

- **Ucp1**
- **Dio2**
- **Ppargc1a**
- **Cidea**
Figure 8

A

iBAT

<table>
<thead>
<tr>
<th>Casein</th>
<th>High sucrose</th>
<th>High Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork</td>
<td>High sucrose</td>
<td>High Protein</td>
</tr>
</tbody>
</table>

B

Gene expression iBAT (relative expression)

- **Ucp1**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Dio2**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Ppargc1a**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Cidea**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Cpt1b**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork

- **Leptin**
  - HF/HS Casein
  - HF/HP Casein
  - HF/HS Pork
  - HF/HP Pork
Figure 8

C

iBAT

Casein

High sucrose

High Protein

Pork

High sucrose

High Protein

UCP1

GAPDH

Relative UCP1 level

HF/HS Casein  HF/HP Casein  HF/HS Pork  HF/HP Pork

UCP-1 staining iBAT (%)
Supplementary figure 2