1. Preface

The present master thesis (60 etcs), "Establishment of an adipocyte cell-based model to investigate the effect of short-chain fatty acids, especially butyrate, on insulin sensitivity and glucose homeostasis in fat tissue", was carried out at Aarhus University, Faculty of Science and Technology, at the Department of Animal science, in the period November 2012 to November 2013. The project was made with supervision from Lotte Bach Larsen, Stig Purup, Tina Skau Nielsen, and Peter Kappel Theil.

1.1 Acknowledgement

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2. Abstract

The prevalence of obesity is expanding worldwide. Obesity is characterised by increased storage of fatty acids in an expanded adipose tissue mass and is associated with a number of health problems, summarised together as the metabolic syndrome and includes type 2 diabetes (T2D). The incidence of T2D has been increasing in the last 20 years, in part due to unlimited access to food combined with a sedentary lifestyle. This does not seem to change in the near future and approaches to prevent the increasing incidence of T2D are needed. The preferable way to minimise the damages of this condition, other than losing body weight, is to increase tissue insulin sensitivity and thereby prevent the development of T2D. This might be achieved by increasing and improving the daily amount of dietary fibers (DF), since studies have shown insoluble non-viscous DF to improve insulin sensitivity and glucose homeostasis. Insoluble DF has no effect on the digestive events in the small intestine. The beneficial effects of insoluble DF on insulin sensitivity and glucose homeostasis may involve colonic metabolism, by which the outcome is short-chain fatty acids (SCFA). Increased synthesis of SCFA in the colon, butyrate in particular, has previously been shown to improve insulin sensitivity and glucose homeostasis.

The aim of this study was to establish an adipocyte cell-based model in order to investigate the effect of SCFAs, especially butyrate, on insulin sensitivity and glucose homeostasis. Furthermore, it was investigated how plasma from pigs fed three diets, Western Style Diet (WSD), Arabinoxylan Diet (AX), and Resistant Starch Diet (RS), affected insulin sensitivity of adipocytes and how the three diets affected the size of cells from subcutaneous fat. The effect of SCFAs on insulin sensitivity of SGBS cells, which is a preadipocyte cell line derived from subcutaneous white adipose tissue, was studied. Only acetate was shown to have a significant effect in improving insulin sensitivity, at physiologic concentrations, while butyrate showed a tendency towards improvement on insulin sensitivity. Propionate showed no effect at all. When combining the SCFAs, a significant improvement on insulin sensitivity was seen when increasing the butyrate content in the combination. Of the three diets fed the pigs, the RS diet gave improvement in insulin sensitivity at 10% plasma and a tendency towards improvement at 5% plasma, while AX gave the highest improvement in insulin sensitivity at 1% plasma. Compared with the size of cells from subcutaneous fat, AX gave more of the smaller adipocytes, ranging from size-group 5-15 μm to 45-55 μm, than did either the WSD or the RS diet. The RS diet seemed to give more of the medium sized adipocytes, ranging from size-group 56-65 μm to 106-115 μm, while the WSD diet seemed to give more of the larger adipocytes, ranging from size-group 116-125 μm to ≥156 μm.

It can be concluded that increasing the daily intake of DF, and thereby increasing the synthesis of SCFA by microbial fermentation in the colon, increases the amount of the small adipocytes and improves insulin sensitivity.

3. Resumé

Udbredelsen af fedme er voksende på verdensplan. Fedme er karakteriseret ved stigende lagring af fedtsyrer i en voksende fedtvævs masse, og er associeret med et antal sundhedsproblemer, sammenfattet som det metaboliske syndrom og inkluderer type 2 diabetes (T2D). Forekomsten af T2D har været stigende i de sidste 20 år, delvis på grund af ubegrænset adgang til mad kombineret med en inaktiv livsstil. Dette ser ikke ud til at ændre sig i den nærmeste fremtid og, fremgangsmåder til at undgå stigende forekomster af T2D er nødvendige. Den mest favorable måde til at minimere skaderne ved denne tilstand, udover at tabe kropsvægt, er at øge insulin sensitiviteten i væv og dermed forebygge udviklingen af T2D. Dette kan opnås ved at øge og forbedre det daglige indtag af kost fibre (DF), da studier har vist, at uopløselige ikke-viskøse DF forbedrer insulin sensitiviteten og glukose homeostase. Uopløselige DF har ingen effekt på fordøjelsessystemets begivenheder i tyndtarmen. De uopløselige DF gavnlige effekter på insulin sensitivitet og glukose homeostase kan involvere tyktarmsmetabolisme, hvorved resultatet er kortkædede fedtsyrer (SCFA). Øget SCFA syntese i tyktarmen, særligt butyrat, har tidligere vist at forbedre insulin sensitiviteten og glukose homeostase.

Formålet med dette studie var at etablere en adipocyt cellebaseret model, med henblik på at undersøge effekten af SCFAs, specielt butyrat, på insulin sensitiviteten og glukose homeostase. Endvidere blev det undersøgt, hvordan plasma fra grise fodret med tre forskellige diæter, Western Style Diet (WSD), Arabinoxylan Diet (AX), og Resistant Starch Diet (RS), påvirkede insulin sensitiviteten af adipocyter og, hvordan de tre diæter påvirkede størrelsen af celler fra subkutant fedt. Effekten af SCFAs på insulin sensitiviteten af SGBS celler, hvilket er en preadipocyt cellelinje fra subkutant hvidt fedtvæv, blev undersøgt. Kun acetat viste sig at have en signifikant effekt på forbedringen af insulin sensitiviteten, ved fysiologisk koncentration, mens butyrat viste en tendens mod forbedring af insulin sensitiviteten. Propionat viste ikke nogen effekt overhovedet. Når man kombinerer SCFAs, ses en signifikant forbedring i insulin sensitiviteten, når man øger indholdet af butyrat i kombinationen. Af de tre diæter, grisene blev fodret med, gav RS diæten forbedring af insulin sensitiviteten ved 10 % plasma og en tendens mod forbedring af insulin sensitiviteten ved 5 % plasma, mens AX gav den største forbedring i insulin sensitiviteten ved 1 % plasma. Sammenlignet med størrelsen af celler fra subkutant fedt, gav AX flere af de små adipocyter, rangerende fra størrelses-gruppe 5-15 µm til 45-55 µm, end enten WSD eller RS diæten gjorde. RS diæten synes at give flere mellem størrelses adipocyter, rangerende fra størrelses-gruppe 56-65 µm til 106-115 µm størrelses-gruppen, mens WSD diæten synes at give flere af de store adipocyter, rangerende fra størrelses-gruppe 116-125 µm til ≥156 µm størrelses-gruppen.

Det kan konkluderes, at en øgning i det daglige indtag af DF, og dermed en øgning i syntesen af SCFA ved mikrobiel fermentering i tyktarmen, øger mængder af de små adipocyter og forbedrer insulin sensitiviteten.

4. Abbreviations

ATGL	Adipose triglyceride lipase
AX	Arabinoxylan
BMI	Body mass index
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
DAG	Diacylglycerol
DF	Dietary fibre
DMEM/F-12	Dulbecco's Modified Eagle Medium:Nutrien Mixture F-12
FCS	Foetal calf serum
FFA	Free fatty acid
GLUT4	Insulin-dependent glucose transporter 4
Glycerol-3-P	Glycerol-3-phosphate
GPR	G protein-coupled receptor
HBS	Hepes buffered saline
HMW	High molecular weight
HSL	Hormone sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IL-6	Interleukin-6
LMW	Low-molecular weight
MAG	Monoacylglycerol
MMW	Medium molecular weight
NSP	Nonstarch polysaccharide
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PPARγ	Peroxisome proliferator-activated receptor gamma
RS	Resistant starch
SCFA	Short chain fatty acid
SEM	Standard error of the mean
SGBS	Simpson-Golabi-Behmel syndrome
SREBP-1c	Sterol regulatory element binding protein 1c
T2D	Type 2 diabetes
T3	3,3',5-Triiodo-L-thyronine
TAG	Triacylglycerol
TG	Triglyceride
TNF-α	Tumor necrosis factor-α
VLDL	very low-density lipoproteins
WHO	World healt organisation
WSD	Western style diet

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5. Objective and hypothesis

The objective of this master thesis was to study the effect of SCFAs, in particular butyrate, for their *in vitro* effect on insulin sensitivity of adipocytes. There has been an increased incidence of T2D over the last 20 years due to unlimited access to food combined with a sedentary lifestyle (Bano 2013). This does not seem to change in the near future and approaches to prevent the increasing incidence of diabetes are needed. Studies have emphasised the importance of insoluble non-viscous dietary fiber (DF) for improving insulin sensitivity and glucose homeostasis (Gao et al. 2009;Priebe et al. 2010;Robertson et al. 2005;Robertson 2007;Robertson et al. 2003). Effects that are ascribed colonic metabolism (Gao et al. 2009;Robertson 2007;Robertson et al. 2003). This lead to the hypothesis that:

Increased production of SCFA, in particular butyrate, during microbial fermentation of DF in the colon may protect against lifestyle diseases such as T2D.

To test the hypothesis, an *in vitro* model was established, in order to investigate the effect of SCFAs, especially butyrate, on insulin sensitivity and glucose homeostasis. Furthermore, it was investigated how plasma from pigs fed three diets, WSD, AX diet, and RS diet, affected insulin sensitivity of adipocytes and how the three diets affected the size of adipocytes from subcutaneous fat.

6. Introduction

Obesity is a medical condition in which excess body fat has accumulated to the extent that it may have an adverse effect on health, leading to reduced life expectancy and/or increased health problems. Obesity is defined by a body mass index (BMI) of ≥ 30.0 (table 1), where BMI is defined as the subjects mass divided by the square of their height and expressed as kilograms per square meter. The most commonly used definitions, established by the World Health organisation (WHO), published in 2000, and are listed in table 1:

Table 1: The most commonly used definitions for BMI, established by WHO.

BMI	Classification
< 18.5	Underweight
18.5-24.9	Normal weight
25.9-29.9	Overweight
30.0- 34.9	Obese
35.0-39.9	Severe obese
≥ 40	Morbid obese

The associated health problems can often be categorised under the metabolic syndrome that involves the development of insulin resistance, T2D, cardiovascular disease and fatty liver disease (Galic, Oakhill, and Steinberg 2010;Gray and Vidal-Puig 2007). Obesity is characterised by increased storage of fatty acids in an expanded adipose tissue mass and is closely associated with the development of insulin resistance in peripheral tissue such as skeletal muscle and the liver (Galic, Oakhill, and Steinberg 2010;Gray and Vidal-Puig 2007). Furthermore, obesity has been associated with a pro-inflammatory state in which plasma concentrations of inflammatory mediators are increased (Roelofsen, Priebe, and Vonk 2010). The preferable way to minimise this condition, other than losing body weight, is to increase tissue insulin sensitivity and thereby prevent the development of T2D (Robertson et al. 2005).

For many years the adipose tissue has been considered as a simple storage organ for triacylglycerol (TAG). However, increased interest in the biology of adipose tissue has proven this to be wrong. Adipose tissue is now recognised as an important organ of a complex network that participates in the regulation of a variety of diverse functions (Coelho, Oliveira, and Fernandes 2013). It serves as an endocrine organ and is dynamically involved in the regulation of cell functions through a complex network of endocrine, paracrine, and autocrine signals that influence the response of many tissues (Coelho, Oliveira, and Fernandes 2013).

An unhealthy dietary pattern, defined as WSD, is regarded as a significant risk factor for developing one of the lifestyle diseases associated with the metabolic syndrome (Priebe et al. 2010;Robertson 2007). Epidemiological studies have suggest that higher levels of DF intake plays a significant protective role with respect to diabetes that is independent of other dietary factors (Anderson et al. 2009). The content of DF in a WSD diet is low. Insoluble DF has no effect on the digestive events in the small intestine, the beneficial effects of insoluble DF on insulin sensitivity and glucose homeostasis may involve colonic metabolism (Priebe et al. 2010;Robertson 2007), of which the outcome is SCFAs (Louis et al. 2007). The dietary intake of fermentable carbohydrates is known, from many studies, to influence SCFA synthesis in the large intestine. Resistant starch has consistently been shown to have butyrogenic effects on the colonic microbiota, both *in vitro* and *in vivo* (Louis et al. 2007). Increased SCFA production in the colon and butyrate in particular, has previously been linked to improvements in insulin sensitivity and glucose homeostasis (Gao et al. 2009;Priebe et al. 2010;Robertson et al. 2005;Robertson 2007;Robertson et al. 2003). The mechanisms behind this are not entirely clear but seem to involve adipose tissue metabolism (Robertson et al. 2005;Robertson 2007). Furthermore, improved colonic fermentation has been shown to increase plasma free fatty acid release, decrease fat deposition, and reduce adipogenesis which results in smaller adipocytes (Robertson 2007).

In the present master thesis an adipocyte cell-based model are established. The effect of SCFAs along with plasma from pigs fed three diets; a WSD, an AX diet, and a RS diet, on improvements on insulin sensitivity were studied. Furthermore, it was studied how the size of cells from subcutaneous fat from pigs fed one of the three diets was affected.

7. Dietary aspects

Analysis made by United States Department of Agriculture of data tracking, for a period of 30 years (1970-2000), revealed that diets have changed both in terms of overall caloric intake and the relative amounts of different food items (Kau et al. 2011). This is consistent with estimates from the US National Health and Nutrition Examination Survey, which indicate that adult men and women increased their daily caloric intake by 6.9% and 21.7%, respectively, during the same period (Kau et al. 2011). Intake of undigestible carbohydrates (dietary fiber, DF) provides many health benefits including reducing the development of hypertension, obesity, diabetes and certain gastrointestinal disorders. Unfortunately, less than half of the daily recommended levels of dietary fibre are consumed (Anderson et al. 2009).

Epidemiological studies have pointed to whole-grain cereal products as factors that may protect against cardiovascular disease and T2D by improving insulin sensitivity (Theil et al. 2011;Slavin 2004). A lower glycaemic index[1], as observed with cereal-based foods, is usually related to soluble DF, β-glucan and arabinoxylans (AX), as these polysaccharides may interfere with the digestion and absorption processes either by delaying gastric emptying or by slowing the uptake of nutrients from the small intestine (Theil et al. 2011). Whereas the refining of grains tends to increase glycaemic response (Slavin 2004). More recent studies have emphasised the importance of insoluble non-viscous DF for improving insulin sensitivity and glucose homeostasis (Theil et al. 2011;Robertson 2007;Priebe et al. 2010). Since insoluble DF has little or no influence on digestive events in the small intestine, the beneficial effects of DF on insulin sensitivity and glucose homeostasis must involve colonic metabolism (Theil et al. 2011;Robertson 2007;Priebe et al. 2010). The metabolism of DF in the large intestine leads to the production of SCFAs, such as acetate, propionate and butyrate, through microbial fermentation (Theil et al. 2011;Bach Knudsen et al. 2003). These SCFA are rapidly absorbed from the gut lumen, a fraction is metabolised by the colonic epithelium, but a significant part enters the portal and peripheral circulation (Theil et al. 2011;Bach Knudsen et al. 2003). Enhanced production of SCFA, in particular butyrate, has been linked to improved insulin sensitivity and glucose homeostasis (Theil et al. 2011;Robertson et al. 2005;Gao et al. 2009).

Cereal foods are an important contributor of carbohydrates and DF, particularly in Northern Europe (Theil et al. 2011). The consumption pattern has changed toward more white breads, based on refined wheat starch instead of whole-grain cereal breads (Theil et al. 2011). DF and whole grains contain a unique blend of bioactive components including resistant starch, vitamins, minerals, phytochemicals and antioxidants (Lattimer and Haub 2010). DF has traditionally been defined as the proportions of plant foods that were

[1] The glycaemic index is defined as the incremental area under the blood glucose response curve for the test food divided by the corresponding area after an equicarbohydrate proportion of white bread multiplied by 100.

resistant to digestion in the small intestine by the human digestive enzymes. This definition has more recently been expanded to include oligosaccharides and resistant starches (Anderson et al. 2009). Dietary fibers can be classified chemically as either soluble or insoluble, based on chemical, physical, and functional properties. Soluble fibers are water soluble, have viscous properties and are fermentable by microorganisms within the gastrointestinal tract. They cause a well-documented reduction in the rate of glucose absorption which reduces the glycaemic index excursion after carbohydrate intake (Robertson et al. 2005;Anderson et al. 2009;Lattimer and Haub 2010). Insoluble fibers are not water soluble in the human gastrointestinal tract, they are non-viscous due to their water insolubility and fermentation is limited (Lattimer and Haub 2010). Insoluble fibers have no effect on glucose absorption, yet they have been shown in short-term human studies (Robertson et al. 2003) to increase insulin sensitivity (Robertson et al. 2005). Most fiber containing foods include approximately one-third soluble and two-third insoluble fibers (Lattimer and Haub 2010).

7.1 Types of diet

The pig, as a model for humans, was in this master thesis used to study the effect of three diets; WSD, AX diet, and RS diet, on insulin sensitivity and the size of adipocytes from subcutaneous fat. The pig is an optimal model, since it is closely related with human physiology and it consumes human foods readily (Topping and Clifton 2001). The pig has been used as a model to examine the effects of numerous human foods and food ingredients, such as rye and starches, on large bowel SCFA production (Topping and Clifton 2001).

7.1.1 Western style diet

The diet of many people in affluent societies like the Danish is often referred to as the WSD. It is characterised by being high in fat, protein and refined carbohydrates, and low in DF (Anderson et al. 2009). The average human diet in European Western societies contains approximately 20-25 g fiber/day (den et al. 2013), while the typical US diet contains approximately 15 g fiber/day (Cordain et al. 2005). The fiber content in the typical US diet is considerably lower than the recommended values of 25-30 g/day (Cordain et al. 2005). This type of diet has been associated with an increased risk of developing T2D in women (Lattimer and Haub 2010).

7.1.2 Arabinoxylan

Cereal AX are the major component of the dietary fiber fraction of cereal grains, together with cellulose, β-1,3:1,4-glycan, arabinogalactan peptide and lignin (Broekaert et al. 2011). AX, as a constituent of hemicellulose, is comprised of a xylose backbone with arabinose side chains (Lattimer and Haub 2010). Clear differences in arabinose to xylan ratio can be found between AX in wheat endosperm and that in bran tissues (Broekaert et al. 2011). Wheat bran, composed of an outer layer of bran and an inner layer of endosperm,

is a rich source of AX. The non-starch polysaccharides (NSP) in wheat bran are approximately 64-69% AX and 15-31% cellulose, whereas NSPs in wheat endosperm are approximately 88% AX (Lu et al. 2000). In the gastrointestinal tract AX acts much like a soluble fiber being rapidly fermented by the microflora of the colon (Lattimer and Haub 2010), but two forms exist: water-soluble and water-insoluble, with the insoluble part being the major fraction and are due to non-covalent interactions with neighbouring AX molecules (Broekaert et al. 2011). Addition of as little as 6 g AX-rich fiber to bread eaten in a breakfast meal, significantly lowered postprandial glucose and insulin response in healthy adults (Lu et al. 2000). The mode of action behind AX improving glucose tolerance is unknown, but since AX acts much like a soluble fiber, it is likely that a high viscosity may slow the rate of gastric emptying and reduce small intestinal motility, which may result in delayed glucose absorption and thereby a flattened blood glucose response (Lu et al. 2000;Lattimer and Haub 2010). In the present thesis the AX diet represented a WSD added a high content of AX in the form of rye flakes and enzyme treated wheat bran. It was expected that the rye flakes and enzyme treated wheat bran would act as a substrate for butyrate production.

7.1.3 Resistant starch

RS behaves as a soluble fiber, but is non-viscous and fermentable, and by definition is resistant to enzymatic digestion in the small intestine and is fermented to SCFAs by the microflora in the large intestine (Robertson et al. 2003;Keenan et al. 2006;Robertson 2012;Lattimer and Haub 2010). RS can be classified into four subtypes depending on botanical source and processing: RS1, starch granules embedded in indigestible plant material such as whole grains; RS2, native granular starch such as found in raw potato or high-amylose maize, wheat etc.; RS3, crystallised starch made by unique cooking and cooling processes and RS4, chemically modified starch typically through esterification, cross-linking or trans-glycosylation and is not found in nature (Robertson 2012;Lattimer and Haub 2010). A majority of human studies involving RS have shown a decrease in postprandial blood glucose and insulin levels. It is though difficult to completely understand these effects due to differences in study design and the type of RS used (Lattimer and Haub 2010). In the present thesis a high content of RS in the diet was obtained by addition of high maize and potato starch. The raw potato starch as well as a great part of the high maize was expected to be fermented in the colon with high yields of butyrate.

7.2 Obesity, glucose homeostasis and type 2 diabetes

Unlimited access to food combined with a sedentary lifestyle has contributed to an increase in the incidence of obesity over time (Bano 2013). Obesity can be described as the accumulation of adipose tissue, is a prevalent health hazard in industrial countries and is closely associated with a number of pathological disorders, including T2D, hypertension, cancer, and atherosclerosis (Gregoire, Smas, and Sul 1998;Bano 2013). Diabetes has been increasing at an alarming rate worldwide, and in the United States almost half of

the individuals have diabetes, pre-diabetes or, are at substantial risk of developing diabetes because of the presence of the metabolic syndrome (Anderson et al. 2009). Of those diagnosed with diabetes, approximately 90% have T2D and around 80% of these are obese (Anderson et al. 2009).

Total blood glucose is the net result of influx of exogenous glucose from the small intestine into the systemic circulation, endogenous glucose production and uptake of glucose into peripheral tissue (Priebe et al. 2010). The normal glucose concentration is usually maintained within a relatively narrow range through an intricate regulatory and counter-regulatory neuro-hormonal system, which may be both insulin-dependent and insulin-independent (Bano 2013;Gerich 1993). The brain and nervous system are insulin independent, whereas muscle and adipose tissue are responsive to insulin (Bano 2013). Both muscle and adipose tissue can use either glucose or ketones and free fatty acids (FFA) as their primary metabolic fuel. The fuel used is primarily determined by the amount of insulin bound to its cell-surface insulin receptors (Bano 2013). In the presence of large amounts of insulin, the cell preferentially uses glucose as their metabolic fuel, taken up by the cell through the glucose-transporter type 4 (GLUT4), metabolises it, and stores it as glycogen in the muscle or as fat in the adipose tissue and thereby lowering postprandial plasma glucose (Bano 2013). When insulin levels are low, the cell switches to ketone/free fatty acid metabolism and thereby reduces uptake of glucose (Bano 2013).

Before the 1990s, it was rare for most paediatrics to see children with T2D. The rate of T2D incidents have, however, been increasing ever since, and if the current rates of obesity continue to increase, 1 in 3 babies born in 2000 will develop diabetes in their lifetime (Lazar 2005;Caprio 2012). T2D is characterised by a decrease in the effect of insulin on peripheral tissue (insulin resistance) and by the inability of the endocrine pancreas to compensate for this resistance (hyperglycemia) (Salmeron et al. 1997;Lattimer and Haub 2010). In the nurses' health study, a positive correlation between glycaemic index and T2D was found (Lattimer and Haub 2010). Several theories have been proposed to understand the physiology behind the relationship of glycaemic index and diabetes. First, carbohydrates with a higher glycaemic index produce higher blood glucose levels. This chronic hyperglycemia is suggested to lead to the dysfunction of beta cells in the pancreas, due to exhaustion, thus decreasing insulin release. Second, due to an overabundance of high glycaemic load, tissues such as skeletal muscle, liver and adipose become resistant to insulin (Lattimer and Haub 2010;Cerf 2013). Obesity is the most important cause in the development of insulin resistance, and it has been demonstrated that the critical determinant of insulin sensitivity is not the degree of obesity, but the distribution of fat partitioning (Caprio 2012). Consumption of dietary fiber also seems to be associated with T2D independent of other compounding factors (such as age, fat intake, smoking, alcohol, exercise, and body weight) (Lattimer and Haub 2010).

Obesity does not, however, always result in diabetes and many people who are very obese are able to maintain normal glucose tolerance (Weyer et al. 2000). The reason why some people with obesity develop T2D and others do not is not known (Weyer et al. 2000;Bano 2013). There is evidence indicating that differences in body fat distribution play a role (Weyer et al. 2000) along with the function of the adipocyte itself (Gray and Vidal-Puig 2007). Nevertheless, it is unlikely to be the only explanation for differences in the propensity to diabetes among people with obesity (Weyer et al. 2000). People with large amounts of abdominal fat accumulated are more likely to have predictable dysfunctional patterns such as insulin resistance, hyperinsulinaemia and glucose intolerance, than those with a more femoro-gluteal pattern (Weyer et al. 2000). It has been hypothesised that people who become obese primarily through an increase in fat cell size ("hypertrophic obesity") are more likely to develop obesity related metabolic disturbances than those in whom the increase in adipose tissue is primarily mediated by an increase in fat cell number ("hyperplastic obesity") (Weyer et al. 2000). Recent studies have identified links between obesity and T2D (Bano 2013). In these studies, increased production of adipokines and cytokines, including tumor necrosis factor-α (TNF-α) and resistin was found to contribute to the development of insulin resistance and to reduce levels of adiponectin (Bano 2013). Inflammatory adipokines such as TNF-α and interleukin-6 (IL-6) suppress the transcription of adiponectin and thereby reduces the levels of adiponectin. With decreasing adiponectin levels, the incidence of insulin resistance seems to be increasing (Deng and Scherer 2010). The hallmark of impaired insulin sensitivity in white adipose tissue is a reduced ability of insulin to inhibit TAG lipolysis, resulting in elevated circulation of FFAs (Konige, Wang, and Sztalryd 2013). High FFA release from white adipose tissue causes insulin resistance in skeletal muscle, liver and other tissues (Konige, Wang, and Sztalryd 2013).

8. SCFA production

SCFAs refer to carboxylic acids with aliphatic tails less than six carbons (Layden et al. 2013). In humans, SCFAs, mainly acetate, propionate, and butyrate, are derived in large part from microbial fermentation of undigested carbohydrates and proteins in the colon. The host is thereby able to salvage energy from foods that cannot be processed enzymatically in the upper parts of the gastrointestinal tract (Layden et al. 2013). Fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolizable energy for microbial growth and maintenance and other metabolic end products for use by the host (Wong et al. 2006). The production of SCFAs are determined by a number of factors, including the numbers and types of microorganisms present in the colon, substrate source, and gut transition (Wong et al. 2006). Bacterial numbers, fermentation, and proliferation are highest in the proximal colon where substrate availability is highest (Wong et al. 2006). The principal site of colonic fer-

mentation, therefore, is the cecum and proximal colon, whereas the distal colon is carbohydrate and water depleted (Wong et al. 2006;Topping and Clifton 2001). Nondigestible food components are a source of substrate for fermentation by anaerobic colonic microflora, because they are resistant to hydrolysis and digestion in the stomach and small intestine and eventually enter the colon for fermentation (Wong et al. 2006). Carbohydrates quantitatively play the most important role in the formation of SCFA (Wong et al. 2006).

8.1 Fermentation

The bacterial population of the human cecum and colon is numerically large and compromise approximately 40-55% of solid stool matter (Topping and Clifton 2001). The dominant organisms in terms of numbers are anaerobes including bacteroides, bifidobacteria, eubacteria, streptococci, and lactobacilli (Topping and Clifton 2001). Some organisms grow on intermediate products of fermentation such as H_2, lactate, succinate, formate, and ethanol and convert these to end products including SCFA, while others metabolise CO_2 either yielding CH_4 or converting CO_2 to acetate (Topping and Clifton 2001). The basic fermentative reaction in the human colon are; hydrolysis of polysaccharides, oligosaccharides, and disaccharides to their constituent sugars, which are then fermented resulting in an increased biomass (Topping and Clifton 2001). The majority of intestinal bacteria use the glycolytic pathway (figure 1) to derive energy from carbohydrates, which are initially converted to pyruvate and acetyl-CoA. These metabolites are key control points in fermentative metabolism, which can be converted to a wide range of products (Macfarlane and Macfarlane 2003).

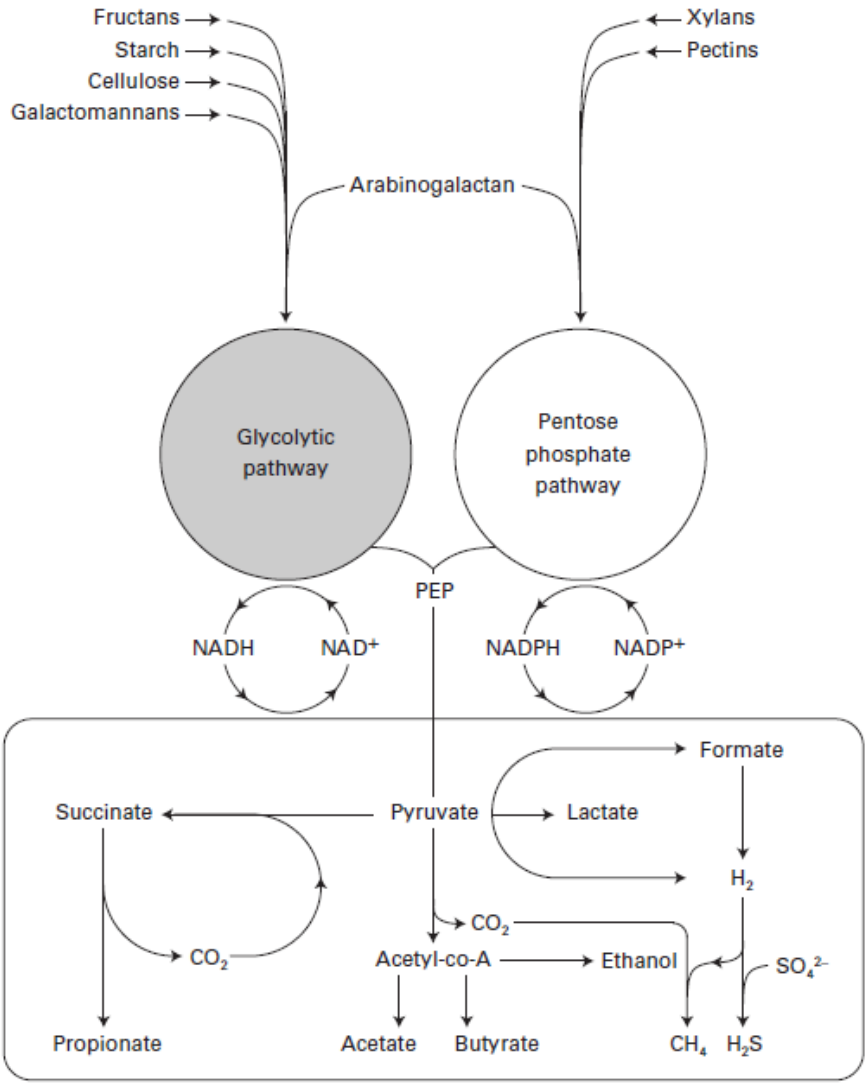

Figure 1: A simplified diagram of polysaccharide breakdown and the main routes of carbohydrate fermentation in the large intestine (Macfarlane and Macfarlane 2003).

The pentose phosphate pathway also occurs in many gut bacteria, where it is used in the metabolism of pentoses (Macfarlane and Macfarlane 2003). Fermentation is regulated by the need to maintain redox balance, which is mediated through the reduction and oxidation of pyridine nucleotides, ferredoxins, and flavins. Synthesis of reduced products including hydrogen, lactate, succinate, butyrate and ethanol is used to effect redox balance during fermentation, whereas the formation of more oxidised products, such as acetate, is associated with ATP production (Macfarlane and Macfarlane 2003).

In humans, the principal products of fermentation are SCFA, especially acetate, propionate, and butyrate, together with gases (CO_2, CH_4, and H_2) and some heat (Topping and Clifton 2001;Bergman 1990). SCFA production represents nearly 75% of the energy content of the carbohydrate in the large intestine; the remaining 25% is used by the microbes for growth or lost as hydrogen and methane (Bergman 1990). Diet can

change the metabolic activities of the microorganisms by providing new or different substrates, and thus diet influences the quantities and nature of the fermentation end products (Bergman 1990). *In vitro* fermentation experiments with faecal bacteria have demonstrated that individual polysaccharides are broken down at different rates, e.g. starch and pectin are degraded more rapidly than xylan and arabinogalactan (Macfarlane and Macfarlane 2003). These studies also demonstrated that fermentation of different polysaccharides gives rise to distinct patterns of SCFA production. For example, acetate was the main product of pectin and xylan breakdown, while large amounts of acetate and propionate were produced from arabinogalactan. Of the four substrates tested, butyrate was only formed in substantial amounts from starch (Macfarlane and Macfarlane 2003).

8.2 Absorption

The major SCFAs acetate, propionate and butyrate are absorbed at comparable rates in different regions of the colon. Once absorbed, SCFAs are metabolised at three major sites in the body: (1) cells of the epithelium that use butyrate as a major substrate for maintenance-energy production pathway; (2) liver cells that metabolise residual butyrate with propionate used for gluconeogenesis and 50-70% of acetate also taken up by the liver; (3) muscle cells that generate energy from the oxidation of residual acetate (Wong et al. 2006). SCFA are absorbed and transported via the portal vein to the liver, and the fraction not utilised is distributed to the other body organs and tissues for metabolism (Topping and Clifton 2001). The liver extracts approximately 75% of acetate, 90% of propionate, and 95% of butyrate from the portal vein. Higher concentrations of SCFAs in the peripheral circulation have, though, been observed after ingestion of non-digestible carbohydrates (Priebe et al. 2010). The concentration found in the peripheral circulation varies with respect to food ingestion, substrates available for fermentation, and time of day measurement. In table 2, different peripheral SCFA concentrations in humans are given. As it appears from table 2, the concentration of acetate in humans varies from 70-156 µM, while propionate and butyrate give variations of 4-8 µM and 1-4 µM, respectively.

Table 2: Human SCFA concentrations (µM) found in the peripheral circulation.

	Acetate (µM)	Propionate (µM)	Butyrate (µM)
(Wolever et al. 1997)	100-150	4-5	1-3
(Cummings et al. 1987)	70	5	4
(Nilsson et al. 2010)	157	8	3

8.3 The individual SCFAs

The role of SCFAs has expanded from being just a source of energy for the colonic epithelium and modulators of colonic and intracellular pH, cell volume, and other functions associated with ion transport, to also being important regulators of proliferation, differentiation, and gene expression (Wong et al. 2006). Acetate is usually present in higher concentrations than the other SCFAs (Bergman 1990). Propionate and butyrate are also present in high concentrations, although their levels can vary considerably with diet (Bergman 1990). Acetate, propionate and butyrate are the predominant forms of SCFA and are produced mainly from the fermentation of plant materials, such as celluloses, fiber, starches and sugars (Bergman 1990).

Acetate (C2) is the principal SCFA in the colon and is readily absorbed and transported to the liver, and therefore is less metabolised in the colon (Wong et al. 2006). The presence of acetyl-CoA synthetase in the cytosol of adipose tissue allow the use of acetate for lipogenesis once it enters the systemic circulation (Wong et al. 2006).

Propionate (C3) is produced via two main pathways: (1) fixation of CO_2 to form succinate, which is subsequently decarboxylated; (2) from lactate and acrylate (Wong et al. 2006). Propionate has been shown to have insulin-like effects; stimulating glycolysis, activating glycogen synthase in isolated hepatocytes and reducing gluconeogenesis, all serving to lower circulating glucose (Robertson et al. 2003).

Butyrate (C4) is the preferred energy source for colonic epithelial cells, but also plays a major role in regulation of cell proliferation and differentiation (Wong et al. 2006). It is the most important SCFA in colonocyte metabolism, where 70-90% of butyrate is metabolised by β-oxidation (Wong et al. 2006;Bach Knudsen et al. 2003). Butyrate is low in its relative concentration but seems to be involved in metabolisms beyond its role as a nutrient. Butyrate has been shown to have several cellular effects, such as influence on cell maturation, cell differentiation, proliferation and in particular cell cycle arrest and apoptosis (Li and Elsasser 2005). Polysaccharides and fractions that stimulate the formation of butyrate are resistant starch and brans from wheat and oats, while xylan, pectin and pectin-rich fractions are all associated with a relatively low formation of butyrate (Bach Knudsen et al. 2003). There will be a substantial change in the amount of butyrate passing from the gut to the bloodstream in response to the type and levels of undigestible carbohydrates that enter the large intestine, as substrate for fermentation (Bach Knudsen et al. 2003).

9. Adipose tissue

For many years adipose tissue was considered a simple storage organ for TAG. Increased interest in the biology of adipose tissue has proven this to be wrong and it is no longer considered to be an inert tissue

that just stores fat. Fat cells are among the largest cells of the body and increase tremendously in size as they differentiate from a fibroblast-like phenotype to an adipocyte by incorporating TAGs into the central droplet (Franck et al. 2007).

In mammals, two types of adipose tissue can be found: white and brown adipose tissue. The adipocytes in the two tissue types exhibit different morphology and function (Coelho, Oliveira, and Fernandes 2013). Brown adipose tissue is almost absent in adult humans and mammals, but is found at birth and is specialised in heat production (Coelho, Oliveira, and Fernandes 2013). The brown adipocytes are in average smaller, 30-40 µm in diameter, than adipocytes of the white adipose tissue (Coelho, Oliveira, and Fernandes 2013;Fonseca-Alaniz et al. 2007). The brown adipose tissue also stores energy in lipid form, as does white adipose tissue. But it more regularly produces heat by oxidation of fatty acids within the cell itself mediated by uncoupling protein-1 in the mitochondria, instead of supplying free fatty acids to be used by other cell types (Coelho, Oliveira, and Fernandes 2013;Sarjeant and Stephens 2012). The function of white adipose tissue is much broader and more comprehensive than the function of brown adipose tissue and it is distributed extensively in the body (Coelho, Oliveira, and Fernandes 2013). White adipose tissue, accounting for the majority of fat present in adult humans, is the critical site for energy homeostasis, insulin signalling, and endocrine action (Sarjeant and Stephens 2012). White adipocytes are generally spherical cells with an average diameter of 10 µm in younger mammals and may increase up to 100 µm (Sarjeant and Stephens 2012). Mature white adipocytes store TAG in a single large lipid droplet that occupies the centre of the cell, accounting for 85-90% of the mass of the cell and dislocates the cytoplasm, nucleus and other organelles to the circumference, where they remain within a thin layer of cytosol (Fonseca-Alaniz et al. 2007). By its ability to accumulate and provide energy when necessary, it adopts the status of the most important buffering system for lipid energy balance, particularly fatty acids, which are an exceptionally efficient fuel storage species (Coelho, Oliveira, and Fernandes 2013).

White adipose tissue is distributed across a large number of different deposits in the body, classified as subcutaneous and visceral adipose tissue (Fonseca-Alaniz et al. 2007). The subcutaneous adipose tissue is primarily accounted for by the deposits below the skin in the abdominal, gluteus and femoral areas, whereas the visceral adipose tissue includes tissue deposited closer to or even inside the viscera of the abdominal cavity (Fonseca-Alaniz et al. 2007). There is a gender difference in the regional distribution of white adipose tissue, with women generally having a greater degree of adipose tissue than men and having a greater proportion of subcutaneous adipose compared to visceral adipose tissue (Rosenbaum et al. 2001;Fonseca-Alaniz et al. 2007). Visceral fat contains higher concentrations of IL-6 and plasminogen activator inhibitor 1 (PAI-1) than subcutaneous fat, whereas in subcutaneous fat a higher concentration of leptin and adiponec-

tin is observed (Rosenbaum et al. 2001;Coelho, Oliveira, and Fernandes 2013). Visceral fat has a higher lipolysis rate than subcutaneous fat and this may be explained by site variations in the functions of receptors for insulin, catecholamines and adenosine (Bano 2013).

In adults, human adipose tissue is very dynamic. As much as 10% of fat cells die and are renewed every year. The fat mass can grow by increasing the size, rather than the number, of fat cells (hypertrophy) or by making many small fat cells (hyperplasia). Individuals with hypertrophy have slower adipogenesis and cell death than those with hyperplasia (Hoffstedt et al. 2010). Increased fat cell volume predicts impaired glucose metabolism, hyperinsulinaemia and insulin resistance and, several earlier studies have suggested that large fat cells are less responsive to insulin (Franck et al. 2007). An association of subcutaneous adipose hypertrophy with decreased insulin sensitivity has been determined in a large population-based sample (Hoffstedt et al. 2010).

9.1 Lipogenesis and lipolysis

Adipose tissue plays a crucial role in the regulation of whole-body fatty acid homeostasis. Adipocytes are the only cells that are specialised and adapted to store lipids without this compromising their functional integrity (Fonseca-Alaniz et al. 2007). In periods of caloric abundance they store FFAs in the form of triglycerides (TG), which are hydrophobic and can be stored in large quantities without water as a solvent and TG contain twice as much energy per unit mass than other nutrients (Fonseca-Alaniz et al. 2007), through their esterification to glycerol and releases them back into the circulation in times of energy shortage (Galic, Oakhill, and Steinberg 2010).

The accumulation of fat is determined by the balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis) depictured in figure 2 (Kersten 2001;Coelho, Oliveira, and Fernandes 2013).

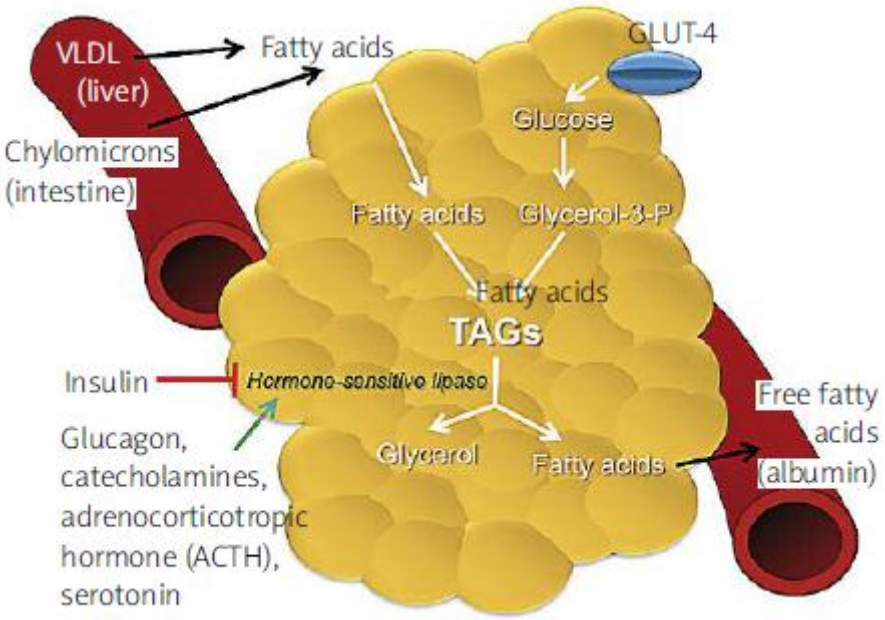

Figure 2: The primary metabolic role of adipose tissue. In the postprandial state, insulin-dependent GLUT4 allows the uptake of glucose from the blood stream and into adipocytes. Glycolysis occurs and produces the substrate required for lipogenesis, glycerol-3-phosphate (glycerol-3-P). Fatty acids carried by very low-density lipoproteins (VLDL) form the liver and chylomicrons from the intestine are esterified with glycerol-3-P to form lipid droplets of TAGs. In the fasting state hormone-sensitive lipase is activated for lipolysis. The production of lipolysis is glycerol, which is transported to the liver and fatty acids which is transported in the bloodstream as free fatty acids bound to albumin to the liver, muscle and other organs to be oxidised (Coelho, Oliveira, and Fernandes 2013).

Lipogenesis occurs mainly in adipose tissue but also takes place in the liver and is the synthesis of fatty acids and subsequent triglyceride synthesis, which is used as energy reserves and is responsive to dietary changes (Coelho, Oliveira, and Fernandes 2013;Kersten 2001). A diet rich in carbohydrates stimulates lipogenesis in both liver and adipose tissue, leading to elevated postprandial plasma TAG levels (Kersten 2001;Coelho, Oliveira, and Fernandes 2013). Plasma glucose levels stimulate lipogenesis via several mechanisms. First, glucose itself is a substrate for lipogenesis. By being glycolytically converted to acetyl-CoA, glucose promotes fatty acid synthesis. Secondly, glucose induces the expression of lipogenic genes and finally, glucose increases lipogenesis by stimulating the release of insulin and inhibiting the release of glucagon from the pancreas (Kersten 2001). By increasing the uptake of glucose in the adipocyte cell via recruitment of glucose transporters to the plasma membrane, as well as activating lipogenic and glycolytic enzymes via covalent modification, insulin potently stimulates lipogenesis (Kersten 2001). For TAG biosynthesis, adipocytes require glycerol-3-P and FFA esterified with coenzyme A. Glycerol-3-P comes from the glycolytic pathway, which involves GLUT4 and is a process controlled by insulin, and FFA is biosynthesised from acetyl-CoA or captured from lipoproteins (chylomicron and VLDL) (Fonseca-Alaniz et al. 2007). In cytosol, FFA is esterified with coenzyme A and TAG is formed through esterification with glycerol-3-P and transferred to lipid droplets (Fonseca-Alaniz et al. 2007). Enlargement of adipocytes might arise from increased

lipogenesis, either from fatty acids taken up from plasma or from those synthesised de novo (Roberts et al. 2009). Roberts et al. has found that small adipocyte size is related to peripheral insulin sensitivity, independently of BMI.

Whereas TAG synthesis occurs in other organs, such as the liver for the production of very low density lipoproteins, lipolysis for the provision of FFAs as an energy source for other organs, is a unique function for adipocytes (Ahmadian, Wang, and Sul 2010). Lipolysis is the hydrolysis of TAG stored in adipose tissue to FFAs and glycerol to be used as an energy source by other tissues (Coelho, Oliveira, and Fernandes 2013) during time of energy shortage (Ahmadian, Wang, and Sul 2010). Lipolysis is strongly regulated by hormones (figure 3) (Ahmadian, Wang, and Sul 2010).

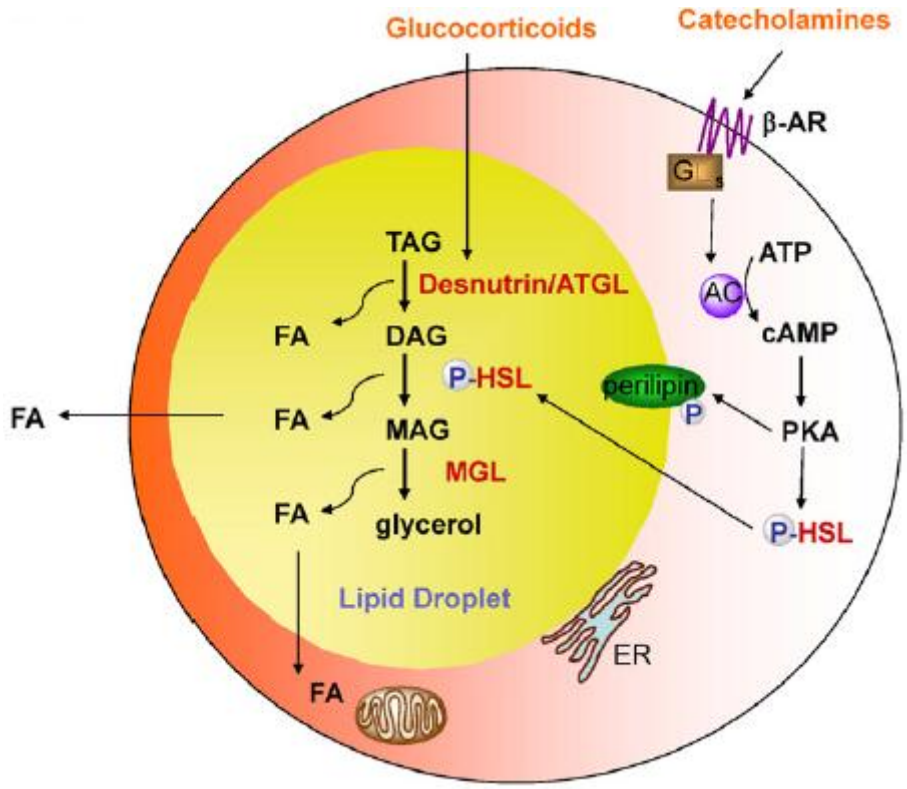

Figure 3: Regulation of lipolysis in adipocytes. Desnutrin/adipose triglyceride lipase (ATGL) initiates lipolysis by hydrolysing TAG to diacylglycerol (DAG). Hormone-senstivite lipase (HSL) hydrolyses DAG to monoacylglycerol (MAG), which is subsequently hydrolysed by MAG lipase to generate glycerol and three fatty acids. The fatty acids generated during lipolysis can be released into the circulation for use by other organs or oxidised within adipocytes (Ahmadian, Wang, and Sul 2010).

Lipolysis proceeds in an orderly and regulated manner, with different enzymes acting at each step. TAG is hydrolysed sequentially by desnutrin/ATGL to form DAG, then MAG, by HSL, with the liberation of a fatty acid at each step. MAG is hydrolysed by MAG lipase to release the final fatty acid and glycerol (Duncan et al. 2007). Glycerol is discharged out of adipocytes via an aquaporin type of transport molecule and shuttled back to the liver for use in oxidation of gluconeogenesis (Coelho, Oliveira, and Fernandes 2013). Outside of

the adipocyte, fatty acids are immediately bound to albumin and via the bloodstream are carried to liver, muscle and other tissues for oxidation (Coelho, Oliveira, and Fernandes 2013). The fatty acid molecules are converted into acetyl coenzyme A molecules through β-oxidation, and the free fatty acids resulting from lipolysis are thereby used as an energy source (Coelho, Oliveira, and Fernandes 2013).

9.2 Adipogenesis

White adipose tissue expansion takes place rapidly after birth as a result of increased fat cell size as well as an increase in fat cell number, and even at the adult stage, the potential to generate new fat cells persist (Murdolo et al. 2012;Gregoire, Smas, and Sul 1998). Adipogenesis refers to the differentiation of preadipocytes into mature fat cells and thereby the development of adipose tissue (Coelho, Oliveira, and Fernandes 2013). Preadipocytes within adipose tissue can differentiate into mature adipocytes throughout life, thereby enabling hyperplastic expansion of adipose tissue when increased storage requirements are needed (Gray and Vidal-Puig 2007). The mature adipocytes present within adipose tissue expand as they take up lipid from the circulation. Additionally, as storage demands increase, preadipocytes differentiate to become mature adipocytes capable of taking up and storing fat (Gray and Vidal-Puig 2007). The developmental origin of fat cells is not known, several studies on multi-potent clonal cell lines have though suggested that the adipocyte lineage derives from an embryonic stem cell precursor with the capacity to differentiate into the mesodermal cell types of adipocytes, chondrocytes, osteoblasts, and myocytes (Fonseca-Alaniz et al. 2007;Gregoire, Smas, and Sul 1998;Musri and Parrizas 2012). This takes place in a series of stages as depicted in figure 4.

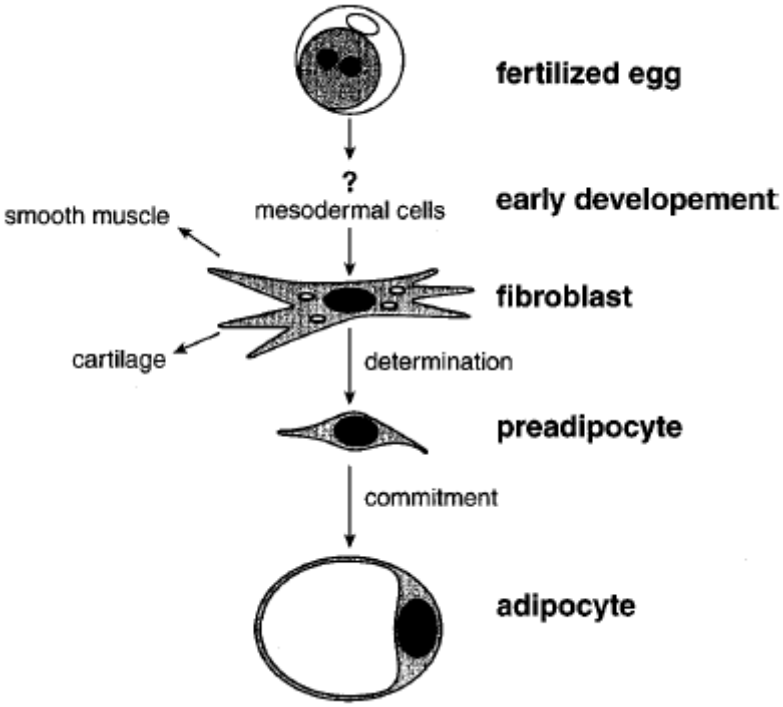

Figure 4: A model for the development of adipocytes from a fertilised mammalian egg. Darkened shapes represent nuclei or pronuclei. The developmental stages of determination and commitment are depicted (Ntambi and Young-Cheul 2000).

A single fertilised egg gives rise to nearly 200 different cell types that make up the multiple developmental linages in multicellular linages (Ntambi and Young-Cheul 2000). The pluripotent fibroblasts have mesodermal origins and can differentiate into committed preadipocytes (Ntambi and Young-Cheul 2000). The first step of adipogenesis is the commitment of these pluripotent precursors to preadipocytes that lose their capacity to differentiate into any other cell type than adipocytes (Musri and Parrizas 2012). The morphological and functional changes that take place during adipogenesis corresponds to a shift in transcription factor expression and activity and leads to a final phenotype characterised by alterations in cell shape and lipid accumulation (Coelho, Oliveira, and Fernandes 2013). Adipogenic transcription factors, including peroxisome proliferator-activated receptor γ(PPARγ), sterol regulatory element binding protein 1c (SREBP-1c) and CCAAT/enhancer binding protein (C/EBP), carry out a key role in the complex transcriptional cascade of adipogenesis (Fonseca-Alaniz et al. 2007;Gray and Vidal-Puig 2007). At confluence, the cell-cell contact induces expression of lipoprotein lipase and type VI collagen genes (Ntambi and Young-Cheul 2000). C/EBP are postulated to be involved in directing the differentiation process, since they are the first transcription factors induced after exposing preadipocytes to the differentiation mixture (Ntambi and Young-Cheul 2000). The activity of C/EBP is thought to mediate the expression of PPARγ, which is transcriptionally induced during day 2 after induction of differentiation and is maximum by day 3-4 (Ntambi and Young-Cheul 2000). SREBP-1c is up-regulated during preadipocyte differentiation and is thought to lead to the produc-

tion of PPARγ ligands required for transcriptional activity (Ntambi and Young-Cheul 2000). Activation of PPARγ is lipogenic, promoting the uptake of FFAs from serum into adipose tissue and the production of TAG inside the adipocyte (Gray and Vidal-Puig 2007).

9.3 Adipose tissue as an organ

Adipose tissue comprises a variety of cell types, including endothelial cells, blood cells, fibroblasts, pericytes, preadipocytes, macrophages and other immune cells, the predominant cells present in adipose tissue is however mature adipocytes (Sarjeant and Stephens 2012). Adipose tissue, other than being the primary site of storage for excess energy, is by now recognised as an important endocrine organ and is capable of synthesising a number of biologically active compounds that regulate metabolic homeostasis. White adipose tissue may represent the largest endocrine tissue of humans (Coelho, Oliveira, and Fernandes 2013). It is dynamically involved in the regulation of cell function through a complex network of endocrine (signals travel through the circulatory system to reach all parts of the body), paracrine (signals sent only to cells close to the cell station), and autocrine (only affecting cells that are the same type) signals that influence the response of many tissues (Coelho, Oliveira, and Fernandes 2013). The discovery of leptin in 1994 opened up a whole field of studies into the biology of adipocytes, their metabolic and endocrine functions and the functional relationship between secretions of adipocytes and peripheral metabolic functions (Coelho, Oliveira, and Fernandes 2013). The adipose tissue secretes a number of bioactive substances (figure 5), known to play a role in immunological responses, vascular diseases and appetite regulation.

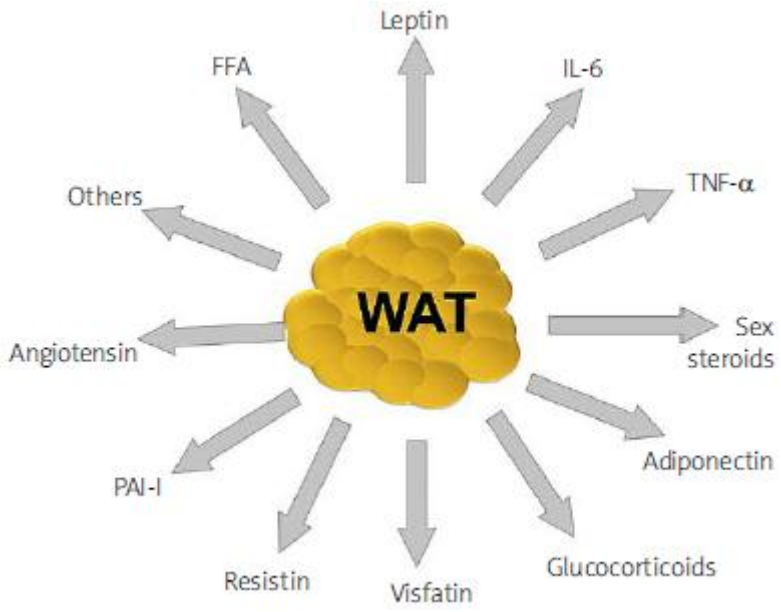

Figure 5: Some of the factors secreted by white adipose tissue, underlying the multifunctional nature of this endocrine organ (Coelho, Oliveira, and Fernandes 2013).

Oversecretion of potentially harmful adipocytokines, such as PAI-1, TNF-α or IL-6 and hyposecretion of potentially beneficial adipocytokines, such as adiponectin might be major mechanisms involved in lifestyle-related diseases, such as diabetes mellitus, hyperlipidaemia, hypertension and atherosclerosis, compromising the metabolic syndrome (Coelho, Oliveira, and Fernandes 2013;Seneff, Wainwright, and Mascitelli 2011). Insufficient adiponectin promotes insulin resistance, while TNF-α and IL-6 are known to be involved in the inflammatory stress response of metabolic syndrome (Seneff, Wainwright, and Mascitelli 2011). Recent studies have shown that obesity has a strong impact on adipokine secretion and insulin resistance (Coelho, Oliveira, and Fernandes 2013).

Increased adipose mass associated with obesity has been linked to low-grade chronic inflammation characterised by altered production of adipokines and an increase in biological markers of inflammation, such as TNF-α and IL-6 (Galic, Oakhill, and Steinberg 2010). Studies in recent years have revealed that mature adipocytes are not the major producer of inflammatory cytokines. Preadipocytes, endothelial cells, fibroblasts, leukocytes and macrophages seem to be responsible for the chronic inflammatory response observed in obesity (Galic, Oakhill, and Steinberg 2010). The expansion of adipose tissue in obesity is associated with and increased infiltration with macrophages of the M1 type which is the "classically activated" macrophage phenotype from the blood. The M1 macrophages are usually recruited to sites of tissue damage and have been reported to be in a pro-inflammatory state with increased expression of TNF-α and inducible nitric oxide synthase (Galic, Oakhill, and Steinberg 2010) (figure 6).

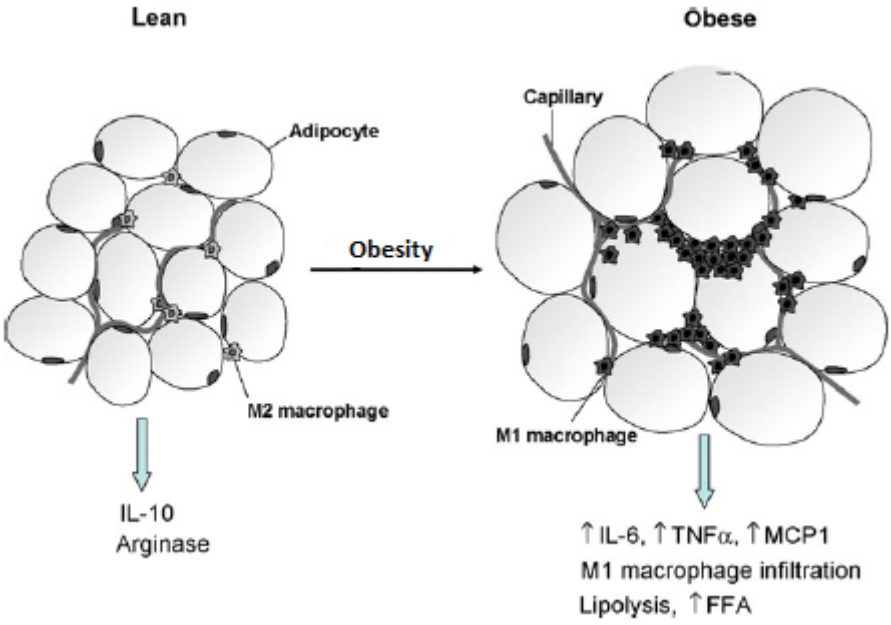

Figure 6: Obesity-induced changes in macrophage infiltration. Adipose tissue macrophages in the lean state show characteristics of "alternative" or M2 activation with increased production of arginase and the anti-inflammatory cytokine IL-10. Expansion of

adipose tissue leads to adipocyte hypertrophy and the release of chemokines that induce increased recruitment of M1 macrophages from the blood stream. M1 or "classically activated" adipose tissue macrophages are characterised by increased production of the pro-inflammatory cytokines TNF-α and IL-6, which promote altered gene expression and insulin resistance in adipocytes. These changes result in altered adipokine secretion, increased lipolysis and excess of circulating non-esterified fatty acids, which may eventually contribute to systemic resistance (Galic, Oakhill, and Steinberg 2010).

Larger adipocytes have more macrophage infiltration and secrete more of the pro-inflammatory cytokines IL-6, TNF-α and angiotensinogen and less of the beneficial adipokines leptin and adiponectin, the larger adipocytes are also indicative of failure in adipose tissue proliferation and/or differentiation (Robertson 2007). The adipocyte size of subcutaneous fat correlates positively with BMI and serum leptin and correlates negatively with age and serum adiponectin (Bambace et al. 2011). The adipocyte growth is different in different fat depots but the adipocyte is though related to insulin resistance independent of localisation (Bambace et al. 2011).

9.3.1 Leptin

Leptin is a small 16 kDa peptide, considered as a pre-inflammatory cytokine (Coelho, Oliveira, and Fernandes 2013), it is a hormone that is primarily made and secreted by mature adipocytes and binds to its receptor in the hypothalamus (Gregoire, Smas, and Sul 1998). Leptin was identified in 1994 as the product of the *ob* gene that had been described in mice (a strain of mice (*ob/ob*) that exhibited a genetic defect resulting in non-production of this protein) (Fonseca-Alaniz et al. 2007). Plasma leptin levels increases with weight gain and decreases with weight loss, consistent with leptins role as a signal of adipose tissue stores (Galic, Oakhill, and Steinberg 2010). There is a positive linear correlation between circulating levels of serum leptin and total body fat mass, which can be explained by increased release of leptin from large compared to small fat cells (Galic, Oakhill, and Steinberg 2010). Nutritional regulation of leptin is mediated at least in part by insulin, as leptin decreases in response to low insulin levels and increases with feeding or in response to insulin stimulation (Coelho, Oliveira, and Fernandes 2013). Leptin is primarily cleared from plasma by the kidney through glomerular filtration followed by proteolytic degradation in the renal tubules (Coelho, Oliveira, and Fernandes 2013), however, with obesity, increased leptin levels are not due to reduced clearance (Galic, Oakhill, and Steinberg 2010).

9.3.2 Adiponectin

Adiponectin is secreted exclusively from adipose tissue and is an abundant plasma protein (Galic, Oakhill, and Steinberg 2010). Adiponectin is a 30 kDa protein and is structurally related to the complement 1q family and contains a carboxyl-terminal globular domain and an amino-terminal collagenous domain and also shares extensive sequence homology with collagen VIII and X (Coelho, Oliveira, and Fernandes 2013;Galic, Oakhill, and Steinberg 2010;Fonseca-Alaniz et al. 2007). The monomers of adiponectin is composed of three domains: a variable N-terminal region, an α-helical collagenous 'stalk' composed of multiple G-X-X

repeats, and a distinctive C-terminal globular domain of approximately 140 amino acids (Coelho, Oliveira, and Fernandes 2013;Galic, Oakhill, and Steinberg 2010). The adipokine circulates in serum in three isoforms: a trimer, of low-molecular weight (LMW), a hexamer (trimer-dimer) of medium molecular weight (MMW) and a multimeric high molecular weight (HMW) iso-form (Coelho, Oliveira, and Fernandes 2013). There is a strong negative correlation between adiponectin concentration in humans and fat mass (Coelho, Oliveira, and Fernandes 2013), with obesity reducing adiponectin levels and weight reduction increasing adiponectin levels (Galic, Oakhill, and Steinberg 2010). Evidence suggests that the HMW complex is the most active form and accounts for the majority of the adipokines' peripheral metabolic effects (Galic, Oakhill, and Steinberg 2010). Adiponectin is associated with T2D, but it is almost exclusively due to a decrease in levels of circulating HMW isoform, without accompanying reduction in levels of the other two oligomeric forms (Coelho, Oliveira, and Fernandes 2013). Adiponectin displays no great fluctuations in the bloodstream, which means that its release is not acute but regulated by long-term metabolic changes (Coelho, Oliveira, and Fernandes 2013). The central actions of leptin and adiponectin have reciprocal functions to provide a homeostatic mechanism to maintain fat levels/energy stores through the suppression or stimulation of appetite and energy expenditure (Galic, Oakhill, and Steinberg 2010).

9.3.3 Resistin

This hormone was first described in 2001 when a relationship between resistin and insulin resistance induced by obesity was demonstrated (Fonseca-Alaniz et al. 2007). This peptide hormone is an adipocyte-derived secretory factor which was first identified as a novel transcript produced exclusively by adipocytes and has been shown to play a significant role in obesity-induced insulin resistance (Galic, Oakhill, and Steinberg 2010). Resistin is expressed within adipocytes of rodents and macrophages of humans and its production is increased with feeding and obesity and decreased by PPARγ ligands (Galic, Oakhill, and Steinberg 2010). Resistin expression is 15 times higher in visceral fat as compared to subcutaneous fat (Fonseca-Alaniz et al. 2007). The structure is strikingly similar to that of adiponectin, it contains an N-terminal helical tail linked to a C-terminal β-sandwich jelly-roll head domain, containing six β-strands (Galic, Oakhill, and Steinberg 2010). Studies demonstrating a causal role of resistin in glucose homeostasis are based on animal models with altered serum resistin levels (Galic, Oakhill, and Steinberg 2010). The importance of resistin in humans is less clear as not all studies report increases in serum resistin in obese T2D (Galic, Oakhill, and Steinberg 2010).

9.3.4 Tumor necrosis factor α

TNF-α is synthesised as a 26 kDa transmembrane protein that undergoes cleavage by a metalloproteinase to be released into the circulation as a 17 kDa soluble TNF-α molecule (Coelho, Oliveira, and Fernandes 2013). TNF-α is an immunomodulatory and pro-inflammatory cytokine (Fonseca-Alaniz et al. 2007). Isolated

and differentiated adipocytes are capable of producing TNF-α but they are not the major source, rather macrophages from the stromal vascular fraction are the primary source of adipose derived TNF-α (Coelho, Oliveira, and Fernandes 2013). The first adipose derived factor suggested representing a link between obesity, inflammation and diabetes was TNF-α. Studies showed that mRNA expression levels of TNF-α in adipose tissue in obesity was strongly implicated in the pathogenesis of insulin resistance (Hotamisligil, Shargill, and Spiegelman 1993); this is because it has been demonstrated that TNF-α can impair insulin signalling in hepatocytes and adipose tissue (Coelho, Oliveira, and Fernandes 2013). The mechanisms of TNF-α induced insulin resistance include: acceleration of lipolysis, increasing free fatty acids in circulation, reduction of GLUT4 synthesis, and expression of insulin and insulin receptor substrate-1 (Fonseca-Alaniz et al. 2007). TNF-α neutralisation in obese T2D humans does not appear to improve glucose tolerance or insulin sensitivity. However, in individuals without established T2D, prolonged treatment does improve insulin sensitivity (Coelho, Oliveira, and Fernandes 2013).

9.4 Adipocytes grown in culture

In order to test the hypothesis; that increased production of SCFA, in particular butyrate, during microbial fermentation of DF in the colon may protect against lifestyle diseases such as T2D, an *in vitro* cell-based model was established. It is of great importance to choose the most applicable model since many adipocyte cell models exists. The cells available all have unique advantages and disadvantages that should be taken into consideration when selecting cells(Poulos, Dodson, and Hausman 2010).

Classic cell types used in studies, such as adipogenesis, are the established cell strains 3T3-F442A and 3T3-L1, which were clonally isolated from Swiss 3T3 cells derived from disaggregated 17- to 19-day mouse embryos (Gregoire, Smas, and Sul 1998;Green and Meuth 1974;Green and Kehinde 1975;Poulos, Dodson, and Hausman 2010). Because the stage of differentiation and the lineage of preadipocyte cell lines have not been well established, primary cultures have been particularly useful for validating results obtained in preadipocyte cell lines (Gregoire, Smas, and Sul 1998). An advantage in using the primary cell lines is that the primary cells are diploid and may therefore reflect *in vivo* context better than aneuploid cell lines (Gregoire, Smas, and Sul 1998). Another advantage in using the primary cell lines is that they can be derived from adipose tissue obtained from various species at different postnatal stages of development and from various adipose depots (Gregoire, Smas, and Sul 1998). This may also be a disadvantage, since tissue sampling from different donors and possibly from different anatomical sites, may lead to unpredictable variation in the characterisation of the cells (Wabitsch et al. 2001). A drawback with the use of primary cultures is, that large amount of fat tissue is required because preadipocytes only constitutes a small fraction of total fat tissue (Ntambi and Young-Cheul 2000). As mentioned, most insights into adipogenic differentiation

were obtained from primary cultures of human preadipocytes as well as murine model systems, such as 3T3-L1 and 3T3-F442A to study adipose differentiation and metabolism (Wabitsch et al. 2001;Fischer-Posovszky et al. 2008). Human adipose tissue material is limited with respect to preadipocyte number and differentiation capacity (Rosenow et al. 2010), a human preadipocyte cell strain with high capacity for adipose differentiation is therefore much needed (Wabitsch et al. 2001).

9.4.1 Mouse fibroblasts (3T3-L1 cells)

The established mouse fibroblast line 3T3 was evolved under defined culture conditions. The cloned 3T3-L1 form accumulates a great deal of lipid when they enter the resting state (Green and Meuth 1974). The 3T3-L1 cells, from the fibroblast line 3T3, have undergone determination (see figure 4) and can either remain as preadipocytes or differentiate into adipocytes (Ntambi and Young-Cheul 2000). When their growth rate is slower they begin to accumulate multiple fat droplets and the cells to some degree resembles brown adipose cells; later the droplets fuses to create a single large central fat droplet surrounded by cytoplasm and a nucleus, which resembles the typical appearance of a white adipose cell (Green and Kehinde 1975). 3T3-L1 cells may be propagated indefinitely in culture, but if allowed to reach an essentially resting condition, have a high probability of converting into adipose cells, this seems to be triggered by a reduction in growth rate (Green and Kehinde 1975). In order for 3T3-L1 cells to differentiate more fully, the concentration of calf serum in the media has to be increased to a concentration of 20-30%, since the serum stimulate cell multiplication to a greater saturation density before the resting state (Green and Meuth 1974). The accumulation of fat also takes place much more rapidly and to a greater extent with increased serum concentrations than in media supplemented with 10% calf serum (Green and Meuth 1974).

9.4.2 Human preadipocyte cells from a patient with Simpson-Golabi-Behmel syndrome (SGBS)

In 2001 the preadipocyte SBGS cell line was introduced. This cell line is derived from subcutaneous white adipose tissue of a patient with Simpson-Golabi-Behmel syndrome which is a rare and complex X-linked disorder characterised by pre- and postnatal overgrowth (Wabitsch et al. 2001). The SGBS cells are morphologically, biochemically and functionally similar to primary preadipocytes and have been used in several other human adipocyte biology studies (Rosenow et al. 2010). The SGBS cells has been shown to proliferate for up to 50 generations and still retain their capacity for adipose differentiation (Wabitsch et al. 2001;Fischer-Posovszky et al. 2008) as opposed to primary human preadipocytes, which quickly loses their ability to differentiate when they are cultured *in vitro* (Fischer-Posovszky et al. 2008). The differentiated SGBS cells behave biochemically and functionally like human adipocytes differentiated in primary culture (Wabitsch et al. 2001). Adipogenic differentiation is achieved under serum-free culture conditions with the adipogenic factors insulin, triiodothyronine, and cortisol in the adipogenic media, further addition of a PPARγ agonist to the adipogenic media stimulates adipose differentiation in subcultures in a dose-

dependent manner, with a maximal effect at 2 µM/L, thereby inhibiting a fast decline of differentiation and preserving a high capacity for differentiation over many generations (Wabitsch et al. 2001;Fischer-Posovszky et al. 2008). With addition of IBMX and dexamethasone for the first three days to the adipogenic media, differentiation is further stimulated (Wabitsch et al. 2001). Over the years the protocol for SGBS cell differentiation has been developed and optimized (figure 7) and it has made it possible to achieve an adipogenic differentiation rate of > 90% up to generation 50 (Fischer-Posovszky et al. 2008).

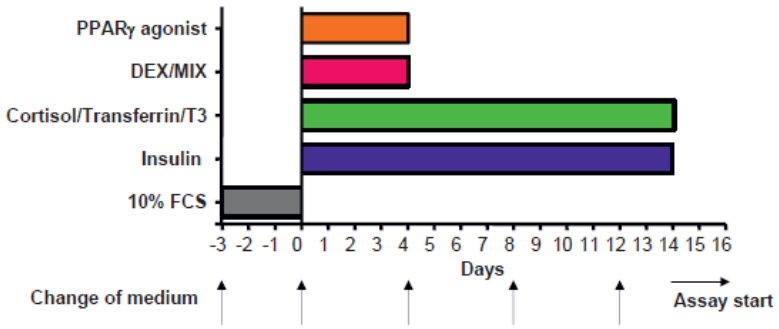

Figure 7: The optimal differentiation protocol for human SGBS cells from (Fischer-Posovszky et al. 2008). The differentiation process is started at day 0 by incubating cells in serum-free differentiation media (10 µM transferrin, 20 nM insulin, 0.1 µM hydrocortisone, 0.4 nM 3,3',5-Triiodo-L-thyronine (T3), 25 nM dexamethasone, 0.5 mM 3-isobutul-1-methylxanthine (IBMX), and 2 µM rosiglitazone). After 4 days, media is changed, and cells are further cultured in serum-free culture media supplemented with 10 µM transferrin, 20 nM insulin, 0.1 µM hydrocortisone and 0.4 nM T3. Media is further changed every 2-3 days of culture (Fischer-Posovszky et al. 2008).

The pattern and time-course of gene expression in SGBS cells during differentiation is comparable to the findings in human preadipocytes in primary culture (Wabitsch et al. 2001;Allott et al. 2012). However, the SGBS cells express increased levels of adipocyte differentiation-specific genes (leptin, GLUT4, lipoprotein lipase and adipocyte protein 2) compared to the murine cell line 3T3-L1 (Allott et al. 2012). A major advantage of the SGBS model system is that adipogenic differentiation is performed in a chemically defined serum- and albumin-free media, in contrast to using 3T3-L1 cells, which need FCS in order to differentiate (Fischer-Posovszky et al. 2008). A feature of the mature adipocyte is the development of an insulin-responsive glucose transport system, with maximal glucose uptake more than 10-fold higher in differentiated SGBS adipocytes compared to preadipocytes (Wabitsch et al. 2001). When performing functional studies with insulin, the cells are washed and deprived of insulin for at least 24 hours prior to the experiment (Fischer-Posovszky et al. 2008). The *in vitro* differentiated SGBS adipocyte is shown to functionally not distinguish from human adipocytes (Wabitsch et al. 2001). An experiment with incorporation of [^{14}C]-labelled D-glucose into cellular lipids showed a dose-dependent stimulation of glucose uptake in differentiated SGBS adipocytes and no detectable incorporation of glucose in undifferentiated SGBS cells. This indicated that accumulation of lipids in differentiated SGBS cells was due to lipogenesis from glucose (Wabitsch et al. 2001). Apart from the SGBS cells having a high capacity for adipose differentiation, they also behave similar

to human preadipocytes in primary culture, such as in doubling time and density at confluence (Wabitsch et al. 2001). The similarity between the SGBS cell strain and human preadipocytes, as well as preadipocyte cell strains from rodents, allows the SGBS cells to be used as a model system of human adipocytes which may offer advantage, such as fewer limitations in terms of cell resources and high homogeneity between passages (Wabitsch et al. 2001).

10. Interaction of SCFAs with adipose tissue

The family of G protein-coupled receptors (GPR) are important in cellular function. It is one of the largest families of proteins in the mammalian genome and they share a conserved structure composed of seven transmembrane helices that can activate heterotrimeric G proteins, for example Gs, Gi, and Gq (Ge et al. 2008;Hong et al. 2005). SCFAs have been shown to bind to the G protein-coupled receptors GPR41 and PGR43 (Robertson et al. 2005). Each ligand of the GPR binds specifically (Hong et al. 2005). Acetate and propionate are the most potent activators of GPR43, whereas acetate is not as potent as propionate and butyrate in activating GPR41 (Ge et al. 2008;Le et al. 2003). Expression of GPR43 has been reported to be highest in immune cells, however, GPR43 is also expressed in a number of other tissues including adipocytes (Ge et al. 2008), whereas GPR41 has been reported to be predominantly expressed in adipose tissue (Garland 2011)

GPR43 is induced during the adipocyte differentiation process and increased during high-fat feeding, suggesting that GPR43 may affect adipocyte function (Ge et al. 2008). Hong et al. has shown an increase in the level of GPR43 during adipocyte differentiation with a peak at day 7. It has further been shown that Oil red O staining of 3T3-L1 cells treated with propionate or acetate for 7 days increased the level of adipocyte differentiation compared with a control (Hong et al. 2005), indicating that acetate and propionate may stimulate adipogenesis via activation of GPR43 (Roelofsen, Priebe, and Vonk 2010). *In vitro*, SCFAs, produced from fermentation of resistant starch, has been shown to inhibit adipose tissue lipolysis, but an *in vivo* effect of dietary RS intake has not yet been shown (Robertson et al. 2005). The natural GPR43 ligands acetate and propionate has been shown *in vivo* and *in vitro* to inhibit adipose tissue lipolysis, resulting in a suppression of both non-esterified fatty acids and glycerol levels (Robertson 2007;Roelofsen, Priebe, and Vonk 2010;Ge et al. 2008;Hong et al. 2005). Butyrate has been shown to significantly reduce the release of FFAs and glycerol and inhibit total lipase activity and the expression of ATGL, HSL, and phosphor-HSL in co-cultured adipocytes (Ohira et al. 2013).

Leptin has been implicated in body weight regulation through its central effects on the hypothalamus. The presence of leptin receptors in peripheral tissues, such as adipose tissue, indicate that these tissues are also

targets for the action of leptin (William, Jr., Ceddia, and Curi 2002). Propionate, as a ligand of GPR41, stimulated leptin expression in both a mouse adipocyte cell line and mouse adipose tissue in primary culture (Hong et al. 2005;Xiong et al. 2004). Leptin has been found to decrease insulin-stimulated *de novo* synthesis from glucose in adipocytes *in vitro*, which is in accordance with earlier findings (William, Jr., Ceddia, and Curi 2002). This study showed that leptin down-regulates *de novo* fatty acid synthesis, which depends on the rate limiting enzymes acetyl-CoA carboxylase and fatty acid synthase. These enzymes may be down-regulated by leptin, since it decreases the incorporation of acetic acid into lipids (William, Jr., Ceddia, and Curi 2002). *In vivo*, leptin has been shown to alter fatty acid flux in adipocytes, stimulating both TAG synthesis and breakdown. As a result FFA are exported to non-adipose cells, such as skeletal muscle and liver, where they can be oxidised at a higher rate (William, Jr., Ceddia, and Curi 2002).

Fatty acid metabolism is a key feature in determining tissue insulin sensitivity. Abnormalities in fatty acid storage and lipolysis in insulin-sensitive tissue with increased flux from adipose to non-adipose tissue such as skeletal muscle may be a critical event in the development of insulin resistance (Robertson et al. 2005). Increases in FFA in the blood can inhibit glucose metabolism through the inhibition of GLUT4 transporters. Therefore, SCFAs, by way of decreasing serum FFA, may reduce blood glucose levels through the competition in insulin-sensitive tissues (Lattimer and Haub 2010). Insulin did, however, not induce an increase in GLUT4 at the plasma membrane in large fat cells, whereas, in the smaller fat cells from the same subjects, on average, a doubling of GLUT4 on the cell surface was observed (Franck et al. 2007). This suggests that glucose uptake mainly takes place in smaller adipocytes and points to a causal relationship between the accumulation of large fat cells and reduced insulin sensitivity in obese subjects (Franck et al. 2007).

11. Materials and methods

11.1 Human preadipocyte cell culture

The human preadipocyte cell line SGBS, used in the following cell assays, were kindly donated by Dr. Martin Wabitch (Pediatric Endocrinology University for Children's and Adolescent Medicine, Ulm, Germany). The cells were maintained in 75 cm^2 cell culture flasks in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM/F-12, Gibco, USA) supplemented with 33 mM biotin(Sigma Aldric, USA), 17 mM pantothenate(Sigma Aldrich, USA), 1% Pen-Strep (Gibco, USA, 10.000 U/mL penicillin, 10 mg/mL streptomycin) and 10% fetal calf serum (FCS, Gibco, USA). Cells were passaged using 0.05% Trypsin-EDTA (Gibco, USA) when reaching 80-90% confluence and maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

11.2 Establishment of optimal culture conditions for SGBS cells

11.2.1 Number of cells per well and optimal level of FCS

Cell proliferation with varied cell density and FCS concentrations were made in order to determine the optimal cell density and FCS concentration used in the assays. Cell proliferation was measured using the AlamarBlue reagent, which contains the cell permeable redox indicator resazurin. When resazurin is dissolved in physiological buffers it gets a deep blue colour which turns into pink in cells with an active metabolism, caused by the reduction of resazurin into the resorufin product. The quantity of resorufin produced is proportional to cell number which can be measured using a microplate fluorometer with excitation and emission set at 560 nm and 590 nm respectively. The required incubation period is usually one to four hours depending on the metabolic activity of the particular cell type, cell density per well and other assay conditions such as the type of culture media (Riss et al. 2004).

Cells were seeded in 96 well plates at a density of 1000, 2000 or 3000 cells/well in 200 µL 10% FCS culture media for 24 hours prior to the experiment. After 24 hours, treatment media (200 µL/well) was added to the individual wells and cells were incubated for 72 hours. Treatment media contained increasing concentrations of FCS (0, 0.313, 0.625, 1.25, 2.5, 5 and 10%). Cell proliferation was determined using AlamarBlue (Invitrogen, USA). The medium was removed and the AlamarBlue reagent was added to the wells (110 µL AlamarBlue in PBS, 1:10) and measured fluorometrically after one, two and three hours of incubation. Measurements were performed on an EnVision 2103 Multilabel Reader (PerkinElmer, USA). Excitation wavelengths were set at 560nm and emission was recorded at 590nm.

11.2.2 Growth curve

A growth curve with varied cell density and FCS concentrations were made in order to determine the optimal cell density and FCS concentration used in the assays. Cell growth was terminated on one plate per day for eight consecutive days. The growth curve were made in order to ensure the cells were in the sigmoid phase when components to be tested were added to the culture media. Cell growth was measured using the PicoGreen reagent, which is a fluorochrome that selectively binds double-stranded DNA. It has an excitation maximum at 480 nm and an emission peak at 520 nm. When bound to double-stranded DNA, fluorescence enhancement of PicoGreen is exceptionally high; little background occurs since the unbound dye has virtually no fluorescence. PicoGreen is very stable to photobleaching, allowing longer exposure times and assay flexibility (Ahn, Costa, and Emanuel 1996).

The method has previously been described in (Purup, Larsen, and Christensen 2009). Cells were seeded in 96-well plates at densities 1000 and 2000 cells/well in medium containing 10% FCS for 24 hours prior to the experiment. After 24 hours, treatment media was added to individual wells and cells were incubated for a total of eight days, with media changed every second day. Treatment media contained increasing concentrations of FCS (0, 0.625, 1.25, 2.5, 5 and 10%). Cell growth was terminated in one plate per day for eight consecutive days. When terminating cell growth, media were removed and each well was added 100 µL TE buffer (20x, PicoGreen dsDNA Assay Kit, Invitrogen, USA). The plate were wrapped with vita-wrap and placed at -80˚C. All cells were lysed by repeated thawing and freezing (three times) of the plates. Cell growth was quantified using the PicoGreen method. 100 µL PicoGreen was added (200x, PicoGreen dsDNA Assay Kit, Invitrogen, USA) and the plates were incubated for 5 minutes at room temperature, protected from light. Fluorescence was measured using an EnVision 2103 Multilabel Reader (PerkinElmer, USA). Excitation wavelengths were set at 480 nm and emission was recorded at 520 nm.

11.3 Preadipocyte proliferation following SCFA treatment

Cells were cultured in 96 well plates (1500 cells/well) in 10% FCS culture media for 24 hours prior to the treatment. Then cells were exposed to treatment media for 72 hours containing increasing concentrations (0, 0.001, 0.005, 0.01, 0.05, 0.1, 1, 5 and 10 mM) of sodium butyrate (Sigma-Aldrich, USA), sodium acetate (Sigma-Aldrich, USA) and sodium propionate (Sigma-Aldrich, USA), as well as two SCFA combinations, given in table 3:

Table 3: The two SCFA combinations used throughout this thesis. The two combinations were made with a total concentration of 100 mM.

	92.5/3/2/2.5	90.5/3/4/2.5
Acetate (Merck, USA)	92.5 mM	90.5 mM
Propionate (Merck, USA)	3 mM	3 mM
Butyrate (Merck, USA)	2 mM	4 mM
Isobutyric acid (Fluka, USA)	0.6 mM	0.6 mM
Isovaleric acid (Acros organics, Belgium)	0.83 mM	0.83 mM
Valeric acid (Acros organics, Belgium)	0.79 mM	0.79 mM
Caprionic acid (Fluka, USA)	0.29 mM	0.29 mM

All solutions were sterile-filtered through a minisart-plus filter (Sigma-Aldrich, USA) with a pore size of 0.20 µm before added as treatments to the cells. Cell proliferation was determined using the AlamarBlue reagent (Invitrogen, USA). Media were removed and the AlamarBlue reagent were added to all wells (110 µL AlamarBlue in PBS, 1:10) and measured after one, two and three hours of incubation. Measurements were performed on an EnVision 2103 Multlabel Reader (PerkinElmer, USA). Excitation wavelengths were set at 560 nm and emission was recorded at 590 nm.

11.4 Preadipocyte differentiation protocol

The method for differentiating SGBS cells has earlier been described in (Fischer-Posovszky et al. 2008). Cells were grown in culture media containing 10% FCS until 80-90% confluence. Differentiation of preadipocytes to adipocytes was initiated at day 0, starting with washing the cells with PBS (Gibco, USA) after which they were cultured in serum-free differentiation media (10 µM transferrin (Sigma-Aldrich, USA), 20 nM insulin (Sigma-Aldrich, USA), 0.1 µM hydrocortisone (Sigma-Aldrich, USA), 0.4 nM 3,3',5-Triiodo-L-thyronine (T3, Sigma-Aldrich, USA), 25 nM dexamethasone (Sigma-Aldrich, USA), 0.5 mM 3-isobutul-1-methylxanthine (IBMX, Sigma-Aldrich, USA) and 2 µM rosiglitazone (Cayman, USA)). After four days, media was changed and the cells were further cultured in serum-free media supplemented with 10 µM transferrin, 20 nM insulin, 0.1 µM hydrocortisone and 0.4 nM T3. For the next ten days media was changed every 2-3 days

11.4.1 Effect of SCFAs and their combinations on cell differentiation

Cell differentiation with SCFAs and their combinations were made in order to determine the effect of SCFAs and their combinations on the cells level of differentiation. Cell differentiation was performed using the

AdipoRed reagent. AdipoRed is a solution of the hydrophilic stain Nile Red and it will fluoresce in the confines of a lipid hydrophobic environment. When using wavelengths of ≤ 570nm, the interaction of Nile Red with extremely hydrophobic environments is preferred and the interaction with cellular membranes is minimized. Some limitations when using this dye is, that Nile Red also fluoresce in the presence of fatty acid-free albumin indicating that proteins containing hydrophobic domains, in addition to lipids, may induce Nile Red fluorescence (Greenspan, Mayer, and Fowler 1985).

Cells were cultured in 48 well plates (5000 cells/well) in 10% FCS culture media and allowed to differentiate according to the differentiation protocol described above. On days 0, 4, 7, 9, and 11 after adding differentiation medium, treatment media (350 µL/well) were added to the individual wells and incubated for a total of two to three days. Treatment media contained increasing concentrations (0.0005-1 mM) of sodium acetate, sodium propionate, sodium butyrate, and the two SCFA combinations (table 3) in serum-free culture media supplemented with 10 µM transferrin, 20 nM insulin, 0.1 µM hydrocortisone and 0.4 nM T3. All solutions were sterile-filtered through a minisart-plus filter (Sigma-Aldrich, USA) with a pore size of 0.20 µm before treatment of cells. Cell differentiation was stopped in one plate on day 0, 4, 7, 9, 11, and 14, respectively. When cell differentiation was terminated, plates were removed from the incubator and cooled to room temperature. Media was removed and cells were washed with PBS (Gibco,USA) at room temperature and 500 µL PBS and 15 µL AdipoRed was added to each well (Lonza, Switzerland), quickly mixed and incubated at room temperature for 10 minutes. Fluorescence was measured using an Envision 2103 Multilabel Reader (PerkinElmer, USA). Excitation wavelengths were set at 485nm and emission was recorded at 572nm.

When fluorescence were measured the AdipoRed-PBS media was removed and cells were washed with PBS (Gibco, USA) and each well was added 250 µL TE buffer (20x, PicoGreen dsDNA Assay Kit, Invitrogen, P7589). Plates were wrapped with vita-wrap and placed at -80˚C. All plates were lysed by repeating thawing and freezing three times. At the day of DNA measurement the plates were thawed and 100 µL from each well was transferred to a 96 well plate in duplicates and 100 µL PicoGreen was added (200x, PicoGreen dsDNA Assay Kit, Invitrogen, USA). The plates were incubated for 5 minutes at room temperature, protected from light. Fluorescence was measured using an EnVision 2103 Multilabel Reader (PerkinElmer, USA). Excitation wavelengths were set at 480 nm and emission was recorded at 520 nm.

11.5 Proliferation of adipocytes following SCFA treatment

Cells were cultured in 96 well plates (1500 cells/well) in 10% FCS culture media. Cells were allowed to differentiate, according to the differentiation protocol, described above. At differentiation day 8 treatment media (200 µL/well) were added to the individual wells and incubated for a total of 72 hours. Treatment

media contained increasing concentration (0-10 mM) of sodium butyrate (Sigma-Aldrich, USA), sodium acetate (Sigma-Aldrich, USA) and sodium propionate (Sigma-Aldrich, USA), as well as the two SCFA combinations (table 3) in serum-free culture media supplemented with 10 µM transferrin, 20 nM insulin, 0.1 µM hydrocortisone and 0.4 nM T3. All solutions were sterile-filtered through a minisart-plus filter (Sigma-Aldrich, USA) with a pore size of 0.20 µm before treatment of cells. Cell proliferation was determined using AlamarBlue (Invitrogen, USA). Media were removed and the AlamarBlue reagent were added to all wells (110 µL AlamarBlue in PBS, 1:10) and measured after one, two and three hours of incubation. Measurements were performed on an EnVision 2103 Multlabel Reader (PerkinElmer, USA). Excitation wavelengths were set at 560 nm and emission was recorded at 590 nm.

11.6 Insulin sensitivity following SCFA treatment

The following protocol was used to determine the insulin sensitivity in adipocytes following SCFA treatment. The method has previously been described in (Bhattacharya et al. 2013). Cells were cultured in 48 well plates (5000 cells/well) in 10% FCS culture media. Cells were allowed to differentiate until day 8, according to the differentiation protocol described earlier. Then treatment media (350 µL/well) was added to individual wells and incubated for 48 hours. Treatment media contained increasing concentrations (0.001-1 mM) of sodium butyrate, sodium acetate, sodium propionate, and the two combinations (table 3) in serum-free culture media supplemented with 10 µM transferrin, 20 nM insulin, 0.1 µM hydrocortisone and 0.4 nM T3. All solutions were sterile-filtered through a minisart-plus filter (Sigma-Aldrich, USA) with a pore size of 0.20 µm before added to the cells.

After 48 hours media was removed and serum-free culture medium supplemented with 10 µM transferrin, 0.1 µM hydrocortisone and 0.4 nM T3 and +/- 100 nM insulin were added to each well (700 µL/well). After one hour of incubation cells were washed three times with Hepes Buffered Saline (HBS, 20 mM HEPES (Sigma-Aldrich, USA), 140 mM NaCl (Merck, USA), 5 mM KCl (Merck, USA), 2.5 mM $MgSO_4$ $7H_2O$ (Merck, USA), mM $CaCl_2$ $2H_2O$ (Fluka, USA) at pH 7.4) and incubated for 30 minutes with 2-Deoxy-[^{3}H]-Glucose (PerkinElmer, USA) in HBS + 0.1% Bovine Serum Albumin (BSA, Sigma-Aldrich, USA). Cells were washed four times with ice cold PBS and lysed by adding warm 0.05 M NaOH (Merck, USA) and rotated for 30 minutes. Cell lysate were mixed and 200 µL cell lysate were transferred into scintillation tubes (VWR, USA) which were added 2 mL Scintillation mix (Ultima Gold, PerkinElmer, USA). Cell lysates (50µL) were stored for later protein determination. The incorporated 2-Deoxy-[^{3}H]-glucose was determined by liquid scintillation counting (Wallac Winspectral 1414 liquid scintillation counter, PerkinElmer, USA) for 2 minutes/tube. The set-up is depictured in figure 8

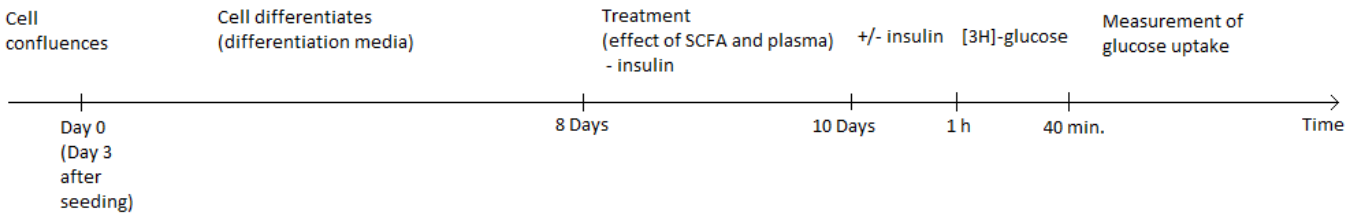

Figure 8: The set-up for measuring insulin sensitivity. Cells were allowed to differentiate until day 8, according to the differentiation protocol described earlier. Then treatment media was added and cells were incubated for 48 hours. After 48 hours, media was removed and serum-free culture medium +/- 100 nM insulin were added. After one hour of incubation cells were washed with HBS and incubated for 30 minutes with 2-Deoxy-[^{3}H]-Glucose in HBS + 0.1% BSA. Cells were then washed with ice cold PBS and lysed by adding NaOH and rotated. Cell lysate were mixed and transferred into scintillation tubes. The incorporated 2-Deoxy-[^{3}H]-glucose was determined by liquid scintillation counting for 2 minutes/tube.

11.7 Insulin sensitivity following plasma treatment

Cells were cultured in 48 well plates (5000 cells/well) in 10% FCS culture media. Cells were allowed to differentiate for 8 days according to the differentiation protocol. Treatment media were added to the individual wells and incubated for a total of 48 hours. Treatment media contained increasing concentrations (1, 5 and 10%) of plasma obtained from pigs fed three different diets, a WSD, a diet containing RS, and a diet containing AX. After 48 hours media were removed and the same procedure as described in the previous section (depictured in figure 8) was applied to wells to determine insulin sensitivity.

10 pigs were assigned to each diet, of those only plasma from 6 pigs from each diet were used in the study. The 6 pigs were chosen on the basis of the insulin content in the blood, previously measured. The two pigs with the highest and lowest insulin contents measured from each diet were discarded. The three diets used in the experiment varied in types and content of fiber. The energy, fat, and protein contents were the same in the diets. The energy from fiber made up 9% in the high-fiber diets (AX and RS) and 3.5% in the WSD diet. The WSD was composed of high fat, protein, degradable carbohydrates, and low fiber content. The WSD diet had a high content of wheat flour as starch source. The AX diet represented a WSD added a high content of AX in the form of rye flakes and enzyme treated wheat bran. The RS diet represented a WSD diet made with a high content of resistant starch by addition of high maize and potato starch.

11.8 Protein determination

The protein was determined following insulin sensitivity measurements. The content of protein in each well was determined using Pierce BCA Protein Assay Kit (Thermo scientific, USA) according to the manufacturer. For one of the standard curves, the following standard solutions were used, 2000 µg/mL, 1500 µg/mL, 1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, and 50 µg/mL. Standard solution and samples (10 µL) were transferred to a 96 well plate in duplicates and 200 µL BCA-detection reagens + $CuSO_4$ (50:1) were added each well and mixed on rotation board and incubated for 30 minutes at 37˚C. For the other standard curve,

the following standard solutions were used, 500 µg/mL, 250 µg/mL, 200 µg/mL, 125 µg/mL, 50 µg/mL, and 25 µg/mL. Standard solution and samples (25 µL) were transferred to a 96 well plate in single determinations. Each well were added BCA-detection reagens + $CuSO_4$ (50:1) and the plate were mixed on rotation board and incubated for 30 minutes at 37˚C. Absorbance was measured using an EnVision 2103 Multilabel Reader (PerkinElmer, USA) at 562 nm.

11.9 Adipocyte size in pigs fed three different diets

Abdominal adipose tissue samples were obtained from 30 pigs fed one of the three diets WSD, RS or AX in order to determine the adipocyte size and test the diet effect on the adipocyte size. The method used has been described in (Tchoukalova et al. 2003;Bambace et al. 2011). Abdominal adipose tissue samples (200-600 mg) from 30 pigs were obtained at slaughter and kept in 4 ml PBS for maximum 3 hours before further processing. Adipose tissue samples were weighed before digestion with 1 mg/ml collagenase type XI (Sigma-Aldrich, USA) in HEPES buffer [0.1 M HEPES (Sigma-Aldrich, USA), 0.12 M NaCl (Merck, USA), 0.05 M KCl (Merck, USA), 0.005 M glucose, 1.5% W/v BSA (Sigma-Aldrich, USA), $CaCl_2$ (Fluka, USA) (pH 7.4)] in 6 wells plates in a 37˚C rotating incubator for 45 min. The cell suspension was mixed twice during this period and after 45 minutes cell suspension was transferred to 4.5 ml tubes (Nunc, USA). The cell suspension was centrifuged for 5 minutes at 400 rcf at room temperature. The top layer (100 µL) were added to 400 µL of 0.2% methylene blue/HEPES solution for nuclei staining and incubated for 15 minutes at 37˚C. 15 µL from the cell suspension were placed two separate places on a glass slide and representative pictures (Leica DFC 320) of the cells were taken to obtain diameters of 300 cells. Fat cells from the digital images were analysed using the public domain image analysis program developed at the US National Institutes of Health (Scion).

11.10 Calculations

11.10.1 Insulin sensitivity

The insulin sensitivity was calculated as the difference in 2-Deoxy-[^{3}H]-glucose incorporation between cells treated with and without insulin (an example is presented in figure 9A). Then, the calculated insulin sensitivity for any given treatment was set relative to the insulin sensitivity calculated for the negative control treatment (no treatment 0=100, figure 9B).

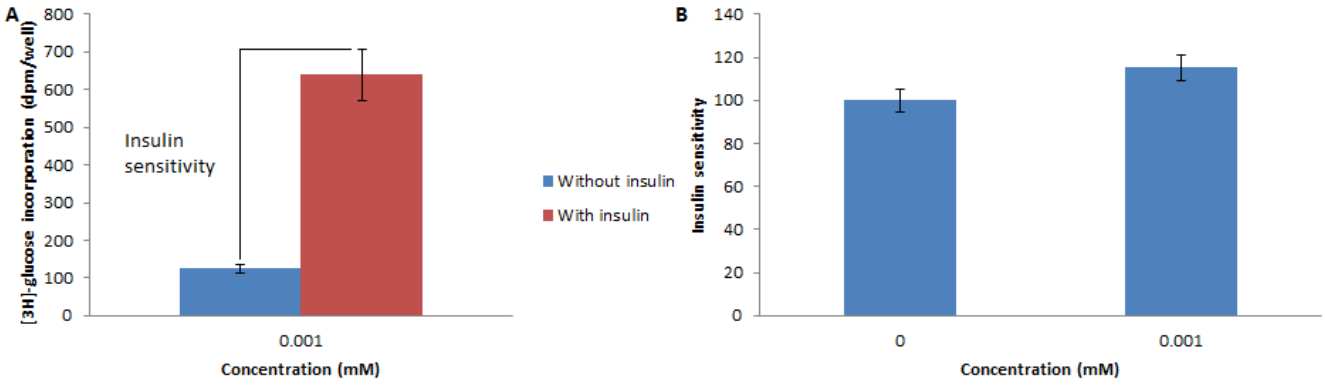

Figure 9: Example of insulin sensitivity calculation. A: Insulin sensitivity was defined as the difference in 2-Deoxy-[^{3}H]-glucose incorporated into the cells with and without insulin treatment for one hour. B: The calculated insulin sensitivities (data from quadruple wells/treatment) are then set relative to the insulin sensitivity calculated for the negative control treatment (no treatment = 100).

11.11 Statistical analysis

Results from proliferation, differentiation, insulin sensitivity, and adipocyte size assay are presented as LSMEANS $\pm$ standard error of the mean (SEM), relative to the solvent control (= 100) and day 0 (= 100). Data were analysed using the general linear model procedure (PROC GLM) of Statistical Analysis Software (SAS institute Inc., Cary, NC, USA, version 9.3) with concentration and assay number and their interaction as fixed effects for proliferation, differentiation, and insulin sensitivity and with size-group as fixed effect for adipocyte size. Normality and equal variance were applied and data was logarithmic transformed if needed (effects of butyrate on proliferation of preadipocytes, effect of acetate and propionate on insulin sensitivity and all effects on adipocyte size). Effect of concentration within each treatment and effect of size-group within each diet were tested using the following model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable, μ is the overall mean, α_i is concentration or size-group (i = 0 mM, 0.001 mM, 0.005 mM etc. or 5-15 µm, 16-25 µm etc.), β_j is assay number (j = 1-4), $(\alpha\beta)_{ij}$ is 2-factor interaction between concentration and assay number or size-group and pig number, and ε_{ij} $(0,\sigma_2)$ = residual error. The $(\alpha\beta)_{ij}$ interaction was eliminated from the model when no statistical significance was evident. Outliers were identified using residual plots and plots of cooks distance. Residual plots were used to confirm whether linear regression models were appropriate for the data analysis.

The two SCFA combinations (table 3) as well as the effect of plasma from pigs receiving the three diets WSD, AX and RS were tested for effects on proliferation, insulin sensitivity and adipocyte size. This was performed using the MIXED procedure of SAS. Data were analysed according to the following statistical model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ij}$$

where Y_{ij} is the observation, μ is the overall mean, α_i is the effect of treatment (i = 92.5/3/2/2.5, 90.5/3/4/2.5 or WSD, AX, RS, plasma week 0 and plasma week 3), β_j is the effect of concentration or size-group (j = 0 mM, 0.01 mM etc. or 5-15 µm, 16-25 µm etc.), γ_k is the random effect of assay number or pig number (k = 1-12 or 1-30), and ε_{ij} is the residual error. A significant effect was reported at $P < 0.05$ and a tendency at $P < 0.10$.

12. Method development

12.1 Establishment of optimal culture conditions for SGBS cells

Cell proliferation with varied cell density and FCS concentrations, as well as a growth curve with varied cell density and FCS concentrations were made in order to determine the cell density and FCS concentration used in the assays. A proliferation assay with varied cell density, given in figure 10 was made in order to determine the cell density to be used in the following assays to be made.

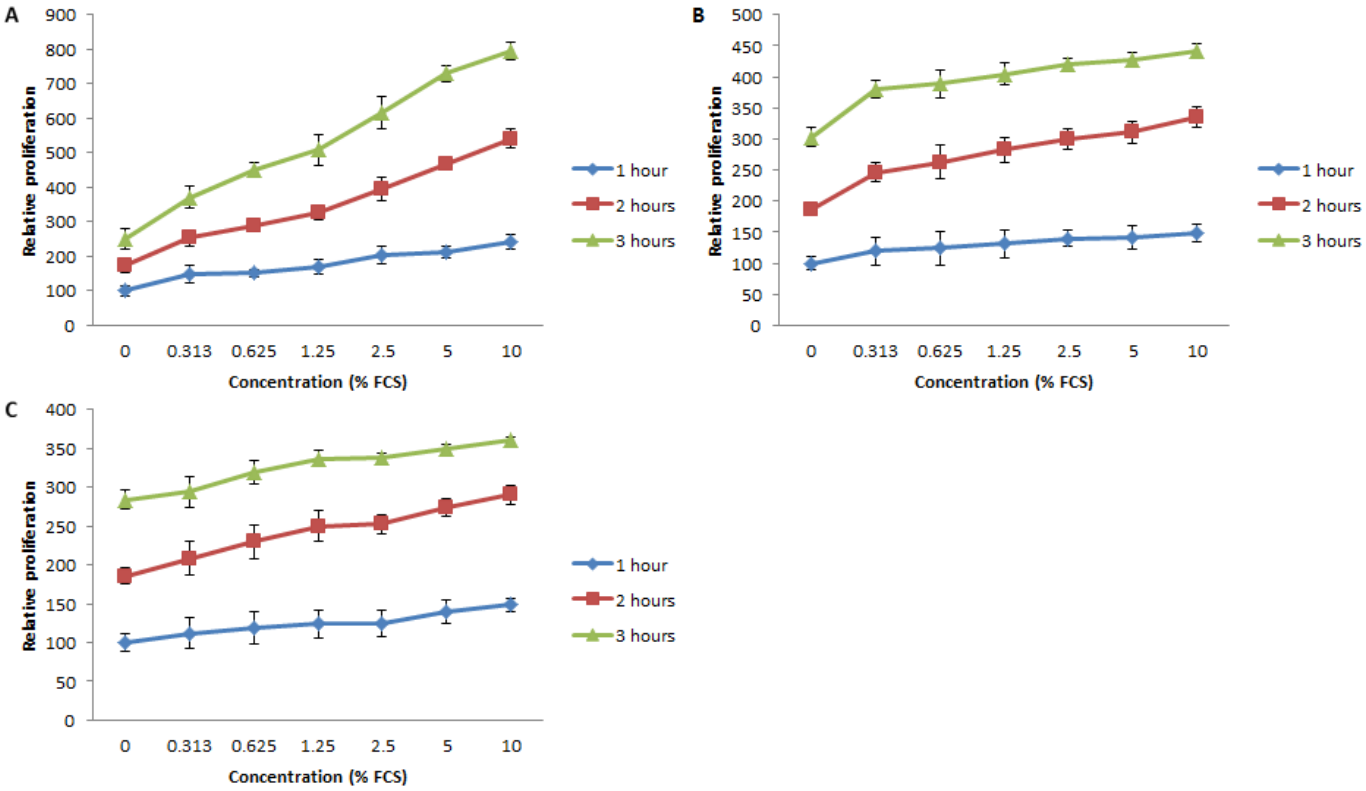

Figure 10: Proliferation curve with varying cell density, A: 1000 cells/well, B: 2000 cells/well, C: 3000 cells/well, and varying FCS concentration. Fluorescence was measured after one, two and three hours of incubation with AlamarBlue. Results are from one experiment with quadruple wells and presented as mean values ± SEM relative to 0% FCS at 1 hour.

Proliferation with varied cell density and FCS concentrations showed that 1000 cells/well resulted in the most optimal curves since it does not peak too fast and thereby it is possible to observe an effect of the treatment given. With cell densities of 2000 or 3000 cells/well the curves peak too fast and it is more difficult to observe an effect of the treatment given. Based on this assay 1000 cells/well was the optimal cell density to use in further experiments. From the proliferation assay, two hours of incubation with AlamarBlue seems to be enough to observe an effect of proliferation. A growth curve, depictured in figure 11, were made with varied cell density and FCS concentration to determine the optimal cell density to be used and determine the optimal amount of FCS to be used in treatment media in the following assays.

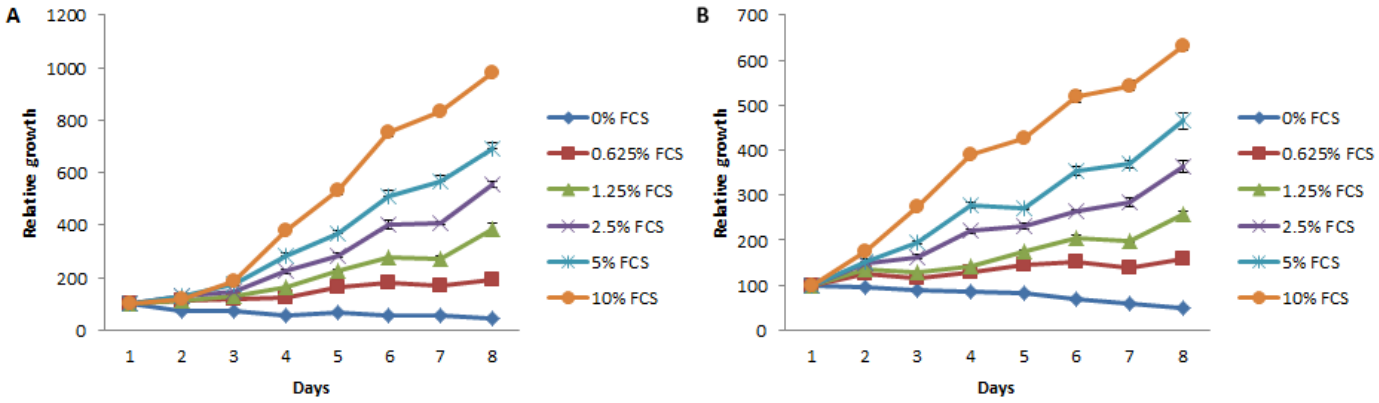

Figure 11: Growth curve with varied cell density, A: 1000 cells/well, B: 2000 cells/well, and varied FCS concentration. A plate was stopped at eight consecutive days and fluorescence was measured using PicoGreen. Results are from one experiment with quadruple wells and presented as mean values ± SEM relative to day 1 for the individual FCS concentrations.

The growth curve (figure 11) showed that SGBS cells cultured in media containing FCS were in their exponential growth phase from day 3 and forward (for 1000 cells/well) and from day 1 and forward (for 2000 cells/well). The cells did not enter the stationary phase of cell growth at all during the eight days of measurement. The curve showed that FCS improves cell proliferation, but does not seem to be vital for cell survival. When working with preadipocytes, treatments were planned to be within the first five days and FCS is therefore not needed in the media, since the cells seems to survive without FCS within the first days. When comparing cell density between the two growth curves, 2000 cells/well resulted in a faster increase in growth, an effect that was already shown at day two, compared to 1000 cells/well, where an increase were shown at day 4. When using 2000 cells/well it was possible to detect an effect of treatment faster than when using 1000 cells/well. On the basis of the proliferation assay, which showed that 1000 cells/well is optimal, and the growth curve, which showed that 2000 cells/well is optimal, we decided to continue experiments with a density of 1500 cells/well in 96 well plates. Proliferation assays with 1500 cells/well and increasing FCS concentrations were made (data not shown) to show that the suggested cell density and FCS concentration in the media used for further cell assays was suitable. When using other plates than 96 well plates, for example 48 well, the density was corrected accordingly by using the plates' area.

12.2 Cell differentiation

Cells were cultured and allowed to differentiate under normal conditions. On days 0, 4, 7, 9, and 11 after adding differentiation media, cells were treated with sodium acetate, sodium propionate, sodium butyrate, as well as two SCFA combinations (table 3). On days 0, 4, 7, 9, 11, and 14 after adding differentiation media, cell differentiation were stopped using AdipoRed to measure level of differentiation.

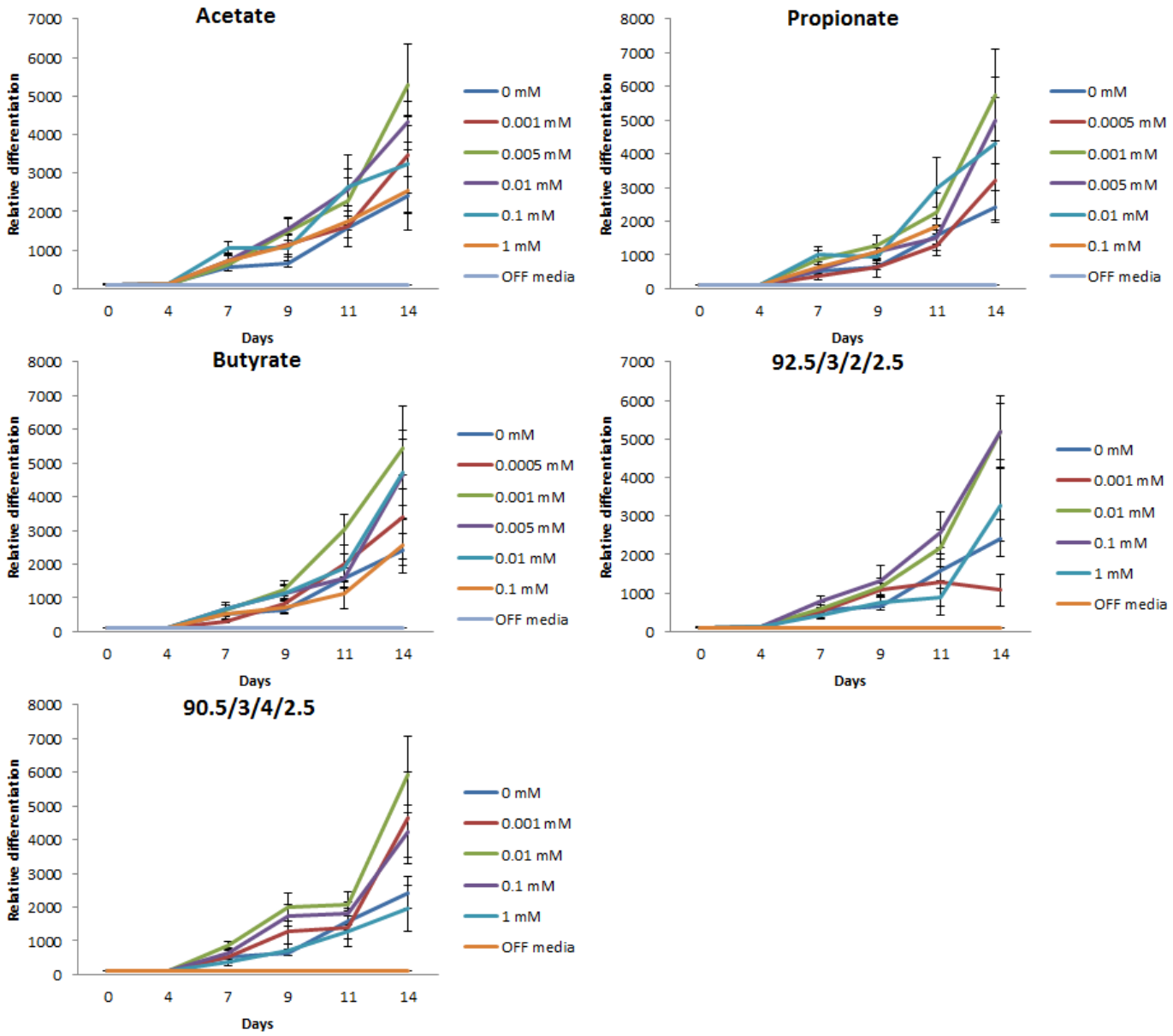

Figure 12: Cell differentiation with different treatments, acetate, propionate, butyrate and the two SCFA combinations 92.5/3/2/2.5 and 90.5/3/4/2.5. OFF media was serum-free culture media supplemented with transferrin, insulin, hydrocortisone, and T3. A plate was stopped at day 0, 4, 7, 9, 11, and 14 and fluorescence was measured using AdipoRed. Results are from two experiments with triplicate wells and presented as mean values ± SEM relative to day 0.

The differentiation curves with the OFF media (serum-free culture media supplemented with transferrin, insulin, hydrocortisone, and T3) were performed to show the importance of the peroxisome proliferator-activated receptor gamma (PPARγ) agonist in the serum-free culture media in order to initiate cell differentiation. A differentiation curve with addition of 10% FCS to the differentiation media were also made (data not shown), to show that FCS were not needed in order for the cells to differentiate. The curve made with 10% FCS culture media resembled the OFF curve and cells did not differentiate at all and at the end of the experiment cells died.

In general, large variations were observed within each assay and also between the two assays (figure 12). In order to minimize the variation we considered to relate the AdipoRed to the amount of cells (measured as amount of DNA using PicoGreen) in the specific wells. Two assays where the amount of DNA was measured using PicoGreen after AdipoRed measurement, were performed

By comparing results from four assays where only AdipoRed were measured with two assays where also PicoGreen was measured, almost no difference was observed. However, the SEM for the four assays where only AdipoRed were measured was a bit lower than the two assays where also PicoGreen was measured. The assays with PicoGreen measurements were not used in further differentiation curves, only AdipoRed measurements were used.

13. Results

13.1 Cell proliferation

The three SCFAs acetate, propionate, and butyrate as well as two SCFA combinations (table 3) were tested on SGBS cells to elucidate how they affected proliferation of both preadipocytes and maturely differentiated adipocytes. A graphical illustration of proliferation after two hours of incubation for preadipocytes and adipocytes are given in figures 13 and 14 respectively.

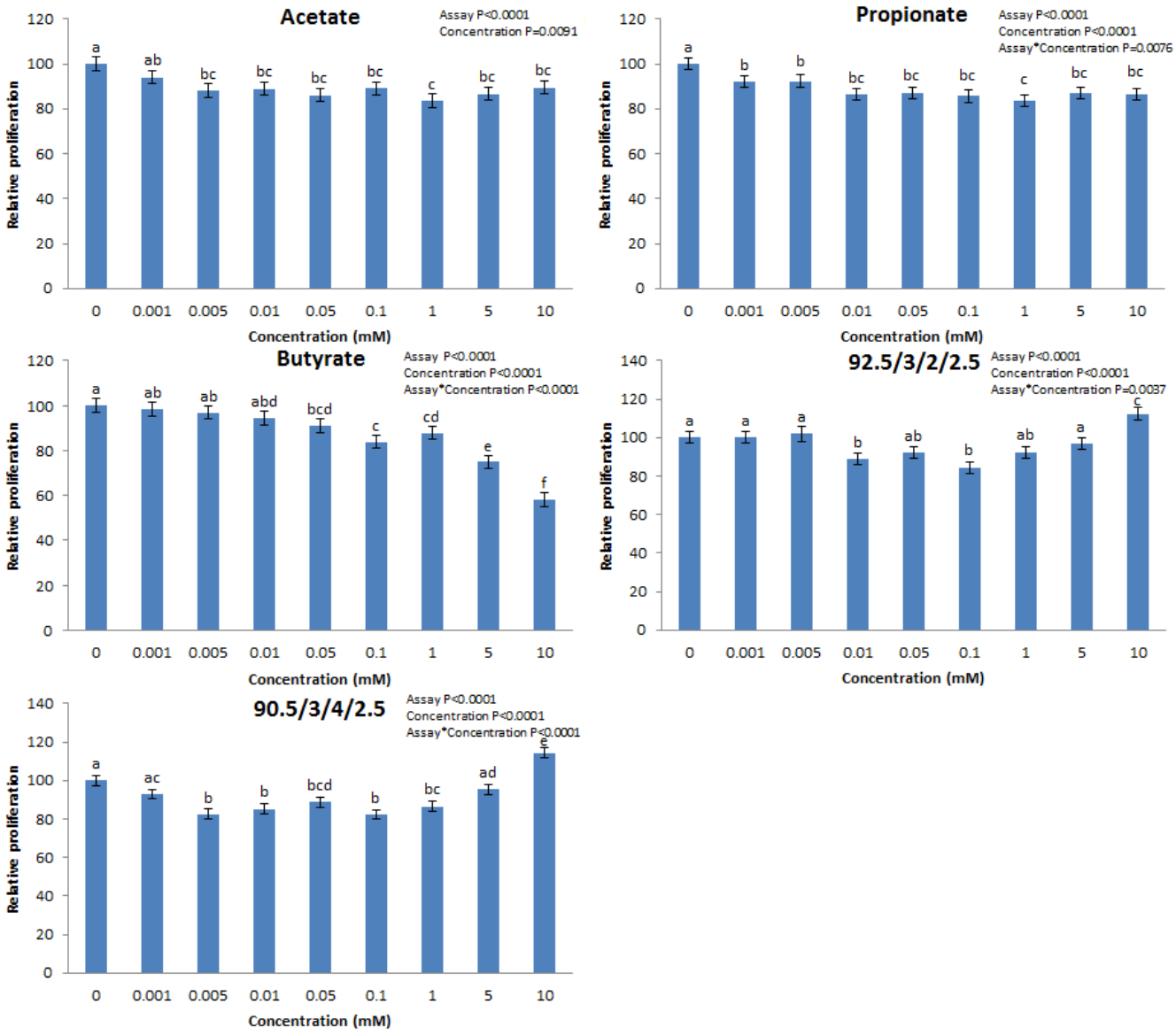

Figure 13: Effect of acetate, propionate, butyrate and the two SCFA combinations 92.5/3/2/2.5 and 90.5/3/4/2.5 on proliferation of SGBS preadipocytes. Cells were treated with culture media containing 10% FCS for 24 hours before replacing the media with medium without FCS but containing 0-10 mM sodium-acetate, sodium-propionate, sodium-butyrate or one of the two combinations (92.5/3/2/2.5 or 90.5/3/4/2.5). Measurements of proliferation were obtained after a three-day treatment period, and after two hours of incubation with AlamarBlue. Results are from three independent experiments with quadruple wells and presented as LSMEANS ± SEM, relative to the level with no treatment (0 mM = 100). Lowercase letters (a-e) indicate significant differences between concentrations, at P < 0.05.

The result for treatment with acetate showed a small negative effect on proliferation of preadipocytes. This effect seemed to be independent of concentration and a negative effect of approximately 10% was the highest negative effect observed (with 1 mM acetate). For treatment with propionate, butyrate and the two SCFA combinations a significant effect of the interaction between assay and concentration was observed, meaning that different effects of treatment were observed within the assays. It was therefore not possible to conclude on significant effects without taking the assay into consideration. Treatment with pro-

pionate, though, seemed to have the same small negative effect on proliferation, as treatment with acetated showed. This effect, as treatment with acetate, seemed to be independent of concentration and a negative effect of approximately 10% was observed for 1 mM propionate treatment. For treatment with butyrate a negative effect was observed with increasing concentrations. At 10 mM butyrate, a decrease of approximately 40% in proliferation was observed. Treatment with the two combinations (92.5/3/2/2.5 and 90.5/3/4/2.5) had either no effect or a small negative effect on proliferation of preadipocytes until a concentration of 5 mM. At 10 mM a significant increase in proliferation was observed for both combinations, approximately 12% for the 92.5/3/2/2.5 combination and approximately 14% for the 90.5/3/4/2.5 combination. It was statistically tested if a significant difference between the two combinations was observed, but no such significant differences between the concentrations and combinations were found (data not shown).

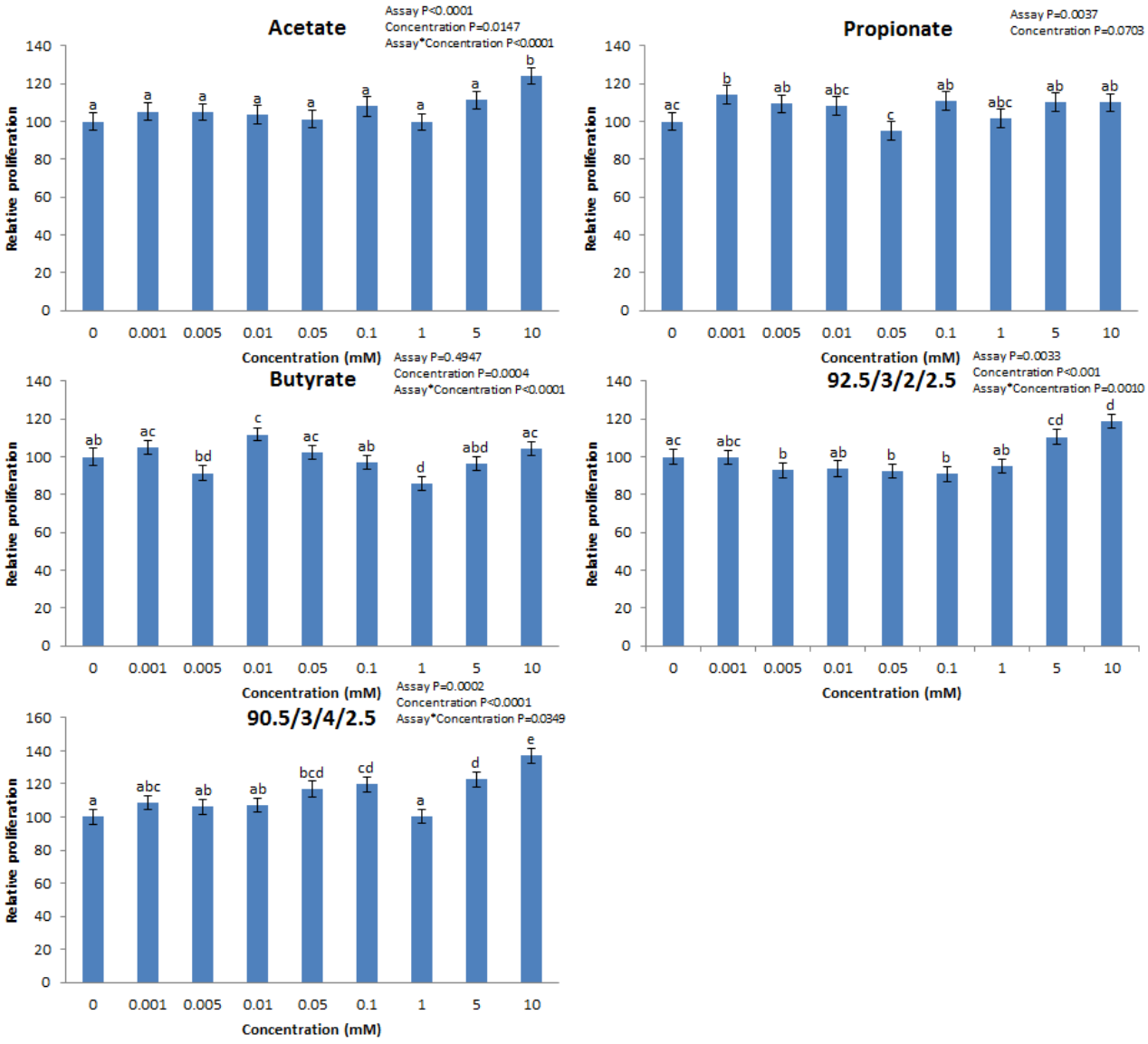

Figure 14: Effect of acetate, propionate, butyrate and the two SCFA combinations 92.5/3/2/2.5 and 90.5/3/4/2.5 on proliferation of SGBS adipocytes. Cells were treated with 10% FCS culture media for three days before replacing the media with serum-free differentiation media for eight days before replacing the media with 0% FCS media containing 0-10 mM sodium-butyrate, sodium-acetate, sodium-propionate or one of the two combinations (92.5/3/2/2.4 or 90.5/3/4/2.5). Measurements of proliferation were obtained after a three-day treatment period, and two hours of incubation with AlamarBlue. Results are from two independent experiments with quadruple wells and presented as LSMEANS ± SEM, relative to the level with no treatment (0 mM = 100). Lowercase letters (a-e) indicate significant differences between concentrations, at P < 0.05.

The results of treatment with propionate had no significant effect on proliferation of mature adipocytes for all concentrations but 0.001 mM, where a 15% increase in proliferation was observed. For treatment with acetate, butyrate and the two SCFA combinations a significant effect of the interaction between assay and concentration was observed, meaning that different effects of treatment were observed within the assays. Therefore, it was not possible to conclude on significant effects without taking the assay into consideration. Treatment with acetate had no significant effect on proliferation of adipocytes for all but one concentra-

tion. At a 10 mM acetate concentration a 25% increase in proliferation was observed. Treatment with butyrate had both negative and positive effects on proliferation of adipocytes. For most of the concentrations used, no significant effect of treatment with butyrate was observed; only a 0.01 mM butyrate concentration gave a significant effect on proliferation, an effect of approximately 10% increase in proliferation. The combination 93.5/3/2/2.5 gave a significant decrease in proliferation of adipocytes for the concentrations 0.005 mM, 0.05 mM, 0.1 mM and a significant increase in proliferation for the concentrations 5 mM, and 10 mM. The combination 90.5/3/4/2.5 gave a significant increase in proliferation of adipocytes for the concentrations 0.05 mM, 0.1 mM, 5 mM, and 10 mM. It was statistically tested whether the two combinations were significant different within the given concentrations (data not shown). It was found that the combination 90.5/3/4/2.5 gave a significant larger increase in proliferation of adipocytes than the combination 92.5/3/2/2.5 for the concentrations 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, and 5 mM.

13.2 Cell differentiation

The effect of acetate, propionate, and butyrate and the SCFA combinations 92.5/3/2/2.5 and 90.5/3/4/2.5 on SGBS cell differentiation was tested. Cell differentiation was measured at days 0, 4, 7, 9, 11, and 14 using the stain AdipoRed. A graphical illustration of SGBS cell differentiation after treatment with SCFAs is given in figure 15.

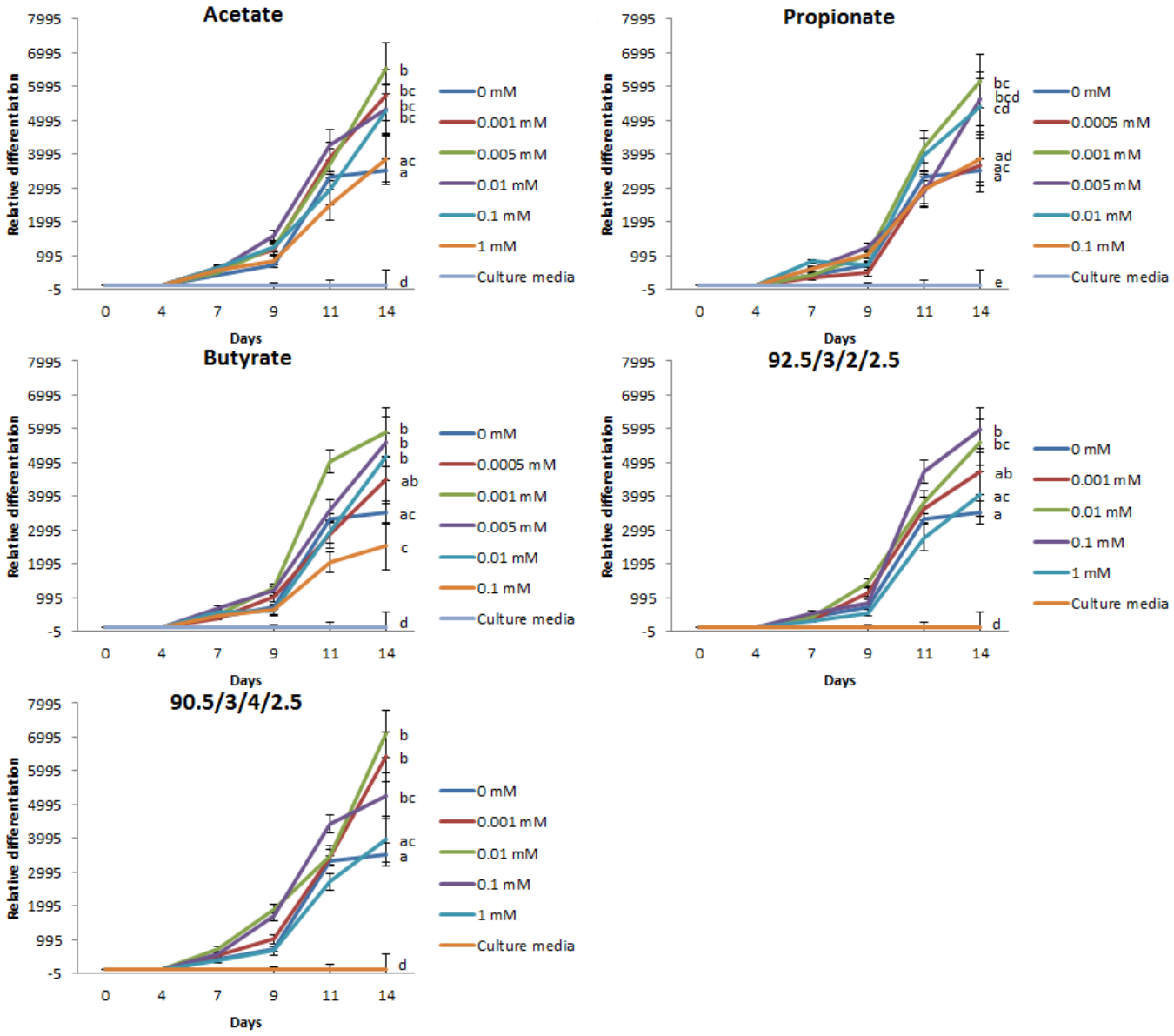

Figure 15: Effect of acetate, propionate, butyrate and the two SCFA combinations 92.5/3/2/2.5 and 90.5/3/4/2.5 on differentiation of SGBS cells. Cells were treated with 10% FCS culture media for three days before replacing the media with serum-free differentiation media supplemented with 0.0005-1 mM sodium-butyrate, sodium-acetate, sodium-propionate, or one of the two combinations; 92.5/3/2/2.5 and 90.5/3/4/2.5. The culture media is serum-free culture media supplemented with 10 µM transferrin, 20 nM insulin, 0.1 µM hydrocortisone and 0.4 nM T3. Measurements of cell differentiation were obtained at days 0, 4, 7, 9, 11, and 14 after 10 minutes incubation at room temperature with AdipoRed. Results are from four independent experiments with triple wells and presented as LSMEANS ± SEM, relative to day 0 (day 0 = 1). Lowercase letters (a-e) indicate significant differences between concentrations at day 14, at P = 0.05.

For all treatments and concentrations, except the serum-free culture media, a significant increase in the differentiation rate was seen from differentiation day 4. Treatment with serum-free culture media showed the same differentiation level throughout the whole experiment and a significant lower level of differentiation at day 14 than any of the other treatments and concentrations. When treating SGBS cells with acetate a significant higher level of differentiation was obtained with the concentrations 0.005 mM, 0.001 mM, 0.01 mM, and 0.1 mM when compared to cells without acetate treatment. The highest level of differentia-

tion was obtained with the concentration 0.005 mM, this increase was though not significantly higher from that observed with the concentrations 0.001 mM, 0.01 mM, and 0.1 mM acetate. Propionate gave the highest level of differentiation with the concentrations 0.001 mM, 0.005 mM, and 0.01 mM when compared to cells with no propionate treatment. The highest level of differentiation was observed when using a propionate concentration of 0.001 mM, this increase in differentiation, compared to no treatment, was though not significantly higher than the concentrations 0.005 mM and 0.01 mM. When treating with butyrate at concentrations 0.001 mM, 0.005 mM, and 0.01 mM a higher level of differentiation was obtained compared to cells without butyrate treatment. The highest level of differentiation was obtained with a concentration of 0.001 mM, the increase observed was though not significantly different from that observed with 0.005 mM, and 0.01 mM butyrate treatment. The combination 92.5/3/2/2.5 at the concentrations 0.1 mM and 0.01 mM gave a significant higher level of differentiation when compared to controls, 0 mM. The highest increase in differentiation was observed at the concentration 0.1 mM, this was though not significantly higher than the increase observed at the concentration 0.01 mM. The combination 90.5/3/4/2.5 at the concentrations 0.01 mM, 0.001 mM, and 0.1 mM gave a significant higher level of differentiation when compared to no treatment, 0 mM. The highest increase in differentiation was observed at the concentration 0.01 mM, this was though no significantly higher than the increase observed at the concentrations 0.001 mM and 0.1 mM.

13.3 Insulin sensitivity

Acetate, propionate, and butyrate and the two SFCA combinations, 92.5/3/2/2.5 and 90.5/3/4/2.5, were tested on SGBS cells to elucidate how SCFA affect insulin sensitivity in the cells. The2-Deoxy-[^{3}H]-Glucose uptake in the cells was measured after a 48 hours and 72 hours of incubation of incubation. No difference between the two incubation times was observed and hence a 48 hour incubation time was preferred for further work. A graphical illustration of insulin sensitivity in SGBS cells after treatment with SCFAs are given in figure 16.

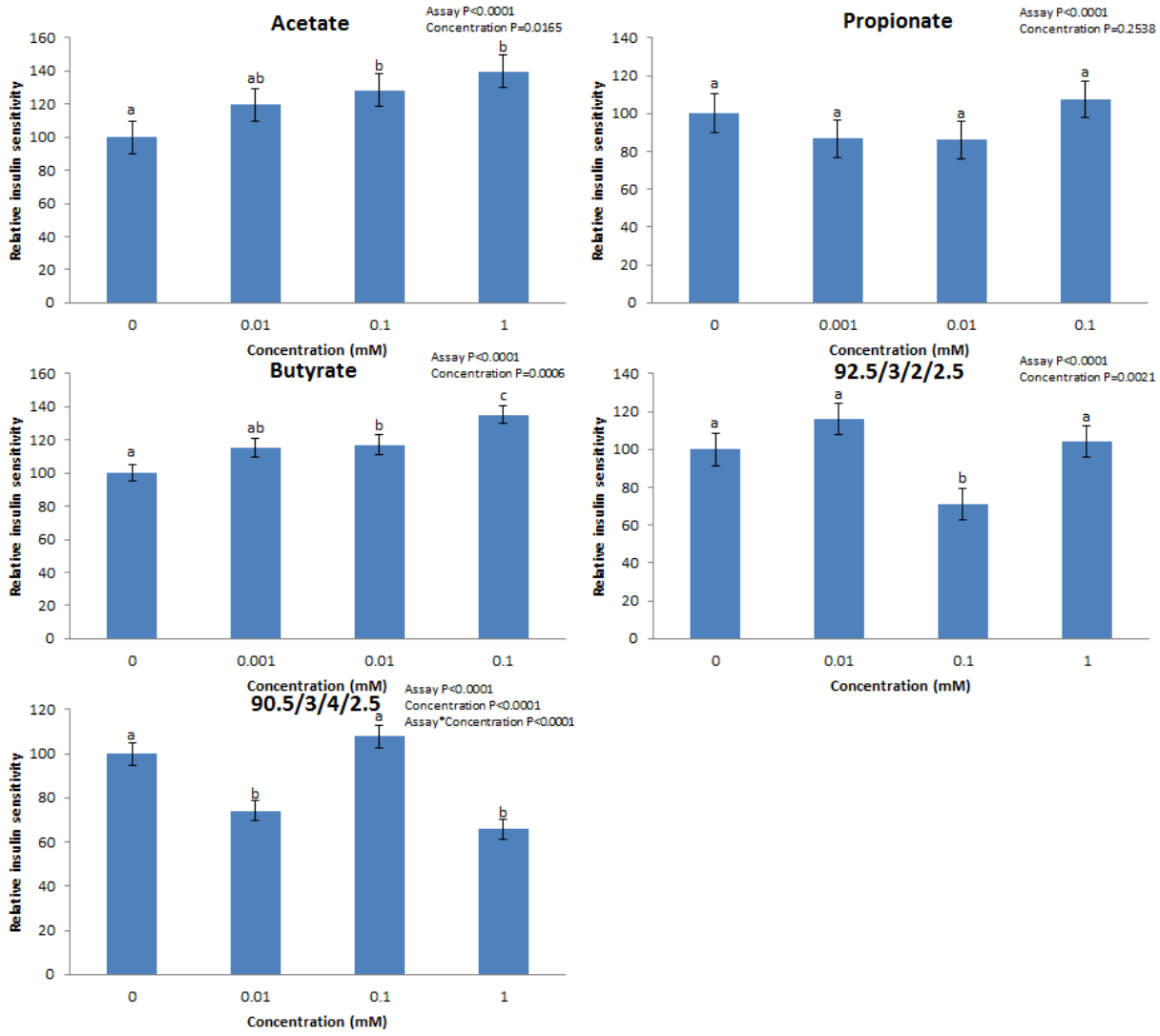

Figure 16: Effect of acetate, propionate, butyrate and the two SCFA combinations 92.5/3/2/2.5 and 90.5/3/4/2.5 on insulin sensitivity of SGBS cells. Cells were treated with 10% FCS culture media for three days before replacing the media with serum-free differentiation media for eight days and replaced with serum-free differentiation media, without insulin, supplemented with 0.001-1 mM sodium-butyrate, sodium-acetate, sodium-propionate or one of the two combinations (92.5/3/2/2.5 or 90.5/3/4/2.5). Measurements of insulin sensitivity were obtained after a two-day treatment period. The insulin sensitivity is the difference in measured [3H]-glucose incorporated into the cells with and without insulin treatment. Results are from three independent experiments with quadruple wells and presented as LSMEANS ± SEM, relative to insulin sensitivity level with no treatment (0 mM = 100). Lowercase letters (a-c) indicate significant differences between concentrations, at P < 0.05.

The results of treating SGBS cells with butyrate and acetate indicated that insulin sensitivity was positively affected with increasing concentrations. At 0.001 mM butyrate, a tendency toward an improvement in insulin sensitivity was observed and at the concentrations 0.01 mM and 0.1 mM, a significant improvement in insulin sensitivity was observed. At 0.01-1 mM acetate significantly improved insulin sensitivity. However, treatment of cells with propionate showed no effect on insulin sensitivity. The combination 92.5/3/2/2.5 gave a significant reduction in insulin sensitivity at the concentration 0.1 mM, whereas no

effect on sensitivity was observed at other concentrations. For the combination 90.5/3/4/2.5 a significant effect of the interaction assay and concentration was observed, meaning it was not possible to make any conclusions about the effect of the concentrations without taking the assay into consideration. Treatment with the combination 90.5/3/4/2.5 gave a significant reduction in insulin sensitivity at the concentrations 0.01 mM and 1 mM, whereas no effect on insulin sensitivity was observed at a concentration of 0.1 mM. It was statistically tested whether the two combinations were significantly different within the given concentrations (data not shown). The combination 92.5/3/2/2.5 resulted in significantly improved insulin sensitivity compared with the combination 90.5/3/4/2.5 for the concentrations 0.01 mM and 1 mM; the opposite was applicable for the concentration 0.1 mM.

The insulin sensitivity of mature SGBS adipocytes were also measured following treatment of cells with plasma from pigs fed; either a WSD, AX diet, or RS diet. The blood used was collected at week 0 and week 3 during the experimental period, after changing the diet from a control diet to the WSD, AX diet, and RS diet. A graphical illustration of insulin sensitivity in SGBS cells after treatment with plasma are given in figure 17.

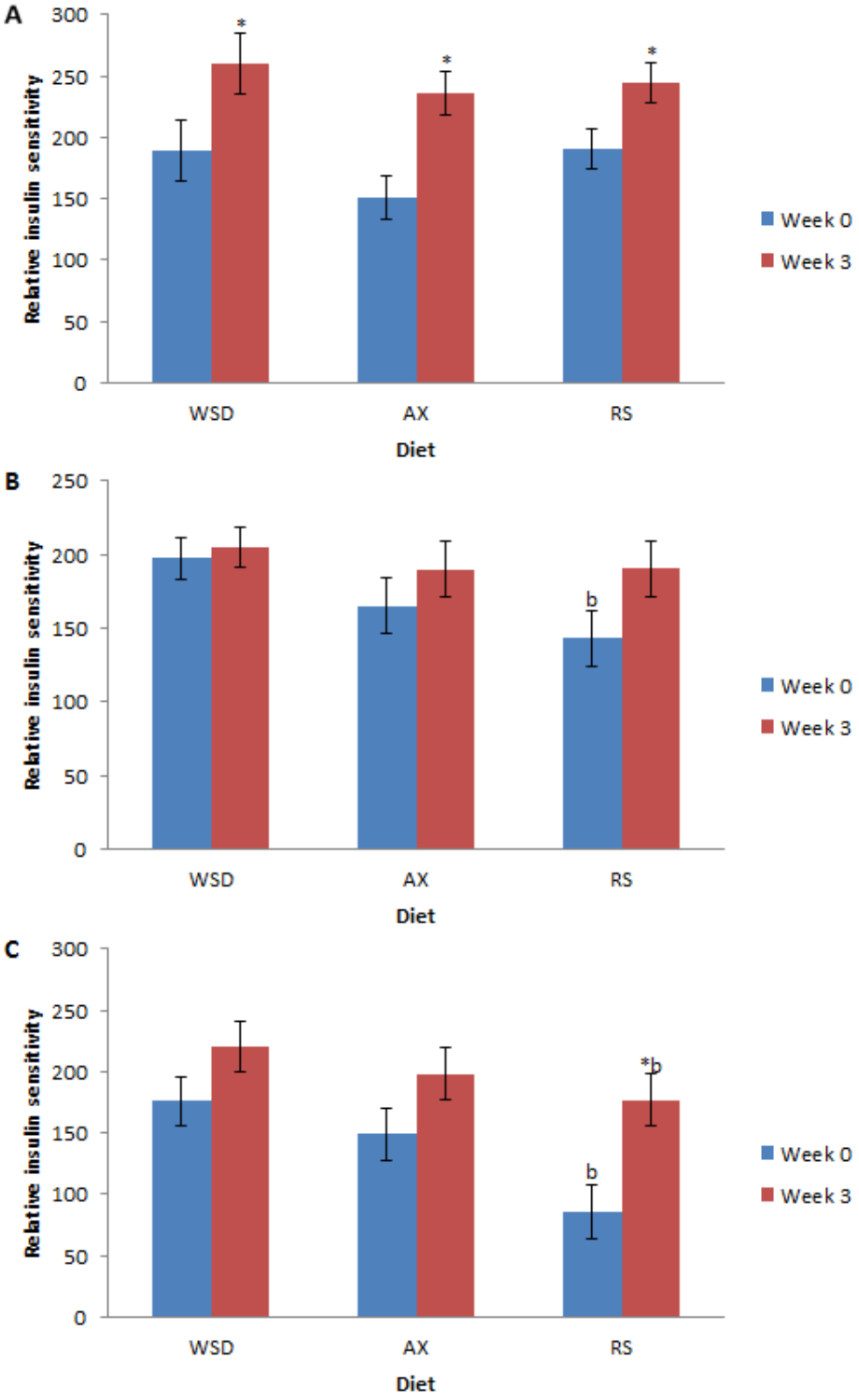

Figure 17: Effect of the three diets WSD, AX, and RS on insulin sensitivity of SGBS cells in concentrations A: 1% plasma, B, 5% plasma, and C: 10% plasma. Cells were treated with culture media with 10% FCS for three days before replacing the media with serum-free differentiation media for eight days and then replaced with serum-free differentiation media, without insulin, supplemented with 1-10% plasma from pigs fed the diets WSD, AX, and RS. Measurements of insulin sensitivity were obtained after a two-day treatment period. Insulin sensitivity is the difference in measured 2-Deoxy-[^{3}H]-Glucose incorporated into cells with and without insulin treatment. Results are from 6 independent assays for both plasma weeks with double wells and presented as LSMEANS $\pm$ SEM. Lowercase letters (b) indicate significant differences between the three diets within either plasma from week 0 or week 3. * indicate significant differences between plasma from week 0 and plasma from week 3, at $P < 0.05$.

All diets improved the insulin sensitivity compared to the standard diet the pigs were fed before treatment (week 0), but only 1% plasma showed a significant improvement for all diets. At 1% plasma there was no significant difference between the tree diets for both week 0 and week 3 plasma. The AX diet induced a larger increase in insulin sensitivity than the WSD diet did. 5% plasma significantly lowered insulin sensitivity for the RS diet compared with the WSD for plasma from week 0, the WSD and AX diet showed no significant difference in insulin sensitivity. At plasma from week 3 for 5% plasma, no significant difference was observed among the tree diets. Although no significant increase in insulin sensitivity was observed for 5% plasma, the RS diet though showed a tendency (P=0.08) towards an improvement in insulin sensitivity. 10% plasma significantly lowered insulin sensitivity for the RS diet compared with the WSD and AX diet for plasma from week 0, the WSD and AX diet showed no significant difference in insulin sensitivity. At plasma from week 3, the RS diet showed significant lower insulin sensitivity than the WSD, the WSD and AX diet showed no significant difference in insulin sensitivity. At 10% plasma a significant increase in insulin sensitivity was only observed for the RS diet, the increase in insulin sensitivity observed for the WSD and AX diet was neither significant nor did it show a tendency.

13.4 Adipocyte size

The size of adipocytes in pigs fed WSD, AX diet, or RS diet, was measured. The pig experiment was previously conducted at Aarhus University Foulum. The fat cell isolations and size measurements were conducted by Annette K. Nielsen and Stig Purup. The focus here will be on the analysis and interpretation of data. A graphical illustration of variation in cell size with the three different diets is given in figure 18.

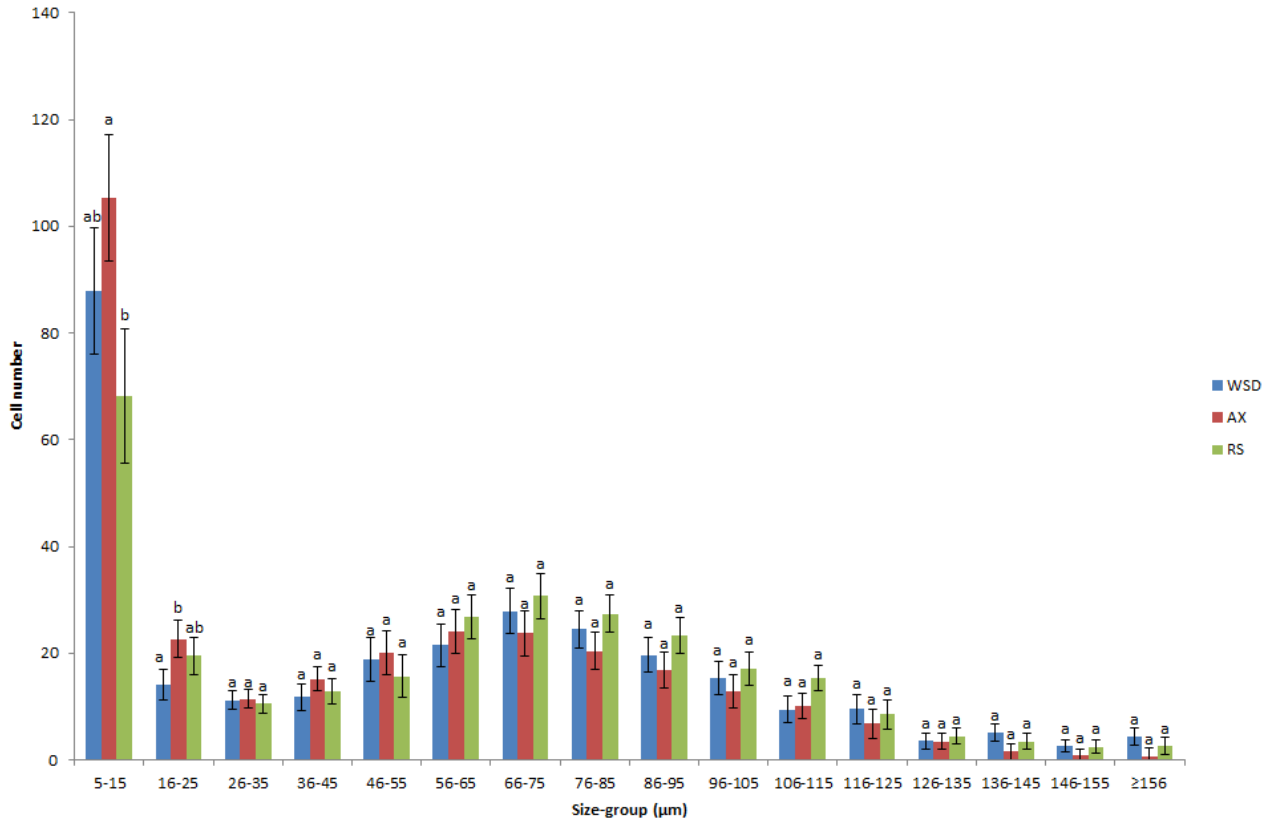

Figure 18: Effect of the three diets; western style diet (WSD), arabinoxylan diet (AX), resistant starch diet (RS), on the adipocyte size from 30 pigs. Abdominal adipose tissue samples, collected after slaughter, were digested with collagenase for 45 minutes before staining with methylene blue. Representative pictures of the cells were taken and adipocyte size was measured. 300 cells/pig was measured and presented as LSMEANS ± SEM. Lowercase letters (a-b) indicates significant differences between the three diets within each size-group.

A significantly higher number of cells were counted in the size-group 5-15 µm than any other size-groups. For all three diets a drop in the amount of cells counted is observed from the 5-15 µm size-group to the 16-25 µm size-group, and again to the 26-35 µm size-group. Next after the small cell size-groups, most cells counted were observed in the size-groups 56-65 µm, 66-75 µm, 76-85 µm and were afterwards falling in the amount of cells counted in the remaining size-groups. When comparing the three diets it was seen that the AX diet had a significant increase in the amount of cells counted compared to the WSD for the size-group 16-25 µm. For the RS diet a significant lower amount of cells than for the AX diet was observed in the size-group of 5-15 µm and a tendency toward a higher amount of cells in the 106-115 µm size-group when compared to the WSD was observed. Otherwise no significant differences between the adipocyte cell size and the distribution of the cells were observed between the three diets.

14. Discussion

The concentrations of the two SCFA combinations (table 3) used throughout this study were determined based on previous pig experiments made at Aarhus University Foulum. On the basis of infusion studies (Serena, Jorgensen, and Bach Knudsen 2009) and plasma concentrations of SCFA from studies with pigs fed diets WSD, AX, and RS (Theil et al. unpublished), the combination were determined to be 92.5/3/2/2.5, representing a low butyrate content diet, and 90.5/3/4/2.5, representing a high butyrate content diet. The last 2.5 in each combination represents other SCFAs, such as isobutyric acid, isovaleric acid, valeric acid, and caprionic acid. On the basis of table 2 physiologic SCFA concentrations reaching adipocytes were estimated to be about 0.1 mM for acetate, 0.005 mM for propionate, 0.003 mM for butyrate, and 0.1 mM for the two SCFA combinations. The physiologic concentration for the two combinations was estimated to be about 0.1 mM, since the contribution from propionate and butyrate was almost negligible compared to the contribution from acetate.

14.1 Establishment of an adipocyte cell-based model

Adipocyte biology is intensely studied, due to a global rise in obesity-associated health issues(Poulos, Dodson, and Hausman 2010). In the present study focus was on establishing an adipocyte cell-based model in order to investigate the effect of SCFAs on insulin sensitivity. It is of great importance to choose the most suitable model, since many adipocyte cell models exists and have been used over time. The cells available all have unique advantages and disadvantages that one should be aware of when selecting cells(Poulos, Dodson, and Hausman 2010). The SGBS cells are of newer origin, as they were introduced in 2001. It is a human cell line derived from subcutaneous white adipose tissue and they are morphologically, biochemically and functionally similar to primary adipocytes (Wabitsch et al. 2001). In the present study the cell density in 96 well plates were determined to be 1500 cells/well. This was determined on the basis of a proliferation assay, which showed that 1000 cells/well would be optimal, and a growth curve, which showed that 2000 cells/well would be optimal. Differentiation assays were made in order to determine optimal differentiation conditions. It was shown that a PPARγ agonist was essential in order to initiate differentiation, and that differentiation should be achieved under serum-free conditions, since cells were unable to differentiate in the presence of serum. This has previously been shown by Wabitsch et al. and Fischer-Posovszky et al.. An advantage with the SGBS cells is their capacity for proliferation for up to 50 generations and still being able to differentiate (Wabitsch et al. 2001;Fischer-Posovszky et al. 2008), oppose to primary human preadipocytes which quickly loses their ability to differentiate when they are multiplied *in vitro*(Fischer-Posovszky et al. 2008). Another major advantage is their ability to differentiate under serum-free condition, in contrast to the 3T3-L1 cells, which need FCS in order to differentiate (Fischer-Posovszky et al. 2008).

14.2 Proliferation

Butyrate was shown to have a concentration-dependent effect on proliferation of preadipocytes and at a concentration of 10 mM a negative effect was observed, however at the estimated physiological concentration (0.003 mM) no effect on proliferation was observed. Butyrate have previously been shown to have apoptotic effects and to affect proliferation in bovine kidney epithelial cells (Li and Elsasser 2005) and treatment of 3T3 cells with 10 mM sodium butyrate have previously been shown to be toxic to these 3T3 cells (Toscani, Soprano, and Soprano 1990). It might therefore be possible that the negative effect on proliferation of preadipocytes observed is due to an apoptotic effect. The apoptotic effects were also observed in earlier studies to be concentration-dependent. The ability of butyrate to cause apoptosis may be linked with the activation of intrinsic apoptotic pathways, in that butyrate can affect gene expression level and protein ratio of pro- and anti-apoptotic proteins (Wu et al. 2012). Since apoptosis was not measured in the present study, the negative effect seen might also be due to a toxic effect of butyrate. Acetate and propionate were, in the present study, shown to have similar effects on proliferation of preadipocytes and no difference between the two SCFA combinations was observed.

When preadipocytes differentiate into mature adipocytes, no more cell-division and increase in cell number should be observed, all energy is used on differentiation of preadipocytes. When treating adipocytes with SCFAs and measuring proliferation, it may therefore be expected that no effect on proliferation would be observed. Acetate, propionate, and butyrate showed no effect on proliferation of adipocytes at their respective estimated physiologic concentrations. Acetate, though, showed a significantly positive effect on proliferation of adipocytes at a concentration of 10 mM. This concentration is though at a so high millimolar concentration that it is physiologically irrelevant. The two SCFA combinations, 92.5/3/2/2.5 and 90.5/3/4/2.5, showed almost the same effect with the given concentrations. At the estimated physiological concentration about 0.1 mM, the combination with low butyrate content (92.5/3/2/2.5) though showed a small negative effect on proliferation, whereas the combination with high butyrate (90.5/3/4/2.5) treatment showed a significant positive effect on proliferation of SGBS adipocytes. For both SCFA combinations, a statistically significant effect of the interaction between assay and concentration was observed, indicating that differences in the effect of concentrations among assays were observed. This may very well also be applicable to the physiological concentration (0.1 mM). It is therefore impossible to make any significant statements about the effect of increasing the butyrate content in the SCFA combination on the proliferation of adipocytes, even though the results implies an stimulation of proliferation when increasing the butyrate content.

14.3 Cell differentiation

When adipocytes have differentiated they start to accumulate lipids in the form of triglycerides and therefore the accumulation of intracellular triglycerides has previously been used as a marker of differentiation (Allott et al. 2012) and was also used as so in this study. When visualising lipid accumulation Oil red O staining has mostly been used. Oil red O, as a hydrophobic stain, partitions into intracellular lipid droplets and quantification requires manual counting of stained cells when viewed in a microscope. However, as an alternative to Oil red O, AdipoRed has been used. AdipoRed is a reagent that enables the quantification of intracellular lipid droplets in a high-throughput manner (Greenspan, Mayer, and Fowler 1985).

A failure in the recruitment of new fat cells, due to impaired differentiation, may lead to a reduction in the capacity of the adipose tissue to accumulate lipids (Lundgren et al. 2007) and it is therefore of importance that the adipogenesis works properly. The impaired differentiation and accumulation of lipids may be paralleled by enlargement of existing fat cells and also spill-over and ectopic accumulation of lipids in other tissues, which in turn contribute to insulin resistance (Lundgren et al. 2007). A suggested solution is an activation of preadipocytes to differentiate into adipocytes, i.e. recruitment of new, small fat cells (Lundgren et al. 2007). It was therefore attempted to study the effect of SCFA on the differentiation of SGBS preadipocytes, to see if they may affect the differentiation of preadipocytes. In the present study it was shown that the three SCFA treatments, acetate, propionate, and butyrate, and the two SCFA combinations gave a significant increase in the level of differentiation at their respectively estimated physiologic concentrations. Staining with Oil red O has shown that treatment with propionate or acetate for 7 days increased the level of adipocyte differentiation of 3T3-L1 cells compared with a control (Hong et al. 2005). Butyrate has also been shown to mediate adipocyte differentiation in 3T3 cells. They have even been shown to be able to substitute for either insulin or dexamethasone of these cells, with a maximum response obtained following treatment with 5 mM sodium butyrate and insulin (Toscani, Soprano, and Soprano 1990). RT-PCR analysis further showed that propionate treatment increased the levels of GPR43 mRNA (Hong et al. 2005), indicating that the SCFAs stimulate adipogenesis via activation of GPR43 (Roelofsen, Priebe, and Vonk 2010).

When comparing the two SCFA combinations at their physiological concentration (0.1 mM) at day 14 of differentiation, to see if an increase in butyrate content had an influence on the level of differentiation, no significant difference between the two combinations was observed ($P = 0.3551$). This indicates that an increase in butyrate production in colon have no effect on differentiation of preadipocytes into mature adipocytes. Acetate and propionate have previously been shown to have beneficial effects on adipose tissue metabolism, serving to lower plasma fatty acid release, increase fat deposition and, because of changes in

adipogenesis, result in smaller adipocytes that release higher levels of leptin and adiponectin and lower levels of pro-inflammatory adipokines (Robertson 2007).

In general, large variations were observed within each assay, and also between the four assays. Even though four assays were made, large variations were still observed within each assay, and also between the assays. This might indicate that AdipoRed are not a reliable method for measurements in our studies.

14.4 Insulin sensitivity

In the present study insulin sensitivity was measured by the glucose uptake capacity. In some of the previous studies, glucose uptake measurements were related to the protein content of the cells embedded in the wells of the assay plates (Biswas et al. 2010), while in other studies only the glucose uptake itself was used as a measure (Kraus et al. 2005). It was therefore decided to try to make an estimation of the protein content in each well after glucose uptake measurements, to see if a higher or lower amount of protein in the wells was observed. For protein determination using a high standard curve (2000-62.5 µg/mL) all measurements were in the low concentration end of the standard curve, and it was therefore tried to determine the protein content using a lower standard curve (500-25 µg/mL). When using the low standard curve, all measurements were still in the low concentration end of the standard curve, meaning the standard curve is very inaccurate. With the high standard curve, only two replicates per well were made and with the low standard curve, only one replicate per well were made, making the measurements even more inaccurate. It was therefore decided to discard the idea of setting the glucose uptake measurements relative to protein content in the wells.

Glucose uptake capacity in adipose tissue is low when compared with that in muscle, for example (Lundgren et al. 2007). It has, however, been shown that modest changes in adipose tissue glucose turnover can have large secondary effects on whole-body glucose metabolism. Small alterations in subcutaneous glucose uptake capacity may therefore also be of importance in humans (Lundgren et al. 2007). In the present study treatment of SGBS cells with acetate was shown to significantly improve insulin sensitivity at the estimated physiological concentrations (about 0.1 mM). Treatment with propionate showed no effect on insulin sensitivity at the estimated physiologic concentration (about 0.005 mM). Butyrate showed no significant effect on insulin sensitivity at the estimated physiologic concentration (about 0.003 mM), but did, however, show a tendency towards an improvement in insulin sensitivity ($P = 0.0577$). Butyrate supplementation at 5% wt/wt in high-fat diet, fed diet-induced obese mice, has been shown to prevent development of dietary obesity and insulin resistance and has further been shown to reduce obesity and insulin resistance in obese mice (Gao et al. 2009). Of the two SCFA combinations only the combination 92.5/3/2/2.5 affected insulin sensitivity at the estimated physiological concentration. However, this effect was not posi-

tive, in that a significant impairment in insulin sensitivity was observed. When comparing the two combinations at their physiological concentration (about 0.1 mM), a significant improvement in insulin sensitivity was observed. This indicates that increasing the production of butyrate in the colon, may have a positive effect on insulin sensitivity in adipocytes and thereby improvements in insulin sensitivity of adipose tissue.

To further examine if the effect of increased butyrate production in the colon has an effect on insulin sensitivity, a pig study with pigs fed three diets, WSD, AX diet, and RS diet were made. Since, insoluble non-viscous DF has been shown to be important for improving insulin sensitivity and glucose homeostasis. This effect seems to be due to SCFA produced by fermentation of nondigestible carbohydrates by colonic microbiota (Priebe et al. 2010). It may be achieved by increasing the intake of resistant starch and other fermentable fibers (Robertson et al. 2003;Robertson et al. 2005), such as arabinoxylan. Improvements in glucose tolerance during a single day has previously been shown with high-fiber foods, in these studies the effect of indigestible carbohydrates could though, not be differentiated from those of glycaemic index (Robertson et al. 2003). In the present study, treatment of SGBS cells with plasma from pigs fed the diets WSD, AX diet or RS diet showed that the RS diet significantly improved insulin sensitivity after a three weeks treatment period for 10% plasma treatment. The RS diet was further showed to give a tendency towards an improvement in insulin sensitivity for 5% plasma treatment. While both WSD and AX diet showed no significant improvements in insulin sensitivity at these plasma concentrations. This is in accordance with a previous study showing that acute ingestion of a high-RS diet for as little as 24 h induced changes in tissue insulin sensitivity. These results indicated that a very high intake of RS had metabolic effects linked to improvements in postprandial insulin sensitivity in normal healthy humans (Robertson et al. 2003). This is also in accordance with another study showing that RS intake increases insulin sensitivity in non-insulin resistant subjects by changing both adipose tissue and skeletal muscle metabolism (Robertson et al. 2005). At 1% plasma concentration the effect of the RS diet on insulin sensitivity though, seemed to be lowest, with the AX diet showing the highest significant improvement. All diets, at 1% plasma treatment, though, gave significant improvements on insulin sensitivity.

14.5 Adipocyte size

In morbid obesity, adipose tissue hyperplasia, with many small adipocytes, is mainly associated with better glucose, insulin, and lipid profiles than adipose tissue hypertrophy, with large adipocytes (Rizkalla et al. 2012). Adipocyte size is not the best predictor of metabolic disorders during obesity, Rizkalla et al., though, likely extends the importance of individual adipocyte variation as a marker associated with changes in insulin sensitivity and abdominal obesity in weight-loss programs, with a significant contribution shown by a low-calorie, high-protein, low-glycaemic index diet (Rizkalla et al. 2012). The AX diet in this study tended to

give more of the small adipocytes, ranging from size-group 5-15 µm to the 54-55 µm size-group, than did either the WSD or the RS diet. since, AX diet gave significantly more adipocytes in the 5-15 µm and 15-25 µm size-groups than the RS diet and WSD did. The RS diet seemed to give more of the medium sized adipocytes, ranging from size-group 56-65 µm to the 106-115 µm size-group, than did either the AX or the WSD diets. While the WSD diet seemed to give more of the larger adipocytes, ranging from size-group 116-125 µm to the ≥156 µm size-group, than did either the AX or the RS diets. The small cells have decreased production and secretion of inflammatory factors and are more insulin responsive than are large adipocytes (Hoffstedt et al. 2010). Larger adipocytes have more macrophage infiltration and secrete more of the pro-inflammatory cytokines, such as IL-6, and less of the beneficial adipokines, such as leptin and adiponectin (Robertson 2007). These cytokines and adipokines seems to be related to fat cell size, and the fat cell size may thus influence hormonal signalling within the adipose tissue itself and to other organs and may therefore also affect insulin sensitivity (Lundgren et al. 2007). Smaller fat cells have been shown to have a doubling of GLUT4 on the cell surface compared to larger fat cells from the same subject. These findings suggests that glucose uptake mainly takes place in smaller fat cells in the fat tissue and, points to a causal relationship between the accumulation of large fat cells and reduced insulin sensitivity in obese subjects (Franck et al. 2007). When comparing this with the results from the insulin measurements of cells treated with plasma from pigs fed diets WSD, AX diet, and RS diet, there seems to be accordance in that the RS diet gave the highest improvement in insulin sensitivity at 5% and 10% plasma concentrations and AX gave the highest improvement in insulin sensitivity at 1% plasma concentrations. This indicates that, increasing and improving the SCFA produced in the colon, influences the adipocyte cell size and also the insulin sensitivity of those cells.

15. Conclusion and perspective

The overall aim of this study was to develop an *in vitro* model to investigate the effect of SCFAs, especially butyrate, on insulin sensitivity and glucose homeostasis and next to investigate the effect of the diets WSD, AX diet and RS diet on insulin sensitivity of adipocytes and the size of fat cell from subcutaneous fat.

Butyrate was shown to have a concentration-dependent negative effect on proliferation of preadipocytes as has previously been shown with other cell lines. When looking at the effect of SCFAs and the two SCFA combinations on the differentiation of preadipocytes to mature adipocytes, it was shown that all treatments gave significant improvements in the level of differentiation at their respective physiologic concentrations. This indicated that the SCFAs may be used as activators of preadipocytes to differentiate into mature adipocytes and, thereby help in remedy impaired differentiation and accumulation of lipids that are paralleled with enlargement of existing cells and, thereby contributes to insulin resistance. Supplementation with butyrate has previously been shown to improve insulin sensitivity and this was in part also shown in this study. Treatment of SGBS cells with SCFA showed that acetate significantly improved insulin sensitivity at physiologic concentrations, while butyrate only showed a tendency towards improvement in insulin sensitivity and propionate did not show an effect at all. Increasing the butyrate content in the SCFA combination was shown to significantly improve insulin sensitivity, in part confirming the hypothesis that, increased production of SCFA, and butyrate in particular, during microbial fermentation in the colon protects against lifestyle diseases such as T2D. In order to further examine the effect of improved SCFA production in the colon, the effect of plasma from pigs fed three diets WSD, AX, and RS were shown. Both of the diets, with increased DF content when compared to the WSD diet, showed improvements in insulin sensitivity. With the RS diet showing to significantly improve insulin sensitivity for 10% plasma treatment and a tendency towards improvement for 5% plasma treatment, and the AX diet showing the highest significant improvement in insulin sensitivity for 1% plasma treatment. When comparing this with the size of fat cells from subcutaneous fat, where smaller fat cells have been shown to have a doubling of GLUT4 on the cell surface compared to larger fat cells, and thereby suggesting that glucose uptake mainly takes place in smaller fat cells, it was seen that the AX diet tended to give more of the smaller adipocytes. The AX diet was followed by the RS diet, giving more of the medium sized adipocytes, and last the WSD diet, giving more of the large adipocytes. None of this was significant, but an image of high DF content in foods giving smaller adipocytes was shown, corresponding well with the same two diets, containing increased DF, giving improved insulin sensitivity. It can be concluded that increasing the daily intake of DF, and thereby increasing the synthesis of SCFA by microbial fermentation in the colon, increases the amount of the small adipocytes and improves insulin sensitivity.

The incidence of type 2 diabetes has been increasing in the last 20 years due to unlimited access to food combined with a sedentary lifestyle (Bano 2013). This does not seem to change in the near future and approaches to prevent the increasing incidence of diabetes are needed. In this study, increasing the intake of DF and thereby increasing the microbial fermentation of SCFAs, butyrate in particular, was shown to positively affect the size of subcutaneous fat cells and improve insulin sensitivity. No gene expression was made in this study and it could be interesting to see how the SCFAs and the two SCFA combinations affected the up and down regulation of different adipokines. Since, leptin and adiponectin have been shown to have reciprocal functions in providing a homeostatic mechanism to maintain fat levels/energy stores through the suppression or stimulation of appetite and energy expenditure (Galic, Oakhill, and Steinberg 2010). Even though an *in vitro* study confirms the hypothesis, further research including *in vivo* studies is needed in order to elaborate the significance of increasing DF intake in treating and preventing lifestyle diseases such as type 2 diabetes.

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