Metabolic adaptation of white adipose tissue to nutritional and environmental challenges

Femke P.M. Hoevenaars

Thesis committee
Promotors
Prof. Dr J. Keijer
Professor of Human and Animal Physiology
Wageningen University

Co-promotor
Dr E.M. van Schothorst
Assistant Professor, Human and Animal Physiology Centre
Wageningen University

Other members
Prof. Dr A.H. Kersten, Wageningen University
Dr P. Oliver Vara, Universitat de les Illes Balears, Palma de Mallorca, Spain
Prof. Dr E.C.M. Mariman, Maastricht University
Dr W.J.J. Gerrits, Wageningen University

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS)

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Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 25 April 2014
at 1.30 p.m. in the Aula.

Femke P.M. Hoevenaars
Metabolic adaptation of white adipose tissue to nutritional and environmental challenges,
168 pages.
PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in Dutch and English
ISBN 978-90-6173-916-2

CONTENT

CHAPTER 1

GENERAL INTRODUCTION

ADIPOSE TISSUE, AN ORGAN

White adipose tissue is an organ which consists primarily of lipid filled adipocytes (approximately 50% of the cellular content) which are held together by a network of collagen fibres. Other cell types, collectively called the stroma vascular fraction, are found in between adipocytes and within the adipose tissue are fibroblasts, stromal cells, pre-adipocytes, leukocytes and macrophages [1](Figure 1).

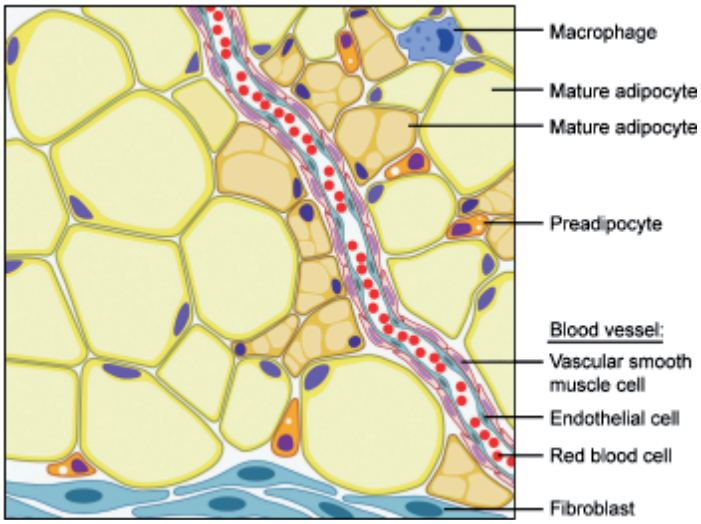

Figure 1 Structure of white adipose. (adapted from [1])

Until 1994 adipose tissue was primarily viewed as a depot for storage of triglycerides during energy consumption and fatty acid release when energy expenditure exceeded energy intake. Next to that, its second function was that of an insulating layer under the skin which helps to regulate body temperature and to cushion and protect body parts. Since the discovery of the obesity gene leptin in 1994 [2], it became clear that white adipose tissue also has an important metabolic and immune function, which involved the secretion and perception of peptide hormones and cytokines.

Energy storage

White adipose tissue is the major site in the body for storage of surplus energy. Energy can be stored in the form of triglycerides in various anatomical depots i.e. subcutaneous, and visceral fat depots and a small portion of around 5% will be stored in the liver or in muscle. Storage capacity is regulated by either growth or shrinkage of the adipose tissue mass. Growth can take place via adipogenesis,

a tightly regulated cellular differentiation process from a fibroblast like pre-adipocyte to a mature lipid loaded adipocyte [3]. So, adipocyte morphology and adipocyte number are able to adapt in response to the demands of the energy balance [4]. The balance is determined by the amount of fat synthesis (lipogenesis) and fat break down (lipolysis). In lipogenesis, fatty acids are synthesized from acetyl-CoA. This process is responsive to nutritional and hormonal regulation [5]. In lipolysis, triacylglycerol molecules are hydrolysed into free fatty acids and glycerol so they can be used as an energy source for β-oxidation in liver, muscle and other tissues. Some of the released free fatty acids are re-esterified to triglycerides in adipocytes. This process is important to be able to directly respond to changes in peripheral energy homeostasis and concomitant changes in fatty acid requirements [6]. In obesity, levels of free fatty acids are elevated in plasma of most subjects [7]. High levels of these circulating free fatty acids have been associated with insulin resistance and atherosclerosis [8]. These high circulating levels lead to an increased lipid accumulation into non-adipose tissues such as muscle, liver, pancreas and heart and may lead to cellular dysfunction or even cell death [9, 10].

Endocrine organ

Some twenty years ago it has been reported that adipose tissue played a role in the secretion of factors that influence appetite and sex steroid metabolism [11, 12]. In 1994 the 'obese gene' leptin was discovered as the first secreted peptide hormone by adipose tissue [2]. This progress stimulated the discovery of more new adipose tissue secreted protein signals and factors also called adipokines or adipocytokines [13, 14]. They function as part of a complex set of physiological control systems that regulate local tissue and systemic physiology. A listing with their functions is shown in table 1. Dysregulation of adipokine secretion plays a fundamental role in the pathogenesis of obesity [15]. Expression of several of these adipokines (Tumor necrosis factor alpha (TNF-α), interleukin-6 (Il-6), adiponectin and C - reactive protein (CRP)) is often dependent on cell size or volume and location [16].

In general, most adipokines are more abundantly expressed and secreted in relation to the amount of fat mass. Two well described adipokines are leptin and adiponectin. Leptin serves as a metabolic signal which regulates energy homeostasis via inhibiting food intake and increasing energy expenditure [17]. Next to that, its levels are proportional to the amount of adipose tissue mass [18]. In obesity, leptin levels are therefore high, however usually there is no reduction in feed intake, which is attributed to leptin resistance. Serum leptin levels are linked to meal times via circadian oscillation and could be a bridge between energy homeostasis and circadian control [19]. Adiponectin levels are negatively correlated to adipose tissue mass, and are decreased upon obesity [20]. However, an increase in adiponectin is also shown in targeted interventions studies which did not induce weight loss [21]. Adiponectin is a modulator of glucose regulation;

it increases rates of fatty acid oxidation, improves insulin sensitivity and reduces inflammation vascular injury [22].

Table 1 Major adipokines secreted by adipose tissue.

Adipokine	Gene symbol	Primary source (s)	Function
Leptin	Lep	Adipocytes	Satiety signal
Adiponectin	Adipoq	Adipocytes	Insulin sensitizer; anti-inflammatory
Resistin	Retn	Adipocytes	Increases insulin resistance; promotes endothelial dysfunction
Adipsin/complement factor D	Cfd	Adipocytes, monocytes, macrophages	Rate limiting enzyme in complement pathway, stimulates triglyceride storage
Acylation stimulating protein	C3	Adipocytes	Stimulates triglyceride storage
Apelin	Apln	Adipocytes	Dependent on location of receptor, role in cardiovascular physiology
Serum amyloid A	Saa	adipocytes	Systemic inflammation and atherosclerosis
Vaspin	Serpina 12	Adipocytes	Insulin sensitivity
Omentin	Itln1	Stromal vascular cells	Role in insulin mediated glucose-uptake
Zinc-alpha2-glycoprotein	Azgp1	Adipocytes	Lipid mobilization
Lipocalin 2	Lcn2	Adipocytes, macrophages	Promotes insulin resistance and inflammation through TNF secretion from adipocytes
Secreted frizzled-related protein 5	Sfrp5	adipocytes	Insulin mimetic effects
Retinol binding protein-4	Rbp4	Liver, adipocytes, macrophages	Implicated in systemic insulin resistance
Chemerin	Rarres2	Adipocytes	Role in adipogenesis and glucose uptake
Plasminogen activator inhibitor-1	Pai-1	Stromal vascular cells	Fibrinolysis
Visfatin	Nampt	Adipocytes, macrophages and other cells	Monocyte chemotactic activity
Angiopoietin-like protein 2	Angptl-2	Adipocytes, other cells	Local and vascular inflammation
Monocyte chemo-attractant protein 1	Mcp-1		Recruits monocytes to sites of injury and inflammation

Table 1 Major adipokines secreted by adipose tissue. (*Continued*)

Adipokine	Gene symbol	Primary source (s)	Function
Pro inflammatory cytokines			
Tumor necrosis factor-alpha	Tnf-α	Stromal vascular fraction cells, adipocytes	Inflammation, antagonism of insulin signaling
Angiotensinogen	Agt	Adipocytes	Adipocyte differentiation, size and insulin sensitivity
Interleukin-6	Il-6	Adipocytes, stromal vascular fraction cells	Changes with source and target
Interleukin-18	Il-18	Stromal vascular fraction cells	Broad-spectrum inflammation
CC-chemokine ligand 2	Ccl2	Adipocytes, stromal vascular fraction cells	Monocyte recruitment
CXC-chemokine ligand 5	Cxcl5	Stromal vascular fraction cells (macrophages)	Antagonism of insulin signaling through the JAK-STAT pathway

Based on [15, 23, 24]

ENERGY BALANCE

In biology energy balance is the homeostasis of energy which is often represented by a simple formula: energy intake = energy expenditure + energy stored. Essentially this is just a restatement of thermodynamics, since energy cannot be created nor destroyed. Food is the main source of our energy. Energy expenditure can be compartmentalized into 3 main categories: resting metabolic rate (i.e. energy needed for maintenance of main physiological functioning), adaptive thermogenesis (i.e. energy expended to digest, metabolize, and store ingested macro nutrients or energy to adjust to environmental temperature), and physical activity (i.e. increase in metabolic rate caused by use of skeletal muscles for any type of physiological movement) [25, 26]. Remarkably, despite substantial daily variation in energy intake and expenditure, most adults obtain a stable body weight. Individual body weight variance is around 0.5% over periods of 6-10 weeks [27]. Net energy balance is counteracted by changes in food intake and/or energy expenditure to minimize changes in body weight [28]. Weight stability suggests homeostatic control, but homeostasis implies active regulation towards a certain physiological condition or set-point. From the late 1970's / early 1980's the percentage of overweight people started to rise and it seems that we are not able to 'auto regulate' our body weight around a fixed point any longer. This casts some doubt on the fixed set point theory as the set point seems to move upward.

One way to explain the obesity epidemic is by the thrifty gene hypothesis. It starts from the assumption that through natural selection we evolved to be efficient in energy storage for times of famine [29]. Stimulated by overabundance of food (introduction of fast food and soft drinks), a sedentary lifestyle (transportation systems) and the availability of new technologies (labor saving devices) this makes us susceptible to development of obesity and its associated pathologies. This is not only the case in the Western world but it has become a global problem, 'globesity'. Interaction of all these parameters results in a dynamic equilibrium 'settling point' which is dependent on current input [30]. This principle is used by two different models, which will be discussed in more detail.

Set point hypotheses

The **set point model** can be considered as a thermostat; when the system is disrupted by either losing or gaining weight, the weight will be either regained or lost to approximate the original settings. Body weight regulation might be secondary to regulation of a component of our body composition, such as fat. This led to the 'lipostatic' theory in which total body fat regulation determines body weight [31]. The set point model represents a closed loop model in which the controlled quantity can be fat mass, body weight or even fat free mass which sends a signal to the central control system (Figure 2). The value of the set-point is independent of the operation of the system, and, once adjusted, remains the same until readjusted [30]. It is now clear that this might be an oversimplified model as more factors become known which are able to influence body weight and composition.

In the **settling point model** it is assumed that there is little active regulation of body weight, but that environmental and socioeconomic factors such as diet and lifestyle are predominant. Precise regulation takes place without an exact predefined set-point, rather they settle at a certain number. An analogy for body weight regulation and its energy stores is the levels of water in a lake [32]. A natural equilibrium is present in a reservoir, here a lake, due to extra inflow of rain and equalisation of outflow to inflow of rain (Figure 3A). When translated into regulation of body weight, body energy stores represent the lake while rain is translated into input, and depth at outflow is represented by expenditure (Figure 3B).

Several weight loss strategies for humans exist, some more successful than others. Reduced energy diets or weight loss medication are generally successful

Figure 2 Set point models for the regulation of body weight.

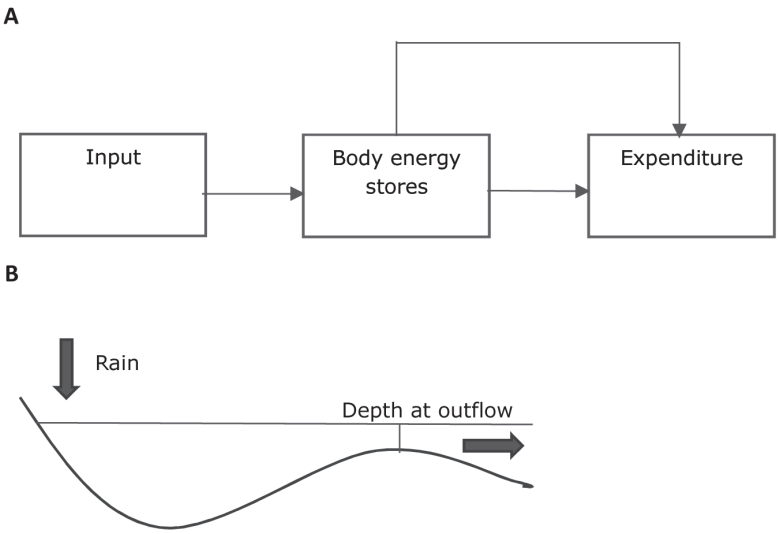

Figure 3 Settling point models for the regulation of body weight. A) Level of water in a lake B) A parameter of interest (body energy stores) is regulated by input and expenditure. Adapted from [32]

in reaching a body weight loss of 5-9% with weight plateauing after 6 months [33]. Also reduction of a specific nutrient such as fat or carbohydrates (Atkins diet) is considered as an effective tool for weight loss [34-35]. The biggest problem is maintenance of the lower body weight, short term effectiveness is high but long term maintenance seems to be more difficult. In a meta-analysis of body weight reduction studies it was observed that after 48 months half of the reduction had disappeared [33]. Successful maintenance of body weight reduction is dependent on several factors. It starts with continued dietary restraint, but it also includes eating breakfast every day, frequent self-monitoring, undertaking regular exercise, and limiting television viewing [36].

Adiposopathy

Recently, obesity has been recognized as a disease. It became clear, that the presence of excessive adipose tissue leads to pathological disruption of adipose tissue metabolic processes. This has been called adiposopathy, 'sick fat' [37]. Several phenotypic changes can be observed: the anatomy of white adipose tissue changes by hypertrophy of adipocyte cells, in particular visceral adipose tissue accumulates and ectopic lipid storage is seen. Physiology is affected by changes in metabolism (increased secretion of free fatty acids), signalling (adipokines) and immune homeostasis (altered secretion of adipocytokines). Disturbances in cross talk with other organs contribute to the development of metabolic diseases. Important was the realization that white adipose depots became inflamed when excessively

enlarged (as in obesity). This chronic inflammation is considered the foundation for the progression of obesity associated diseases such as insulin resistance, type 2 diabetes, cardiovascular disease and some forms of cancer [38]. The next question asked was why this inflammatory state was aroused. It was proposed that due to the excessive expansion of the adipose tissue, hypoxia developed at tissue level [39]. Alternative theories for development of adipose tissue inflammation are endoplasmic reticulum stress [40] or oxidative stress [41].

Measuring the energy balance, indirect calorimetry

Indirect calorimetry is a method in which total energy expenditure as well as relative and absolute substrate specific energy expenditure rates can be measured *in vivo* by means of gas exchange measurements [42]. Specifically designed equipment monitors gas volume of oxygen (O_2) and carbon dioxide (CO_2) at the inlet and outlet vents of a chamber which is ventilated with fresh ambient air at a known flow rate by positive pressure (Figure 4).

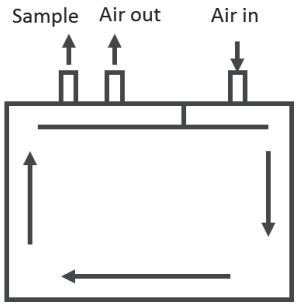

Figure 4 Indirect calorimetric airflow.

The ratio between carbon dioxide production (VCO_2) and oxygen consumption (VO_2) reflects the nutrient mixture which is oxidized on a whole body level, the respiratory exchange rate (RER). Actual substrate oxidation at the tissue level is called the respiratory quotient (RQ). The three main energetic substrates that are oxidized for energy production are carbohydrates, fat and proteins. Proteins only contribute to a small extent to the variation in total daily energy expenditure [43], therefore protein is often not included in substrate specific calculations. Therefore, RER represents a measure for the relative participation of fat and carbohydrate oxidation to total energy expenditure. The general equation is

$$RER = \frac{VCO_2}{VO_2} \qquad 1$$

The overall chemical equation for the metabolism of glucose is

$$C_6H_{12}O_6 + 6(O_2) \rightarrow 6(H_2O) + 6(CO_2) \quad 2$$

The overall equation for a typical fatty acid, palmitic acid is

$$C_{16}H_{32}O_2 + 23O_2 \rightarrow 16(H_2O) + 16(CO_2) \quad 3$$

From the equations Eq1 and 2, RER associated with only glucose oxidation equals 1. For fat, it would equal approximately 0.7. RER is different for each consumed nutrient mixture because the ratio C to O is higher in fat, hence more oxygen is needed for complete oxidation (53.8ml O_2 vs. 47.5ml O_2) but also more energy is generated (39 kJ/g vs. 16 kJ/g) [43]. Also the oxygen cost of ATP production differs between the two substrates: 3.93 l/mol vs. 3.72 l/mol respectively.

Four of the most popular equations to calculate the energy equivalent of oxygen have been described by Brouwer (16.07 VO_2 + 4.69 VCO_2), Elia (15.8 VO_2 + 5.18 VCO_2), Lusk (15.79 VO_2 + 5.09 VCO_2), and Weir (16.3 VO_2 + 4.57 VCO_2) [44-47]. Even and Nadkarni showed that the deviation between these formulas is not more than 1% [43], so all are considered to be suitable. In this thesis, energy expenditure calculations are based on the Weir equation, as this is the most popular and used equation. The general equation is

$$EE = VO_2 * [3.815 + (1.232 * RER)] \quad 4$$

ADIPOSE TISSUE AND DIET INDUCED OBESITY (MOUSE AS A MODEL)

It is often difficult to perform experiments in humans as protocols are often unethical or unfeasible. The mouse has been acknowledged as a suitable model for man as its genome is about 90% identical. Next to that, it is possible to make use of knockout and overexpression models of specific genes to produce models of human disease, or use phenotype driven models. In all these approaches, changes in normal functioning of the whole organism can be studied which leads to insights in underlying mechanisms of malfunctioning. Eventually this can even lead to potential treatments. Some more advantages are the standardizations of performed experiments which lead to increased power and usage of less mice. Furthermore, many tools (whole gene expression microarrays, antibodies) are available for research on mice and due to their small size they are relatively cheap.

Mouse models have become a useful tool for the characterization of molecular and physiologic aspects of obesity. An appropriate mouse model for studying

adipose tissue metabolism in obesity is the C57BL/6J mouse. This strain is known for its capacity to develop obesity, hyperglycaemia, and insulin resistance by high fat feeding [48-50], but it remains lean when fed a low fat chow diet. The progression from being just overweight to obesity and metabolic syndrome closely parallels obesity development in humans [51]. High fat diet and time are able to develop obesity and insulin resistance gradually [48]. An important role in the development of obesity associated diabetes type II is the distribution of fat. Abdominal fat surrounding the organs has been shown to be a good predictor for diabetes via waist circumference; subcutaneous fat underneath the skin however does not increase the risk [52-54]. In C57BL/6J mice an increase in visceral (abdominal) fat upon high fat feeding has been shown [49, 55, 56]. Adipocytes grow; develop white adipose tissue inflammation, hypoxia, all like humans. Altogether, the C57BL/6J strain is a very suitable model to study adipose tissue metabolism.

Two other factors which play an important role in the development of obesity are environment and diet. Therefore it is important to take these conditions into account when designing a mice intervention. Ambient housing temperature of mice was shown to have large effects on physiology. Normal standard laboratory environments are kept at 20-24°C. This is below the lower critical temperature of mice which is between 29-34°C [57]. As a result this will for example influence energy balance due to an increase in metabolic rate to cope with the lower temperature. When comparing mice to humans it is recommended to house them within the thermo neutral zone to avoid cold stress, as we humans also prefer thermoneutrality [43, 58, 59]. However this is debatable. Speakman and Keijer argue for a housing temperature of 23-25°C which represents a temperature at least 3°C below the lower critical temperature [60]. Unfortunately only few investigators have taken notice of the importance of housing temperature. Consequently, very little is known about the effects on metabolic response.

High availability of high fat / energy dense foods in modern society is a large component in the obesity epidemic. This is also mimicked in mouse by feeding a westernized diet to induce diet induced obesity. Therefore, it is important to consider the composition of the diet. For example, dietary fat source of the diet has shown to reshape gut micro biota and alter host adipose tissue inflammatory profile [61]. But also macronutrients are important, i.e. protein to vcarbohydrate ratio in a high fat diet can delay development of adiposity [62] and even different types of sugar contribute to this [63]. Besides composition, also purity of the diet is important. Many interventions make use of a chow control. Unfortunately chow has a high variability in its nutritional content as it is composed of various plant materials. Next to that, the ingredients often contain non-nutritive components, phytoestrogens in soy, which can influence study results. To be able to observe functional effects of different nutrients it is therefore essential to use standardized semi-purified diets.

AIMS AND OUTLINE OF THIS THESIS

Together with the development of the obesity epidemic, it became clear that (dys)-functioning of adipose tissue is a major contributor to the associated adverse health effects. The focus of this thesis is on the adaptive capacity of white adipose tissue metabolism to improve the understanding of adipose tissue functioning. This will help in finding strategies to fight metabolic disease. There are many possibilities for challenging adipose tissue; this thesis focusses on investigating three basic questions.

1. Does a body weight set-point exist?
2. How is the diet-induced metabolic response affected by housing at thermoneutrality?
3. Does oxygen restriction induce inflammation in white adipose tissue?

Energy content and complex interactions between macronutrients are the basis for the ability to adapt our metabolism to various nutritional challenges. To investigate the existence of a body weight set-point **chapter 2** describes a nutritional intervention study which investigates if dietary history influences present metabolism and homeostatic settings. In **chapter 3** we increased adipose tissue stress by using two standard weight loss strategies; a reduction in fat intake and a reduction in energy intake. Thereby it was examined if these two strategies influenced metabolic regulation differently.

Environmental challenges also result in adaptation of metabolism. Thermoneutrality has a significant impact on metabolism and physiology, i.e. increased body weight gain. In **chapter 4** it is investigated to which extent thermoneutrality affects metabolic health parameters imposed by two different semi-purified diets, and if it allows for more sensitive detection of biomarkers.

Lastly we challenged adipose tissue by exposure to oxygen restriction in **chapter 5** to investigate if oxygen restriction leads to inflammation in white adipose tissue. We accommodated mice at thermoneutrality and combined this with feeding a high fat diet to induce severe diet-induced adiposity without inflammation.

Realizing that comparisons with other studies are hampered by differences in dietary compositions. A standard purified diet was designed which is described in **chapter 6.**

The discussion, the final chapter of this thesis, summarizes and discusses all findings in this thesis and concludes with recommendations for future research.

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CHAPTER 2

EFFECTS OF DIETARY HISTORY ON ENERGY METABOLISM AND PHYSIOLOGICAL PARAMETERS IN C57BL/6J MICE

Femke P.M. Hoevenaars, Jaap Keijer, Hans J. Swarts, Sophie Snaas-Alders, Melissa Bekkenkamp-Grovenstein, and Evert M. van Schothorst*

Human and Animal Physiology, Wageningen University, Wageningen, Netherlands.

* To whom correspondence should be addressed: Dr. Evert M. Van Schothorst, Human and Animal Physiology, Wageningen University, De Elst 1, 6708 WD, Wageningen, PO box 338, 6700AH Wageningen, The Netherlands
Email: Evert.vanschothorst@wur.nl

Published in: Experimental Physiology (2013) 98(5):1053-1062
DOI: 10.1113/exphysiol2012.069518

WHAT IS THE CENTRAL QUESTION?

Have intake patterns of diets with a different energy content a lasting effect on body weight persistence?

MAIN FINDING AND ITS IMPORTANCE

After switches between semi-purified diets differing only in the fat/sugar ratio, the last consumed diet determined energy intake, energy expenditure, body weight, body fat stores, circulating hormones and metabolites in C57BL/6J mice. In contrast with persistent effects observed in studies using diets with different ingredients, suggesting diet induced set points, this study shows that body weight and metabolic parameters 'settle' based on current energetic input and output. This supports the settling point theory and underlines the importance of diet choice in physiological studies.

ABSTRACT

Understanding body weight regulation is essential to fight obesity. Mouse studies, using different types of diet, showed conflicting results in terms of bodyweight persistence after changing from *ad libitum* high fat diet to an *ad libitum* low fat diet. In this study, we questioned specifically whether the energy content of the diet has a lasting effect on energy balance and body weight, using multiple switches and 2 purified diets with a different fat/sugar ratio, but otherwise identical ingredients. Young-adult obesity prone male C57BL/6J mice were fed single or double alternations of semi-purified diets with either 10en% fat (LF) or 40en% fat (HF) with starch replaced for fat, while protein content remained equal. After none, one or two diet alternations, energy metabolism was assessed at t=5 weeks, t=14 weeks and t=19 weeks. We observed no systematic continuous compensation in diet and energy intake when returning to LF after HF consumption. Body weight, white adipose tissue mass and histology, serum metabolic parameters, energy expenditure, and substrate usage all significantly reflect current diet intake, independent of diet alternations. This contrasts with studies that used diets with different ingredients and showed persistent effects of dietary history on body weight, suggesting diet dependent metabolic set points. We conclude that body weight and metabolic parameters 'settle', based on current energetic input and output. This study also highlights the importance to consider the choice of diet in physiologic and metabolic intervention studies.

Keywords: Indirect calorimetry, high fat diet, adipose tissue

INTRODUCTION

Obesity is characterized by a surplus amount of white adipose tissue (WAT). This excess WAT results from a positive energy balance; an excess energy intake relative to energy expenditure (EE). WAT has a major role in energy storage, but also fulfils an active metabolic signalling function [1]. One of the signalling molecules secreted by WAT is the peptide hormone leptin, that controls adipose tissue mass via a negative feedback loop. Upon weight loss, fat mass and leptin levels decrease [2], stimulating appetite and suppressing EE to counteract weight loss. Likewise, when fat mass increases, leptin levels increase thus suppressing appetite to counteract weight gain [3]. In humans, maintenance of a 10% or greater reduction in body weight (BW) is accompanied by a reduction in EE beyond that predicted by loss of body mass [4-6]. Addition of exogenous leptin is able to counteract this decrease in EE that resulted from BW loss [7]. Together, this suggests homeostatic control of BW. This, has been developed into the set-point model [8]. The set-point model is supported by more extensive analyses for both humans [9] and rodents [10].

The concept that a BW set-point protects against weight loss is widely taken for granted and is often used to explain BW gain after BW loss. A meta-analysis of BW reduction trials showed that half of the BW reduction disappeared within 48 months [11], despite moderate BW losses. In addition, a substantial number of people fully regained BW. In the NHANES study, of those who lost 10% BW, 35% regained BW after one year [12]. In an analysis of predictors of BW gain, it was concluded that maintenance of a lower BW depends on a number of factors, including a genetic component [13], but it always includes continued attentiveness to match dietary intake to energy balance [14]. This and many other observations [15-17] indicate that once weight is gained, it is very difficult to lose it and to remain on the new, lower, BW.

One can wonder how the homeostatic set-point for BW is determined. It has been suggested that this results from previous consumption of a high caloric diet. This was previously examined using rats and mice. In particular C57BL/6J mice, which are sensitive to diet induced weight gain, are considered a good, human relevant model to study pathogenesis of diet induced obesity [18]. Performing dietary interventions in this mouse model, as compared to humans, allows for full control of diet and physical environment, without influence of social factors. Thus, only internal metabolic pressure and stresses applied by the study design are accountable for alternations in phenotype. Two studies have been performed in C57BL/6J mice to assess homeostatic BW regulation. In both studies, a high caloric diet was used to increase BW followed by *ad libitum* feeding with a low caloric diet. In the most recent study reported by Guo *et al.* [19], two different high caloric semi-purified conditions were used, which each were compared to a low caloric non-purified chow diet. In both high caloric conditions, the diet contained 59en% fat, 27en% carbohydrate and 14en% protein, and in one condition an additional high energy drink was provided. After reversal to the low caloric chow diet, a persistent higher weight was observed in both groups that were first on a high caloric diet, compared to the group that

was continuously on the chow diet [19]. These results are consistent with the set-point model, but differ from those of the study by Parekh *et al*. [20]. In this earlier study by Parekh *et al*. [20], semi-purified low caloric and high caloric diets were used with carbohydrates and fat being exchanged to alter only the energetic content. The single alternation from *ad-libitum* high caloric diet to *ad libitum* low caloric diet resulted in a stable lower BW and normalization of metabolism, comparable to the BW of the continuous low fat control group [20]. Identical results were also obtained using obesity resistant A/J mice [20]. These results argue against a diet-induced BW set-point and are consistent with an alternative model for regulation of body adiposity, being the settling point model [21].

Thus, two highly controlled studies in mice have examined whether previous diet intake patterns have a lasting effect on a newly achieved BW, but resulted in different outcomes. The study designs were similar, but only in one case, and not in the other, the low caloric diet matched the composition of the high caloric diet. Differences in the type of dietary constituents, such as macronutrients or bioactive food components, can potentially influence homeostatic control of BW. For that reason, we hypothesize that the different outcomes are due to the difference in dietary constituents. Physiological changes induced by the dietary constituents, other than the caloric content, may also provide the basis for an explanation why the body would protect against BW loss in an overweight condition. To confirm that the caloric content of the diet per se does not lead to changes in BW set-point, we here re-examined the effects of previous dietary patterns on energy balance and BW using purified diets. Carbohydrates and fat were exchanged to provide energetic differences, but of identical composition, thus excluding effects of differences in dietary constituents. In addition, compared to the previous study (Parekh et al., 1998), we extended our study by examining not only one, but also two dietary alternations and included additional measurements of energy homeostasis.

MATERIALS AND METHODS

Ethical approval

All animal care and use was according to the guidelines for use and care given by the Dutch Animal Experimentation Act (1996). Permission for this study was granted by the Animal Ethical Committee of Wageningen University (DEC 2009146). Male wild-type C57BL/6JOlaHsd mice were purchased from Harlan (Horst, The Netherlands) at 8 weeks of age. After a 4 week acclimatization period to the animal facilities and a purified low fat diet (LF), mice were stratified to mean BW into a T=0wk (n=12), a LF-fed group L (n=36) and a HF-fed group H (n=84).

Animal study and diets

Access to water and food (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) was *ad libitum* and renewed on a weekly basis. BW and food

consumption were measured once a week. Animals were fed either a purified high fat diet (HF) or purified LF (table 1). Both diets contain exactly the same ingredients, and are equally supplemented with vitamins and minerals. However, the amount of carbohydrate and fat has partly been exchanged. The amount of 40en% fat in the HF is based on the average human intake across Europe [22]. Furthermore, the diets have a well-balanced fat composition, containing saturated as well as unsaturated fat. The T=0wk group was sacrificed at T=0wk for baseline measurements (n=12). At T=5wk, 12 mice of each diet group were sacrificed. Groups L and H were then each divided into two groups: half of the group remained on the consumed diet, and half changed to either LF or HF, which resulted into four groups LL, LH, HL, and HH. At T=14wk, another 12 animals per dietary group were sacrificed. Remaining mice from groups HL (n=24) and HH (n=24) were subdivided for a second time on LF or HF, which resulted in four groups: HHH, HHL, HLH, and HLL. At T=19wk, all animals were sacrificed. This study design is schematically shown in Fig. 1. Analyses are based on n=12 per dietary group, except for indirect calorimetry (n=9) and WAT histology (n=6) for which animals and samples were randomly selected within the diet group.

Table 1 Composition of purified low fat diet (LF) and high fat diet (HF).

Composition	LF	HF
Crude protein	220	267
l-cystine	3	3
Starch	386.5	172.5
Maltodextrin	100	100
dextrose	50	50
Sucrose	100	100
Cellulose	50	50
Coconut oil	6.5	31.5
Sunflower oil	36.5	178.5
Mineral premix AIN-93G	35	35
Vitamin premix AIN-93	10	10
Choline bitartrate	2.5	2.5
Cholesterol	0.03	0.097
Energy (kcal/kg)	3865	4700
Energy% from carbohydrate	66.9	36.8
Energy% from fat	10.0	40.2
Energy% from protein	23.1	23.0

Values are in g/kg, unless stated otherwise.
Energy content is adjusted for energy from vitamin mix.

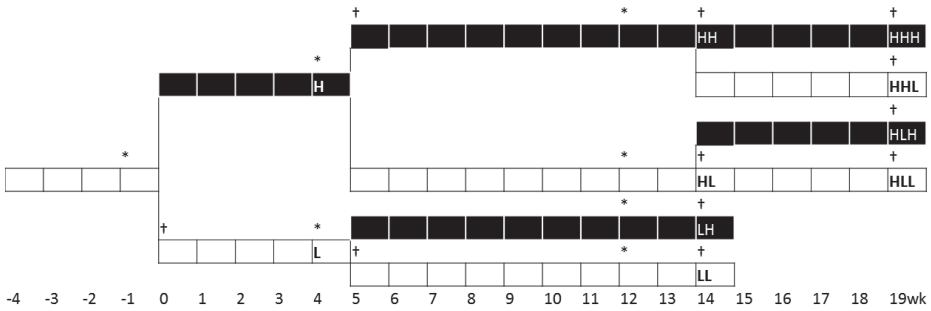

Figure 1 Study design. All mice were fed a purified low fat diet (LF) during a four week acclimatization period. Twelve mice were sacrificed at the beginning of the intervention (T=0wk), remaining mice were randomly divided into intervention groups. T=5wk, L and H, T=14wk, LL, LH, HL, and HH, and T=19wk HLL, HLH, HHL, and HHH (*n*=12/group) in which L (low fat diet, 10en% fat) and H (high fat diet, 40en%fat) indicate the order of consumed diet(s). Indirect calorimetry was performed longitudinally for the same nine mice at T=-1wk, T=4wk and T=12wk represented by an asterisk (*). Mice were sacrificed at T=0wk, T=5wk, T= 14wk, and T=19wk, represented by †.

Tissue collection

At T=0wk, T=5wk, T=14wk and T=19wk, food was removed 2h before sacrifice in the morning (light phase). Mice were anesthetized with 5% isoflurane inhalation and blood was collected in Mini collect serum tubes (Greiner Bio-one, Longwood, FL) via orbital exsanguination. After blood collection, mice were killed by cervical dislocation. Epididymal white adipose tissue (eWAT) was rapidly isolated, weighed (left depot) and one portion was snap frozen in liquid nitrogen. The other portion was fixed overnight in 4% paraformaldehyde, washed with 70% ethanol, and paraffin-embedded for histological analysis.

Indirect calorimetry

Energy expenditure (EE) was evaluated at T=-1wk, T=4wk, and T=12wk from the last 24hours data of an 48 hour measurement by an open circuit LabMaster Metabolism Research Platform (TSE systems GmbH, Bad Homburg, Germany) with airflow of 400ml/min. Mice remained in their home cage which is placed within the system in order to reduce potential stress. As our system can handle 12 mice simultaneously, subsequent sub-runs were performed with an equal number of mice from all dietary groups. We calibrated the O_2 and CO_2 sensors before each experiment with two highly defined mixtures of O_2 and CO_2. Rates of oxygen consumption (Vo_2) and carbon dioxide production (Vco_2) were monitored for 1 minute every 13 minutes for each animal or reference cage. Respiratory exchange ratio (RER) is defined as Vco_2 divided by Vo_2, and EE was calculated with the equation [3.815 + (1.232 x RER)] x Vo_2 [23]. Carbohydrate (CH_{ox}) and fat (F_{ox}) oxidation rates were calculated for every time point according to the table of Peronnet [24]. As all diets have 23en% protein content it is therefore unlikely that protein will affect RER values. Measured parameters included real-time measurements

of food and water intake. Relative cumulative frequency (RCF) curve analysis, which is a quantitative approach to enable detection of small differences in large datasets, was used to transform RER data [25] which was justified as data showed a normal Gaussian distribution; the 50th percentile equals mean 24h RER. BW was measured before and after the indirect calorimetry measurements for all mice to evaluate energy balance. Individual mice were followed longitudinally. Voluntary activity was assessed during the calorimetric assessments at T=19 weeks only, due to delivery of the equipment, and only in a subset of mice. This indicated that no significant differences in activity were present (data not shown).

Serum measurements

Serum levels of leptin and insulin were measured using the mouse serum adipokine milliplex multianalyte kit (Millipore Corporation, Billerica, MA) according to the manufacturers's protocol and analyzed using the Bio-Plex 200 system (Biorad, Veenendaal, The Netherlands). Serum glucose was determined by the GOD-PAP colorimetric assay (Roche diagnostics, Woerden, The Netherlands). Serum free fatty acids (FFA) levels were measured using the NEFA-C kit (Wako chemicals, GmbH, Neuss, Germany). Volumes were scaled down to analyze samples with a microplate reader (BioTec Synergy HT, Bad Friedrichshall, Germany). All measurements were performed in duplicate and averaged. Finally, homeostatic assessment of insulin resistance (HOMA2-IR) was calculated using fasting glucose and insulin levels as published using the excel program (http://www.dtu.ox.ac.uk/homacalculator/index.php) [26].

Histology

Paraffin embedded eWAT was sectioned at 5µm and stained by PAS-H (periodic-acid-schiff-hematoxylin) staining. Slides were coded and blind-scored by eye by two independent researchers with a raster on adipocyte size.

Statistical analysis

Data are shown as means ± SE. Significance of difference between groups is determined by analysis of variance (oneway ANOVA) in combination with a Tukey's post-hoc test using Graphpad Prism 5.04 (GraphPad Software Inc., San Diego, CA). Data were normally distributed except for HOMA2-IR. When appropriate, a two-tailed Student's *t*-test was performed when means of two groups were compared. Differences were considered significant at $P < 0.05$.

RESULTS

Diet induced differences in physiological parameters

Purified high fat diet (HF) feeding increased BW significantly from week 2 onwards compared to control low fat diet (LF) feeding (Fig. 2a). After the first diet alternation (T=5wk), body weight (BW) of group HL became significantly lower from week 7

onwards compared to groups LH and HH, and matched BW of group LL. After the second diet alternation (T=14wk), the groups appeared to be divided into two significantly different weight groups HLL and HHL vs. HLH and HHH from T=17wk onwards (Fig. 2a). There was no effect of dietary history on BW when alternated groups are compared to their non-alternated HF or LF controls (t=14wk HL vs. LL and LH vs. HH, t=19wk HLH vs. HHH and HHL vs. HLL). Changes in BW are reflected by adipose tissue mass; adiposity was modulated according to BW at all data points (Fig. 2b and 2c). This was confirmed by histological staining of the epididymal adipose tissue (Fig. 3), which showed no difference in adipocyte size between the alternated fed groups and the corresponding continuously fed group at T=14wk.

Dietary energy intake slightly increased throughout the intervention for both LF and HF diets when mice did not alternate diets (Fig. 2d). However, when animals alternated from diet at T=5wk, their dietary energy intake showed compensation before it adjusted to the energy intake of the control group. This was seen for the first dietary alternation which resulted in a week over- (HF) or under- (LF) compensation

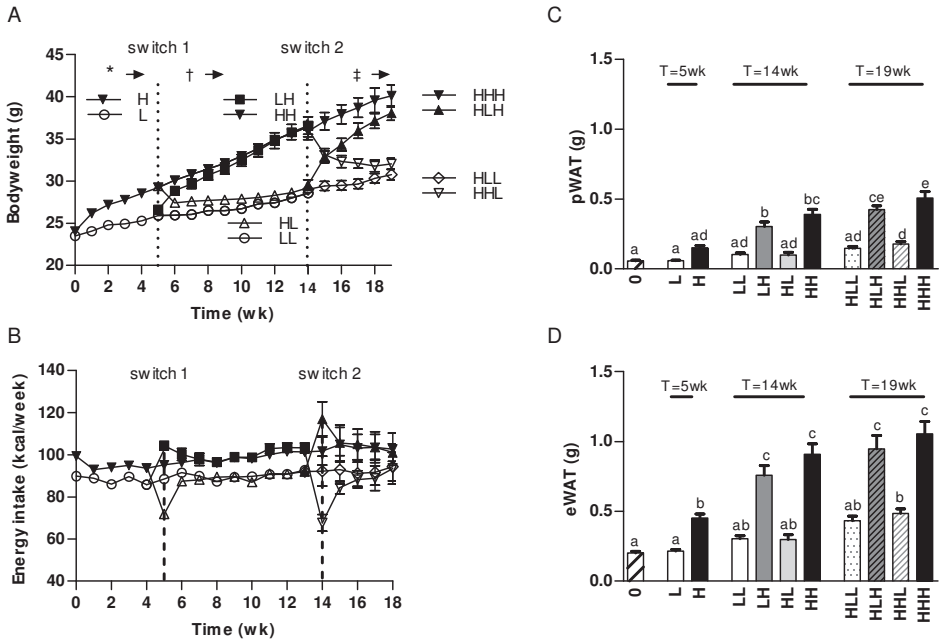

Figure 2 Animal characteristics. Body weights (a), and energy intake (b) are shown for the 19 week intervention period. At time points T=5wk and T=14wk, sub-sets of mice alternated diets (dashed line). Diet patterns are represented by letter codes L (10en% fat, low fat diet) and H (40en% fat, high fat diet; see table 1 for dietary composition). LF fed animals had significant ($p<0.05$) lower body weights than HF fed animals from week 2 onwards (*), from week 7 onwards (†) and from week 17 (‡) onwards. White adipose tissue mass of peri-renal (pWAT; c) and epididymal (eWAT; d) origin at sacrification. Different letters denote significant differences ($p<0.05$; One-Way ANOVA and Tukey's post hoc) between fat masses. Data are means ± SEM (n=12).

depending on the newly consumed diet versus those mice that continued on the same diet (Fig. 2d). This was also seen after the second dietary alternation for all diet groups: the first week after the alternation their energy intake pattern was disturbed (over- or under- compensation) and thereafter there was no difference between continuously fed and alternated fed groups of mice on either LF or HF (table 2). In general energy intake was larger than EE in all groups (table 2, 3). BW increased more in the HF fed groups although the difference in intake and EE was equal at T=4wk for L and H and at T=12wk for LL and HH.

Cumulative energy intake (CEI) was equal at T=14wk for group HL vs. group LL, and for group LH vs. group HH, although groups HL and LH had a different dietary intake history (table 2). Although diet was provided *ad libitum* to all mice, mice alternating from HF to LF (HL and HHL) did not compensate intake to match the dietary energy intake levels of the HF group. Their energy intake level was decreased to the level of the continuously LF-fed group. It appeared that based on BW patterns and CEI, mice follow a pattern related to present consumed diet. Moreover, diet history had no effect on final BW and adiposity. To substantiate this phenomenon into more detail, we analyzed several serum parameters which are indicative of metabolic health and metabolic signaling, including leptin.

Effects of diet intervention on serum metabolic parameters

Fasting serum glucose levels were not significantly different between intervention groups, although diet groups (H, LH and HH) who consumed 40en% HF tended to show higher levels at each time point. This was also observed for insulin levels (table 2), and as a result, assessment of insulin resistance using the surrogate marker

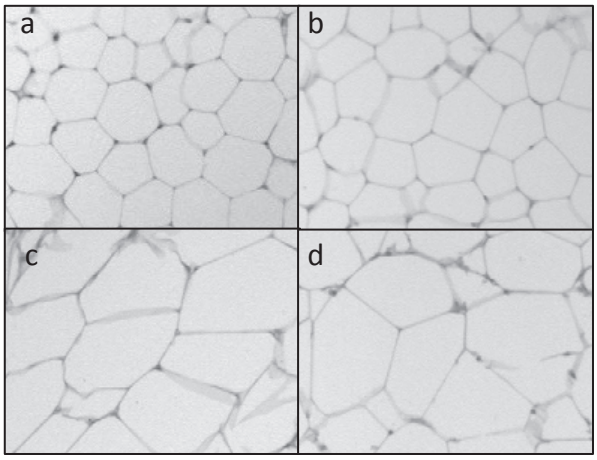

Figure 3 Epididymal adipose tissue histology. Histology of epididymal adipose tissue at T=14wk from dietary groups LL (a), HL (b), LH (c), HH (d). Tissue was sliced at 5μm and subjected to PAS-H staining. See Figure 1 for letter codes. Magnification 10x; Bar denotes 100 μm.

HOMA2-IR index increased significantly suggesting increased insulin resistance in all HF-fed groups independent of dietary history (table 2).

Leptin levels showed a significant diet dependent difference at T=5wk, T=14wk, and T=19wk. Mice fed LF had significantly lower levels of leptin than mice fed HF, independent of dietary feeding in the preceding period (table 2). BW and eWAT mass are both significantly correlated with serum leptin levels in most groups (data not shown). Fasted free fatty acid (FFA) levels were similar in all groups (table 2).

Fuel utilization

To assess whether dietary history influenced whole body energy metabolism, indirect calorimetric measurements were performed. Respiratory exchange ratio (RER) as a measure for metabolic substrate usage showed a significant difference as expected between LF and HF mice at T=4wk, with a higher carbohydrate (CHO) oxidation rate and lower fat oxidation rate for the LF-fed mice (table 3). This was also observed at T=12wk (table 3). There was no effect of time (T=4wk to T=12wk) on substrate utilization for L vs. LL groups and H vs. HH groups (data not shown). Relative cumulative frequency (RCF) curves of RER for LH and HH as well as HL and LL groups align perfectly, which fits the observation that the RER represents current dietary intake independent of dietary history (Fig. 4). Matching fuel oxidation (RER) to fuel availability is defined as a measure for metabolic flexibility [27] and in particular for the HL group this suggests that their metabolic flexibility was preserved despite previous HF consumption.

EE remained similar between all groups at T=4wk, even though the HF-fed mice showed an increased BW. At T=12wk, LF consuming groups LL and HL were equal in their mean EE, but both HF consuming groups LH and HH showed a significant increase in EE. Again, no significant difference in EE was observed between the mice that were continuously fed the same diet as compared to the mice that alternated diets (table 3). Interestingly, adiposity (Fig. 2c and 2d) showed a significant difference between LF fed groups and HF fed groups, suggesting that fat mass could be responsible for the observed difference in EE. When corrected for BW by covariance analysis, the differences in EE by diet group were no longer significant. Unfortunately, we lack carcass analyses so we cannot fully exclude a contribution of (small) differences in lean mass, if at all present.

DISCUSSION

In this study we examined whether previous purified diet intake patterns have a lasting effect on a newly achieved energy balance and BW. We studied one and two dietary alternations and observed that energy intake was dependent of the current diet and not influenced by a previous diet. BW, WAT mass and histology, serum metabolic parameters, EE and RER levels depend on the current diet, without any effect of a previous diet.

Table 2 Metabolic parameters of intervention groups.

	5wk*		14wk†				19wk			
	L	H	LL	LH	HL	HH	HLL	HLH	HHL	HHH
Energy intake (kcal/wk)	85.82±1.560*	93.59±0.966	92.70±1.394^{a}	103.3±2.351^{b}	92.01±1.043^{a}	101.4±1.063^{b}	94.29±1.944^{a}	101.0±1.205^{b}	93.56±2.186^{a}	102.7±2.220^{b}
Cumulative energy intake (kcal)	440.4±3.684*	467.6±5.003	1255±9.671^{a}	1352±22.36^{b}	1272±11.32^{a}	1349±10.27^{b}	1722±26.21^{a}	1816±21.63^{bc}	1780±21.15^{ab}	1875±27.53^{c}
Average energy intake (kcal/d)	12.64±0.08*	13.64±0.11	12.81±0.10^{a}	13.83±0.22^{b}	13.06±0.11^{b}	13.76±0.10^{c}	13.02±0.20^{a}	13.88±0.20^{b}	13.05±0.14^{a}	14.08±0.21^{b}
Glucose (mmol/L)	11.5±0.46	11.8±0.37	11.9±0.62	13.3±0.72	12.0±0.83	13.6±0.64	12.8±0.60	14.8±0.70	13.1±0.78	13.7±1.13
Insulin (ng/ml)	0.36±0.09*	1.24±0.17	1.11±0.47^{ac}	1.59±0.36^{ac}	0.51±0.17^{a}	2.15±0.44^{bc}	0.29±0.09	2.34±1.41	0.51±0.13	1.77±0.47
HOMA2-IR‡	1.4±0.4*	4.6±0.6	4.1±1.6^{a}	6.4±1.4^{a}	2.3±0.8^{ab}	8.8±1.^{6ac}	1.6±0.5^{a}	4.5±1.3^{ab}	2.1±0.5^{ab}	7.1±1.8^{bc}
Leptin (ng/ml)	2.09±0.32*	9.45±1.76	3.85±0.69^{a}	16.5±2.39^{b}	3.82±0.68^{a}	20.5±2.18^{b}	6.62±1.60^{a}	22.1±1.71^{b}	6.80±0.78^{a}	25.0±2.11^{b}
FFA (mmol/L)	0.73±0.02	0.73±0.05	0.85±0.04	0.71±0.06	0.70±0.04	0.72±0.02	0.87±0.04	0.74±0.03	0.86±0.04	0.92±0.05

* H was greater than L at T=5wk ($p<0.05$, Student's *t*-test). † Different superscript letters denote differences ($p < 0.05$) between the diet groups within one time-point T=14wk and T=19wk (One way ANOVA and Tukey's post hoc). ‡ Difference for HOMA2-IR ($p < 0.05$) was calculated by Kruskall Wallis followed by Dunn's post-hoc tests because of non-normal distribution.

Table 3 Effect of diet on whole body energy metabolism.

	4wk*		12wk†			
	L	H	LL	LH	HL	HH
Respiratory Exchange Ratio (RER)						
24h	0.948±0.006*	0.823±0.002	0.979±0.005[a]	0.869±0.002[b]	0.984±0.005[a]	0.874±0.002[b]
12h light	0.912±0.008*	0.815±0.003	0.963±0.007[a]	0.870±0.003[b]	0.972±0.008[a]	0.870±0.003[b]
12h dark	0.984±0.005*	0.832±0.003	0.995±0.005[a]	0.868±0.003[b]	0.997±0.006[a]	0.879±0.002[b]
Substrate Utilization						
CHO (%)	76.22±5.615*	39.90±2.051	80.79±4.151[a]	55.61±1.841[b]	81.93±2.983[a]	56.09±2.578[b]
FAT (%)	23.77±5.614*	60.10±2.051	17.15±3.415[a]	44.39±1.841[b]	18.07±2.983[a]	43.91±2.578[b]
Energy expenditure (kcal/h)						
24h	0.511±0.005	0.520±0.004	0.500±0.003[a]	0.563±0.004[b]	0.497±0.004[a]	0.543±0.003[c]
12h light	0.468±0.004*	0.486±0.003	0.469±0.003[a]	0.540±0.003[b]	0.471±0.004[a]	0.511±0.003[c]
12h dark	0.547±0.004	0.555±0.003	0.520±0.002[a]	0.580±0.003[b]	0.536±0.003[c]	0.565±0.002[d]
Average EE (kcal/d)	11.99±0.42*	13.04±0.21	11.99±0.42[a]	13.52±0.65[a]	11.93±0.56[a]	13.04±0.21[a]

* H was greater than L at T=4wk ($p<0.05$, student's *t*-test). † Different superscript letters denote differences ($p < 0.05$) between the diet groups within one time-point T=12wk (One way ANOVA and Tukey's post hoc). Substrate utilization (%) is calculated over 24hr based on RER-values and the table of Peronnet (Peronnet & Massicotte, 1991).

Our study, although more rigorous in the sense that we used two dietary alternations, confirmed previous findings in that no effect of dietary history was seen when mice are fed purified diets with identical ingredients. This setup minimizes possible other effects of diets, such as differences in growth due to differences in amount or composition of amino acids, micronutrients or bioactive food components. Only differences due to energy content and changed amounts of fatty acids and starch were assessed. This contrasts with the results of another study in which a purified high fat diet and a non-purified chow low fat diet were used [19, 28]. Due to the different nature of the diets it cannot be excluded that the persistent higher weight in the intervention group is due to differences in the type of ingredients, such as protein, which was substantially different, or the presence of bioactive food components such as phytoestrogens in the soy bean meal and soy oil, or to any of the other differences in the diets, such as cholesterol [29]. This further supports efforts of many groups to use identical purified diets in order to exclude unintended effects due to differences in diet composition [30, 31]. Experimental data from studies with rats also report controversial results with respect to persistency of obesity. Both persistent obesity has been reported [32, 33] as well as reversal of obesity [33, 34] sometimes even in the same report

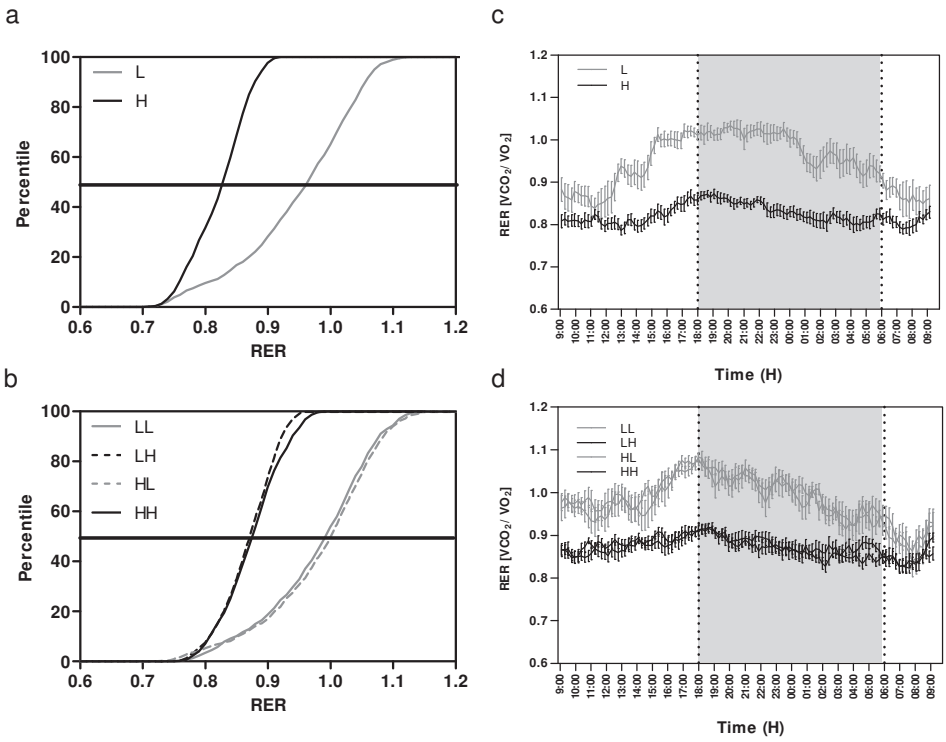

Figure 4 Respiratory exchange ratio (RER). Pooled (n=9) 24h RER data transformed into relative cumulative frequency (rcf) distributions in week 4 (a): groups L (grey line), and H (black line), and in week 12 (b): groups LL (grey line), LH (dashed black line), HL (dashed grey line), and HH (black line). See Figure 1 for letter codes. In each graph the solid line marks the 50th percentile. An asterisk indicates a significant higher mean RER for LF fed mice at T=4wk (L) and at T=12wk (LL and HL, $p<0.05$). Mean 24h RER curves at week 4 (c) and week 12 (d). The grey area represents the 12 hour dark period. At weeks 4 and 12, HF fed mice have a lower RER than LF fed mice ($p<0.05$).

using chow as control (Hill *et al.*, 1989). Our study suggests that dietary composition may have a role and it is of interest to specifically assess the role of various nutrients, in particular those that induce metabolic alterations, such as diets with saturated fats in future studies.

To analyze the effects of dietary history in more depth, we measured several serum metabolic and hormonal parameters. The lack of significant differences in time in the serum glucose, insulin, and FFA profiles of the mice may be related to the healthy fatty acid composition of the diet. The diet only contains 5% of saturated fats as compared to high fat palm oil and lard diets, which contain a high amount of saturated fat, in particular palmitic acid [35]. Saturated fatty acids are considered to be more obesogenic. This obesogenic potential increases BW gain, particularly via adipose tissue, which increases the risk of adipose tissue dysfunction. However, when we analyze our data using the HOMA2-IR index, an initial insulin resistance is

suggested in the current HF-fed groups, in agreement with expectation, independent of dietary history (table 2).

The decreased EE that is seen during weight loss may be a relative short term effect, induced by lower levels of leptin. Lowering of leptin levels is known to increase food intake, which is mediated via AMPK in the hypothalamus [36]. Injection of leptin was shown to counteract the reduced EE [37]. The effects of leptin can fit a set-point as well as a settling theory, depending on the time scale of the effects. Our data show that leptin levels in mice fed LF after a previous period of HF feeding return to levels corresponding with control (LL) mice (Table 2), and are significantly different from HF-fed mice. Dietary history did not influence this reversal of leptin levels. As an alternative model for BW regulation Flatt proposed that BW is determined by the past history of energy imbalance [38]. This idea does not explain why a body would defend an unhealthy overweight or obese state. Our data are best explained by the theoretical settling point model. This non-regulated passive model is, as is the set-point model, based on engineering control systems. The system 'settles' at a point defined by the unregulated parameter (inflow being nutritional input or outflow being EE). The level of the reservoir, here WAT mass, settles to an equilibrium determined by inflow, here diet. If we place our data into the context of this model, we observe a different settling point in BW for either LF and HF consumption, each being independent of previous dietary history. We observed a rise in EE when BW and fat mass increased, and a decrease when BW and fat mass declined as a result of different dietary intake, which follows the model. This is however not necessarily due to an increase in lean body mass as suggested by Speakman et al. [39]. It could also be just due to an increase in fat mass [40]. Nevertheless, both are sound with the expectation that EE follows weight changes [4], and moreover, that correction of EE by BW might mask true biological differences [41]. Thus, we empirically show that this settling point model is valid and that there is no effect of diet history on this equilibrium.

In conclusion, our results indicate that physiological parameters related to BW depend on the current dietary intake, with no indication of an effect of diet history in this specific time frame. A settling point is reached for each of these specific diets, LF and HF, respectively. Our data implicate that adherence to a healthy energy balanced diet will reverse a higher BW and may counteract possible harmful effects of a previous consumed unhealthy diet that provides excess energy and vice versa in C57BL/6J mice. The latter is supported by normalization of adipocyte size and number, and normalization of HOMA index. These aspects may be considered in effective BW loss and BW maintenance strategies.

ACKNOWLEDGMENTS

We thank Inge Palm, Elise Mennes, and Andries Olie of the department of Human and Animal Physiology for their helpful contributions, and everyone from the animal facility who was involved in this study.

GRANTS

FH is financial supported by WIAS Graduate School, Wageningen University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

FH, EvS and JK conception and design of research; FH, HS, SS-A, and MB-G performed experiments; FH and EvS analysed data; FH, EvS, and JK interpreted results of experiments; FH drafted manuscript, FH, EvS, and JK edited and revised manuscript; JK obtained funding, FH, HS, SS-A, MB-G, EvS, and JK approved the final version of the manuscript.

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CHAPTER 3

ADIPOSE TISSUE METABOLISM AND INFLAMMATION ARE DIFFERENTLY AFFECTED BY WEIGHT LOSS IN OBESE MICE DUE TO EITHER A HIGH FAT DIET RESTRICTION OR CHANGE TO A LOW FAT DIET

Femke P. M. Hoevenaars, Jaap Keijer, Laure Herreman, Inge Palm, Maria A. Hegeman, Hans J.M. Swarts, Evert M. van Schothorst[1]

Human and Animal Physiology, Wageningen University, Wageningen

[1]Correspondence: Evert M. van Schothorst, Human and Animal Physiology, Wageningen University, De Elst 1, PO box 338, 6700 AH Wageningen, The Netherlands, E-mail: Evert.vanschothorst@wur.nl

Accepted in adapted form in Genes and Nutrition

ABSTRACT

Restriction of a high fat diet (HFD) and a change to a low fat diet (LFD) are two interventions that were shown to promote weight loss and improve parameters of metabolic health in obesity. Examination of the biochemical and molecular responses of white adipose tissue (WAT) to these interventions has not been performed so far. Here, male C57BL/6JOlaHsd mice, harboring an intact nicotinamide nucleotide transhydrogenase gene, were fed a purified 40 energy% HFD for 14 weeks to induce obesity. Afterwards, mice were divided into three dietary groups: HFD (maintained on HFD), LFD (changed to LFD with identical ingredients), and HFD-CR (restricted to 70% of the HFD). The effects of the interventions were examined after 5 weeks. Beneficial effects were seen for both HFD-CR and LFD (compared to HFD) regarding physiological parameters (body weight and fat mass) and metabolic parameters, including circulating insulin and leptin levels. Macrophage infiltration in WAT was reduced by both interventions, although more effectively by HFD-CR. Strikingly, molecular parameters in WAT differed between HFD-CR and LFD, with increased activation of mitochondrial carbohydrate and fat metabolism in HFD-CR mice. Our results confirm that restriction of the amount of dietary intake and reduction of the dietary energy content are both effective in inducing weight loss. However, the larger decrease in WAT inflammation and increase in mitochondrial carbohydrate metabolism emphasize potential superior effectiveness of dietary restriction in weight loss strategies.

Keywords: Caloric restriction, fat restriction, nutrition, metabolic health, white adipose tissue

INTRODUCTION

Obesity would be less of a problem if it was not a major risk factor for non-communicable diseases such as cardiovascular diseases, type 2 diabetes, and some sorts of cancer [1]. Obesity is a condition of superfluous white adipose tissue (WAT) mass. Next to its function as an energy reservoir, WAT is recognized as an endocrine organ responsible for secretion of adipocytokines [2, 3]. Changes in this secretory function of adipose tissue have been associated with obesity and with dysfunction of the adipose tissue. Adipose tissue dysfunction may result in ectopic lipid deposition which is detrimental for normal tissues [4]. Together with a state of chronic low grade inflammation, an increased risk for development of non-communicable diseases is created.

To improve or even restore health in an obese condition, a negative energy balance is needed, leading to fat mass reduction and thus to weight loss. Indeed, a small weight reduction of 5% already leads to improved health outcomes [5].This can be achieved by either restriction of calorie intake maintaining the same dietary composition, or by a reduction of fat intake, that is, by changing the macronutrient composition of the consumed diet. Both have been shown to be successful in humans: caloric restriction (CR) improves glycaemic control [6] and reduction of fat intake, without intentional restriction of energy intake, induces weight loss [7]. The optimal macro nutrient composition of weight reducing diets is still under debate.

When allowed *ad libitum* access to a high fat diet (HFD), C57BL/6J mice develop insulin resistance and obesity in resemblance to disease progression in humans [8]. Like in humans, weight loss in diet induced obese rodents can be obtained with various diet intervention strategies. However, only few studies investigated this from a nutritional point of view. Reduction in body weight, fat mass, and several metabolic parameters were obtained using a standard percentage of CR, a 30% reduction, of a HFD [9], so without changing the macronutrient composition. A substantial improvement of an obese condition, reduction of body weight and improvement of insulin sensitivity, was also obtained by a reduction of the fat percentage in the diet (i.e. by a change from a HFD to a low fat diet (LFD) [10]. These studies showed that dietary energy restriction and dietary fat reduction are both effective in weight reduction in diet-induced obesity (DIO). However, a number of questions remain. In particular, it is not known how these interventions impact adipose tissue at the functional and molecular level and to which extent this differs between these intervention strategies. This information is important in the development of dietary anti-obesity interventions.

The aim of this study is to compare the effects on adipose tissue of two different body weight loss strategies; a standard 30energy% (en%) CR (i.e. energy restriction of HFD) and a change from *ad libitum* HFD to *ad libitum* LFD (i.e. restriction of fat intake). These interventions were done using purified diets containing the same ingredients, with only the amounts of carbohydrate and fat differing between the diets. C57BL/6JOlaHsd mice were used. Similar to other C57BL/6J sub-strains,

C57BL/6JOlaHsd is sensitive to DIO [8]. This sub-strain has an intact nicotinamide nucleotide transhydrogenase (*Nnt*) gene, which is in contrast to most C57BL/6J sub-strains that were used in other studies investigating weight loss by HFD restriction or a change to LFD. Intact and functional NNT was recently shown to be important for cellular redox control [11].

In this study the interventions started from an obesogenic condition, that is, after 14 weeks of HFD feeding. The effects of both interventions were examined after 5 weeks, which we expected to be at the end of the adaptation period based on previous studies [12].

We report here that a 30en% restriction of a HFD induces more pronounced effects than a change to LFD, in particular regarding WAT inflammation and expression of mitochondrial carbohydrate metabolism genes.

MATERIALS AND METHODS

Animal study and diets

Male wild-type C57BL/6JOlaHsd mice were purchased from Harlan (Horst, The Netherlands) at 8 weeks of age and were housed individually in a controlled environment (12h light/dark cycle, 55% humidity, temperature at 22°C). Access to water and food (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) was *ad libitum* and renewed every week. Mice were acclimatized to the housing conditions for four weeks on a low fat 10en% purified diet (LFD, Supplemental table 1; as published [12]). During a 14 week run-in, mice were given a 40en% high fat purified diet (HFD, Supplemental table 1; as published [12]) to induce obesity. After the run-in, 36 mice were stratified on body weight into 3 groups: HFD continued on HFD, LFD was changed to the purified LFD, and HFD-CR received 70% of the individual HFD consumption —as measured over weeks 10-12 during the run-in— on a daily basis 2 hours before the start of the dark phase. After 5 weeks of intervention at the age of 31 weeks, all mice were killed. All analyses are based on 12 mice per dietary group, except for histological analysis (n=5-6). Body weight and food intake were monitored weekly. The experimental protocol was approved by the animal welfare committee of Wageningen University, Wageningen, The Netherlands.

Tissue collection

Food was removed 2 hours before mice were killed in the morning. Mice were anesthetized by 5% isoflurane inhalation. Blood was collected in Mini collect serum tubes (Greiner Bio-one, Longwood, FL) via orbital exsanguination, and processed as published [12]. After blood collection, mice were killed by cervical dislocation. The left portion of epididymal and peri-renal WAT (eWAT and pWAT, resp.) were isolated, weighed and snap frozen in liquid nitrogen, and stored at -80°C. The right-sided portions of eWAT were fixed overnight in 4% paraformaldehyde, washed with 70% ethanol, and subsequently embedded in paraffin.

Adipose tissue morphology

Paraffin embedded eWAT was sectioned at 5µm and immunohistochemically stained to measure mean adipocyte area as published [13]. Macrophage infiltration was determined as a marker for tissue inflammation. Briefly, eWAT sections were deparaffinized and incubated with a monoclonal antibody against the macrophage marker MAC-2 (Cedarlane Laboratories Limited, Burlington, Ontario, Canada), using chemicals from Vector laboratories, Burlingame, California, United states, unless stated otherwise. Briefly, endogenous peroxidase was inactivated by incubation for 30 minutes in 1% H_2O_2 in methanol. Sections were rinsed with PBS after which they were blocked with 5% normal goat serum in PBS-BSAc (Aurion, Wageningen, the Netherlands). Sections were incubated overnight at 4°C with a monoclonal anti-MAC2 antibody (diluted 1:5000 in PBS-BSAc). Next, sections were rinsed in PBS and incubated for 60 min at room temperature (RT) with a secondary goat anti-rat biotinylated antibody diluted 1:200 in PBS BSAc, rinsed again, and incubated for 60 min at RT with Vectastain elite ABC reagent (dilution 1:1000). After rinsing, the sections were incubated for 2 min at RT with 3, 3'-diaminobenzidine solution (dilution 1:200), rinsed, counter stained with haematoxylin QS and mounted with vectamount. Cells were considered to stain positively for MAC-2 when a brown substrate was present in the cytoplasm of the cells. Tissues were examined under an Axiokop 2 light microscope (Zeiss, Göttingen, Germany) and digital images were taken using an Axiocam MRC 5 camera (Zeiss). The number of crown like structures (CLS) were counted per 1000 adipocytes per mouse and macrophage infiltration was expressed as a total number of CLS per 100 adipocytes.

Serum measurements

Serum levels of leptin and insulin were measured using the mouse serum adipokine milliplex multianalyte kit (Millipore Corporation, Billerica, MA) according to the manufacturers' protocol using the Bio-Plex 200 system (Biorad, Veenendaal, The Netherlands). Serum glucose was determined by the GOD-PAP colorimetric assay (Roche diagnostics, Woerden, The Netherlands). Serum free fatty acids (FFA) levels were measured using the NEFA-C kit (Wako chemicals, GmbH, Neuss, Germany). Volumes were scaled down to analyze samples with a micro plate reader (BioTec Synergy HT, Bad Friedrichshall, Germany). All measurements were performed in duplicate and averaged. Homeostatic assessment of insulin resistance (HOMA2-IR) was calculated using fasting glucose and insulin levels as published using the excel program (http://www.dtu.ox.ac.uk/homacalculator/index.php; [14]).

RNA/DNA isolation and cDNA synthesis

RNA isolation was performed as published [15]. All samples were purified with RNeasy columns (Qiagen) according to the manufacturers' instructions.

Total DNA was extracted from homogenized eWAT by digestion with Proteinase K in a lysis buffer (50 mM Tris-HCL, pH 7.5, 0.5% SDS and 12.5 mM EDTA, pH 8.0).

Homogenate samples were incubated overnight at 56°C. Proteinase K was inactivated by a 10-minutes incubation at 70°C. Samples were then incubated with 40 µg/ml RNAse A (Sigma-Aldrich, St. Louis, USA) for 1h at 37°C. After centrifugation, the aqueous phase was mixed and extracted with an equal amount of phenol-chloroform-isoamylacohol and twice with chloroform. DNA was precipitated by 96% ethanol and sodium acetate (3M, pH 5.2). DNA was washed with 750 µl of cold 70% ethanol, air-dried and re-suspended in 30 µl of RNAse DNAse free water. RNA/DNA concentration and purity were assessed with the Nanodrop spectrophotometer (IsoGen Life Science, Maarsen, The Netherlands). RNA integrity was checked by capillary zone electrophoresis (Experion, Bio-Rad). DNA concentration was adjusted to 40 ng/µl with RNAse DNAse free water. RNA of all individual samples was reversely transcribed using the iScript cDNA synthesis kit (Bio-Rad).

Gene expression by quantitative RT-PCR (qRT-PCR)

Differential transcript expression was assessed by qRT-PCR using iQ SYBR Green Supermix (Bio-rad) and the MyIQ single-color real-time PCR detection system (Bio-rad). A standard curve for all transcripts including reference transcripts was made using serial dilutions of a pool prepared from all cDNA samples. Relative levels of gene expression were obtained using two reference genes (Ribosomal protein s15 (*Rps15*) and Calnexin (*Canx*)) in duplicate and averaged. Target genes, primer sequences, and annealing temperatures are shown in table 1. The expression of the gene of interest was normalized against the geometrical mean of the reference genes *Rps15* and *Canx,* which were chosen based on stable gene expression levels, previous experience (i.e. Duivenvoorde, et al. 2011) and geNorm (Ghent University Hospital, Ghent, Belgium) analysis).

Mitochondrial density

Relative mitochondrial density was determined as the ratio of mitochondrial DNA to nuclear DNA, as published [16]. The mean of the control group (HFD) was set as 1.0. The primer pair's sequences used were as follows: mitochondrial DNA (forward) 5'-CCGCAAGGGAAAGATGAAAGAC-3' and (reverse) 5'-TCGTTTGGTTTCGGGGTTTC-3' [16]; nucleic DNA (forward) 5'-CTTAGAGGGACAAGTGGCGTTC and (reverse) 5'- CGCTGAGCCAGTCAGTGTAG-3' [17].

Citrate synthase activity

Snap frozen eWAT was homogenized in liquid nitrogen and diluted to a fixed protein concentration of 0.8 µg/µl in PBS-0.5% Triton X-100 buffer containing 1% protease inhibitor cocktail. Citrate synthase (CS) activity was measured spectrophotometrically using the CS Assay Kit (Sigma Aldrich) according to the manufacturers' protocol. Briefly, eWAT homogenates were transferred to 10mM DNTB (5, 5'-dithiobis-(2-nitrobenzoic acid)) and 30mM acetyl-CoA. CS activities were measured for 60 min at 412 nm at 25°C after addition of 10 mM oxaloacetate. CS activities were then

Table 1 Primer sequences and annealing temperature of primers used for quantitative RT-PCR.

Genes	Forward primer 5'-3'	Reverse primer 5'-3'	T(C°)
Canx*	GCAGCGACCTATGATTGACAACC	GCTCCAAACCAATAGCACTGAAAGG	58.5
Cpt1a	AAAGATCAATCGGACCCTAGACA	CAGCGAGTAGCGCATAGTCA	60.0
Cpt1b	ACCCCTAAGGATGCCATTCTTG	GCGGAAGCACACCAGGCAGTA	57.0
Esrra	ATCCAGGGGAGCATCGAGTA	AAAGGCAAAGGGTCCACCTC	60.0
Fasn	AGTTAGAGCAGGACAAGCCCAAG	TTCAGTGAGGCGTAGTAGACAGTG	60.0
Mpc1	GACTTTCGCCCTCTGTTGCT	GCAGATGGCCGCTTACTCAT	58.5
Mpc2	ACCTACCACCGACTCATGGA	AGTTTCTCTGCAGGTCTGGC	60.0
Lipe	TGGAACTAAGTGGACGCAAGCC	TCAAGGTATCTGTGCCCAGTAAGC	58.0
Pdha1	CTGCCTATTGCAGGTCTGGT	CTTCTCGAGTGCGGTAGCTT	60.0
Pdhb	GAAAGGCAAGGGACCCACAT	CCTCCTTCCACAGTCACGAG	60.0
Pdk1	GCTACTCAACCAGCACTCCTT	GGTCGCTCTCATGGCATTCT	60.0
Pnpla2	ACCACCCTTTCCAACATGCTACC	GCTACCCGTCTGCTCTTTCATCC	58.0
Ppargc1a	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC	60.0
Rps15*	CGGAGATGGTGGGTAGCATGG	ACGGGTTTGTAGGTGATGGAGAAC	58.5
S100a8	ACTTCGAGGAGTTCCTTGCG	TGCTACTCCTTGTGGCTGTC	58.0

* Genes denoted with an asterisk were used as reference genes.

calculated using the slope of the linear increase according to the manufacturer's protocol. The mean of the control group (HFD) was set as 1.0.

Liver triglycerides

Triglyceride kit reagent (triglycerides Liquicolor kit, Human, Wiesbaden, Germany) was used according to protocol as described [18]. Briefly, snap frozen liver was homogenized and dissolved in a 10mM Tris, 2 mM EDTA, and 0.25M sucrose buffer with pH7.5. Input was corrected for wet tissue weight.

Statistical analysis

Data are expressed as mean ± SEM; statistical analyses were performed with GraphPad Prism 5.04 (Graphpad software, San Diego, CA, USA). All measurements within the different treatment groups were checked for normality with D'Agostino & Pearson normality test. qRT-PCR data was log2 transformed before statistical analysis. Normal distributed data was analyzed by One way analysis of variance and Tuckey's multiple comparison *post hoc* analysis. Test results of serum FFA, and gene expression data of *Esrra*, *Ppargc1a*, *Mpc1*, *Mpc2*, *Pdha1*, *Pdhb*, *Pdk1* and *Fasn* were not normally distributed and therefore analyzed by non-parametric Kruskall-Wallis test and Dunn's multiple comparison *post hoc* test. $P<0.05$ was considered significant.

RESULTS

LFD and HFD-CR decreased body weight and adiposity

Body weight of DIO mice declined immediately after the change from HFD to either HFD restriction (HFD-CR) or LFD. Mean body weight stabilized after 3 weeks in LFD mice, while it further declined till the end of the 5-week intervention period in HFD-CR mice (Figure 1A). There were no differences in eWAT and pWAT weights between LFD and HFD-CR mice, but both were significantly lower than control HFD mice (Figure 1B, 1C). In contrast, weight gain/loss was significantly different between all group comparisons; HFD gained 4.2±0.5g, while LFD and HFD-CR lost 4.1±0.4g

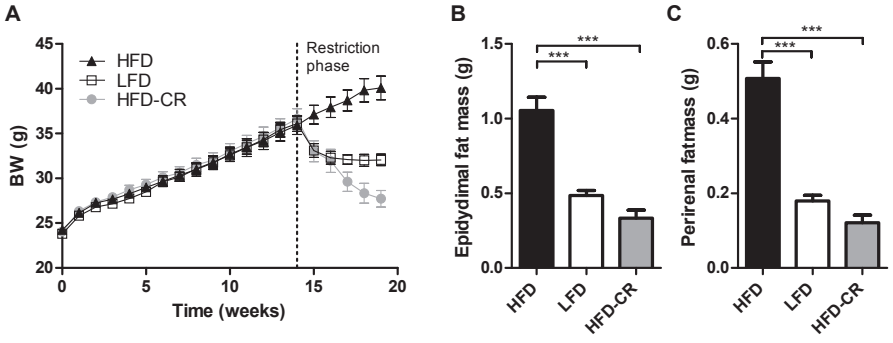

Figure 1 Changes in body weight (A) and adipose tissue mass of 14-week DIO mice, followed by a 5-week diet intervention of *ad libitum* high fat diet (40en% fat, HFD), *ad libitum* low fat diet (10en% fat, LFD) or restricted high fat diet (30% restriction of HFD, HFD-CR). B and C represent white adipose tissue mass of epididymal (B) and peri-renal (C) origin immediately post mortem. Data are means ± SEM (n=12). *** P<0.001

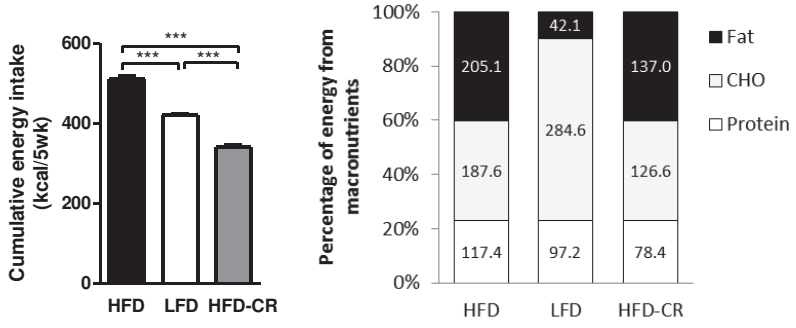

Figure 2 Cumulative energy intake (A) and percentage of energy intake from macronutrients (B, fat, carbohydrate (CHO), protein) during 5-week intervention. Numbers in bars represent corresponding energetic value (Kcal) of various macronutrients. Data are means ± SEM (n=12). *** P<0.001

and 9.0±0.5g ($p<0.0001$), respectively, due to the intervention. Cumulative food intake (Kcal) during the 5-week intervention was also different between all group comparisons (Figure 2). Although mean dietary energy intake level was the lowest in HFD-CR, the contribution of lipids to total energy availability from the diet remained clearly higher for HFD-CR mice in comparison to LFD mice (Figure 2).

Adipocyte area and number of macrophages were decreased

To quantify the effects of the dietary interventions on the morphology and inflammatory status of eWAT, adipocyte area and macrophage infiltration were investigated in accordance with weight loss. Adipocyte area was significantly reduced in LFD and HFD-CR mice in a similar manner (Figure 3A). Strikingly, inflammatory status of eWAT was considerably improved by a reduction of CLS by LFD, with an even further significant reduction by HFD-CR (Figure 3B). Representative pictures of these CLS are shown in Figure 3C.

Serum parameters

Health status was also determined by measuring serum levels of insulin, glucose, leptin, and FFA. Serum insulin levels were significantly lowered in LFD and HFD-CR (Figure 4A). Glucose levels were significantly lower for HFD-CR (Figure 4B). The

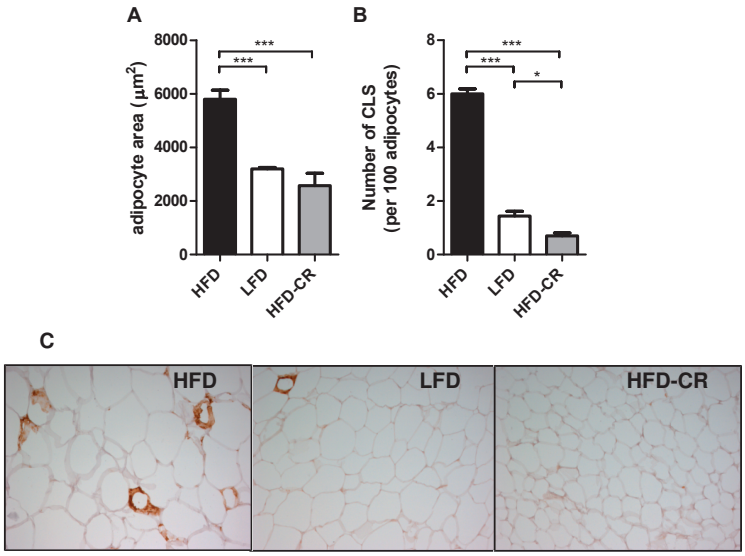

Figure 3 Adipose tissue morphology. (A) Mean adipocyte area after a 5-week diet intervention of high fat *ad libitum* diet (40en% fat, HFD), low fat *ad libitum* (10en% fat, LFD) or high fat restriction (30% restriction of HFD, HFD-CR). (B) Observed number of crown like structures (CLS) expressed per 100 adipocytes, as a measure for macrophage infiltration. (C) Representative pictures of MAC-2 stained white adipose tissue for HFD, LFD, and HFD-CR. Grey area represents dark phase. Data are means ± SEM (n=5-6). *** $P<0.001$

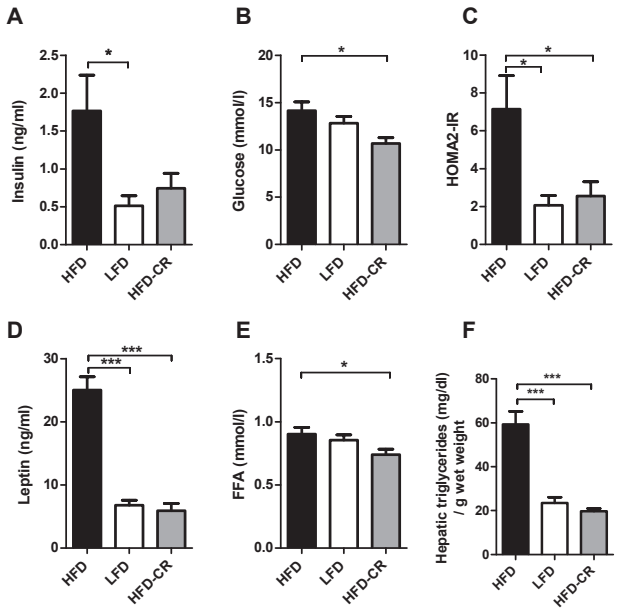

Figure 4 Serum and tissue parameters. (A) Insulin levels, (B) glucose levels, (C) calculated HOMA2-IR insulin resistance index, (D) leptin levels, and (E) free fatty acids (FFA) levels in serum, and (F) hepatic triglycerides levels of mice after a 5-week diet intervention of high fat *ad libitum* diet (40en% fat, HFD), low fat *ad libitum* (10en% fat, LFD) or high fat restriction (30% restriction of HFD, HFD-CR). Data are means ± SEM (n=12, except for liver triglycerides n=8). * P<0.05 *** P<0.001

calculated HOMA2-IR insulin resistance index showed a similar significant reduction for LFD and HFD-CR (Figure 4C) indicating a similar improvement in glucose homeostasis for the two dietary interventions. Moreover, a significant reduction was observed for serum leptin levels and FFA (Figures 4D and 4E), suggesting improvement in lipid homeostasis as well. This is supported by reduced hepatic triglycerides levels in both groups (Figure 4F).

Mitochondrial density was not affected by both interventions

Caloric restriction of HFD or a switch to LFD did, unexpectedly, not affect eWAT mitochondrial density (Figure 5A). This was also reflected by equal levels of eWAT CS activity (Figure 5B).

Gene expression in eWAT was altered mainly by HFD-CR

To investigate the molecular consequences of the dietary interventions for metabolic functions in WAT, we selected target genes for metabolism related pathways: mitochondrial biogenesis, carbohydrate metabolism, fat metabolism and inflammation. HFD-CR has been reported to increase mitochondrial metabolism [19,

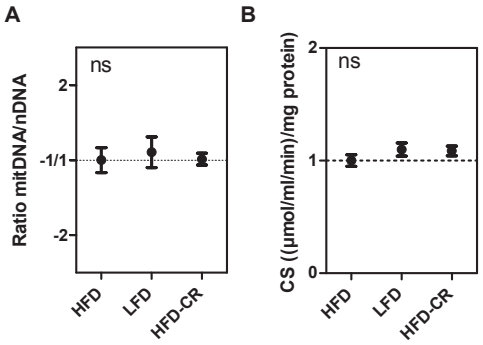

Figure 5 Mitochondrial density in eWAT after a 5-week diet intervention of high fat *ad libitum* diet (40en% fat, HFD), low fat *ad libitum* (10en% fat, LFD) or high fat restriction (30% restriction of HFD, HFD-CR) as measured by the mitochondrial DNA copy number versus the nuclear DNA by q-PCR and citrate synthase (CS) activity assay. Data are means ± SEM (n=10). Ratio of mtDNA/nDNA and CS activity levels of HFD mice was set to 1.0.

20] which is induced by estrogen related receptor alpha (*ESRRA*), which is linked to oxidative metabolism, and peroxisome proliferator activated receptor gamma co-activator 1 alpha (*PPARGC1A*), which is a master regulator of mitochondrial biogenesis [21]. We observed increased expression of *Ppargc1α* for HFD-CR and LFD (compared to HFD) with the largest increase in HFD-CR. In contrast, *Esrrα* expression levels were not significantly changed relative to the HFD control, although HFD-CR showed significantly higher expression relative to LFD (Figure 6A). Pyruvate is the end product of glycolysis and a major substrate for the tricarboxylic acid (TCA) cycle in mitochondria, which leads to ATP formation, fatty acid synthesis, or via malate to glyceroneogenesis. Recently, the genes and transcripts for the heterocomplex of mitochondrial pyruvate carrier and importer (MPC) have been identified [22]. We analyzed the nutritional transcript regulation for both isoforms *Mpc1* and *Mpc2*. Here, only a significant up regulation of *Mpc1* was found as a result of HFD-CR (Figure 6B). *Mpc2* expression levels were not significantly changed relative to the HFD control, although HFD-CR showed a higher expression relative to LFD (Figure 6B). After import into mitochondria, pyruvate needs to be converted into acetyl-CoA for use in the TCA cycle. Pyruvate dehydrogenase E1 alpha 1 (*Pdha-1)* and pyruvate dehydrogenase (lipoamide) beta (*Pdhb)* catalyze this reaction and we observed an up regulation of both *Pdha-1* and *Pdhb* in HFD-CR but not in LFD mice, compared to the HFD control. Previous studies in our lab have shown a strong up-regulation of pyruvate dehydrogenase kinase, isoenzyme 1 (*Pdk1*) as a result of long term HFD-CR [20]. In support, 5 weeks of HFD-CR also showed a significant up regulation of *Pdk1*, but not in LFD (Figure 6B). Next to carbohydrate metabolism, we investigated crucial transcripts of fat metabolism. As a measure of fat synthesis we measured fatty acid synthase (*Fasn*), which showed an up regulation upon HFD-CR only. We found no effects by either dietary intervention on mitochondrial import of fatty acids preceding

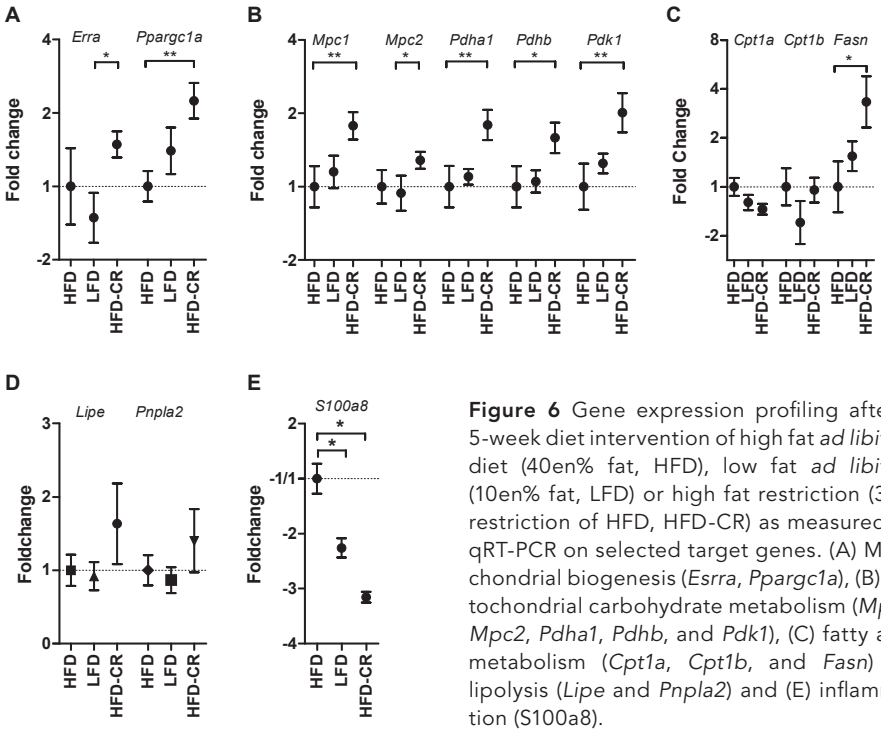

Figure 6 Gene expression profiling after a 5-week diet intervention of high fat *ad libitum* diet (40en% fat, HFD), low fat *ad libitum* (10en% fat, LFD) or high fat restriction (30% restriction of HFD, HFD-CR) as measured by qRT-PCR on selected target genes. (A) Mitochondrial biogenesis (*Esrra*, *Ppargc1a*), (B) mitochondrial carbohydrate metabolism (*Mpc1*, *Mpc2*, *Pdha1*, *Pdhb*, and *Pdk1*), (C) fatty acid metabolism (*Cpt1a*, *Cpt1b*, and *Fasn*) (D) lipolysis (*Lipe* and *Pnpla2*) and (E) inflammation (S100a8).

mitochondrial fatty acid oxidation, assessed by carnitine palmitoyl transferase 1 alpha and beta (*Cpt1a* and *Cpt1b*, resp.) gene expression levels (Figure 6C).

In adipose tissue the rate limiting enzyme for lipolysis is encoded by adipose triglyceride lipase, which officially is named patatin-like phospholipase domain containing 2 (*Atgl*/*Pnpla2*). Its expression is regulated by nutritional status and is associated with rates of adipose tissue lipolysis. Levels of hormone sensitive lipase (*Hsl*/*Lipe*) are known not to be regulated by nutritional status and are decreased in acute fasting and increase after prolonged restriction [23]. Here, assessing *Atgl* as well as *Hsl* expression, we show that lipolysis is not affected, although there seems to be a tendency for increased transcript levels in the HFD-CR mice as compared to the LFD mice (Figure 6D).

S100 calcium binding protein a8 (*S100a8*) transcript levels were investigated as a marker for inflammatory status of the adipose tissue. We observed a reduction by both weight loss strategies LFD and HFD-CR (Figure 6E).

DISCUSSION

In this study, a 14 week period of high fat feeding was used to induce DIO in male C57BL/6JOlaHsd mice. From this starting point, we intervened with a standard

percentage of restriction (30%) of intake of a purified high fat diet (40en% fat, HFD-CR) or with a change to a purified low fat diet (10en% fat, LFD). These interventions strategies were applied for 5 weeks to mediate weight loss and improve health. Our findings confirmed the beneficial effects of both strategies, HFD-CR and LFD, with regards to physiological (body weight and adiposity) and metabolic parameters (insulin, HOMA2-IR, leptin, and FFA). Macrophage infiltration in eWAT was reduced by both interventions, but, remarkably, more by HFD-CR, whereas the reduction in adipocyte size is comparable. Similarly, eWAT expression of in particular mitochondrial carbohydrate metabolism genes was more affected by HFD-CR than by LFD.

Recent studies have shown that adipose tissue inflammation plays an important role in the pathogenesis of obesity as reviewed by Goossens [24]. As there was no statistical difference in fat mass and insulin resistance between HFD-CR and LFD mice, we expected to find no differences in eWAT infiltration by macrophages. However, we noticed that there was a higher reduction of CLS by HFD-CR compared to LFD which corresponded with reduced transcript levels of the inflammatory marker *S100a8*. This confirms the findings of Wang *et al.* who showed a reduction by HFD-CR of chemokines CCL2/monocyte chemoattractant protein-1 (*MCP-1*) and CXCL2/macrophage inflammatory protein-2 (*MIP -2*) mRNA expression levels (which act on monocytes and neutrophil granulocytes respectively), and CD11c+/CD11b+ cells (marker for activated macrophages or dendritic cells), independent of body weight [25].

Despite the same amount of fat mass after the restriction period, HFD-CR mice did not only display a stronger reduction in macrophage infiltration, but also had a more prominent regulation of mitochondrial biogenesis, lipid synthesis and, especially, carbohydrate metabolism. HFD-CR did not result in a change in intake of a specific macronutrient, but rather reduced overall macronutrient intake, resulting in a lower energy intake. The change to LFD on the other hand changed macronutrient intake and concomitantly lowered energy intake. While carbohydrate intake was higher in the LFD than in HFD-CR (and in HFD for that matter), fat intake was lower (Figure 2). These data strongly suggests that in particular the lowering of energy intake, and not so much the lowering of fat intake, is of primary importance to obtain beneficial metabolic effects of a diet intervention. Our results however, contrast with previous studies showing that when HFD is restricted to the caloric intake of LFD fed mice, insulin resistance and hepatic steatosis are not prevented [26, 27]. The lack of more pronounced effects of the LFD may be due to a stronger decrease in energy intake during HFD restriction compared to the LFD (even though fat intake was higher in HFD-CR).

Alternatively, differences between the previous studies and our study may be explained by the use of different C57BL/6J sub-strains; with (our study) or without a functional Nnt gene. Although all C57BL/6J mice are known for their susceptibility to DIO [28], differences in macronutrient handling are likely to exist between different sub-strains in view of the role of NNT in, for example, the TCA cycle [29] and insulin release from pancreatic beta-cells [30].

A major difference in feeding regimen is whether the animals eat *ad libitum* (LFD mice) or whether they receive their food as a restricted single portion (HFD-CR mice). Feed was provided to the HFD-CR mice once a day prior to the dark phase. The mice consumed their portion very rapidly and were subsequently 'fasted' until the next feeding time. This is supported by indirect calorimetry data showing that restricted mice shift to a cyclic pattern in which the initial phase shows elevated fatty acid synthesis (RER > 1.1), followed by prolonged fatty acid oxidation (RER = 0.7) as observed by ourselves (Supplemental Figure 1) and others [31].

Another possibility is that the rate of restriction or the difference in protein intake is responsible for the differences found between LFD and HFD-CR. In a recent study with rats it was shown that energy restriction, but not diet composition and protein level, impacted weight loss and adiposity [32]. In humans, *ad libitum* access to food with a variety in the macronutrient composition promoted dietary adherence during weight loss and maintenance [33]. Energy restriction, however, has shown consistently to produce weight loss and beneficial health effects. Extrapolation of our results from mice to the human situation remains difficult as we do not really mimic free living conditions in this experiment (i.e. only one kind of diet is available).

The long fasting period due to the feeding regimen is further substantiated by decreased serum levels of FFA (Figure 4E), which have shown to be correlated to the rate of lipolysis and fatty acid flux in adipose tissue [34]. Altogether these findings suggest that food restriction forces the animals into a highly dynamic metabolic pattern, which may contribute to increased metabolic flexibility, possibly contributing to the enhanced health effects in the HFD-CR regimen.

The expression profile of eWAT showed enhanced mitochondrial carbohydrate metabolism as well as increased lipogenesis (Fasn). It is striking that the two intervention groups are not regulated in a similar way, even though LFD mice are in the same physiological state as HFD-CR mice. Our molecular profiles showed no effects on mitochondrial fatty acid beta oxidation as carnitine palmitoyl transferase transcripts were not regulated. But we do see an increase in *Fasn* regulation, indicative of fatty acid biosynthesis. Together with the increase in carbohydrate metabolism this may suggest a shift to metabolism of carbohydrates, possibly to prevent energy depletion from WAT. Alternatively, it may be associated with a higher turnover of triglycerides in WAT as has also been observed after long term HFD-CR (Duivenvoorde et al., 2011).

PPARGC1A is considered to be the master regulator of mitochondrial biogenesis. A strong body of evidence shows that increased expression of *Ppargc1a* is associated with increased mitochondrial density in response to calorie restriction [20, 35, 36]. Here, we show a strong up-regulation of *Ppargc1α* as a response to HFD-CR. However, we do not see this reflected in levels of mitochondria as measured by ratio of mitochondrial to nuclear DNA nor by CS activity. This could indicate that *PPARGC1A* is stimulated via a different regulation pathway. Alternatively, the absence of an increased mitochondrial density may be due to the time-frame of the current study

and it may be speculated that increased mitochondrial density will follow changes in *Ppargc1a* and will become evident only after long term HFD-CR. Although most papers show a strong correlation between measurements of mitochondrial density in WAT, one recent study reported a lack of association between several measured mitochondrial parameters (*Cyt c*, *COXIV*, *Core 1*, *ATPS*, and *Ppargc1a*) in several organs, including WAT upon (LFD) CR in Wistar rats [37]. To be able to interpret markers of mitochondrial density, it is important to understand the relation between these markers in time.

In summary, using a mouse model of DIO, we demonstrated differential effects of LFD and HFD-CR weight loss strategies on inflammatory status of eWAT and on molecular mitochondrial carbohydrate and lipid metabolism of eWAT. From a physiological point of view, it seems that there is little difference in response between LFD and HFD-CR as both interventions mediate a more healthy profile. However, with a focus on molecular regulation, HFD-CR showed a more evident response by increasing both fat and carbohydrate metabolism, together with a more pronounced reduction in eWAT inflammation. Therefore, HFD-CR could be marked as a more effective approach.

ACKNOWLEDGEMENTS

We kindly acknowledge the extensive help of the members of Human and Animal Physiology with tissue harvesting at the necropsy, and everyone at the animal facility who was involved in this study. FH was supported by funding from graduate school WIAS (Wageningen Institute for Animal Science).

CONFLICTS OF INTEREST

The authors have declared no conflicts of interest

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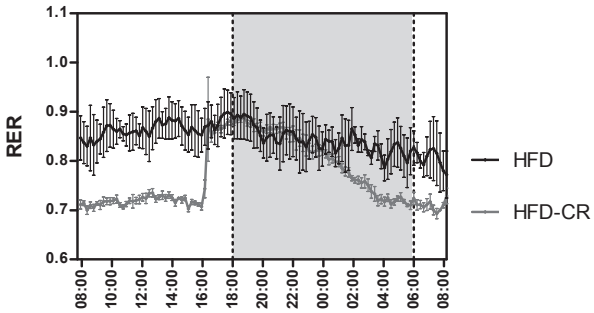
Supplemental table 1 Diet composition.

Component	LFD (g/kg feed)	HFD (g/kg feed)	AL intake LFD 1X	Al intake HFD 1x	HFD-CR 0.7x
Crude protein	*220*	267	*220*	267	154
L-cysteine	3	3	3	3	2.1
Starch	386.5	172.5	386.5	172.5	120.8
Maltodextrin	100	100	100	100	70
Dextrose	50	50	50	50	35
Sucrose	100	100	100	100	70
Cellulose	*50*	50	*50*	50	35
Coconut oil	6.5	31.5	6.5	31.5	22.1
Sunflower oil	36.5	178.5	36.5	178.5	125.0
Mineral premix AIN-93G	35	35	35	35	24.5
Vitamin premix AIN-93	10	10	10	10	7
Choline bitartrate	2.5	2.5	2.5	2.5	1.8
Cholesterol	0.03	0.097	0.03	0.097	0.068
Energy (kcal/kg)	3865	4700			
Energy% from carbohydrate	66.9	36.8			36.8
Energy% from fat	10.0	40.2			40.2
Energy% from protein	23.1	23.0			23.0



Supplemental figure 1 Respiratory exchange ratio (RER) of 14-week DIO mice, followed by a 5-weeks continuation of *ad libitum* high fat diet (40en% fat, HFD), or a 5-weeks restricted high fat diet (30% restriction of HFD, HFD-CR). Data are means ± SEM (n=3-6).

CHAPTER 4

THERMONEUTRALITY RESULTS IN PROMINENT DIET-INDUCED BODY WEIGHT DIFFERENCES IN C57BL/6J MICE, NOT PARALLELED BY DIET INDUCED METABOLIC DIFFERENCES

Femke P.M. Hoevenaars[1,4], Melissa Bekkenkamp-Grovenstein[1,2,4], Rolf J.R.J. Janssen[1], Sandra G. Heil[1,2], Annelies Bunschoten[1,2], Elise F. Hoek-van den Hil[1,3], Sophie Snaas-Alders[1], Katja Teerds[1], Evert M. van Schothorst[1], Jaap Keijer[1,2,5]

[1]Human and Animal Physiology, Wageningen University, Wageningen, and [2]TI-Food and Nutrition, Wageningen, The Netherlands. [3]Toxicology, Wageningen University, Wageningen, [4]equal contribution.

[5]Correspondence: Jaap Keijer, Human and Animal Physiology, Wageningen University, De Elst 1, PO box 338, 6700 AH Wageningen, The Netherlands, E-mail: jaap.keijer@wur.nl

Published in: Molecular Nutrition and Food Research (2013) DOI10.1002/mnfr.201300285 [Epub ahead of print]

ABSTRACT

Scope

Mice are usually housed at 20°C to 24°C. At thermoneutrality (28°C) larger diet-induced differences in obesity leading to increased differences in metabolic health parameters are expected. This is tested.

Methods

We performed a 14 week dietary intervention in C57BL/6J mice at 28°C and assessed adiposity and metabolic health parameters for a semi-purified low fat (10 energy %) diet (LFD) and a moderate high fat (30 energy%) diet (MHFD).

Results

A large and significant diet-induced differential increase in body weight, adipose tissue mass, adipocyte size, serum leptin level, and, to some extent, cholesterol level was observed. No adipose tissue inflammation was seen. No differential effect of the diets on serum glucose, free fatty acids, triacylglycerides (TAG), insulin, adiponectin, resistin, PAI-1, MMP-9, sVCAM-1, sICAM-1, sE-selectin, IL-6, ApoE, fibrinogen levels or HOMA index was observed. Also in muscle no differential effect on mitochondrial density, mitochondrial respiratory control ratio or mRNA expression of metabolic genes was found. Finally, in liver no differential effect on weight, TAG level, aconitase / citrate synthase activity ratio was seen.

Conclusions

LFD and MHFD induce prominent body weight differences at thermoneutrality, which is not paralleled by metabolic differences. Our data rather suggest that thermoneutrality alters metabolic homeostasis.

Keywords: obesity, metabolic health, adipose tissue function, biomarkers, serum parameters.

INTRODUCTION

Obesity is a major societal and health care problem that is still increasing [1]. Obesity is strongly associated with a number of multifactorial chronic diseases, including type II diabetes mellitus, cardiovascular diseases (CVD) and even several types of cancer. Obesity predisposes to these chronic diseases, but the relation is not absolute. Indeed, healthy obese individuals, without type II diabetes and CVD, exist. Similarly, a substantial number of cardiometabolic patients are lean [2, 3]. This suggests modulating effects of environmental and genetic factors.

Obesity is the consequence of chronic excess energy intake relative to energy expenditure. The excess energy is stored as tri-glycerides (TAG) in white adipose tissue (WAT). The main determinants of energy expenditure are maintenance of the organism (determining the basic metabolic rate), heat production to maintain body temperature (thermogenesis) and the energy that is used for different activities, such as physical activity and digestion of food [4]. The amount of energy that is used for thermogenesis is strongly dependent on the environmental temperature and decreases with increasing temperature until the lower critical temperature, where the thermoneutral window starts [5]. At thermoneutrality, the energy that is required to maintain a normal body temperature is at its lowest. Thermoneutrality will therefore increase the propensity to become obese. Conversely, cold exposure can reduce obesity [6]. Although it is clear that thermoneutrality will increase obesity, the metabolic consequences of temperature on obesity development are less clear and the interaction between thermoneutrality and diet has hardly been investigated.

To unravel the relation between nutrition and obesity and its vascular and metabolic co-morbidities, mouse models are widely used. One of these is C57BL/6J, a mouse strain that, like humans, is sensitive to diet-induced obesity. Indeed, on high fat/high carbohydrate 'Western' diets C57BL/6J mice accumulate an excess amount of WAT [7]. They gradually develop insulin resistance, mitochondrial dysfunction, hepatosteatosis and at a later stage, systemic inflammation. This results in a phenotype that is similar to humans with metabolic syndrome, although in many ways the mouse phenotype seems milder. Mice have their lower critical temperature at 28°C ([5] and references therein), but are usually housed at temperatures between 20°C and 24°C. At these housing temperatures a substantial part of the ingested energy is used for thermogenesis to keep up body temperature. This may mask, for example, bioenergetics aspects of weight reduction [8]. It also influences diet-induced obesity, since C57Bl/6J mice show a larger difference in adiposity between a high fat diet and a low fat diet at thermoneutrality compared to the difference in adiposity that was seen at a normal housing temperature [9]. If this increased differential effect of metabolism of diet on the development of obesity would be accompanied by pronounced differential effects on metabolic parameters related to insulin resistance and CVD, it would allow to more sensitively examine the relationship between diet, obesity, insulin resistance and vascular disease.

This would facilitate more sensitive detection of biomarkers that could be used to substantiate health claims.

To investigate whether housing at thermoneutrality will not only result in large diet-induced differences in adiposity and but also in pronounced differences in associated metabolic parameters in serum and the major metabolic organs, adipose tissue, liver and muscle, we performed a dietary intervention in C57BL/6J mice at 28°C and assessed energy metabolism, serum and metabolic parameters for a low fat (10 energy% (en%)) diet (LFD) and a moderate-high fat (30 en%) diet (MHFD). For the dietary intervention we used semi-purified AIN93-based diets with a fat composition that is based on the average human fat intake in The Netherlands, therefore containing both saturated and unsaturated fats [10]. Thirty en% fat is the average human fat intake, while 10 en% is standard for a mouse diet. We observed large differences in WAT mass, adipocyte size and leptin levels, but hardly any differences in a large number of other metabolic parameters in serum, WAT, liver and muscle.

MATERIALS AND METHODS

Animals, diets, study design

Ten-week-old wild-type male C57BL/6JOlaHsd mice (Harlan, Horst, The Netherlands), were individually housed at 28°C, humidity 40-55%,12h light-dark cycle (7.00 h lights on) with *ad libitum* food and water. The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC number 2008127). The mice were fed AIN93 based semisynthetic diets (Table S1), differing in the amount of fat (replacing carbohydrates); 10 en% (low fat diet; LFD) versus 30 en% (moderate-high fat diet; MHFD). Twelve animals were sacrificed at t=0 wks (at the time of diet switch, but after 3 weeks at 28°C) and another 12 per dietary group at t=14 wks. Mitochondrial respiration analysis was performed at t=14 weeks in LFD and MHFD groups of 6 animals that were run in parallel. Further details in supplementary materials and methods (MM).

Indirect Calorimetry

O_2 consumption, CO_2 production, food, and water intake were measured as described before [11]. Details in supplementary MM.

Sacrifice

From 08:00 h onwards, mice were fasted for 2 hours and killed after isoflurane anesthesia (4%). Blood collection was performed by orbital exsanguination and serum was prepared in serum collection tubes (Greiner Bio one B.V., Alphen aan de Rijn, The Netherlands). Organs were isolated, weighed and either immediately snap-frozen in liquid nitrogen and stored at -80°C untill analyses or placed in 4% buffered paraformaldehyde for 24h. Details in supplementary MM. For respirometry measurements, mice were sacrificed by cervical dislocation and the entire leg

muscle was dissected from both hind limbs. The tissue was further processed and mitochondria were isolated as described [12].

Histological analyses

Paraffin-embedded eWAT was sectioned at 5µm and stained with Periodic Acid Schiff reagent and counterstained with haematoxylin. Average adipocyte size was determined of ≥ 400 adipocytes per animal in at least two different sections using Zeiss Axiovision software (release 4.7). Fixed hepatic sections were stained with Oil red O (Sigma) for neutral lipids and quantified as described [13]. Details in supplementary MM.

High-resolution respirometry

Freshly isolated leg muscle mitochondria were subjected to high-resolution respirometry using an Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria). Details in supplementary MM.

Serum parameters

Serum levels were measured in duplicate using commercial kits for glucose (GOD-PAP kit (Roche, Woerden, The Netherlands)), free fatty acids (FFA, NEFA HR(2) kit (Wako Chemicals GmbH, Neuss, Germany)), glycerol (glycerol kit (Instruchemie, Delfzijl, the Netherlands)), triacylglyceride (TAG; Triacylglyceride liquicolor kit, Human GmbH, Wiesbaden, Germany), HDL-cholesterol (HDL-Cholesterol kit, Human GmbH) and total-cholesterol levels (total-cholesterol liquicolor kit, Human GmbH). Hepatic TAG were measured in triplicate after tissue homogenizatrion and dilution (Triacylglyceride liquicolor kit, Human GmbH). Serum levels of leptin, resistin, insulin, monocyte chemoattractant protein-1 (MCP-1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and plasminogen activator inhibitor-1 (PAI-1), soluble vascular cell adhesion molecule 1 (sVCAM-1; CD106), soluble intercellular adhesion molecule 1 (sICAM-1, CD54), soluble E-selectin, (sE-selectin) and matrix metallopeptidase 9 (MMP9), apolipoprotein E (ApoE), fibrinogen, and adiponectin were measured in duplicate with mouse serum kits (Millipore corporation, Billerica, USA) according to the manufacturer's protocol using the Bio-plex 200 system with Bio-plex manager software (Biorad Laboratories, Veenendaal, The Netherlands).

ADDITIONAL INFORMATION IN SUPPLEMENTARY MM.

Mitochondrial DNA quantification

Quantitative real time RT-PCR was performed with primers as described [14] and IQ SYBR Green supermix in an IQ5 (Biorad) with a program of 3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 45 seconds at 60°C. Data are expressed as amount of mitochondrial DNA over nuclear DNA, with the mean of t=0 group set at 1.00. Additional information in supplementary MM.

Activity of mitochondrial enzymes

Aconitase activity was determined essentially as described [15]. Citrate synthase is measured using the Citrate Synthase assay kit (Sigma-Aldrich, St. Louis, USA). Details in supplementary MM.

Real-time quantitative polymerase chain reaction (Q-PCR)

RNA isolation from muscle and gene expression analysis were performed as described [16]. Details in supplementary MM.

Statistics

Data are shown as mean ± SEM. Statistical analyses were performed using Graphpad Prism v5.04 (Graphpad Software Inc., San Diego, CA, USA). Data were checked for normality using the Shapiro-Wilk test. The qRT-PCR data were log2 transformed and tested. Statistically significant differences were analyzed by analysis of variance (ANOVA) and post-hoc Tukey tests, or with Student's unpaired *t*-test if means of 2 groups were compared. $P < 0.05$ was considered significant.

RESULTS

Mice were housed at 28°C and were fed for a 14 weeks (wks) intervention period either a low fat diet (LFD) or a moderate-high fat diet (MHFD), resulting in a prominent weight increase in both groups (Figure 1A) and gave rise to a significant difference in body weight gain (Figure 1B). Indeed, from week 11 on mean body weights differed significantly between the dietary groups. It should be noted that the mean body weight increase on the LFD at 28°C was already more than the mean body weight increase on the MHFD at 21°C (body weight of the same strain of mice at 21°C on MHFD increased with 3.5 ± 0.4 g between t=0 wks and t =15 wks; data from [17]). Energy intake was significantly 4.4% higher on the MHFD (Figure 1C), which most likely explains the significant difference in weight gain (Figure 1B). As expected, the difference in macronutrient composition between the diets (LFD vs MHFD) resulted in a significant difference in the respiratory exchange ratio (RER, Figure S1A), with the RER 50th percentile of relative cumulative frequency over 24 hours being 0.92 for the LFD and 0.86 for the MHFD (Figure S1B). Assuming that there was no difference in protein metabolism between the diets, this constitutes a significant change from a mean 75% glucose and 25% fat oxidation on the LFD to 58% glucose and 42% fat oxidation on the MHFD, showing that the diets enforced a different metabolic substrate use.

The increased body weight is matched by a significantly increased amount of adipose tissue, as shown for the epididymal fat pad (Figure 1D). Absolute liver weight is increased with time, but no difference is seen between the LFD and the MHFD (Figure 1E). Not only the amount of WAT is increased, also the mean size of adipocytes is significantly increased in the MHFD group compared to the LFD

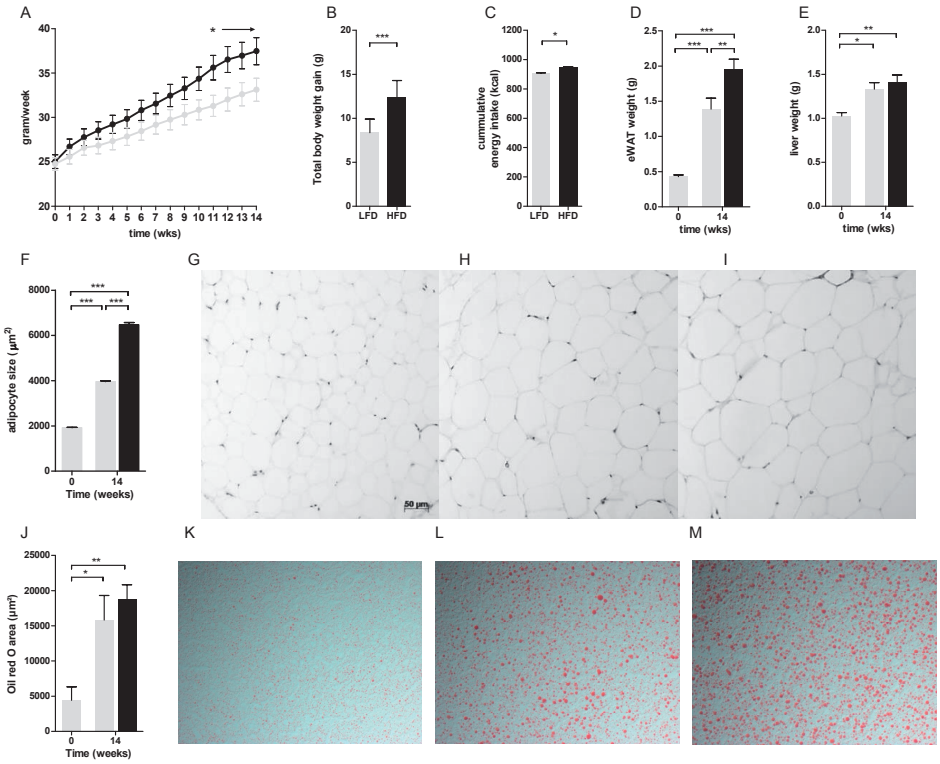


Figure 1 Mean body weight with time (A). Total body weight gain (at 14 weeks (wks) compared to t=0) (B), cumulative (t=0 to t=14wks) energy intake (C), mean epididymal white adipose tissue (eWAT) weight (D), and mean liver weight (E) of C57BL/6J mice kept at 28°C on low fat diet (LFD, grey) and on moderately high fat diet (MHFD, black). *: p<0.05, **: p<0.01, ***: p<0.001. Data are shown as mean ± SEM (n=12). Mean epididymal white adipocyte size (t=0: n=4; t=14wks LFD: n=4; t=14wks MHFD: n=4) (F) Representative images of epididymal white adipose tissue (G-I) at t=0 LFD (G), t=14wks LFD (H) and t=14wks MHFD (I). Bar is 50µm and G-I are at equal magnification. Mean hepatic lipid droplet area (t=0: n=4; t=14wks LFD: n=4; t=14wks MHFD: n=8) (J). Representative images of liver at t=0 (K), at t=14wks on LFD (L) and at t=14wks on MHFD (M) stained for lipid droplets.

group (Figure 2). Epididymal WAT was further stained for macrophage infiltration to examine adipose tissue inflammation, which was expected in view of the increased adipose tissue mass and adipocyte size. However, no signs of inflammation and no visual differences between the LFD and the MHFD groups could be observed in the number of MAC positive cells at t=14wks, or when either of these groups is compared to t=0 LFD. In all cases MAC positive cells were rarely seen (data not shown).

After establishing that the LFD and the MHFD impose substantial differences in adiposity and metabolic substrate use, we assessed whether this would be reflected by substantial differences in vascular and metabolic disease-risk biomarkers. Therefore, serum levels of various adipokines and proteins associated

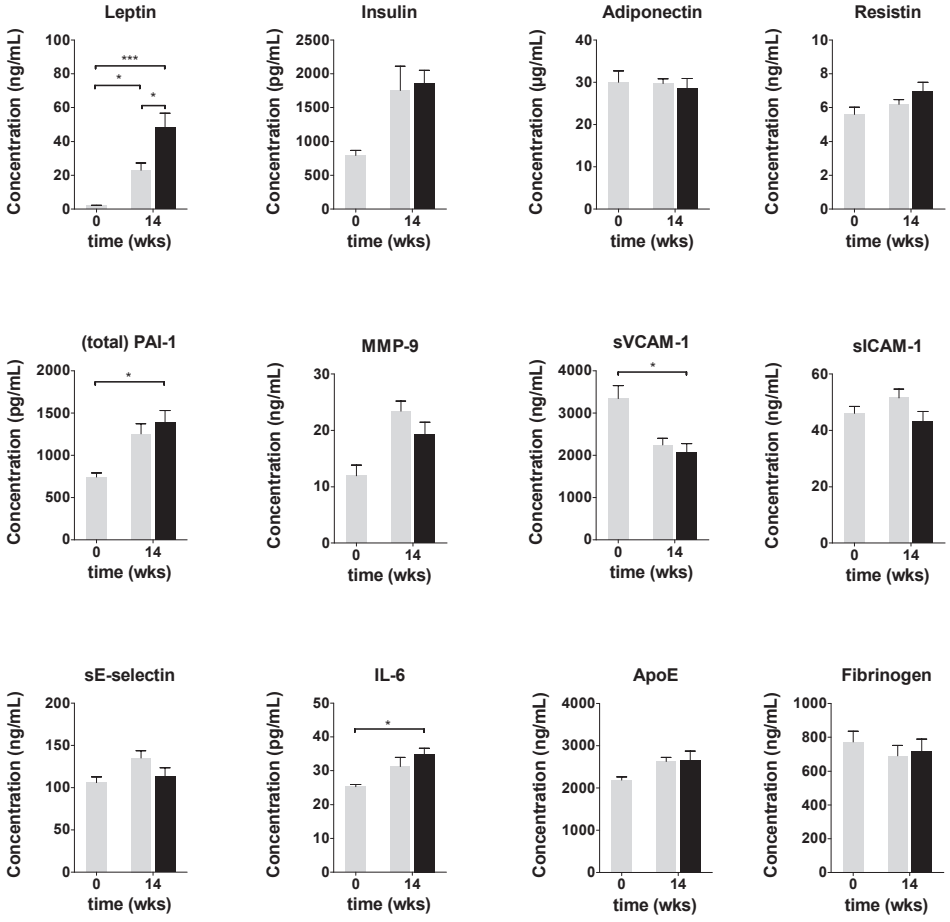


Figure 2 Mean serum peptide parameters of C57BL/6J mice kept at 28°C, at t=0, at t=14wks on a low fat diet (LFD, grey; n=12) and at t=14wks on a moderately-high fat diet (MHFD, black; n=12). *: $p<0.05$; ***: $p<0.001$; only significant differences are indicated. Data are shown as mean ± SEM.

with endothelial activation/cardiovascular risk or inflammation were analyzed and as expected, a significant difference between the LFD and the MHFD at t=14wks is seen for leptin levels (Figure 3), reflecting the differences in adiposity. Contrary to expectations, no difference between LFD and MHFD was seen at t=14wks for any of the other serum proteins that were analyzed; we observed no difference in the serum levels of insulin, adiponectin, resistin, plasminogen activator inhibitor-1 (PAI-1), matrix metallopeptidase 9 (MMP-9), soluble vascular cell adhesion molecule 1 (sVCAM-1; CD106), soluble intercellular adhesion molecule 1 (sICAM-1, CD54), soluble E-selectin, (sE-selectin), interleukin-6 (IL-6), apolipoprotein E (ApoE) or fibrinogen (Figure 3). The levels of MCP-1 and tumor necrosis factor alpha (TNFα)

were below the detection limit. The levels of these parameters were also compared to t=0, i.e. analyzed with time. For mice on the MHFD, sVCAM was significantly decreased, while an increase was observed for leptin, PAI-1 and IL-6. Leptin was also increased in mice fed the LFD (Figure 3). Levels of IL-6 and MMP-9 were and stayed very close to the detection limit, while MCP-1 and TNFα remained below the detection limit, which indicates absence of inflammation in the animals, in agreement with the histological data of WAT.

Little difference in serum adipokines and proteins associated with cardiovascular and metabolic risk were seen between the LFD and the MHFD. We therefore examined the effect of thermoneutrality on serum parameters of metabolism, i.e. glucose and lipids intermediates, which are associated with cardiovascular and metabolic risk. There was however no difference in serum glucose levels between LFD and MHFD, nor did we observe a change with time (Figure 4A). The calculated HOMA2-IR index, a measure indicative for insulin resistance, did not differ between the LFD and the MHFD at t=14wks, but is significantly higher than at t=0, due to a 40% increase in insulin levels between these time points (Figure 4B). No difference in FFA levels between the LFD and the MHFD groups at t=14wks was seen, with slightly, but non significantly, lower levels compared to t=0 (Figure 4C). There is no difference in TAG levels between the LFD and the MHFD groups and neither with time (Figure 4D).

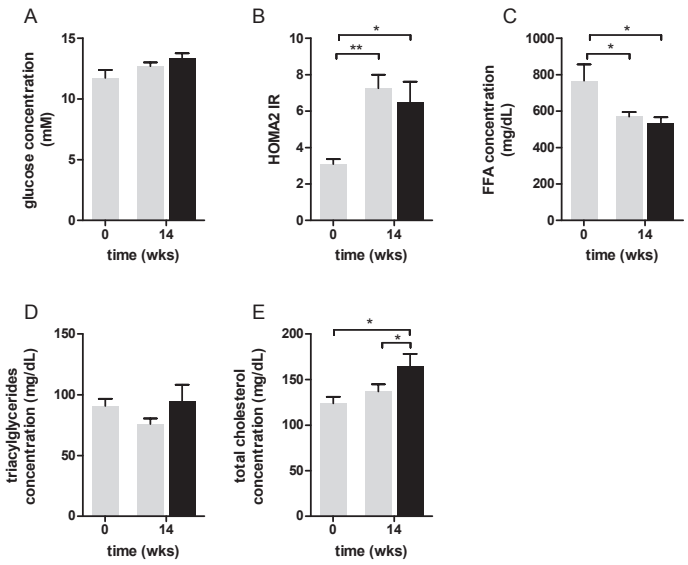


Figure 3 Mean serum glucose concentration (A), HOMA2-IR index (B), mean free fatty acids concentration (C), mean triacylglycerides concentration (D) and mean total cholesterol (E) concentrations of C57BL/6J mice (n=12) kept at 28°C, at t=0, at t=14wks on low fat diet (LFD, grey; n=12) and at t=14wks on moderately-high fat diet (MHFD, black; n=12). *: $p<0.05$, **:$p<0.01$; only significant differences are indicated. Data are shown as mean ± SEM.

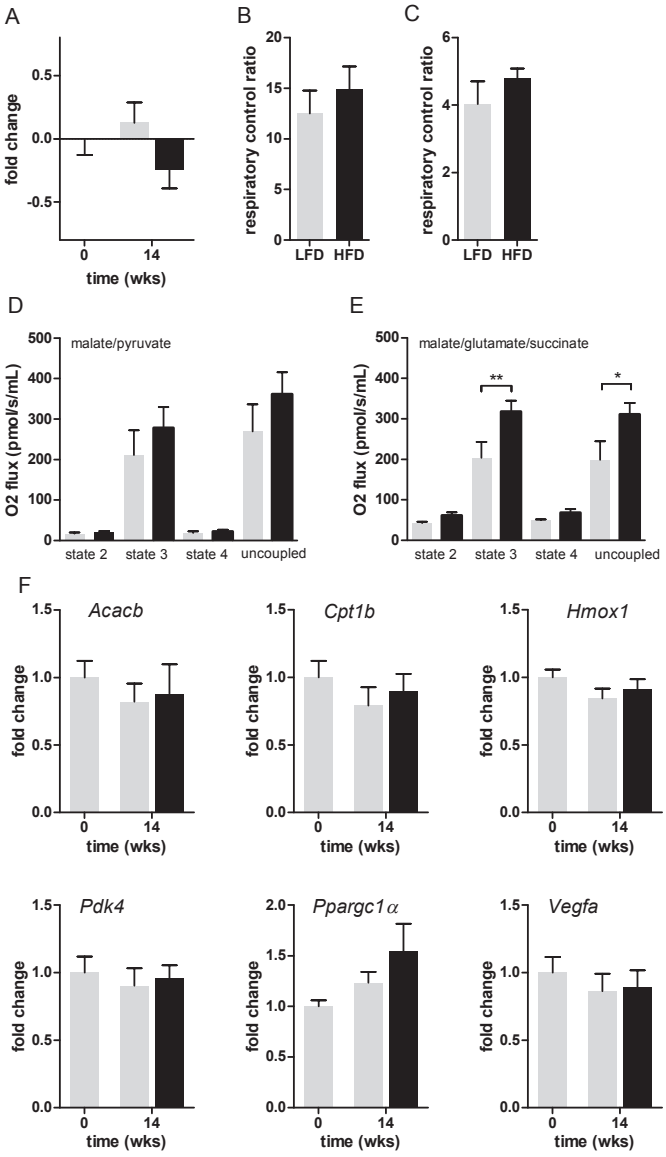


Figure 4 Muscle. Mean muscle mitochondrial density of C57BL/6J mice kept at 28°C, at t=0, at t=14wks on low fat diet (LFD, grey; n=12) and at t=14wks on moderately-high fat diet (MHFD, black; n=12) (A). Mean respiratory control ratio (RCR) on malate/pyruvate (B) and on malate/glutamate/succinate (C) and respiration on malate/pyruvate (D) and malate/glutamate/succinate (E) of isolated muscle mitochondria of C57BL/6J mice kept at 28°C, at t=14wks on low fat diet (LFD, grey; n=6 except LFD GMS traces n=4) and at t=14wks on moderately-high fat diet (MHFD, black; n=6). Mean relative mRNA expression levels (F) of *Acacb* (acetylCoA carboxylase beta), *Cpt1b* (muscle carnitine palmitoyltransferase 1), *Hmox1 (*heme oxygenase 1), PGC1α (*Ppargc1a*, peroxisome proliferator-activated receptor gamma coactivator 1-α), *Pdk4* (mitochondrial pyruvate dehydrogenase kinase isozyme 4), and *Vegfa* (vascular endothelial ▶

Total cholesterol levels were significantly different between the LFD and the MHFD groups at t=14wks. On MHFD, this increase was also significant compared to t=0, while the LFD did not differ from t=0 (Fig 4E). The higher cholesterol levels in mice on the MHFD most likely reflect the increased diet cholesterol content. For HDL-cholesterol, no difference with time nor between LFD and MHFD was observed (data not shown). With the exception of total cholesterol, the examined parameters of metabolism were hardly affected by the diet at 28°C.

A significant decrease in mitochondrial density in muscle has been reported in C57BL/6J mice on high fat diets at ambient temperatures [14, 18]. Since mice kept at 28°C gained substantial weight, more than in the referenced studies, we analyzed mitochondrial density in muscle tissue. In contrast to the referenced studies, no difference was observed in our C57BL/6J mice housed at 28°C in muscle mitochondrial density, not with time nor between LFD and MHFD (Figure 5A). Furthermore, no qualitative differences were seen between mitochondria isolated from muscle of mice fed LFD or MHFD; the respiratory control ratios on malate/pyruvate as well as on malate/glutamate/succinate were not significantly different between these two dietary groups (Figure 5B, 5C). Also no difference in mitochondrial respiration on malate/pyruvate male (Figure 5D) was seen. However, a difference between LFD and MHFD in state 3 and in maximal mitochondrial respiration was seen on malate/glutamate/pyruvate as substrates (Figure 5E). These results indicate an adaptation of muscle mitochondrial substrate metabolism to the diet. Because also indirect calorimetry showed a significant difference in substrate metabolism between the two diets on a whole body level, we investigated the metabolism of skeletal muscle in more detail as this tissue is the largest metabolic organ. We did this by assessing the expression of a number of genes crucially involved in substrate metabolism. No difference was observed, neither between MHFD and LFD groups nor with time, in the expression of the genes acetyl-CoA carboxylase beta (*Acacb*, rate limiting in fatty acid synthesis), carnitine palmitoyltransferase 1b (*Cpt1b*, encoding the protein that determines lipid import into mitochondria for beta-oxidation), heme oxygenase 1 (*Hmox1*, an indicator of oxidative stress), pyruvate dehydrogenase kinase isozyme 4 (*Pdk4*, a regulator and inhibitor of glucose metabolism), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Ppargc1a/PGC1α*, which governs mitochondrial biogenesis) and vascular endothelial growth factor (*Vegfa/VEGF*, a signal molecule that stimulates angiogenesis) (Figure 5F).

▶ growth factor a) of muscle of C57BL/6J mice kept at 28°C, at t=0, at t=14wks on low fat diet (LFD, grey; n=12) and at t=14wks on moderately-high fat diet (MHFD, black). Gene expression was determined by real-time quantitative RT- PCR using *Rps15* (Ribosomal protein S15) and *Hprt* (Hypoxanthine phosphoribosyltransferase 1) as stable reference genes. Expression at t=0 is set at 1.00. *: $p<0.05$; **: $p<0.01$; only significant differences are indicated. Data are shown as mean ± SEM (n=12, except for MHFD *Ppargc1a* which is n=5).

Besides WAT and muscle, also liver plays a crucial role in lipid and glucose metabolism. Liver is among the first organs that are differentially affected by long term high fat diets, giving rise to lipid accumulation, ultimately leading to hepatosteatosis. However, no significant differences were observed for hepatic TAG levels between the LFD and MHFD groups as measured either by histology (Figure 6A - 6D) or enzymatic assay (Figure 6E). We also assessed hepatic aconitase activity. Aconitase is

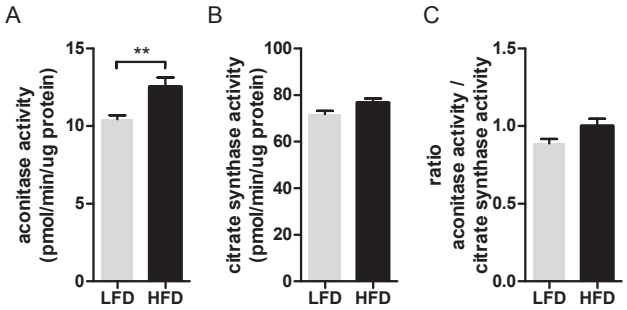


Figure 5 Aconitase levels (A), Citrate synthase levels (B) and Aconitase over citrate synthase ratio (C) of C57BL/6J mice kept at 28°C, at t=0, at t=14wks on low fat diet (LFD, grey) and at t=14wks on moderately high fat diet (MHFD, black). *: p<0.05. **:p<0.01; only significant differences are indicated. Data are shown as mean ± SEM (n=12).

a mitochondrial enzyme that is sensitive to oxidative damage. A decreased aconitase activity is used as a marker for (mitochondrial) oxidative stress and it could be expected that aconitase activity is lower in liver of the MHFD group. In contrast to this expectation, we observed a significant lower hepatic aconitase activity in mice on the LFD compared to the MHFD (Figure 6F). When we normalized this for the activity of another TCA cycle enzyme, citrate synthase (Figure 6G), this difference disappeared (Figure 6H). The most straightforward interpretation of these results is that there is no differential effect of the diets on this marker of oxidative stress in the liver.

DISCUSSION

In the present study, we assessed whether housing at thermoneutrality (28°C) would lead to pronounced differences in metabolic phenotype between a LFD and a MHFD, which would allow for sensitive detection of biomarkers to be used to substantiate claims on food. As expected, we observed a large difference in body mass, in adipose tissue mass, in leptin levels, in adipocyte size, and in 24-h substrate use between the two dietary groups of mice kept at thermoneutrality. The latter is reflected in a difference in maximal muscle mitochondrial oxygen consumption on malate/glutamate/pyruvate. However, we saw hardly any difference in a range of metabolic parameters at 28°C. The metabolic parameters that were measured include various

adipokines and metabolic and peptide serum markers related to CVD/metabolic syndrome, adipose tissue inflammation, parameters of muscle substrate and energy metabolism (mitochondrial density, mitochondrial respiratory control ratio and levels of mRNA of metabolic genes) and metabolic parameters of the liver (TAG levels and aconitase/citrate synthase). Our data thus showed that thermoneutrality resulted in large diet-induced differences in body composition, but this was not paralleled by large diet-induced differences in metabolic parameters.

The absence of metabolic differences between the diets could be due to several reasons. One of these is a smaller differential effect of the diets at 28°C on body weight and adiposity. Although a perfect comparative experiment that compares LFD and MHFD in the same mouse strain at 21°C is not available, this seems not to be the case, since not only the body weight, but also the difference in body weight increase between the LFD and the MHFD at 28°C is larger than that of mice of the same strain and age which were fed the same MHFD for a similar amount of time (15 weeks, including 3 week run in), but were housed at 21°C (118%, based on data from [17]). Similarly, the difference in leptin levels, a biomarker for adiposity, between the LFD (23.0 ± 4.3 ng/ml) and the MFHD (48.4 ± 8.2 ng/ml) was 7.5 fold larger than the leptin levels on the MHFD at 21°C (3.4 ± 0.4 ng/ml). Both parameters show that thermoneutrality increases body weight and adiposity differences that are larger than those induced at 21°C, since the increase on MHFD at 21°C without subtracting the increase on LFD, is larger than the difference between LFD and MHFD at 28°C. One might argue that body weight increase and leptin levels on the MHFD was small at 21°C, however in a second experiment using a MHFD with the same en% fat, but of a different carbohydrate composition, the increase in body weight and leptin level at 21°C was comparable [16]. The diets induce a larger differential effect at 28°C than at 21°C, but it may be that the en% fat in the MHFD is too low to induce differential metabolic effects, also at 21°C. Therefore, our data may be explained by the fact that we did not reach a critical threshold, despite the level of adiposity and the differences in adiposity.

How do our data at 28°C on the MHFD compare to data of mice of the same strain and age on the same diet at 21°C [13, 17]? We see no difference at t=0 wks or t=14 wks for circulating PAI-1, adiponectin, TNFα (below background) or MCP-1 (below background). The FFA and TAG levels were even slightly, but non significantly, lower at 28°C compared to the levels at 21°C, both at t=0 wks and t=14 wks (data at 21°C from [13, 17]; data not shown). On the other hand, insulin levels, which did not differ at t= 14 wks between the LFD and the MHFD (1.76 ± 0.36 ng/ml and 1.86 ± 0.19 ng/ml, respectively; figure 3), were significantly higher at t=14 wks at 28°C, than either at t=0 wks (after 3 weeks at 28°C; 0.80 ± 0.07 ng/ml) or at 21°C on the MHFD both at t=0 wks (0.16 ± 0.02 ng/ml) and at t=15 wks (0.29 ± 0.08 ng/ml) (data at 21°C from [17]). The difference in insulin levels between t=0 wks and t=14 wks was also statistical significant at 21°C. Levels of resistin (7.0 ± 0.5 vs 1.4 ± 0.7 ng/mL) and IL-6 (35.0 ±1.7 vs 5.5 ± 0.6) at 14 wks on the MHFD were five times higher at

28°C compared to 15 wks at 21°C (data at 21°C from [13, 17]). In fact, resistin and IL-6 levels were already higher after the run in period of 3 weeks at 28°C on the LFD (Figure 3). Similarly, already after the run in period of 3 weeks at 28°C on the LFD, hepatic TAG was more (oil red O area 4421 ± 1903 μm^2) than at 21°C after 15 weeks on the MHFD (oil red O area 321 ± 155 μm^2; data taken from [13]. Again, the difference between the LFD and the MHFD was not significant (Figure 6). These data suggest that the thermoneutral temperature alters metabolic homeostasis, but allows accommodation of differences in diet-induced body composition. This is supported by an observed hepatic TAG content (13,151 ± 1,800 μm^2) of mice of the same strain at 21°C fed a similar 40en% semi purified diet [19], which is in the same range as that of mice on a MHFD (30en% fat diet, hepatic TAG: 18,832 ± 2,041 μm^2) at 28°C, but in contrast to 28°C (Figure 6D), at 21°C a clear difference is seen with the LFD (hepatic TAG: 516 ± 111 μm^2).

Based on our results, thermoneutrality does not seem to be a condition that will allow for more sensitive detection of biomarkers as compared to standard housing temperatures.

The question arises which temperature provides the best comparison to humans. This has been recently discussed [5]. Briefly, the lower critical temperatures of mice is about around 28°C [5]. However, humans preferably reside 3 - 5°C below their critical temperature. This suggests that for comparison mice should be housed at 23°C - 25°C. These temperatures should be further decreased to 20°C - 22°C when bedding is available, or for group housing [5]. It thus seems that standard housing temperatures are well suited both for the identification of diet-sensitive biomarkers as well as for translatability to humans. Although other views exist on the optimal temperature for comparison to humans [20-22], our results agree with existing data that show that environmental temperatures can have a profound effect on physiology (e.g. [8, 9, 23]). This implies that environmental temperature should carefully be controlled (and properly described), in nutrition and pharmaceutical studies in rodents, and likely also in humans. It also implies that interventions at various environmental temperatures may increase our understanding of metabolism.

Housing at thermoneutrality results primarily in an increase in WAT mass. WAT is well equipped to store excess energy; it has an enormous capacity to enlarge. WAT enlargement is the result of hyperplasia as well as hypertrophy of adipocytes [24]. Normally in obesity, a condition of chronic energy excess, functional failure of WAT initiates a vicious cycle that includes pro-inflammatory signaling [25]. Leptin has been suggested to play a chemotactic role in macrophage recruitment to adipose tissue [26], but our data do not support this. Despite enhanced obesity, the thermoneutral condition seems to allows for continued WAT functionality. This may be the result of a lower metabolic rate due to a reduced level of thermogenesis at thermoneutrality, compared to mice that are kept at 20°C - 24°C [27]. It may be speculated that cold stress at normal housing temperatures together with the energy excess of in particular (M)HFD will ultimately result in allostatic overload [28], which will lead to adipose

tissue dysfunction and, subsequently, metabolic complications, which will be easier attained and consequently be more pronounced in animals on a MHFD relative to a LFD. However this continued WAT functionality may also be explained by the fact that the adipose tissue did not yet met its expansion limit. Human adipocytes have a large capacity to expand up to 150 (or even 200) μm in diameter [29], which is highly similar to mouse adipocyte sizes [30]. The diffusion distance of oxygen in WAT lies around 100μm [31] When we assume that adipocytes are perfect spheres, the average adipocyte area in this study is 3,957μm^2 for LFD which equals a diameter of 71.0μm and an average of 6,469 μm^2 for MHFD equals a diameter of 90.6μm. These numbers suggest that in our study adipose tissue has not yet reached the diffusion limit of oxygen and therefore complications of local tissue hypoxia are maybe not yet present. This is supported by the absence of differences in expression of *Hmox1* and *Vegf,* which display hypoxia sensitive expression ([32] and [33], respectively), in muscle (Figure 5). Moreover, housing at thermoneutral temperature facilitates enlargement of adipocytes but without signs of inflammation as we show here, while previous findings in mice kept at 21°C showed indeed infiltration of macrophages to be positively associated to adipocyte size, which were in the same size range as we observed [34].

Thermoneutrality apparently protects WAT against dysfunction, while cold stress predisposes to adipose dysfunction. A this moment it is debated which mechanisms underlie adipose tissue dysfunction and several hypotheses have been postulated. One recent hypothesis focuses on adipose tissue lipid flux [35]. In this hypothesis a reduced lipid flux in and out of adipocytes, but in particular a lower lipid re-cycling, is thought to underlie the metabolic complications associated with obesity. Regretfully, we have not assessed adipose tissue lipid flux in our animals and thus cannot evaluate this hypothesis. However, it seems likely that the higher levels of thermogenesis that are required in animals at normal housing temperatures will impose a higher metabolic flux, compared to animals that are kept at thermoneutrality. This is supported by the higher energy intake (MHFD: 85.2 ± 2.5 kcal/week) and leaner phenotype of the mice kept at 21°C relative to mice kept at 28°C (MHFD: 67.7 ± 3.4 kcal/week). Our data therefore do not seem to support this hypothesis.

In conclusion, based on our results we reject the hypothesis that thermoneutrality will enhance diet-induced differences in metabolic parameters. While diet-induced body weight and adiposity differences were enhanced, this was not the case for metabolic differences. Our data rather suggest that thermoneutral housing alters metabolic homeostasis.

ACKNOWLEDGEMENTS

We kindly acknowledge the extensive help of the members of Human and Animal Physiology with tissue harvesting of the animals at necropsy. JK was supported by funding from the European Union's Seventh Framework Programme FP7 2007-2013

under grant agreement n° 244995 (BIOCLAIMS Project). FH was funded by WIAS and EH-vdH was funded by VLAG.

CONFLICT OF INTEREST

None to declare. This research was supported by Human and Animal Physiology of Wageningen University and by TI-Food and Nutrition.

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SUPPORTING MATERIAL

Table S1 Composition of the low fat diet (LFD) and the moderately-high fat diet (MHFD).

Ingredients	LFD	MHFD
Acid casein	220.0	220.0
L-Cystine	3.0	3.0
Carbohydrates	634.5	534.5
Cornstarch 63%		
Maltodextrin 21%		
Sucrose 16%		
Cellulose (Arbocel B800)	50.0	50.0
Lard	31.5	101.5
Corn oil	13.5	43.5
Mineral premix AIN-93G	35.0	35.0
Vitamin premix AIN-93	10.0	10.0
Choline bitartrate	2.5	2.5
Total (g)	1,000.0	1,000.00
Energy density (kcal/kg)	3875	4375
Carbohydrate (en%)	67	50
Fat (en%)	10	30
Protein (en%)	23	20

Values are g/kg, unless stated otherwise.

Table S2 Nucleotide sequences of the primer pairs used for quantitative real time RT-PCR.

Gene	forward (5′-3′)	reverse (5′-3′)
Acacb	ACTGTGGAGTATCTCGTTAACCTTCTG	GGACTGTCGGGCTACCTTGAG
Cpt1b	GGCCAGAGAGGCCAGACCCAT	CCTGGGGTTTATTCAGCACAGCTCAG
Hmox1	ATTGAGCTGTTTGAGGAG	TGCAGGGGCAGTATCTTG
Ppargc1a	CACGCAGCCCTATTCATTGTTCG	GACTCCCGCTTCTCGTGCTCTTT
Pdk4	TCAGTGACTCAAAGACGGGAAACC	TGTGGTGAAGGTGTGAAGGAACG
Vegfa	AAGCCAGCACATAGGAGAGATGAG	TCTTTGGTCTGCATTCACATCTGC
Rps15*	CGGAGATGGTGGGTAGCATGG	ACGGGTTTGTAGGTGATGGAGAAC
Hprt*	TGACACTGGTAAAACAATGCAAACTTTG	GAGGTCCTTTTCACCAGCAAGCT

Acacb is acetylCoA carboxylase beta; *Cpt1b* is muscle carnitine palmitoyltransferase *1*; *Hmox1* is heme oxygenase *1*; *Ppargc1a* (*PGC1α*) is peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *Pdk4* is mitochondrial pyruvate dehydrogenase kinase isozyme 4, and *Vegfa* (*VEGF*) is vascular endothelial growth factor *a*. *Rps15* is Ribosomal protein S15; *Hprt* is hypoxanthine phosphoribosyltransferase *1*. * indicates reference genes

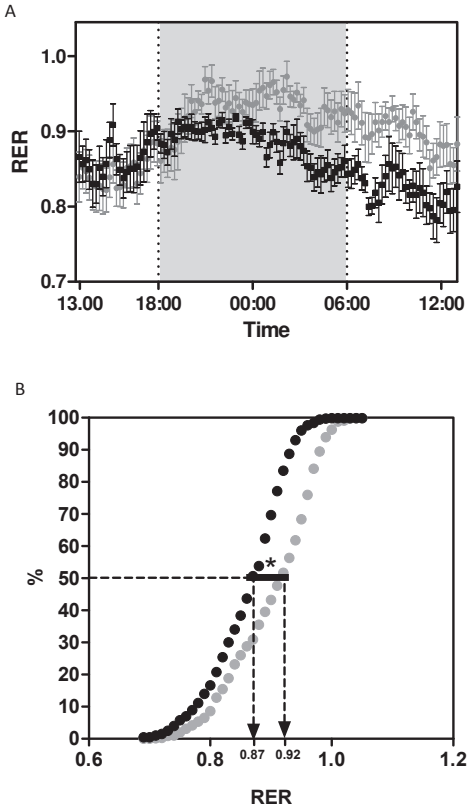

Figure S1 Respiratory Exchange Ratio (RER) plotted with time (A) and as percentage cumulative relative frequency (PCRF) curve at 12 weeks (B) of C57BL/6J mice kept at 28°C on low fat diet (LFD, grey, n=6) and on moderately high fat diet (MHFD, black, n=6). B: arrows indicate average 24h RER. *: $p<0.05$.

SUPPORTING MATERIALS AND METHODS

Animals, diets, study design

Ten-week-old wild-type male C57BL/6JOlaHsd mice (Harlan, Horst, The Netherlands) were housed individually in macrolon type 2 cages. This strain of C57BL/6J mice has the functional Nnt gene encoding nicotinamide nucleotide transferase which is absent in many other C57BL/6J strains (1). The housing environment was kept within the thermoneutral zone (28°C), with a humidity of 40-55% and a 12h light-dark cycle (7.00 h lights on). The experimental protocol was approved by the Animal Welfare Committee of Wageningen University, Wageningen, The Netherlands (DEC number 2008127). Food and water were given *ad libitum* and were replaced once a week, and concomitantly food intake and body weight were measured. The mice were given AIN93 based semisynthetic diets (Table 1; Research Diet Services B.V., Wijk bij Duurstede, The Netherlands), which differed in the amount of fat; 10 en% (low fat diet; LFD) versus 30 en% (moderate-high fat diet; MHFD). The en% was calculated using 38 kJ/gr fat and 17 kJ/g for protein and carbohydrate. The fat consisted of 70% lard and 30% corn oil. In the MHFD, the fat replaced a part of the carbohydrates. The mice (males, 10 weeks (wks) of age) were fed the LFD during the three-week acclimatization period at 28°C, at the end of which they were stratified over the treatment groups according to their body weight. Twelve animals were sacrificed at t=0 wks (at the time of diet switch, but after 3 weeks at 28°C) and another 12 of each of the dietary groups at t=14 wks. Mitochondrial respiration analysis was performed at t=14 weeks in LFD and MHFD groups of 6 animals that were run in parallel. All analyses were performed on n=12, except for Q-PCR of PGC1α, which was n=5, indirect calorimetry which was n=6, mitochondrial respirometry which was n=6, except for LFD on malate/glutamate/succinate which was n=4 and histological analysis (n given below).

Indirect Calorimetry

To measure O_2 consumption, CO_2 production, and, simultaneously, food and water intake, mice (at t = 12 wks) were housed for 48h at 28°C in a laboratory animal monitoring system that allows the continuous measurement of these parameters (LabMaster, TSE Systems GmbH, Bad Homburg, Germany). Indirect calorimetry was performed as described before (2). Data obtained in the first 24h were considered as adaptation and were excluded from data analyses. Rates of oxygen consumption (VO_2, ml/h) and carbon dioxide production (VCO_2, ml/h) were calculated by TSE software using the reference cage as measurement of oxygen and carbon dioxide inflow, and used to calculate the respiratory exchange ratio (RER = VCO_2/VO_2). Percent relative cumulative frequency (PRCF) curve analysis was used to transform 24h RER data for quantitative analysis (3). RER values were converted to glucose and lipid oxidation using the table of Peronnet (4).

Sacrifice

From 08:00 h onwards, mice were fasted for 2 hours and then anesthetized with a mixture of oxygen and nitric oxide (1:1) with isoflurane (4%). Blood collection was performed by orbital exsanguination, whereafter the mice were sacrificed by cervical dislocation. Organs were isolated, weighed and either immediately snap-frozen in liquid nitrogen or placed in 4% buffered paraformaldehyde. Serum was prepared by incubating the blood for one hour on ice in serum collection tubes (Greiner Bio one B.V., Alphen aan de Rijn, The Netherlands), centrifuged at 1520g for 10 minutes and subsequently collected and aliquoted. Serum and frozen organs were stored at -80°C for subsequent analyses. The organs were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 24h at 4°C, then incubated for another 24h at 4°C in PBS, and then transferred to 70% alcohol for further histological analyses. For the respirometry measurements, mice were sacrificed by cervical dislocation and the entire leg muscle was dissected from both hind limbs. The tissue was further processed and mitochondria were isolated as described (5).

Histological analyses

Fixed pieces of epididymal white adipose tissue were dehydrated, cleared and paraffin-embedded. Five µm sections were cut using a Reichert microtome (Reichert-Jung 2030, Cambridge Instruments, Heidelberg, Germany) and mounted on Superfrost plus slides (Menzel-Gläser, Menzel GmbH & co KG, Braunschweig, Germany). The sections were stained with Periodic Acid Schiff (PAS) reagent and counterstained with haematoxylin. Tissues were examined under an Axiokop 2 light microscope (Zeiss, Göttingen, Germany) and digital images were taken using a Axiocam MRC 5 camera (Zeiss). Average adipocyte size was determined on the digital images by measuring the bound width, bound height and surface area of 400 adipocytes per animal in at least two different sections using Zeiss Axiovision software (release 4.7). Frozen liver sections (7 µm) were fixed with 3.7% buffered formalin. Neutral lipids were stained with Oil red O (Sigma). The stained areas were quantified as described previously using Photoshop software (Version 12.0.4, Adobe, San Jose, CA, USA). Briefly, contrast was enhanced with automatic contrast tool, red pixels were selected with the colour range selecting tool, and total selected area was measured in μm^2 (6). Ten pictures taken from two different sections per animal (n=4 for t=0 and LFD and n=8 for MHFD) were quantified.

High-resolution respirometry

Freshly isolated leg muscle mitochondria were subjected to high-resolution respirometry using an OROBOROS Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria) by incubation of 0.2 mg of mitochondrial protein in mitochondrial respiration buffer (100 mM sucrose, 50 mM KCl, 20 mM TES, 1 mM EDTA, 4 mM KH_2PO_4, 2 mM $MgCl_2$, 0.1% (w/v) FA-free BSA, 3 mM malate; at pH 7.2) in two oxygraph chambers at 37°C (2 mL). After successive addition of the following substrates

the corresponding respiration states were measured: 5 mM pyruvate (malate/pyruvate) or 10 mM glutamate plus 10 mM succinate (malate/pyruvate/glutamate) for measurement of state 2 respiration; 1 mM adenosine-di-phosphate (ADP) for state 3 respiration; cytochrome c addition after state 3 assessment was done to confirm intactness of mitochondria; and 1 µg/mL oligomycin was used for state 4 respiration. Maximal respiratory capacity, state uncoupled, was obtained by titration with the chemical uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 0.5 µM/step). All chemicals used were obtained from Sigma-Aldrich, Zwijndrecht, The Netherlands.

Serum parameters

Serum levels were measured using commercial kits for free fatty acids (FFA, NEFA HR(2) kit (Wako Chemicals GmbH, Neuss, Germany), glycerol (glycerol kit (Instruchemie, Delfzijl, the Netherlands)), triacylglyceride (TAG) and hepatic TAG (triacylglyceride liquicolor kit), and HDL-cholesterol and total-cholesterol (HDL-cholesterol or total-cholesterol liquicolor kit (all from Human GmbH, Wiesbaden, Germany)). All analyses were performed according to the manufacturers' protocols, but the volumes were scaled down to allow for analysis with a plate reader (BioTek Synergy HT, Bad Friedrichshall, Germany). For hepatic TAG measurement, approximately 30 mg liver tissue was ground in liquid nitrogen, weighed, added to homogenization buffer (100 mM Tris, 2 mM EDTA, 0.25M sucrose, pH 7.5) and further homogenized using a disposable pestle in a Eppendorf tube. Tissue was diluted in water (20 µg/µl) and 4 µl sample or standard was added to a 96-wells plate in triplicate. 100 µl kit reagent was added followed by an incubation for 45 minutes at room temperature. Serum samples were measured in duplicate and averaged, and concentrations were calculated using standard curves. Serum levels of leptin, resistin, insulin, monocyte chemoattractant protein-1 (MCP-1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and plasminogen activator inhibitor-1 (PAI-1) were measured using the mouse serum adipokine kit (Millipore corporation, Billerica, USA). Soluble vascular cell adhesion molecule 1 (sVCAM-1; CD106), soluble intercellular adhesion molecule 1 (sICAM-1, CD54), soluble E-selectin, (sE-selectin) and matrix metallopeptidase 9 (MMP9) were measured with mouse cardiovascular disease panel 1, and apolipoprotein E (ApoE), fibrinogen, and adiponectin with mouse cardiovascular disease panel 2 (Millipore corporation, Billerica, USA). The sera were diluted 5 times (adipokine kit), 100 times (CVD1 kit), or 5000 times (CVD2 kit). The assays were conducted according to the manufacturer's protocol and measured using the Bio-plex 200 system with Bio-plex manager software (Biorad Laboratories, Veenendaal, The Netherlands). For glucose analysis, sera were 10 times diluted in 0.3 M trichloroacetic acid (Merck, Darmstadt, Germany) and centrifuged for 5 minutes at 1750 g. The supernatant was 1:5 mixed with glucose oxidase solution (GOD-PAP kit, Roche, Woerden, The Netherlands) in a 96-wells plate and after 30 minutes incubation at room temperature, the extinction at 490 nm

was measured using a 96 wells plate reader (BioTek). Concentrations were calculated using a glucose standard curve (Sigma-Aldrich, Zwijndrecht, The Netherlahnds).

Mitochondrial DNA quantification

After grinding leg muscle tissue in liquid nitrogen, DNA was isolated with the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands). DNA was quantified using the Nanodrop ND-1000 spectrophotometer (Isogen Life Sciences, Maarssen, The Netherlands). Quantitative real time RT-PCR was performed with primers as described (7) and IQ SYBR Green supermix in an IQ5 (Biorad) with a program of 3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 45 seconds at 60°C. Single product amplification was verified by an integrated post-run melting curve analysis. Exponential amplification efficiency was verified during each PCR run using a standard dilution series made from pooled DNA samples. Data are expressed as amount of mitochondrial DNA over nuclear DNA, with the mean of t=0 group set at 1.00.

Activity of mitochondrial enzymes.

Liver tissue was ground in liquid nitrogen and further homogenized using a disposable pestle in a microtube with ice cooled 50 mM Tris-HCl, pH 7.4. Protein concentration was measured using the DC Protein Assay (Bio-Rad). For both activity assays, all steps were performed on ice, until the final measurement. Maximal aconitase activity was determined as described (8) with minor modifications. Briefly, aconitase activity was determined by conversion of citrate to into isocitrate and then to α-ketoglutarate. The resulting formation of NADPH is determined. For this, the liver homogenate was diluted in TrisHCl (50 mM, pH 7.4) to a final concentration of 0.2 µg/µl. An equal amount of assay mixture was added, containing TCM buffer (50 mM TrisHCL, 50 mM citric acid monohydrate, 1 mM MnCl2; pH 7.4), 0.4 mM $NADP^+$ and $NADP^+$ isocitrate dehydrogenase (4U/ml). Absorbance at 340 nm was measured during 1 hour at 37°C in a plate reader (BioTek) in 4 replicates and averaged. The aconitase activity is calculated from the slope of the linear part of the curve for each sample. Citrate synthase is measured using the Citrate Synthase assay kit (Sigma-Aldrich, St. Louis, USA). The analysis is performed according to the manufacturer's protocol and measured in triplicate in a plate reader (BioTek) for 1 h at 25°C. For the assay 8 µg of protein is diluted in 186 µL of assay buffer, then 2 µL of 30 mM acetyl CoA and 10 mM DTNB are added and the reaction was started with the addition of 10 µl of oxaloacetic acid. Citrate synthase activity was calculated from the slope of the linear part of the curve and averaged per sample.

Real-time quantitative polymerase chain reaction (Q-PCR)

RNA isolation from muscle was performed as described (9). cDNA was synthesized with the iScript kit (Biorad) according to the manufacturer's protocol. Quantitative PCR was performed using the conditions described above using the primers listed in table 2. PCR amplification of the target genes and 2 reference genes (*Rps15* and

Hprt) to obtain relative levels of gene expression, was performed in duplicate and averaged. The expression of the genes of interest was normalized for the reference genes using the GeNorm normalization factor (10).

Statistics

All data are shown as mean ± standard error of the mean. Statistical analyses were performed using Graphpad Prism v5.04 (Graphpad Software Inc., San Diego, CA, USA). Data were checked for normality using the Shapiro-Wilk test. The qRT-PCR data were log2 transformed and tested. Statistically significant differences were analyzed by analysis of variance (ANOVA) and post-hoc Tukey tests, or with Student's unpaired *t*-test if means of 2 groups were compared. $P < 0.05$ was considered significant.

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CHAPTER 5

MILD OXYGEN RESTRICTION REVEALS SERUM BRANCHED CHAIN AMINO ACIDS AND CCDC3 IN WHITE ADIPOSE TISSUE AS BIOMARKERS, WITHOUT AN INCREASE IN INFLAMMATION

Femke P. M. Hoevenaars[1], Jaap Keijer[1], Ondrej Kuda[2], Inge van de Stelt[1], Robin van Nes[1], Jan Kopecky, Evert M. van Schothorst[1,3]

[1]Human and Animal Physiology, Wageningen University, Wageningen, The Netherlands
[2]Department of adipose tissue biology, Institute of physiology of the academy of sciences of the Czech Republic, v.v.i., Prague, Czech Republic

[3]Correspondence: Evert M. van Schothorst, Human and Animal Physiology, Wageningen University, De Elst 1, PO box 338, 6700 AH Wageningen, The Netherlands, E-mail: Evert.vanschothorst@wur.nl

In preparation

ABSTRACT

Background

Excessive white adipose tissue expansion as in obesity is generally associated with chronic inflammation of white adipose tissue (WAT) which contributes to obesity associated complications. Low oxygen availability in WAT is hypothesized to be the initiator of this inflammatory response.

Hypothesis

We examined the hypothesis that local tissue hypoxia is responsible for the initiation of inflammation in WAT.

Research design and methods

Diet induced obese male C57BL/6JOlaHsd mice,housed at thermoneutrality were exposed to mild environmental oxygen restriction (OxR, to 13% oxygen) for five days and compared with mice kept at normoxia, after which WAT and serum were collected. Body composition, systemic metabolic parameters, WAT macrophage infiltration as marker for tissue inflammation) and whole genome microarray analysis, and circulating adipokines were measured.

Results

Five days OxR decreased body weight and fat mass, and increased blood levels of haemoglobin and haematocrit, as well as lactate to glucose ratio, which indicated systemic hypoxia. No difference in adipose tissue inflammation was found, which was supported by down regulation of inflammation associated transcript levels of *S100a8*, *Saa1*, and *Saa3*. Serum metabolomics revealed an increase of branched chain amino acid Valine and propionyl-carnitine. Adipokines CCDC3, CCK, and Adiponectin are reduced by OxR on transcript (*Cck*) or serum protein level (Adiponectin), or both (CCDC3).

Conclusions

Mild oxygen restriction does not increase WAT inflammation in obese mice. However, a systemic adaptation together with a metabolic response in WAT was observed.

Keywords: Hypoxia, white adipose tissue, inflammation, metabolomics, microarray

INTRODUCTION

Storage of excess energy leads to increased adipose tissue mass. It is well appreciated that adipose tissue is not merely a storage depot, but that it also produces and secretes a wide range of adipokines which are involved in homeostatic regulation [1, 2]. One of these adipokines is tumour necrosis factor alpha (TNF-α), which is a pro-inflammatory cytokine that is synthesized and secreted by white adipose tissue (WAT), and that provided the first direct link between WAT and low grade inflammation [3]. WAT inflammation is now accepted to contribute to obesity-associated complications like insulin resistance and, hyperinsulinemia leading to type 2 diabetes, and cardio vascular diseases [4]. As these diseases represent the major medical problems associated with obesity, it is of great interest to unravel the mechanisms that underlie the change from healthy WAT to inflamed WAT. Of note, a subset of obese individuals are metabolically healthy, and remain insulin sensitive [5, 6]. This indicates that not the amount of adipose tissue, but another trigger initiates the inflammatory state of WAT. One such a trigger may be WAT hypoxia. Hypoxia occurs when oxygen availability does not meet the needs of the surrounding tissue. In the obese state adipocytes are able to expand to a diameter of 150-200μm, which is larger than the average diffusion distance of oxygen in WAT [7, 8]. This implicates blockage of oxygen diffusion by large adipocytes which results in hypoxic tissue areas. Additionally, a reduction in blood flow can result in adipose tissue hypoxia [9]. Indeed, adipose tissue hypoxia has been demonstrated in several mouse and human studies [10-13]. Co-localization between hypoxic areas and macrophage infiltration suggests a direct link between hypoxia and inflammation of WAT [11], but this has not yet been fully elucidated.

This effect of hypoxia as a trigger for adipose tissue dysfunction has been studied in C57BL/6J male mice that were fed a chow, low fat, diet and housed at 21°C. These mice were exposed to environmental hypoxia (8% O_2) to challenge WAT. Remarkably, these mice showed reduced, rather than increased WAT inflammation, when compared to control mice exposed to an ambient level of 21% O_2. However translation to an obese condition is difficult, since these mice were lean and not obese, and it is expected that adipocyte sizes were in the normal range due to low fat feeding [14].

Within the thermal neutral zone the energy needed for maintenance, basal metabolic rate (BMR) is at its lowest [15]. Indeed, compared to housing of C57BL/6J mice at 22°C [16], housing at thermoneutrality results in increased enlargement of adipocytes and fat mass. However, despite the larger adipocyte sizes, less WAT inflammation was observed at thermoneutrality [17]. This is most likely due to decreased metabolic stress resulting from a decreased thermogenic metabolism.

Here we made use of these observations to study the effects of hypoxia on adipose tissue function. We tested the hypothesis that local tissue hypoxia is responsible for the development of adipose tissue inflammation and dysfunction under conditions of obesity. We used diet induced obese (DIO) male C57BL/6JOlaHsd mice as a

model, which were housed at thermoneutrality to induce a non-inflamed obese state. To challenge adipose tissue, DIO mice were exposed to environmental oxygen restriction (OxR) of 13% O_2 for 5 days to reduce systemic oxygen availability and induce local tissue hypoxia. We choose to use 13% O_2, since this is a level of oxygen that obese individuals may encounter when living at high altitudes (±4000m), flying in an airplane [18], or due to apnoea [19]. We assessed body composition, systemic metabolic parameters, WAT inflammation, and performed a whole genome microarray analysis of WAT to examine effects induced by mild environmental hypoxia.

We report that exposure to 5 days of oxygen restriction in DIO C57BL6J mice resulted in systemic decreased oxygen availability but did not increase inflammation in epididymal WAT. Indicating, that also in obese conditions hypoxia is not the trigger of inflammation. We were able to identify biomarkers of metabolic response to mild oxygen restriction, which are serum levels of branched chain amino acids and adiponectin and WAT levels of CCDC3.

MATERIAL AND METHODS

Animal study

Male wild-type C57BL/6JOlaHsd mice (n=36), aged 9 weeks, were purchased from Harlan (Horst, The Netherlands). Mice were acclimatized to the thermo neutral (29-30°C) animal facility (12h light/dark cycle, 55 ±15% humidity) for 3 weeks. We studied mice at thermoneutrality to exclude effects of thermogenic metabolism as a confounder. During these 3 weeks mice were fed a purified low fat diet (supplemental table 1; [20]), followed by purified high fat diet (supplemental table 1) for 12 weeks. Access to water and food (Research Diet Services BV, Wijk bij Duurstede, The Netherlands) was *ad libitum* and renewed every week. Body weight, food consumption, and body composition (EchoMRI 100V, EchoMedical Systems, Houston,TX) were measured weekly from week 7 onwards. After 11 weeks of high fat feeding the (adult) DIO mice were stratified on fat mass into a control (C, n=18), and an oxygen restriction (OxR, n=18) group. OxR mice were acclimatized to indirect calorimetric chambers for 48h under normal ambient 20.9% oxygen, followed by 120h (5 days) with 13% O_2 as described {Duivenvoorde, (under revision) #890}, and killed directly thereafter (n=18). Activity levels were measured by infrared light-beam frames surrounding the cages. All animal care and use was according to the guidelines for use and care given by the Dutch experimentation act (1996). Permission for this study was granted by the Animal Ethical Committee of Wageningen University (DEC2012056).

Tissue and blood collection

In the light phase food was removed 2h before the mice were scheduled to be killed in the morning. Mice were directly killed by decapitation, and blood was partially collected in 60µl heparinised capillary tube (Hirschmann Laborgeräte, Eberstadt,

Germany) for determination of whole blood haematocrit levels. Capillary tubes were centrifuged in a micro-haematocrit centrifuge at 3000g for 5min. Haemoglobin levels were measured using an automated haemoglobin monitoring system (Hemocue 201 Plus, HemoCue Ltd, Angelholm, Sweden) with 10µl micro cuvettes. Remaining blood was collected via a funnel into a mini collect serum tube (Greiner Bio-one, Longwood, FL, USA) and spun down 10min at 5780g at 4°C. Epididymal white adipose tissue (eWAT) was excised rapidly, weighed (left portion) and snap frozen in liquid nitrogen. The right portion was fixed overnight in 4% paraformaldehyde, washed with PBS and paraffin embedded for histological analysis.

Tissue analysis

Lactate in eWAT was determined using the Lactate Assay Kit II (Biovision, Mountain View, USA). Preparation and measurements of the samples were carried out according to the manufacturer's protocol. The lactate concentration in eWAT is expressed as µmol/mg of total protein.

Citrate synthase and aconitase activity were determined as previously published [17].

Histology

Paraffin embedded epididymal WAT was sliced at 5 µm and stained with a MAC-2 antibody recognizing macrophages for detection of crown like structures (CLS) as published [16].

Serum metabolite analysis

For metabolome analysis, 3 µL of serum samples (n=10) were mixed with 97 µL of methanol solution containing 0,1% formic acid and deuterated internal standards (Aminoacids and acylcarnitines, nonderivatised kit, Chromsystems). The final solutions were vortexed and stored overnight at -80°C. After centrifugation (24, 400 x g, 10 min, 4°C), the 80 µL of supernatant was transferred to a glass vial and used for direct injection mass spectrometry analysis. Remaining supernatant was pooled and used for quality control purposes.

The samples were measured on 5500 QTrap (AB Sciex, CA, USA) by direct analysis infusion with settings as follows: polarity was set to positive mode with ionspray voltage 5500 V, capillary temperature 450°C, Curtain Gas - 25 arb, Ion Source Gas (GS1/GS2) - 35 arb. Methanol containing 0.1% formic acid was chosen as a mobile phase. Flow rate was set at 0.05 mL/min (0.0 – 1.0 min) and 0.30 mL/min (1.0 – 1.5 min). Compounds were measured in multiple reaction monitoring mode under optimized parameters of declustering potential and collision energy for each mass transition. Unit resolution was set for isolation ions in mass analyzer.

Data were processed by software Chemoview 2.0 (AB Sciex, CA, USA). The obtained dataset was interpolated and statistically evaluated in R software (http://www.R-project.org/). First step in the data processing was reduction of systematic error by data interpolation by means of quality control samples applying LOESS

method. Coefficient of variation (CV) was calculated for all compounds in quality control samples. Compounds with values higher than 30% CV were rejected from further processing. Metabolomic data were considered as compositional data and *clr* transformation and centering was applied on the dataset [21]. For statistical evaluation, several approaches were chosen: - an unsupervised method (principal component analysis), and a supervised method (discriminant function analysis). Box plots were generated for all metabolites to compare groups.

Serum adipokine analysis

Adiponectin was determined with a mouse serum kit (Biorad laboratories, Veenendaal, the Netherlands) according to the manufacturer's protocol.

Mouse Coiled Coil Domain Containing Protein 3 (CCDC3) was determined using a sandwich CCDC3 Elisa kit (Mybiosource, San Diego, Ca, USA) according to the manufacturer's protocol, except that serum was diluted 1:2 with serum matrix.

Cholecystokinin (CCK) was determined by a competitive ELISA (Sigma Aldrich) according to the manufacturer's protocol, except that serum was diluted 1:8 using kit 1x assay diluent E.

RNA isolation, cDNA synthesis and microarray hybridization

RNA isolation from eWAT was performed as described (van Schothorst et al 2005), with quality and purity checked and approved as published [22]. For transcriptome analysis, as part of a larger hybridization experiment, Agilent whole mouse genome microarrays were used (G4852A, Agilent Technologies, Santa Clara, CA, USA). Preparation of the samples and the microarray hybridization was conducted according to the manufacturer's protocol with a few modifications as described previously (van Schothorst et al 2007). Briefly, cDNA was synthesized using 200ng eWAT RNA with the Agilent low input Quick amp labelling kit without addition of spikes (10 mice per group). All samples were individually labelled with Cy5, while for the reference pool, 5 random samples per intervention group were pooled on an equimolar basis.

Normalization and statistical analysis of microarray data

Normalization was performed as published [22] using Feature Extraction version 10.7.3. Of the 39430 entrez genes and 16251 linc RNA's, 30734 probes were considered significant. Principal component analysis was performed using GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium). Differential gene expression was analysed by unpaired Student's *t*-test between OxR and Control. P value < 0.01 was considered statistically significant. Fold change is expressed as the ratio of OxR over Control and we focused on the significant genes with an absolute fold change > 1.25. Interpretation of functional changes was essentially done as described [23]. Briefly, pathway analysis was performed by Metacore (GeneGo Inc St. Joseph, MI) and genes were classified based on GO-annotation.

Initial categorization was refined using biological databases (MGI, NCBI, Nextprot, BioCarta, genecards) and scientific literature. As processes overlap, we bundled some processes and renamed them.

Quantitative real time reverse transcriptase-PCR (qRT-PCR)

One µg of total RNA of all individual samples was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands). qRT-PCRs were performed as described (Duivenvoorde et al., 2011). Data were normalized according to the geometrical mean of the reference genes ribosomal protein S15 (*Rps15*) and β-2 microglobulin (*B2m*), which were chosen based on stable and appropriate gene expression levels by microarray analysis, and least CV over all samples. Primers were designed using NCBI Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences and PCR annealing temperatures are in table S2.

Statistical analysis

Data are provided as means±SEM. Significance of difference between Control and OxR was determined by unpaired Student's *t*-test. For comparison within a group (Fig 1A-C) a paired Student 's *t*-test was performed. For comparison over time (Fig 1D-F) a one way ANOVA was performed in combination with Dunnett's multiple comparison *post hoc* test using Graphpad Prism 5.04 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at $p<0.05$.

RESULTS

Five days of oxygen restriction (OxR) decreased body weight and fat mass

After 12 weeks of high fat DIO, all mice were characterized as obese, with a mean fat mass of 41% of body weight. There were no differences between C and OxR mice in body weight, fat mass, and lean mass (Fig 1A, 1B, and 1C) before the start of the intervention. After 5 days OxR intervention, body weight declined significantly, which is accompanied with a significant decline in fat mass (to 39% of body weight) and lean mass (Fig 1A-C). Feed, drink, and activity levels were significantly decreased as a first response to OxR. However, after one to two days all parameters returned to baseline (Fig 1D-F).

OxR increases haemoglobin and haematocrit

As an indication for reduced systemic oxygen supply, we measured haemoglobin and haematocrit levels. These were indeed significant increased as a result of oxygen restriction (Fig 2A and 2B). Furthermore, increased levels of lactate in epididymal WAT suggest a switch to glycolytic metabolism and is indicative for tissue hypoxia and its metabolic adaptations ([24], Fig 2C). This was confirmed by a decrease in blood glucose and an increase in lactate-to-glucose ratio (Fig 2D and 2E).

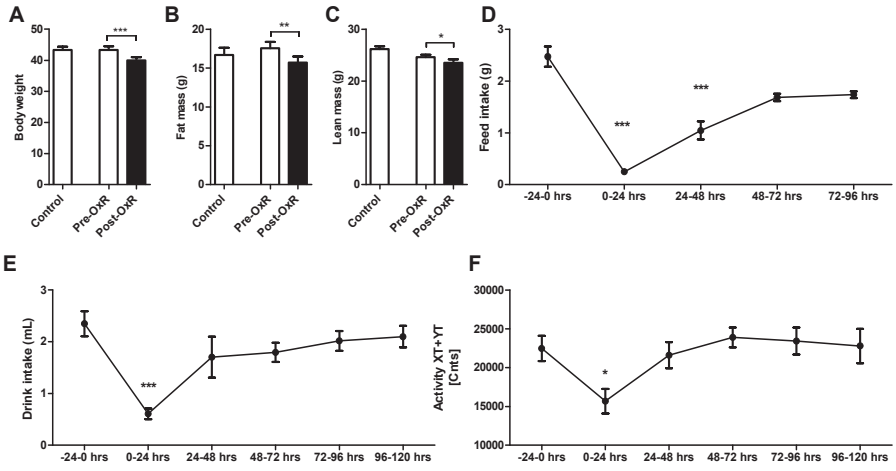

Figure 1 Physiological characteristics of C57BL/6J mice upon oxygen restriction. Body weight (A) fat mass (B) and lean mass (C) of Control (C, n=12) and oxygen restricted (OxR, n=12) C57BL/6J mice. Pre-OxR (n=6) is before intervention with OxR, and Post-OxR (n=6) indicates after OxR intervention. 24h drink (D) and feed (E) intake preceding (-24-0) and following 5 days OxR intervention in indirect calorimetric system (n=6-12). Data are means ± SEM. * p<0.05, ** p<0.01, *** p<0.0001

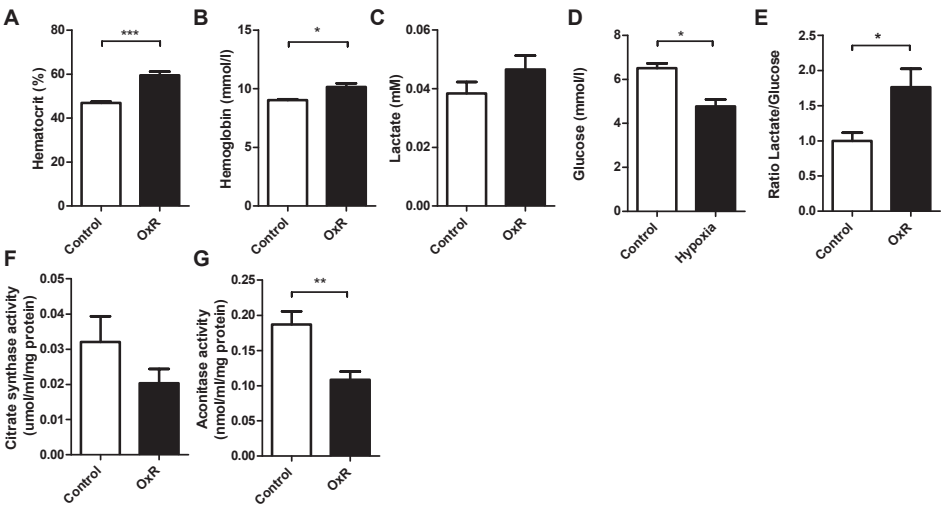

Figure 2 Metabolic effects of OxR intervention. OxR increased hematocrit (A, n=12) and hemoglobin levels (B, n=5-6) in blood, and adaptation in eWAT on lactate levels (C, n=10). Serum glucose levels were decreased (D, n=12), and resulting from that lactate to glucose levels were increased (Control was set to 1.0). Citrate synthase (D, n=6-7) and aconitase activity (E, n=10) tended to decrease. Data are means ± SEM. * p<0.05, ** p<0.01, *** p<0.0001

An increase in macrophage infiltration is absent in OxR

Most adipose tissue macrophages have been found surrounding death adipocytes forming so-called crown-like structures (CLS)[25]. Here, we visualised and quantified the amount of CLS (Fig 3) which showed no difference between the two groups. This suggests an absence of increased inflammation in OxR exposed mice.

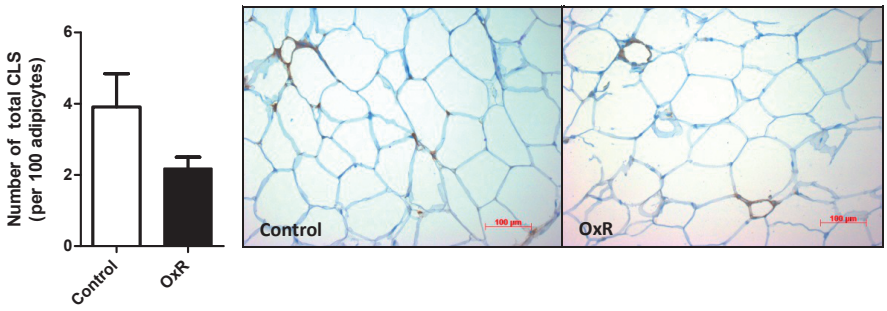

Figure 3 Macrophage infiltration in eWAT. Adipose tissue macrophage infiltration as assessed by quantification of MAC-2 stained crown like structures (CLS) show a tendency of decreased levels by OxR (n=12). Representative pictures illustrate levels of CLS after in Control and OxR.

White adipose tissue metabolism is affected by OXR

Citrate synthase and aconitase are both enzymes of the mitochondrial TCA cycle. Citrate synthase is commonly shown as a quantitative enzyme marker for mitochondrial density. Here we show a reduction of citrate synthase and aconitase in response to OxR, which indicates a reduction in mitochondrial density (Fig 2F and 2G).

Serum metabolomics defines an OxR fingerprint

Amino acids and lipids (carnitines) have been shown to play a potential role in the development of metabolic diseases [26]. Here we investigated whether a specific 'fingerprint' develops by the OxR challenge. The orthogonal partial least squares (OPLS-DA) score plot (Supplemental Fig 1) showed clear separation between control and OxR groups. When analysing the individual metabolites, we found a significant increase of branched chain amino acid Valine ($p=0.0203$), as well as an increase of Leucine/ Isoleucine, touching significance ($p=0.0542$). We also observed significant increased levels of amino acids Alanine, Glutamic acid, Methionine, Proline, and Threonine. Of the carnitines analysed, only propionylcarnitine (C3) was significantly increased while tetradecanoylcarnitine (C14), tetradecenoylcarnitine (C14:1), palmitoylcarnitine (C16) and palmitoleylcarnitine (C16:1) were significantly decreased (Table 1).

Table 1 Serum amino acids and carnitines.

Amino acids	C	OxR	p-values
Alanine	488.3±38.8	780.6±54.1	0.0003*
Arginine	242.7±36.0	331.0±42.7	0.1297
Citruline	66.27±3.3	57.7±2.5	0.0553
Glutamic acid	99.06±14.9	159.5±13.5	0.0082*
Glycine	333.3±13.9	400.6±36.0	0.0875
Histidine	47.3±3.1	56.1±4.0	0.0954
Lysine	393.8±26.1	440.1±33.0	0.2817
Methionine	67.1±7.2	97.6±12.2	0.0422*
Ornithine	116.8±12.8	116.9±10.0	0.9976
Phenyl alanine	101.1±9.0	123.5±8.7	0.0926
Proline	108.6±9.0	189.9±24.4	0.0046*
Threonine	24.0±1.8	34.9±2.5	0.0020*
Tryptophan	42.9±2.5	44.5±3.6	0.7168
Tyrosine	111.2±10.2	145.1±12.9	0.0524
Valine	139.7±10.9	184.7±14.1	0.0203*
Isoleucine/Leucine	309.3±34.8	406.7±31.1	0.0542
Carnitines			
C0	20.3±1.0	22.2±1.1	0.2049
C2	10.1±0.5	9.2±0.8	0.2976
C3	0.19±0.01	0.27±0.02	0.0026*
C14	0.05±0.003	0.04±0.002	0.0008*
C14:1	0.06±0.004	0.05±0.003	0.0094*
C14:2	0.02±0.001	0.02±0.002	0.1500
C16	0.14±0.007	0.12±0.005	0.0214*
C16:1	0.05±0.004	0.04±0.003	0.0101*
C18	0.04±0.002	0.04±0.001	0.1888
C18:1	0.18±0.01	0.16±0.01	0.0894
C18:2	0.11±0.005	0.10±0.008	0.6606

* $p<0.05$

Gene expression in epididymal WAT was altered between OxR and C mice

Gene expression in eWAT was measured after 5 days of oxygen restriction and compared to control mice. 354 probes were differentially expressed by OxR of which 69 unique down-regulated genes and 43 unique up-regulated genes had an absolute fold change >1.25 (tables 2 and 3). Up regulated genes were grouped into five categories: response to OxR, signalling, adipocyte cytoskeleton and matrix proteins,

inflammation, and oxidative stress defence (Fig 4). Down regulated genes were grouped into six main categories: adipocyte cytoskeleton and membrane proteins, metabolism, lipid mediators, inflammation, cholesterol, and histone regulation/ modification (Fig 4).

Table 2 Genes down regulated by OxR with a fold change <-1.25 and p< 0.01.

Gene symbol	Gene name	FC	P value
Adipocyte cytoskeleton and matrix proteins			
1100001G20Rik	RIKEN cDNA 1100001G20 gene	-2.1	0.0073
A2ld1	Gamma-glutamylamine cyclotransferase	-1.3	0.0033
Acta1	Actin, alpha 1, skeletal muscle	-1.7	0.0022
Col4a5	Collagen, type IV, alpha 5	-1.3	0.0070
Gkn3	Gastrokine 3	-1.5	0.0003
Hapln3	Hyaluronan and proteoglycan link protein 3	-1.3	0.0058
Itgbl1	Integrin, beta-like 1	-1.4	0.0030
Krtap11-1	Keratin associated protein 1-1	-1.4	0.0013
Lctl	Lactase-like	-1.6	0.0019
Lrtm1	Leucine richrepeats and transmembrane domains 1	-1.6	0.0004
Maoa	Monoamine oxidase A	-1.3	0.0008
Mme	Membrane metallo endopeptidase	-1.5	0.0009
Myom1	Myomesin 1	-1.3	0.0057
Serpina3b	Serine (or cysteine) peptidase inhibitor, clade A, member 3B	-1.4	0.0071
Serpina3m	Serine (or cysteine) peptidase inhibitor, clade A, member 3M	-1.7	0.0004
Tinag	Tubulointerstitial nephritis antigen	-1.8	0.0013
Tpm1	Tropomyosin 1, alpha	-1.4	0.0026
Trdn	Triadin	-1.9	0.0082
Metabolism			
Acoxl	Acyl-Coenzyme A oxidase-like	-1.4	0.0061
Aldh1a7	Aldehyde dehydrogenase family1, subfamily A7	-1.7	0.0095
Apoc4	Apolipoprotein C-IV	-1.4	0.0005
Atp1a4	ATPase, Na+/K+ transporting, alpha 4 polypeptide	-1.7	0.0087
Bcat1	Branched chain aminotransferase 1, cytosolic	-1.3	0.0066
Cox8b	Cytochrome c oxidase subunit VIIIb	-1.9	0.0055
Fabp12	Fatty acid binding protein 12	-1.4	0.0042
Glul	Glutamate-ammonia ligase (glutamine synthetase)	-1.3	0.0026
Grb14	Growth factor receptor bound protein 14	-1.6	0.0003
Gys2	Glycogen synthase 2	-1.8	0.0029

Table 2 Genes down regulated by OxR with a fold change <-1.25 and p< 0.01. (*Continued*)

Gene symbol	Gene name	FC	P value
Lrpap1	Low density lipoprotein receptor –related protein associated protein 1	-1.3	0.0031
Mpst	Mercaptopyruvate sulfurtransferase	-1.5	0.0020
Nudt14	Nudix (nucleoside diphosphate linked moiety X)-type motif 14	-1.3	0.0001
Ppic	Peptidylprolyl isomerase C	-1.3	0.0028
Rwdd3	RWD-domain containing 3	-1.4	0.0009
Sfxn5	Sideroflexin 5	-1.3	0.0011
Tph2	Tryptophan hydroxylase 2	-2.7	0.0033
Tst	Thiosulfate sulfurtransferase, mitochondrial	-1.4	0.0065
Lipid mediators			
Cyp2c44	Cytochrome P450, family 2, subfamily c polypeptide 44	-1.8	0.0003
D3Bwg0562e	DNA segment, Chr3, Brigham & Women's Genetics 0562 expressed	-1.9	0.0096
Enpp5	Ectonucleotide pyrophosphatase/phosphodiesterase 5	-1.4	0.0099
Hpgd	Hydroxyprostaglandin dehydrogenase 15 (NAD)	-1.3	0.0004
Mgst2	microsomal glutathione S-transferase 2	-1.5	0.0033
Pemt	Phosphatidylethanolamine N-methyltransferase	-1.3	0.0075
Pla2g2e	Phospholipase A2, group IIE	-2.5	0.0001
Pla2g5	Phospholipase A2, group V	-1.9	0.0056
Slco3a1	Solute carrier organic anion transport family, member 3a1	-1.5	0.0003
Inflammation			
Ccl8	Chemokine (C-C motif) ligand 8	-1.8	0.0002
Clec9a	C-type lectin domain family 9, member a	-1.4	0.0014
S100a8	S100 calcium binding protein A8 (calgranulin A)	-2.8	0.0001
Saa1	Serum amyloid A 1	-1.8	0.0077
Saa3	Serum amyloid A 3	-2	0.0025
Cholesterol			
Dhcr24	24-dehydrocholesterol reductase	-1.6	0.0006
Nsdhl	NAD(P) dependent steroid dehydrogenase-like	-1.3	0.0078
Tm7sf2	Transmembrane 7 superfamily member 2	-1.3	0.0001
Histone regulation/modification			
H2-K1	Histocompatibility 2, K1, K region	-1.3	0.0037
H2-Q10	Histocompatibility 2, Q region locus 10	-1.3	0.0060
Zmynd15	Zinc finger, MYND-type containing 15	-1.4	0.0002
Miscellaneous			
Ccdc3	Coiled coil domain containing 3	-1.4	0.0094
Cck	Cholecystokinin	-4.4	0.0041
Hfe	Hemochromatosis	-1.4	0.0034

Table 2 Genes down regulated by OxR with a fold change <-1.25 and p< 0.01. (*Continued*)

Gene symbol	Gene name	FC	P value
Unknown			
6030419C18Rik			
9130204L05Rik		-1.8	0.0047
A_55_P1981992		-1.3	0.0018
B230311B06Rik		-1.3	0.0032
BC054084		-1.4	0.0023
Chr1:138579972-138587381_F		-1.5	0.0026
Chr10:93819821-93976621_F		-1.3	0.0008
chr8:89996710-90049453_F		-1.3	0.0050
ENSMUST00000090166		-1.4	0.0007
NAP060515-1		-1.3	0.0019

Categorized transcripts were subsequently sorted based on alphabetical order

Table 3 Genes up regulated by OxR with a fold change > 1.25 and p<0.01.

Gene symbol	Gene name	FC	P value
Response to OxR			
Alas2	Aminolevulinic acid synthase 2, erythroid	2.1	0.0013
Ankrd37	Ankyrin repeat domain 37	1.6	0.0008
Beta-s	Hemoglobin subunit beta-1-like	2.1	0.0009
Chrdl1	Chordin-like 1	1.9	0.0017
Cish	Cytokine inducible SH2-containing protein	1.7	0.0094
Edn1	Endothelin 1	1.3	0.0067
Gm5226		2	0.0007
Hba-a1	Hemoglobin alpha, adult chain 1	1.9	0.0045
Hba-a2	Hemoglobin alpha, adult chain 2	1.9	0.0073
Hbb-b1	Hemoglobin, beta adult major chain	2	0.0017
Hbb-b2	Hemoglobin, beta adult minor chain	1.9	0.0005
Slc25a37	Solute carrier family 25, member 37	1.3	0.0003
Slc4a1	Solute carrier family 4 (anion exchanger), member 1	1.5	0.0006
Signalling			
1300014I06Rik	PX domain containing 1	1.4	0.0020
Enc1	Ectodermal-neural cortex 1	1.3	0.0087
Hic1	Hypermethylated in cancer 1	1.3	0.0042
Htra3	HtrA serine peptidase 3	1.4	0.0076
Rgs16	Regulation of G-protein sugnalling 16	1.3	0.0082
Tmem198	Transmembrane protein 198	1.3	0.0021

Table 3 Genes up regulated by OxR with a fold change > 1.25 and p<0.01. (*Continued*)

Gene symbol	Gene name	FC	P value
Adipocyte cytoskeleton and matrix proteins			
Has2	Hyaluronan synthase 2	1.3	0.0095
St6galnac5	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl	1.5	0.0044
Zdhhc22	Zinc finger, DHCC-type containing 22	2.1	0.0013
Tubb3	Tubulin, beta 3 class III	1.4	0.0049
Inflammation			
Darc	Duffy blood group, chemokine receptor	1.3	0.0008
Fam46c	Family with sequence similarity 46, member C	1.4	0.0070
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member C	1.3	0.0091
Oxidative stress defence			
Gstm1	Gluthathione S-transferase, mu 1	1.5	0.0023
Gstm4	Gluthathione S-transferase, mu 4	1.4	0.0024
Miscellaneous			
Gnat2	Guanine nucleotide binding protein, alpha transducing 2	1.3	0.0058
Prpf4b	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	1.3	0.0026
Unknown			
2700081O15Rik		1.3	0.0084
4930415C11Rik		1.4	0.0013
chr1:107866276-107887102_R		1.3	0.0059
chr8:106037800-106070025_F		1.3	0.0044
chr8:74431707-74442440_F		1.3	0.0008
chr10:69819062-69871640_F		1.3	0.0052
chr14:28218945-28241903_R		1.3	0.0019
chr17:47006033-47023558_R		1.3	0.0020
Ensmust00000073543		1.3	0.0066
Fam171b		1.3	0.0076
Gm10447		1.3	0.0021
Gm16516		1.3	0.0005
Gm9992		1.7	0.0001

Categorized transcripts were subsequently sorted based on alphabetical order.

Microarray results were confirmed by qRT-PCR

Genes from three different functional categories were selected and their expression was confirmed by qRT-PCR. They represent inflammation (S100 calcium binding protein A8, *S100a8*) and adaptation to oxygen restriction (ankyrin repeat domain 37 (*Ankrd37)* and aminolevulinic acid synthase (*Alas*)) (Fig 5).

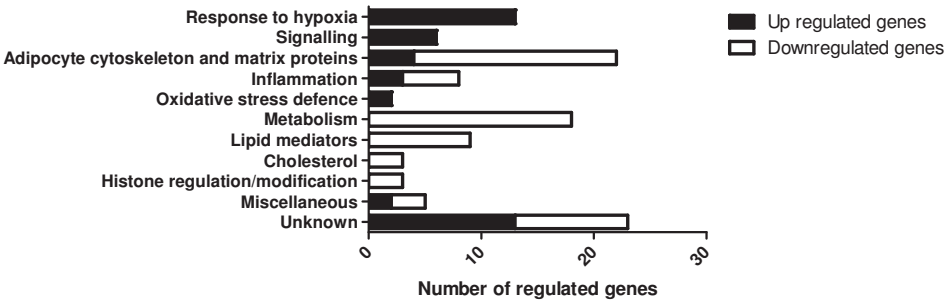

Figure 4 Categorization of the 112 unique genes that were found to be differentially regulated ($p<0.01$, absolute fold change >1.25) by OxR. Filled black bars represent up regulated genes and open bars represent down regulated genes.

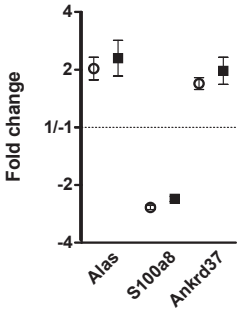

Figure 5 Confirmation of microarray results by real time qRT-PCR. Change in gene expression levels by OxR over values observed in control group of *Alas*, *Ankrd37, and S100a8* was analysed by qRT-PCR (closed circles), relative to stable reference genes *B2m* and *Rps15*. Data obtained with microarray analysis is shown for comparison by open circles. Data are presented as means ±SEM (n=10).

Metabolic serum proteins altered by OxR

We then focuses on proteins with a potential role in systemic regulation; adiponectin, coiled coil domain containing protein and cholecystokinin. Previous studies have shown an inverse relation between serum adiponectin and fat mass [27]. Recently, adiponectin has been appointed as a marker of metabolic dysregulation [28]. Here, we find a reduction of adiponectin by OxR (Fig 6A). *Ccdc3* and *Cck* were further investigated, because OxR resulted in altered transcript levels of these potential peptide hormones CCDC3 (also known as favin) is a newly discovered secretory protein of WAT vessel origin [29]. Serum CCDC3 levels showed a downward trend by OxR corresponding to its gene expression decrease in eWAT (Fig 6B). CCK is well known for its role as peptide hormone of the gastrointestinal system responsible for stimulating the digestion of fat and protein, and as hunger suppressant [30]. Serum levels of CCK were not influenced by OxR (Fig 6C).

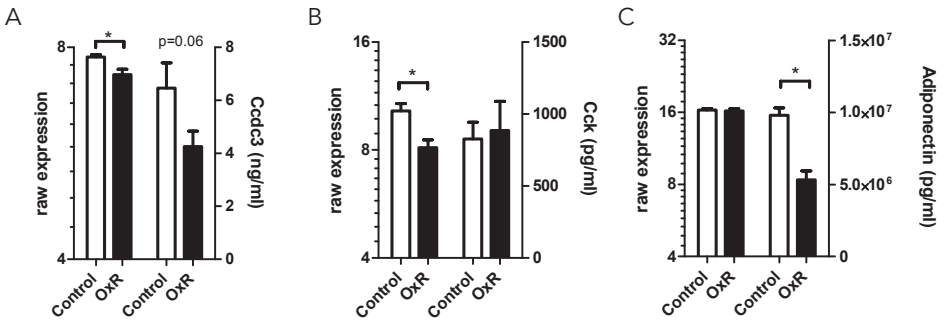

Figure 6 WAT transcript levels and serum levels of peptide proteins. Raw expression of gene transcripts as analysed by microarrays (left) and their serum levels (right) of metabolism associated parameters Ccdc3 (A), Cck (B), and Adiponectin (C) of control (open bar) and OxR (closed bar) mice. Data are means ±SEM (n=10). * $p<0.05$

DISCUSSION

Diet induced obese C57BL/6J mice exposed to ambient OxR (13%) for 5 days had a systemic decrease of oxygen availability as shown by increased circulating haemoglobin and haematocrit levels, and an increased lactate to glucose ratio in WAT. This is further supported by the gene expression profile, where genes that are indicative of limitations in oxygen availability are up regulated, including marker genes *Ankrd37* [31] and *Alas2* [32]. Remarkably, we found in white adipose no difference in crown like structures representing tissue inflammation. This was supported by a decrease in in marker genes of inflammation, including s100a8. These results suggest that hypoxia is most likely not the first step which leads to inflammation of white adipose tissue.

Induction of inflammation related transcripts as *Tnfa*, interleukin 1(*Il-1*), interleukin 6 (*Il-6*), monocyte chemotactic protein 1 (*MCP1*), plasminogen activator inhibitor 1 (*PAI-1*), macrophage migration inhibitory factor (*MIF*), inducible nitric oxide synthase (*iNOS*), matrix metallopeptidase 9 (*MMP9*), and matrix metallopeptidase 2 (*MMP2*) indicate that hypoxia is able to induce inflammation in primary adipocytes and cell lines *in vitro* [10, 12]. However, we find that none of these genes is regulated in WAT due to OxR intervention *in vivo*. We also see no difference in CLS accumulation which is consistent with the down regulation of i.e. inflammation associated genes chemokine (C-C motif) ligand 8 (*Ccl8*), C-type lectin domain family 9 member A (*Clec9a*), S100 calcium binding protein A8 (*S100a8*), serum amyloid A1 (*Saa1*), and serum amyloid A3 (*Saa3*), as macrophages are known for their more active secretion of inflammatory cytokines than adipocytes. These findings fit with recent *in vitro* findings, showing a reduction of the NF-kB signalling and MCP-1 secretion in human primary adipocytes due to hypoxia [33]. Adipocytes are major producers of serum amyloid A (SAA) family members in the non-acute phase in humans [34], especially

hypertrophic adipocytes [35] that are known to be associated with obesity and insulin resistance [36]. Moreover, WAT encoded and secreted SAA3 is linked to attraction of monocytes and thus an inflammatory state [37]. In contrast, in the mouse model containing human SAA1 (hSAA1) expressed specifically in WAT, no evidence was found that adipose tissue-derived hSAA1 influences the development of insulin resistance or obesity-related inflammation [38]. Here, we identified reduced levels of *Saa* transcripts by OxR, as well as of other markers of WAT inflammation, including a downward trend in the number of CLS.

We expected to stress adipose tissue in DIO mice by OxR leading to increased WAT inflammation. But we did not find any other hallmarks for deterioration of adipose tissue health, such as increased secretion of cytokines. However, we do show a serum metabolome profile with increased levels of branched chain amino acids (BCAA; Valine, isoleucine/leucine) and C3 carnitine in OxR mice. This profile matches metabolic serum profiling of obese versus lean humans, where this predictive branched-chain amino acid signature correlates with insulin resistance [26]. In rodents a down-regulation of the expression of adipose tissue BCAA metabolizing enzymes has been shown in obesity and insulin resistance [39]. This confirms our findings of decreased *Bcat1* expression levels in WAT by OxR. Our data may imply that adipose tissue hypoxia is responsible for the serum metabolome profile that is associated with insulin resistance in humans

Hypoadiponectimia is an important biochemical hallmark in the pathogenesis of obesity-related disorders [40, 41]. Our present study demonstrated that serum adiponectin is markedly decreased by OxR. This supports the finding that hypoxia deregulates adiponectin in adipocytes *in vitro* [42] and in adipose tissue *in vivo* [12]. It would be interesting to investigate adiponectin levels in WAT to know whether less adiponectin is produced or less adiponectin is secreted, as *Adipoq* transcript levels remained unaltered.

Coiled-coil domain containing 3 (CCDC3) has recently been identified as an adipocyte secreted protein, with increased levels in a genetic model of obesity as well as during adipocyte differentiation [29]. The expression of CCDC3 is specifically increased in visceral adipose tissue in abdominally obese subjects, and is suggested as potential biomarker for estimating visceral adiposity [43]. Here, we found decreased *Ccdc3* transcript levels in epididymal WAT by OxR, which was similar to decreased CCDC3 serum levels. Why CCDC3 levels are decreased upon lower oxygen availability remains unanswered and needs further investigation.

Presence of cholecystokinin (CCK) in white adipose tissue has been shown first in human visceral WAT of a non-obese subject [44]. CCK is involved in the regulation of appetite through autocrine/paracrine mechanisms. Here, we show a reduction of CCK on transcript level by OxR without an effect on serum level. A well-known symptom of exposure to oxygen restriction is a reduction of food intake [45, 46]. Therefore, a reduction in CCK transcript levels is remarkable as CCK is responsible for short term satiety signalling. However, as there are no changes in serum levels of

CCK it may be that only local regulation in WAT takes place or that nervous signalling is involved. Why CCK levels are decreased in WAT remains unclear. In summary, 5 days of reduced oxygen availability did not increase in inflammation in epididymal WAT, but rather suggests a decrease. Strikingly, OxR did induce a serum metabolome profile of branched chain amino acids, which is associated with insulin resistance in humans. Furthermore, decreased serum levels of adiponectin, an adipose derived peptide hormone with insulin sensitizing properties, were seen. Therefore, it seems that reduced oxygen availability contributes to health deterioration in obesity. Branched chain amino acid, CCDC3 and adiponectin may be markers for systemic or adipose tissue hypoxia.

ACKNOWLEDGEMENTS

We kindly acknowledge the extensive help of the members of Human and Animal Physiology with tissue harvesting at necropsy, and everyone at the animal facility who was involved in this study. FH was supported by funding from the graduate school WIAS (Wageningen Institute for Animal Science).

CONFLICTS OF INTEREST

The authors have declared no conflicts of interest.

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Supplemental table 1 Diet composition.

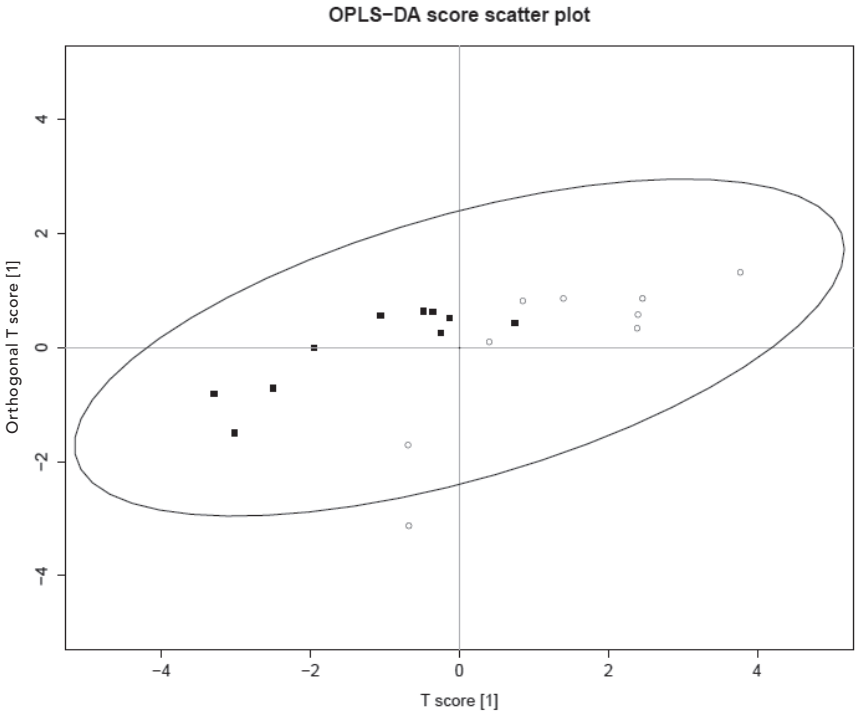
Components	LFD	HFD
Casein	220	267
Wheat starch	386.5	172.5
Maltodextrin	100	100
Dextrose	50	50
Sucrose	100	100
Fat*	43	210
Cholesterol	0.03	0.097
Cellulose	50	50
Mineral mixture	35	35
Vitamin mixture	10	10
Choline bitartrate	2.5	2.5
L-cysteine	3	3
Energy (Kcal kg^{-1})	3865	4700
Percentage of energy from carbohydrate	66.9	36.8
Percentage of energy from fat	10.0	40.2
Percentage of energy from protein	23.1	23.0

Values are in grams per kilogram, unless stated otherwise. Energy content is adjusted for energy from the vitamin mix. *Fats consist of 18% (w/w) coconut oil, 70% sunflower oil, and 12% flaxseed oil. LFD as published (Hoevenaars, et al. 2012.).

Supplemental table 2 Primers and annealing temperature for qRT-PCR.

Gene	Forward 5′-3′	Reverse 5′-3′	T (°C)
Alas	TCAGACACAATGACCCAGGC	CGAAGGTCAGGGCTCCATAC	58
Ankrd37	CAGGTGCTGACCTCAACCAAC	CCAAGCAAGATCTTCAGCTGTC	58
Ccdc3	CCCGGCTCAACCTTACTGG	GAAATTGACTCCGTGTGGCA	56.5
Cck	ACTGCTAGCGCGATACATCC	CATCCAGCCCATGTAGTCCC	58
S100a8	ACTTCGAGGAGTTCCTTGCG	TGCTACTCCTTGTGGCTGTC	58
*B2m**	CCCCACTGAGACTGATACATACGC	AGAAACTGGATTTGTAATTAAGCAGGTTC	60
*Rps15**	CGGAGATGGTGGGTAGCATGG	ACGGGTTTGTAGGTGATGGAGAAC	58

*genes denoted with an asterisk were used as reference genes



Supplemental figure 1 Supervised OPLS-DA score plot of serum metabolome data of control vs. OxR mice. Two component model based on 27 measured serum metabolites. Control mice are represented by filled squares and open circles depict OxR mice. OPLS-DA, orthogonal partial least squares discriminant analysis.

CHAPTER 6

BIOCLAIMS STANDARD DIET (BIOSD): A REFERENCE DIET FOR NUTRITIONAL PHYSIOLOGY

Femke P.M. Hoevenaars[1], Evert M. van Schothorst*[1], Olga Horakova[2], Anja Voigt[3], Martin Rossmeisl[2], Catalina Pico[4], Antoni Caimari[5], Jan Kopecky[2], Susanne Klaus[3], and Jaap Keijer[1]

[1]Department of Human and Animal Physiology, Wageningen University, Wageningen, Netherlands
[2]Department of Adipose Tissue Biology, Institute of Physiology Academy of Sciences of the Czech Republic, v.v.i. Prague, Czech Republic
[3]Group of Energy Metabolism, German Institute of Human Nutrition in Potsdam, Nuthetal, Germany
[4]Molecular Biology, Nutrition and Biotechnology (Nutrigenomics), University of the Balearic Islands, Palma de Mallorca, Spain
[5]Technological Center of Nutrition and Health (CTNS), Reus, Spain

*To whom correspondence should be addressed, Evert.vanschothorst@wur.nl
T: +31-317 483184, F: +31-317 484077 / + 31-317 483962

Published in: Genes and Nutrition (2012) 7:399-404
DOI 10.1007/s12263-011-0262-6

ABSTRACT

Experimental replication is fundamental for practicing science. To reduce variability it is essential to control sources of variation as much as possible. Diet is an important factor that can influence many processes and functional outcomes in studies performed with rodent models. This is especially true for, but not limited to, nutritional studies. To compare functional effects of different nutrients, it is important to use standardized, semi-purified diets. Here, we propose and describe a standard reference diet, the BIOCLAIMS standard diet (BIOsd). The diet is AIN-93 based, but further defined with dietary and experimental requirements taken into account that allow for experiments with bioactive food components and natural (non-expensive) labeling. This diet will be implemented by two European research consortia, Mitofood and BIOCLAIMS, to ensure inter-laboratory comparability.

DISCLOSURES

No conflict of interest or commercial interest are declared by the authors.

Keywords: semi-purified diet, nutrient requirements, rat, mouse

INTRODUCTION

Nutrition will affect functional outcomes in many studies performed with rodent models. This may already be the case with strong pharmaceutical or toxicological interventions, or when dominant gene knock-outs or knock-ins are considered. When we take obesity, type 2 diabetes, or cardiovascular diseases into consideration, which are multifactorial diseases with a strong dietary background, nutrition becomes even more important. Indeed, dietary intervention studies in wildtype mice and rats were found to give relatively small effects on a variety of parameters such as lipoprotein profiles, biomarkers of inflammation and gene expression responses [1, 2]. In addition, 'standard' chow diets usually contain poorly specified ingredients that may strongly vary in composition between batches and between providers. For example, a 5000 fold difference in the level of phytoestrogens has been encountered [3, 4]. Moreover, when a chow diet is compared with a semi-purified diet, a remarkable difference in digestibility and thus effective energy intake has been described [5].

To be able to compare functional effects of different nutrients, it is therefore important to use standardized, semi-purified diets; both as experimental as well as its reference, control diet. This necessity is now widely recognized among researchers employing rodents to unravel molecular mechanisms underlying functional effects of nutritional components [6, 7]. Indeed, semi-purified control and experimental diets based on the recommendations of the American Institute of Nutrition (AIN93) are increasingly used. While of great importance, these guidelines leave substantial room for variation in a number of dietary constituents that are of significant relevance to molecular and health outcomes of dietary intervention studies [1].

A need to further standardize diets is strongly felt in order to improve comparison of study outcomes and to increase efficiency of resources and animals [8]. It is, however, difficult to reach consensus since it implies that individual laboratories have to perform additional initial comparative analyses in ongoing research lines. Despite this, partners in two European research consortia, Mitofood[1] and BIOCLAIMS[2], consisting of respectively 38 and 11 partners spread throughout 24 countries in Europe have proposed a common, defined semi-purified AIN93 based reference diet, the BIOCLAIMS Standard Diet (BIOsd), as well as an experimental high fat diet. Here, we present the choices that have been made, including the underlying rationale, as well as a number of dietary analyses that have been performed on the final formulation of the standard diet.

[1] www.mitofood.eu

[2] www.bioclaims.eu

MATERIALS AND METHODS

Gross energy determination

Approximately 10 grams of BIOsd (Research Diets Services, Wijk bij Duurstede, NL) and standard chow (Teklad global 16% protein rodent diet, 2016) were grinded on a 1.0 mm sieve to prepare a homogenate sample for gross energy determination. Samples were left at room temperature for 24 hours to acclimatize for humidity. Samples were measured by IKA C7000 calorimeter (Staufen, Germany) in triplicate or until the deviation was less than 2%. Polyethylene bags were used as standard.

Kahl pellet hardness tester

Hardness of pellets was assessed by using a Kahl device (Amandus Kahl Nachf., Reinbek/Hamburg, Germany). A pellet is inserted between two bars and due to increasing static pressure applied by a spring, the force (H, kg) necessary for breakage of the pellet is determined. The average of 12 measurements minus the hardest H_{max} and the least hard H_{min} of pellets randomly chosen from the batch, is referred to as 'Kahl-hardness' of the pellet, which is given by the following formulae:

$$\text{Hardness (kg)} = \frac{\sum n_{1\text{-}12}\ \text{Hardness}\ (H)_{1\text{-}12} - \text{H}_{max} - \text{H}_{min}}{10}$$

Here, measured pellets differed however in shape. Regular chow is oval shaped (16 x 10 mm) while the semi-purified diet is round (9mm diameter). Chow was tested for breakage on the 'flat' side.

Animal experiments

Male C57BL/6JRccHsd mice were delivered from Harlan Laboratories GmbH to the German Institute of Human Nutrition (DIfE) at week 4 of age. Mice were housed individually with ad libitum access to food and water at a temperature of 22°C on a 12h light-dark cycle. At 6 weeks of age mice were switched from standard chow diet (Ssniff) to the BIOsd (Research Diets Services, Wijk bij Duurstede, NL). Body weight and composition was determined weekly for 12 weeks using quantitative magnetic resonance (QMR; Bruker´s Minispec MQ10, Housten Texas, USA) as described (Klaus et al. 2005). Animal maintenance and experiments were approved by the animal welfare committee of the Ministry of Agriculture and Environment (State of Brandenburg, Germany).

Male and female C57BL/6J mice (Institute of Physiology Academy of Sciences of the Czech Republic) were maintained at 22°C on a 12 h light–dark cycle (light on from 06:00 hours) and fed Ssniff standard diet (extruded Ssniff R/M-H diet; Ssniff Spezialdieten GmbH, Soest, Germany; containing 25%, 9%, and 66% calories in the form of protein, fat, and carbohydrate, respectively) since weaning. At 6 months of age they were randomly assigned to either the Ssniff standard diet or BIOsd. After 7 weeks of the differential feeding, 10 breeding pairs on each diet were allowed for mating for 1 week, and offspring were followed for 6 weeks. All experiments were performed in

accordance with the directive of the European Communities Council (68/609/EEC), and the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985).

Results and Discussion

An inventory of changes in nutrient composition compared to the original AIN93 diet was made and the grounds for making these changes are given below. The general formula of the BIOsd in comparison with AIN diets is shown in table 1, and the mineral and vitamin mixes of the BIOsd are shown in tables 2 and 3 respectively.

Table 1 BIOCLAIMSsd.

Composition *g/kg diet*	AIN-93G	AIN-93M	BIOsd
Corn starch	397.486	465.692	-
Wheat starch	-	-	386.5
Casein	200	140	220
Dextrinized cornstarch	132	155	-
Maltodextrin	-	-	100
Sucrose	100	100	100
Dextrose	-	-	50
Soybean oil (no additives)	70	40	-
Fats[1]	-	-	43
Fiber[2]	50	50	50
Mineral mix (AIN-93G-MX)[3]	35	35	35
Vitamin mix (AIN-93-VX)[4]	10	10	10
L-cysteine	3	1.8	3
Choline bitartrate	2.5	2.5	2.5
tert-Butylhydroquinone (TBHQ), *mg*	14	8	-
Total energy *kcal/ kg diet*	3766	3601	3865
% as carbohydrates	64.0	75.9	66.9
% as protein	19.3	14.1	23.1
% as fat[1]	16.7	10.0	10.0

[1] Combination of sunflower oil (70%), coconut oil (18%) and flaxseed oil (12%)
[2] Cellulose, [3] For composition see table 2, [4] For composition see table 3

Table 2 Contribution of minerals.

Essential mineral elements	g/kg mix
Calcium, carbonate, anhydrous, 40.04% Ca	357
Potassium phosphate, monobasic, 22.76% P; 28.73% K[1]	196
Potassium citrate, tri-potassium, monohydrate, 36.16% K	70.78
Sodium chloride, 39.34% Na; 60.66% Cl	74

Table 2 Contribution of minerals. (*Continued*)

Essential mineral elements	g/kg mix
Potassium sulfate, 44.87% K; 18.39% S	46.6
Magnesium oxide, 60.32% Mg	24
Ferric citrate, 16.5% Fe	6.06
Zinc carbonate, 52.14% Zn	1.65
Manganous carbonate, 47.79% Mn	0.63
Cupric carbonate, 57.47% Cu	0.3
Potassium iodate, 59.3% I	0.011
Sodium selenate anhydrous, 41.79% Se	0.01025
Ammonium paramolybdate, 4 hydrate, 54.34% Mo	0.00795
Potential beneficial mineral elements	
Sodium meta-silicate, 9 hydrate, 9.88% Si	1.45
Chromium potassium sulfate, 12 hydrate, 10.42% Cr	0.275
Lithium chloride, 16.38% Li	0.0174
Boric acid, 17.5% B	0.0815
Sodium fluoride, 45.24% F	0.0635
Nickel carbonate, 45% Ni	0.0318
Ammonium vanadate, 43.55% V	0.0066
Powdered sucrose (carrier)	221.026

[1] This amount of potassium phosphate supplies only 1561mg P/kg diet. The remainder (1440mg) comes from casein, which contains an average of 0.72% P. The recommended amount of phosphorus in the diet is 3000mg/kg diet (Reeves et al. 1993).

Table 3 Contribution of vitamins.

Vitamin	g/kg mix
Nicotinic acid	3
Ca Panthothenate	1.6
Pyridoxine-HCL	0.7
Thiamin-HCL	0.6
Riboflavin	0.6
Folic acid	0.2
D-Biotin	0.02
Vitamin B-12 (cyanocobalamin) (0.1% in mannitol)	2.5
Vitamin E (all-rac-α-tocopheryl acetate) (500 IU/g)[1]	15
Vitamin A (all-trans-retinyl palmitate) (500IU/g)	0.8
Vitamin D_3 (cholecalciferol) (400IU/g)	0.25
Vitamin K (phylloquinone	0.075
Powdered sucrose (carrier)	974.655

[1] Use of the dry, gelatin-matrix form of these vitamins is recommended

Fiber. Cellulose is used at 50g/kg according to AIN-93G and AIN-93M (table 1).

Macro nutrients

Carbohydrates. In the BIOsd, corn starch has been replaced by wheat starch. This has been done due to a high prevalence of natural stable isotope ^{13}C in corn. This change makes the diet suitable for metabolic tracer studies using natural enriched corn [9, 10]. Furthermore, a combination of maltodextrin, dextrose and sucrose has been used to obtain a pellet with a pleasant hardness for mice [11]; BIOsd vs. chow, table 4).

Table 4 Pellet Hardness (kg).

Diet	Pellet Hardness (kg)
Chow[1]	21.9 ± 0.6
BIOsd	28.3 ± 0.8

[1] Harlan Laboratories, Teklad global 16% protein rodent diet (2016)
Values are means ± SEM

Protein. For the BIOsd a choice has been made to use 220g protein/kg diet, supplemented with 3g L-cysteine, corresponding to a total of 23en% of protein. This protein content avoids potential hyperphagia due to lower protein content [12]. L-cysteine is added to meet the requirements of sulfur amino acid levels [13]. The increase in casein also covers the increase in recommendation of sulfur amino acids according to the National Research Council nutrient requirements of Laboratory Animals [14].

Fat. The choice for fats was based on a number of criteria. First, the diet should provide a balance of the essential fatty acids, linoleic acid (omega-6 fatty acid, n-6;18:2) and linolenic acid (omega-3 fatty acid, n-3;18:3) acid. Bourre et al. suggest a minimal intake of 12g linoleic acid and 2g α-linolenic acid/kg diet for rats, which has been recommended after revision of the original AIN-93 [15, 16]. Secondly, a healthy polyunsaturated over saturated fat (P/S)-ratio of at least 2 should be attained [17]. Thirdly, we selected oils containing neither polyphenols nor carotenes to be able to sensitively intervene with these food bioactive compounds. To meet all these criteria, a combination of sunflower oil (70%), coconut oil (18%) and flaxseed oil (12%) has been selected. This results in a (n-6)/(n-3) ratio of 7.8 and a (P/S)-ratio of 3.1 (table 5). The use of soy oil was considered not appropriate due to its variable polyphenol content.

In addition, it was decided to optionally add 30mg/kg cholesterol to the diet (investigator depended), since human diets usually contain cholesterol. This amount of cholesterol corresponds to the amount of cholesterol present in approximately 30g lard/kg diet, and represents average Dutch human intake [18]. To our knowledge, there are no known side-effects if cholesterol is excluded from the diets.

Table 5 Fatty acid profile (% of gr/kg diet).

	Coconut 18%	Sunflower 70%	Flaxseed 12%	Total 100%
C8:0	7	-	-	1.3
C10:0	6	-	-	1.1
C12:0	47	-	-	8.5
C14:0	19	-	-	3.4
C16:0	10	6	6	6.7
C18:0	3	4	4.5	3.9
C18:1 (n-9)	7	25	25	21.8
C18:2 (n-6)	1	64	17	47
C18:3 (n-3)	-	-	50	6
n-6/n-3 ratio (P/S)-ratio				7.8 3.1

Table 6 Gross energy content kJ/g.

Diet	Calculated	Measured
Chow[1]	12.6	16.8 ± 0.0478
BIOsd	16.2	17.5 ± 0.0016

[1] Harlan Laboratories, Teklad global 16% protein rodent diet (2016)
Values are means ± SEM

Micronutrients

Vitamin and Mineral mix. Vitamin and mineral content follows AIN-93 recommendations and is specified in tables 2 and 3, respectively. The carbohydrate component that is used as a carrier for the vitaminmix provides 40 kcal/kg of energy which has been taken into account for calculating the carbohydrate energy contribution and total energy content (table 1).

Polyphenols and carotenoids. The oils that are used contain no carotenoids. Sunflower oil contains 1mg polyphenols per 100gr oil [19], according to the French national institute for agricultural research (INRA) Phenol Explorer Database [20]. This results in 0.3 mg of polyphenols/kg diet ($3*10^{-5}$%), which is considered to be a negligible amount. Sunflower oil contains 41.08mg/100gr of α-tocopherol (vitamin E) [21].

Performance and development of mice

Using the diet during maintenance, similarly to the use of other purified diets, the mice initially lost some bodyweight, as a consequence of the diet switch. After this the mice displayed a fully normal development and behaviour. This was also the case for rats.

Fig. 1 shows the development of body composition of C57BL/6J mice which were fed the BIOsd diet from 6 weeks of age for 12 weeks. At 6 weeks (wk6) of age mice are not yet fully grown. During feeding of the BIOsd, mice gained body weight due to an increase of lean body mass as well as body fat as expected (body mass (g) wk5: 19.95 ±0.24, wk18: 24.45±0.46; lean mass (g) wk5: 14.65±0.20, wk18: 17.29±0.34; body fat (g) wk5: 5.30±0.11, wk18: 7.16±0.17, data are mean ± SEM, n=12).

For mice we also tested the immediate effect on breeding, after an adaptation period of seven weeks on the BIOsd. Neither breeding performance nor sex ratio of offspring was affected by the BIOsd when compared with non-purified Ssniff standard diet. Thus, 9 of 10 female mice on each diet became pregnant, while average litter size (5.8.±1.1 and 6.0±0.8 pups/litter in the Ssniff diet-fed and BIOsd-fed mice, respectively) and average sex ratio of offspring (2.3±0.5/3.5±0.7.and 2.8±0.5/3.2±1.0.female/male mice born in total in the Ssniff diet-fed and BIOsd-fed mice, respectively) were not significantly different when compared to the BIOsd (data are mean ± SEM). Furthermore, body weight of mice (followed during 4 weeks of lactation and 2 weeks after weaning) was not significantly affected by the BIOsd (data not shown).

The emphasis on a purified diet to reduce experimental variation becomes more and more important due to the use of new technologies such as genomics, proteomics and metabolomics, where small differences can be of major significance for interpretation of the data. Enhanced standardization of general used diets is still necessary. For example, phytoestrogen content of equal open-formula diets can differ up to 3 fold depending on mill date and use of different commodities [4]. Genistein, a phytoestrogen present in soy for example, displays estrogen receptor activation properties which are an unwanted effect in dietary studies [22]. Not only the protein fraction, but also the fat fraction can contain phytoestrogens. Traditional diets often contain soybean oil for its adequate (n-6)/(n-3) ratio. Furthermore, for a proper health and physiological state of the rodents, general rules for n-6 and n-3

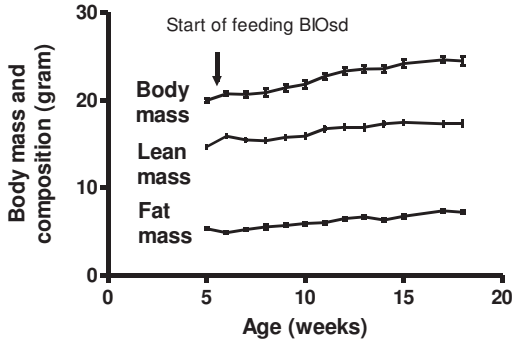


Figure 1 Body composition. Body weight and body composition development of C57BL/6J mice fed BIOsd *ad libitum* for 12 weeks from 6 weeks of age. Data are mean ± SEM, n=12.

fatty acids and (P/S)-ratio exclude usage of for instance palm oil as sole dietary fatty acid ingredient due to a (n-6)/(n-3) ratio of 25:1, which is considered to increase the probability of a number of diseases [23].

As many partners are interested in the use of a high fat diet, we also propose a high fat diet based on the BIOsd, in which part of the carbohydrate content of wheat starch will be replaced by fat to mimic average daily western human intake (40en% fat, 4700kcal/kg).

The initial studies that were performed, and those that are still ongoing, show that the diet can be used for maintenance. Preliminary data suggest no direct adverse effects on mouse breeding using the BIOsd. We have not examined long term or trans-generational effects and therefore at present we do not advise to use the diet as a standard diet in breeding unless further tested. We cannot exclude adverse effects on breeding of other rodent species.

ACKNOWLEDGEMENTS

The authors would like to thank Sven Alferink (Wageningen University) for technical assistance. The research leading to these results has received funding from the European Union's Seventh Framework Programme FP7 2007-2013 under grant agreement n° 244995. We would also like to thank the Mitofood (COST Action FA 0602) for making the formulation of this diet possible.

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CHAPTER 7

GENERAL DISCUSSION

DISCUSSION

In this thesis three basic questions which are of importance for the understanding of adipose tissue functioning were investigated. Namely:

1. Does a body weight set-point exist?
2. How is the diet-induced metabolic response affected by housing at thermoneutrality?
3. Does oxygen restriction induce inflammation in white adipose tissue?

All of these questions explore the metabolic adaptive capacity of the adipose tissue. This chapter reflects on the results of the previous chapters and places the findings in the broader context of weight balance, thermoneutrality and adipose tissue. Finally the implications of our research and main conclusion are provided.

Weight balance

The number of people who are obese is still rising globally. In 2012 approximately 53% of the Dutch male population, above the age of 20, and 44% of the Dutch female population was overweight (body mass index (BMI) >25 kg/m^2). While more male are overweight, more females are obese (BMI>30 kg/m^2): 14% females and 11% males [1]. High prevalence of overweight and obesity increases the number of people attempting to lose body weight. Unfortunately, the main problem with dieting is mostly not the achievement of short term weight loss, but long term body weight maintenance after body weight reduction. Body weight reduction is considered significant when 5-10% of initial body weight is lost as this is associated with an improvement of metabolic and cardiovascular parameters [2, 3]. This is achieved in many instances within 3-18 months [4]. However, long term success rate is substantial lower. After 2.5 year follow-up, participants from four different intervention groups aiming to lose body weight, only lost 1.7 kg compared to their body weight at the start of the intervention [5]. This shows that dieters are not in the advantage in the long run. In the NHANES study 35% of people with significant reduced body weight regained body weight after one year [6]. This is thought to be due to the body retaining a body weight set-point.

In homeostasis, a set-point is a target value of a controlled variable that is maintained by an automatic control system. This is analogues to a cruise control system in which a certain target speed is programmed; when driving up a mountain, the internal control system of the car will hit the gas to retain the target speed, but when driving down the car will hit the brakes to return to its target speed. One big difference between a physiological set-point and the cruise control system is that you can tune the cruise control to your own desire but a physiological set-point is fixed. The fixed set-point also contrasts with the rise in obesity; did we all suddenly released the fixed point and increased our 'fixed' set-point upwards?

In chapter 2 we showed that alterations of purified diets did not result in a persistent elevated body weight set-point. The last consumed diet, either high or low in fat content, determined energy intake, energy expenditure, body weight, body fat store mass and circulating hormones and metabolites. Therefore we concluded that all these parameters 'settle' into a new flexible point determined by energetic input and output which is in contrast with the fixed set point as defined in homeostasis. We thus rejected the set-point theory and support the settling point theory as introduced in the introduction. Nevertheless, also in the settling point theory the question remains: which factors are responsible for establishment of the settling point, in other words: which factors are responsible for keeping a weight balance? It is unlikely that this is a single parameter. Most likely it is the optimal balance, within a certain sphere, between a number of evolutionary determined parameters driven by metabolic rate, energy and nutrient availability, palatability of feed, and homeostatic maintenance of the organism. This is visualised in Figure 1.

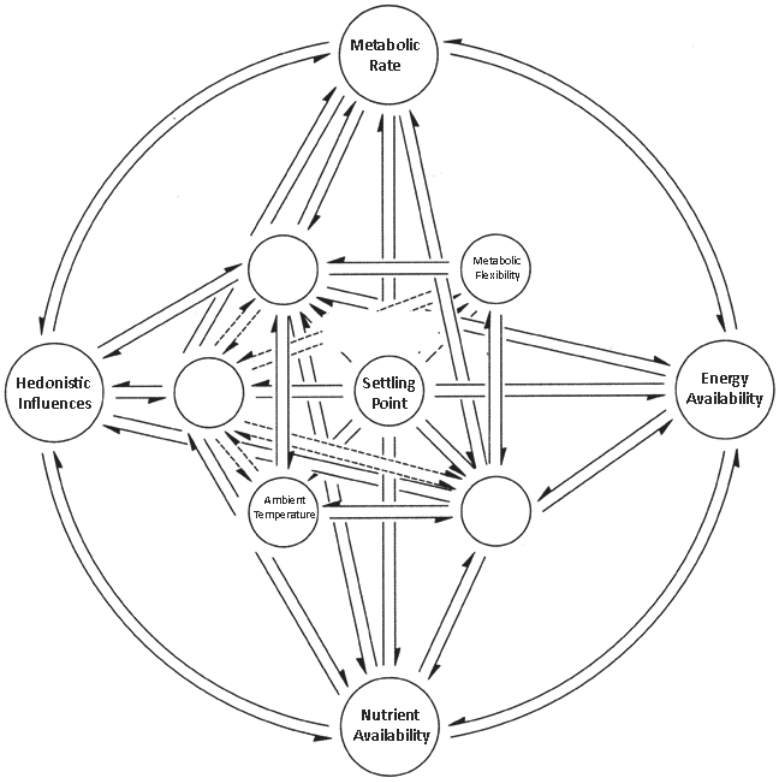

Figure 1 Visualisation of possible forces which are able to influence the dynamic equilibrium of the settling point within this sphere. The position of the settling point represents optimal functioning for a certain set of input forces.

Effects of different forces which are able to influence the settling point such as energy and nutrient availability, metabolic rate, and maintenance of the organism in terms of metabolic flexibility and hedonism will be discussed further.

From the study described in **chapter 2** we learned that diet itself has a major influence on weight balance. There was a diet specific (either high fat or low fat) response for all measured parameters. In **chapter 3** we found that body weight loss can be achieved via two standard used strategies: reduction of portion size (30% energy restriction of previous high fat diet intake) or restriction of fat intake (change from *ad libitum* high fat diet to *ad libitum* low fat diet). These interventions were performed with purified diets containing the same ingredients, with only the amounts (but not the type) of carbohydrate and fat differing between the diets. Both strategies reduced energy intake, with the low fat diet being fed *ad libitum* compared to the high fat diet which was fed in a restricted manner. Our choice for comparing two widely used interventions, rather than focusing on equal energy intake, has led to a different energy intake between the two weight loss groups, in addition to the inherently different macronutrient composition. This is unfortunate as both energy density and macronutrients have been shown to be able to affect energy intake [7]. This was highlighted in a study performed with female mice where reduction of dietary energy density reduced body weight regain after energy restriction [8]. In this study, the energy density of the feed was reduced by the addition of cellulose, which thus resulted in a reduction of net energy intake but also a probable reduction in macro- and micronutrients as well as possible effects on feed transition time. This example indicates the difficulty to really separate all parameters of interest in a dietary intervention study, which is also true for our own intervention as described in **chapter 3**. Our findings suggests that it would have been beneficial to add a pair-fed control group (fed the same amount of calories of the low fat diet as the high fat restricted diet) to be able to determine separate effects of energy density and macronutrients. Assessment of the influence of various dietary factors on body weight and body weight loss and regain is already difficult in mice, most likely this is even more so in humans.

Mice [9] and humans [10-16] display reductions in metabolic rate below the level that would be expected based on their lower body weight as a result of energy restriction. This drop in metabolic rate promotes the conservation of body energy by sparing lean and fat mass. This may predispose them to a positive weight balance when there is no compensation in energy expenditure by for example an increase in physical activity. These body weight loss mediated reductions in metabolic rate have been suggested to remain present in humans for up to six years after body weight loss [17] and perhaps even indefinitely [18]. However, if the new metabolic rate suits the new body weight and when food intake will match metabolic rate it will not predispose to body weight regain [19]. A balanced diet will therefore be of utmost importance. Next to a disproportional reduction of metabolic rate in reaction to energy restriction, leptin levels have also been shown to reduce more,

than predicted by the reduction in body weight [20, 21]. Rosenbaum et al. suggested that this disproportional reduction of leptin would lead to a deficiency of leptin in the brain, which creates as risk for increased appetite in the weight-reduced state [22]. Outcomes of the study performed in **chapter 3** however, sketch a different picture. Epididymal fat mass in this study is reduced by 55% and 73% respectively, in the *ad libitum* low fat diet and restricted high fat diet group, while leptin levels are reduced by 73% and 76%. This may suggest that the response in mice could be somewhat differently regulated compared to the response in humans. However, also other factors such as the change in diet composition (macronutrients) may have played a role in the different results obtained. The results of our study amplify the importance of our understanding of the duration of these kind of disproportional reductions of metabolic rate or/and leptin levels.

Due to the different body weight loss approaches described in **chapter 3,** mice which were exposed to high fat diet restriction experienced longer daily fasting periods than mice fed an *ad libitum* low fat diet. To cope with this major discontinuous supply of energy, mice have to be able to utilize lipid and carbohydrate fuels and transition between them. This feature characterizes the healthy state and can be termed metabolic flexibility [23]. When mice would be metabolically flexible (the ability to switch to appropriate fuel preference in the face of metabolic changes), they should be able to increase lipolysis and fat oxidation. In humans a lower rate of fat oxidation has been shown as a predictor of body weight gain [24]. In **chapter 3** we saw that on a molecular level transcription of fatty acid synthase (*Fasn*) was increased in high fat diet restricted mice but not in *ad libitum* low fat fed mice. This suggests an increase in fat biosynthesis, but no effect was found on import of fatty acids via carnitine palmitoyltransferase 1a (*Cpt1a*) and carnitine palmitoyltransferase 1b (*Cpt1b*) into the mitochondria to increase β-oxidation. Next to that, no effect was found on lipolysis transcripts patatin-like phospholipase domain containing 2 (*Pnpla2*) and hormone sensitive lipase (*Lipe*). Together this suggests that there was no effect of the longer fasting periods compared to *ad libitum* intake on these parameters, which are indicative for lipolysis and fatty acid beta oxidation. Thus, there seems to be no difference in metabolic flexibility between the two diet groups from a molecular point of view.

Besides the underlying regulatory mechanisms, behaviour can also influence the weight balance. Food intake can be recognized as a form of behaviour. You can recognize its structure in frequency of eating and size of portions. The pattern of behaviour (frequency and portion size), nutrient content, and energy density determine the amount of energy that is ingested [25]. Therefore, it seems that we have control of what we eat; however, humans have a hedonistic mind-set. We like to pursuit happiness. Foods rich in sugar and fat are potent rewards [26], which promote eating even without energetic requirements for food [27]. To some extent this is true for mice as well, which, as can be seen in **chapters 2-5**, gain body weight on a high fat diet. On the other hand, rats remain as lean on a standard chow diet as when they are fed a pelleted high fat chow diet, when followed for 15 weeks [28]. However, rats gain

body weight when given a cafeteria diet, a diet in which a free choice of different food items which differ in macro nutrient composition. Thus, in mice and rats the pleasure achieved from eating, like in humans, is an important component in body weight gain [29], although species may have a different hedonistic threshold, which seems lower for mice than for rats. This rat example implicates that dietary variation or food texture might be as much of influence on the weight balance as is food composition.

As visualised in figure 1, many parameters are able to influence the location of the settling point. One of the key experiments to understand the weight balance will be to define the duration of compensating energy loss by an energy saving metabolism. Most mouse experiments are performed in a fixed environment with no choices of food and a standard ambient temperature set to around 22°C. This is clearly not reflective of humans under free living conditions. However, these fixed conditions will result in experiments that unravel underlying mechanisms of weight balance. Mapping the influence of all individual pieces of the puzzle called weight balance will result in understanding the whole picture. This eventually will offer us handles for the discovery of a solution to the obesity epidemic.

Thermoneutrality

An aspect of homeostasis is thermoregulation. Humans, like all mammals are capable of keeping their core body temperature within tight boundaries, independent of environmental temperature. When humans are exposed to temperatures below their

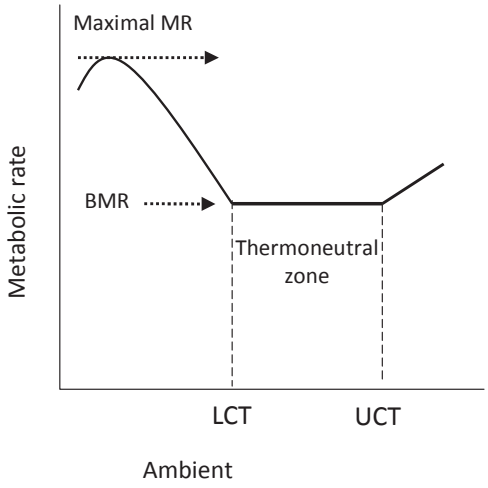

Figure 2 Body temperature of humans is kept as stable as possible to avoid hypothermia or hyperthermia. In the classical heat diagram the influence of ambient temperature (x-axis) on metabolic rate (y-axis) of an endotherm like the mouse is shown. Between the lower critical temperature (LCT) and upper critical temperature (UCT) metabolic rate is equal to the obligatory energy needed for whole body maintenance (BMR). Below LCT and above UCT metabolic regulation and active heat dissipation keep body temperature within the boundaries of thermal homeostasis. Adapted from Hill [32]

thermal neutral zone (<28°C) the body will first respond by an insulative adjustment via peripheral vasoconstriction (blood flow through the skin) to decrease heat loss from the skin. To further protect the body from the cold a metabolic response in the form of heat production is necessary. This can be achieved via shivering thermogenesis, non-shivering thermogenesis, and / or activity-induced thermogenesis, an ultimately at even lower temperatures, by shivering thermogenesis [30]. In terms of energetics, metabolic rate increases when we are below or above our thermoneutral zone and energy costs rise, as shown in figure 2 [31].

Adaptive thermogenesis has been defined as heat production in response to environmental temperature or diet to protect the organism from cold or regulating energy balance after a change in diet [33]. When we look at the effects of diet on metabolism, we saw in **chapter 4** that diet was able to induce large differences in adiposity at thermoneutrality. However, almost no differential effects on serum parameters were observed with the exception of leptin, a peptide hormone that is secreted proportional to adipose tissue mass. Also no changes were seen in a number of metabolic parameters in liver and muscle. Metabolic efficiency appeared increased in mice fed the high fat diet at thermoneutrality as increased storage of fat took place. Remarkably, the increased amount of adipose tissue mass did not influence health status (there were no signs of adipose tissue dysregulation or tissue inflammation).

When we investigated the effect of ambient temperature on metabolism by a reduction from 29°C to 22°C, we saw an increase in energy expenditure due to an increased metabolic rate (figure 3A). A shift takes place from energy deposition to energy release from the adipose tissue upon this shift from thermoneutrality to standard housing conditions.

Part of this increased energy expenditure can be explained by activity induced thermogenesis. Physical activity expressed as counted total beam breaks per

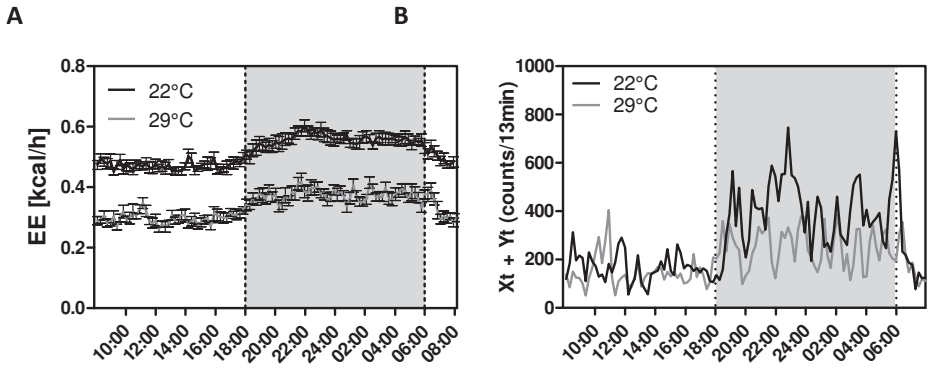

Figure 3 Total energy expenditure and physical activity over 24hours. A The reduction in ambient temperature from 29°C to 22°C raised energy expenditure significantly (P<0.01, mean ± SEM, n=12). B Mean physical activity was increased due to the reduction of ambient temperature (P<0.05, n=8). Grey area indicates night phase in which mice are active.

13 minutes was increased in mice exposed to normal housing conditions (Figure 2B). This significant increase in physical activity was also present during both day and night separately (data not shown). These results clearly show that a reduction in ambient temperature from thermoneutral zone to standard housing temperature for mice increases metabolic rate in C57BL/6J mice; here, a mean 78% increase over 24 hour. This supports previous estimation at an additional ~50% to 100% above basal metabolic rate [34]. Microarray studies of brown adipose tissue and liver have recently shown that metabolic reactions (such as oxidoreductase activity, iron ion binding, endoplasmic reticulum function, lipid metabolic processes and protease inhibitor activity) were reduced by a reduction in ambient temperature from 22°C to 8°C [35]. These studies conclude that mice decrease their energy expenditure on certain metabolic processes to increase energy expenditure for heat production. This was also shown by an increase in expression of key genes involved in thermogenesis being, uncoupling protein 1 (*Ucp-1*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1a*) and CCAAT/enhancer-binding protein beta (*C/ebp beta*) in brown adipose tissue. The question that follows is: what do these metabolic effects on mouse physiology mean for modelling human homeostasis and disease?

Murine models are extensively used for investigating genetic and physiological traits as they closely resemble human metabolism. However, it has become clear that the mouse is not always perfect as a model. Despite promising treatment results in pre-clinical trials with certain drugs, the same drugs are not always translatable to humans. This was also shown by comparing the genetic and proteomic response to acute inflammatory stress between humans and mice, which did not correspond with each other [36]. Although mice might not be perfect as a model, much scientific knowledge has been gained by using mice and rats. An easy improvement for using the mice as a model would be to take housing temperature into account. Temperature is the universal engine which affects all life processes and it is functionally linked to maintenance of body weight. It has become clear that ambient housing temperature influences outcomes via thermoregulation in interventions as shown in **chapter 4** and in figure 1. Several perspectives on housing temperatures have been postulated recently in literature.

In classical non-shivering thermogenesis, Cannon and Nedergaard propose that brown adipose tissue is fully responsible for the elevated metabolic rate when exposed to cold [34]. To mimic a human situation more closely they propose to house mice at thermoneutrality [34]. By doing so, it omits the possibility to be misled by false positive observations when approaching studies of metabolism. This is supported by several occasions; i.e. the fatty acid elongase Elovl3 knock out mouse was described to have an increased metabolic rate. However, this model had fur trouble which led to increased metabolism at normal housing temperatures due to decreased insulation. Upon housing at thermoneutrality it became clear that not the genetic defect was responsible, but the fur [37]. Of course the reverse is also possible, for example when a (mutant) animal shows the same metabolic rate as a wild-type mouse at normal

housing temperature, but metabolism is reduced when housed at thermoneutrality. A specific example is the Ucp1-ablated mouse which does not show an obesity phenotype at normal housing temperature when fed either chow or a high fat diet [38], but it does become obese –even to a higher degree- at thermoneutrality [39]. Many features of mice metabolism become more similar to humans when housed at thermoneutrality; these include control of heart activity changes from sympathetic to vagal tone [40], serum lipid patterns being more alike to humans [34], and effects of pyrogens which are only visible at thermoneutrality [41]. Furthermore, it has been shown that humans also possess brown adipose tissue [42-44]. Therefore, rodent studies performed at thermoneutrality are evidently important for understanding human metabolism.

Speakman and Keijer [45] agree that attempting to mimic human thermal physiology is of importance to model normal human physiology. However, they question if housing within the thermoneutral zone is really the solution. Normal free living humans make use of insulation via clothing which lowers the lower critical temperature by ~5.1°C which results in apparent lower critical temperature of 22.9°C [46]. Normally we do not function at maintenance/basal metabolic rate, but we also have some adaptive thermogenesis. A temperature of 22.9°C would then inflict heat stress, implicating that the apparent lower critical temperature lies even lower, by about 3°C. This results in an ambient preference temperature of 19.9°C in humans. If this observation is extrapolated to mice, this means that they should be housed at 23-25°C, which is approximately 3°C below their lower critical temperature, but mimics the human ambient temperature preference. A further decrease in housing temperature as low as 20-22°C might even be necessary when mice are group housed [47], or are given nesting material as this will reduce heat loss/increase thermal insulation [48].

From these two views it is clear that thermoregulation matters. A clear difference between the two views is whether the mice should be compared at their basal metabolic rate or whether a lower temperature in accord with the human preference to be below the lower critical temperature should be used, so whether 30°C or between 23-25°C is most comparable to the human situation. In a recently performed titration experiment with temperature and nesting material it was shown that mice prefer temperatures around 26-29°C and 10 grams of nesting material [49]. Unfortunately, only behaviour was investigated and not the effects on the level of metabolic rate which could maybe confirm the view of Speakman and Keijer. From the data presented in figure 3A we can conclude that temperature does not has to be adapted between day and night time as difference in energy expenditure between 22°C and 29°C is constant over 24hours.

For future mouse experiments, it is therefore necessary to also think about the effects that ambient housing temperature will have on metabolism and if this is likely in the human situation. Too low ambient temperatures speeds up metabolic rate and may result in thermoregulation dependent effects. In contrast, too high ambient

temperatures may slow down metabolism in such a way that you are looking at basal metabolic rate which is not comparable to normal human living conditions either.

Adipose tissue

Adipose tissue was first only recognized as a passive organ in which adipocytes were considered just as an energy storage place for lipids. It has become clear that adipocytes and the other components present in adipose tissue play an important role in health and obesity. Adipose tissue has a critical role in metabolic control and endocrine functioning [50, 51]. It is however unclear how the tissue turns from healthy and functional to unhealthy and dysregulated in most obese subjects. Like Sun and Scherer we think that dysregulation of adipose tissue is a multi-step process in which several factors could be responsible for the initiation of inflammation in adipose tissue [52, 53]. The existence of a metabolically healthy subset of obese subjects [54, 55] suggests that there must be a certain trigger present in white adipose tissue, which initiates inflammation. A number of factors play a role. One of these factors may be the increase in exposure to free fatty acids in the serum of diabetics [56]. Another harmful factor is the deregulated secretion of adipokines. Most adipokines with pro-inflammatory properties are overproduced with increasing adipose tissue mass [57], while some adipokines with anti-inflammatory, or insulin sensitizing properties, like adiponectin are decreased [58]. Possibly, during extensive growth of adipose tissue mass, adipocytes can reach the diffusional limit of oxygen which can result in local tissue hypoxia [59]. Or hypoxia can develop due to a lag in the development of the vasculature [60], which will stimulate inflammation to promote angiogenesis and increased blood flow. Local tissue hypoxia could also promote oxidative stress and reactive oxygen species (ROS) formation in the cytosol [61, 62]. Alternatively, increased cell death in obese humans and *db/db* mice has been shown to be of influence. It attracts macrophages to clear the debris which increases the inflammatory state of the adipose tissue [63]. Furthermore, macrophages are able to produce ROS [64] and therefore, infiltration of macrophages can also lead to increased ROS production in adipose tissue during the obese state [65]. Indirectly, mitochondrial ROS formation has been shown to increase endoplasmic reticulum (ER) stress [62], and ER stress has been identified to have a direct link with obesity and the development of type 2 diabetes and may contribute to obesity comorbidities [66]. However, which of these steps is first in the delineation process to the development of adipose tissue inflammation is still unknown. Some important determinants in this process which will be discussed below are: protective role of adipose tissue, storage capacity, ER-stress, oxidative stress and adipocyte modulation.

The importance of possessing adipose tissue was shown clearly in a mouse model which lacks adipose tissue [67]. This pathological condition, lipodystrophy, is associated with insulin resistance and hyperglycemia. Likewise, insulin resistance and hyperglycemia is also seen when adipose tissue is extremely enlarged as in obesity. Transplantation of donor fat reversed the diabetic phenotype by increasing

insulin sensitivity, lowering serum insulin, and decreasing amounts of ectopic fat accumulation. This indicates that the absence of adipose tissue is detrimental for health [67]. This has also been shown in humans as recently reviewed by Vatier *et al* [68]. To visualize this phenomenon in figure 4 the U-shaped relationship between BMI and the relative risk of death is shown. Individuals with a low fat mass and individuals with a high fat mass have an increased risk of death.

The common link between obesity and lipodystrophy probably is a defect in storage capacity either due to a lack of adipose tissue or due to saturation of storage capacity [70]. Extensive growth in obesity makes use of two processes, hyperplasia and hypertrophy, to accommodate triglycerides storage. The ability of the adipose tissue to cope with this expansion determines how 'healthy' the tissue is. When coping fails, adipose tissue dysregulates. The adipose tissue can expand via hypertrophy, hyperplasia, or a combination of these two [71]. Hypertrophy is an increase in cell size from pre-existing mature adipocytes, while in hyperplasia the amount of adipocytes increases via adipogenesis from pre-adipocytes present in the stromal tissue. The size and number of adipocytes are of interest as they determine the storage capacity of the adipose tissue. In **chapters 2 and 3** of this thesis we show that adipose tissue mass and adipocyte size of white adipose tissue are able to increase and decrease via hypertrophy and hyperplasia dependent on dietary intake. Which expansion method is the best is from a health perspective discussable. Pharmacological remodelling of adipose tissue by anti-diabetic drugs of the family of thiazolidinediones has shown to promote pre-adipocyte recruitment and adipogenesis by activating peroxisome proliferator activated receptor gamma (PPAR-gamma) in a rodent model which resulted in improved adipose tissue health and glucose homeostasis [72]. Therefore, the presence of more small adipocytes recruited via hyperplasia is suggested to be

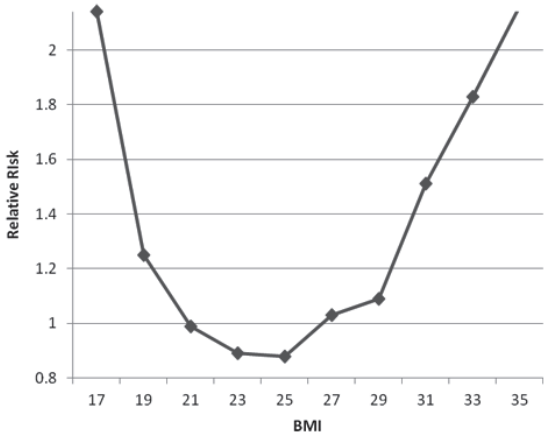

Figure 4 Relative risk of death as a function of body mass index (BMI). Adapted from R. Fogel [69]

healthier. However, another hypothesis postulated is that the number of adipocytes remains constant throughout adulthood, even after excessive body weight loss or body weight gain, meaning that the number of adipocytes can only increase during childhood and adolescence via hypertrophy [73]. This hypothesis leaves no room for hyperplasia in adulthood but suggest that the rapid turnover of the adipocytes is a target for therapeutics. Another possibility is that the down regulation of PPAR-gamma and the inhibition of adipocyte recruitment in a low O_2 environment in adipose tissue mass provides a brake on fat cell recruitment in obesity [74].

Due to the large size of the adipocytes in obesity a low O_2 environment is expected. In **chapter 5** we hypothesized that the first step to development of white adipose tissue inflammation would be the existence of adipose tissue hypoxia. However, we did not find an increase in inflammation of white adipose tissue after exposure to mild oxygen restriction for 5 days. This could be due to some limitations of the study. From the results of the microarray we saw that a specific set of genes was regulated which could be classified as 'response to hypoxia'. In this group we found regulation of a target gene of *Hif1α* (master regulator of O_2 homeostasis), *Ankrd37*, and also genes indicating presence of systemic hypoxia such as *Beta-s* and *Hba-a1*. Unfortunately, we were not able to confirm these findings on a protein level for main regulatory proteins HIF1 and GLUT1, which are known indicators of the local tissue hypoxia response. To asses local tissue hypoxia immunohistochemically, mice were injected with hypoxyprobe-1. Binding of the probe is an indication for presence of hypoxia. Unfortunately, we were not able to make a distinction between the exposed and control mice after the staining. We think that this is due to the starting point of our study, both groups of mice had a diet induced obese phenotype before we exposed them to mild oxygen restriction. Another possibility would be the sensitivity of the probe, which only stains when the partial pressure of oxygen is below 10 mmHg. Besides the presence of a hypoxia response in the microarray, there was also a group of genes regulated which was directed at down regulation of metabolism (i.e. *Gys2*, *Bcat1*, and *Atp1a4*). This suggests a clear adaptive metabolic response to the oxygen restriction exposure. While this confirms expected responses to oxygen restriction exposure, this did not provide the answer to the question what the initiator of the inflammatory cascade was. So, what else could go wrong in the adipose tissue? There are indications for some other determinants which could be of influence in the development of the inflammatory state such as ER stress, oxidant stress, and size or storage location of adipocytes. These parameters will be discussed below.

The ER is a central organelle which is responsible for the biosynthesis of protein and lipid components of a cell. Genetic and environmental factors can cause an accumulation of misfolded proteins in the ER. This causes ER-stress and induces an unfolded protein response [75]. ER-stress and the unfolded protein response are linked to inflammation via JNK-AP1 and NF-kB-IKK pathways [76]. ER-stress and a consequent inflammatory response were recently found in mice fed a high fat diet for 16 weeks [77]. This showed that there indeed is a relation between ER-stress

and inflammation in obesity. However, whether ER-stress is first in the cascade to induce inflammation is at present unclear. To check if ER stress is the trigger for inflammation we checked ER stress in the microarray performed in **chapter 5**. The markers that were analysed were all shown to be increased in white adipose tissue of mice fed a high fat diet for 16 weeks [77]. In table 2, we show that in our study only one transcript, *Chop*, is increased. However, to conclude that ER stress is the activator of the inflammatory response is impossible as no inflammation was present yet. This data might suggest that ER-stress is a secondary reaction to oxygen restriction as there is also a tendency in up regulation of *Atf4*, which is the transcriptional activator of *Chop*.

Table 2 Fold change of ER stress markers of control and oxygen restriction exposed mice.

Full name	Gene symbol	Official gene symbol	Fold change	P-value
Binding immunoglobin protein	Bip	Hspa5	1.0	0.659711
c/EBP-homologous protein	Chop	Ddit3	1.2	0.016196
activating transcription factor 4	Atf4	Atf4	1.1	0.075765
ER degradation enhancer mannosidase	Edem	Edem1	1.0	0.336277
ER DnaJ homolog 4	Erdj4	Dnajb9	-1.1	0.234516
protein disulfide isomerase	Pdi	Pdia3	1.1	0.218959

Another possible 'first step in inflammation' trigger could be oxidative stress. Oxidative stress results from an accumulation of reactive oxygen species (ROS) which exceeds the cell's ability to detoxify [78]. Normally ROS is formed as a by-product during oxygen metabolism and is known to have a role in cell signalling and homeostasis [79]. The production of ROS has also been found in white adipose tissue and shown to induce dysregulated production of adipokines and increased systemic oxidative stress which results in development of insulin resistance [80]. Also elevated levels of free fatty acids, as found in the obese state, are able to induce ROS generation [81]. In the study described in **chapter 5** mice were exposed to oxygen restriction which leads to a reduction in oxygen availability. Since O_2 is a necessary substrate for the formation of ROS, an increase in ROS production during oxygen restricted conditions seems contradictory. How could lower oxygen availability possibly lead to increased formation of ROS? In a hypoxic environment it is possible that accumulation of NADH, substrate to the oxidative phosphorylation, occurs. This results in an increase in the proton gradient across the inner mitochondrial membrane and ROS production occurs more easily upon sudden exposure to O_2 during re-oxygenation [82]. Alternatively, electrons are not able to donate their energy to final electron acceptor oxygen at complex IV. This may result in a discontinuation of the

chain and electrons at complex II, coenzyme Q or complex III will leak from the usual route and create ROS by binding to oxygen directly. Since ROS production mainly occurs in the mitochondria, mitochondrial membranes are prone to sustain damage through increased oxidative stress. This may eventually result in a decrease in mitochondrial density within white adipose tissue. To test this hypothesis, markers of mitochondrial ROS production and mitochondrial density can be measured to assess the level of oxidative stress within the tissue.

Also the size of adipocytes or the storage location are of influence in the process of becoming unhealthy. Before the maximum expandability of the tissue is reached there is the question where to store the fat preferentially: subcutaneously or visceral? Visceral fat has been more strongly associated with a higher risk for insulin resistance compared to subcutaneous adipose tissue [83, 84]. Besides anatomical differences between the two fat depots there are also physiological differences present which help in the explanation why visceral adipose tissue is an increased metabolic risk factor [85]. Normally, lipids are stored in white adipose tissue. But, when adipose tissue has reached its maximal expandability, fat has to be stored somewhere else, for example in non-adipose tissues such as liver, skeletal muscle or pancreatic β-cells. First storage of triacylglycerols will take place but when this is not possible anymore lipids will enter non-oxidative pathways which produce toxic reactive lipid species. This has been shown to contribute to impaired insulin secretion, insulin resistance and to mediate obesity related cardiovascular disease [86]. Our data in **chapter 4** show that there was no 'spilling' of lipids to the liver after 14 weeks of high fat feeding although body weight and epididymal adipose tissue body weight of mice housed at thermoneutrality was higher than body weight and epididymal adipose tissue weight at standard housing conditions. Besides the presence of storage locations also the adipocyte themselves have to be able to expand. Expansion of adipose tissue mass in obesity results in remodelling of the extra cellular matrix which involves stromal cells, preadipocytes, immune cells and endothelial cells. It has been shown that extracellular matrix components are up-regulated during metabolically challenging conditions (i.e. diabetic state) [87]. When the extracellular matrix does not permit expansion and becomes 'stiff', this might lead to higher susceptibility to necrosis, and angiogenesis might be impaired [88]. In **chapter 5** adipocytes enlarged to around 6619μm^2. But we did not see significant increase of transcript levels of genes associated with increased fibrosis in the microarray. Actually, we found a down regulation of 18 genes associated with adipocyte cytoskeleton and matrix proteins. This suggests that in this study adipocytes had not reached their maximum expandability yet.

We discussed multiple factors, (i.e. hypoxia, ER-stress, oxidative stress and adipocyte modulation) which could possibly lead to the initiation of inflammation in adipose tissue. We concluded from our study as described in **chapter 5** that local tissue hypoxia was not the trigger for initiation of inflammation. These mice were housed at thermoneutrality which implies that their metabolic rate is at their lowest, basal, level. As no indicators were found for increased inflammation due to oxygen restriction, it

might be possible that an increased metabolic flux is a necessity for development of inflammation. For example in COPD patients, an increase in resting energy expenditure is associated with increased levels of inflammatory cytokines [89]. A recent hypothesis focusses on adipose tissue lipid flux [90]. In this hypothesis a reduced lipid flux in and out of adipocytes, but in particular a lower lipid recycling is thought to underlie the metabolic complications associated with obesity. It would therefore be interesting to repeat the experiment with an extra group housed at standard housing conditions to investigate the effect of metabolic fluxes on initiation of inflammation.

Implications of our research and main conclusions

It is clear that many factors are able to influence adipose tissue health. Due to the increasing prevalence of obesity it is of great importance to understand and control fat accumulation. As many biological and molecular processes which determine how white adipose tissue functions and responds are still unclear. With this thesis we investigated three basic questions which are involved in adipose tissue metabolism.

1. Does a body weight set-point exist?
2. How is the diet-induced metabolic response affected by housing at thermoneutrality?
3. Does oxygen restriction induce inflammation in white adipose tissue?

We started off with showing that there is no effect of diet history on current metabolism. The major determinant of metabolic outcome in the study presented in chapter 2 was diet composition. Therefore, we concluded that metabolic outcome parameters such as body weight, body fat stores and levels of circulating hormones and metabolites 'settle' into a new active multi-parameter equilibrium in which all competing influences (i.e. genetics, energy density, metabolic rate, metabolic flexibility and cognition) are balanced. This implies that we reject the hypothesis that there is a body weight set-point.

Secondly, we investigated how housing temperature at thermoneutrality affects the diet induced metabolic response. Here we saw that nothing happens on measured parameters of metabolism, although there is a more pronounced obese phenotype present. We concluded that the combination of a low metabolic rate with a high fat diet results in a healthy obese phenotype (there is no indication of inflammation). This model together with a diet induced obese mouse model at standard housing temperature could be used for investigating potential effects of body weight loss on adipocyte morphology and turnover to make a link between morphology and metabolic risk factors.

For the third question we challenged the healthy diet induced obese mouse model with mild oxygen restriction to determine if hypoxia was an initial factor in the development of adipose tissue inflammation. Inflammatory status of the white adipose tissue did not change, when measured as white adipose tissue transcripts

and morphology. Therefore, we concluded that inflammation was not the initial factor in the development of adipose tissue inflammation. However, many other metabolic changes indicated that hypoxia influences metabolic functioning of the adipose tissue. Here, as well as for the set-point theory, it seems that there is a multi-parameter equilibrium in which hypoxia, ER-stress, oxidative stress and adipocyte modulation, play a part in the development of the inflammatory state.

These findings result in recommendations for the execution of future mice experiments. We propose when investigating the body weight balance to think about the composition of the diet as energy density as well as energy composition (levels of macronutrients, fat, carbohydrate and protein) determine outcomes. Next to that, ambient housing temperature of the mice has a big influence on metabolic rate. For comparison with the human situation housing at an elevated temperature, whether at 23-25°C or at thermoneutrality might be better, because then mice metabolism behaves more like human metabolism.

To finish up, this thesis was focused on the metabolic adaptive capacity of white adipose tissue to nutritional and environmental challenges. We can conclude that

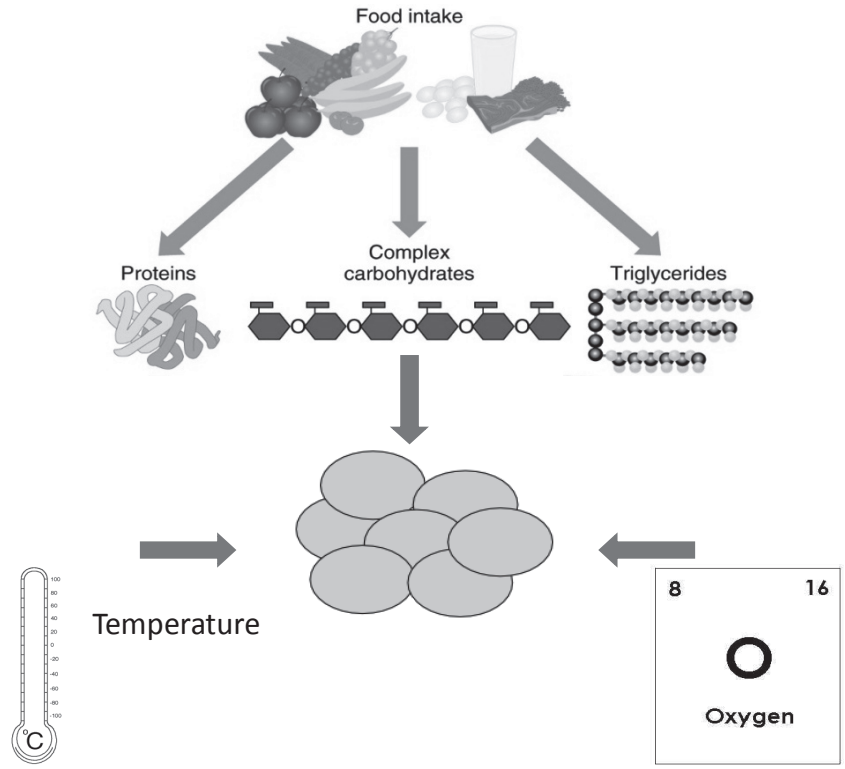

Figure 5 Visualisation of the multi-parameter dynamic equilibrium. Showing the three main effectors of adipose tissue functioning described in this thesis.

in the adipose tissue a multi-parameter dynamic equilibrium of functioning exists, this is captured in figure 5. Three main effectors, diet composition, ambient housing temperature and oxygen availability are shown. However many more processes i.e. storage capacity, ER-stress, oxidative stress and adipocyte modulation are able to influence adipose tissue functioning. This shows the complexity of an organ which is of great interest for obesity and cardiovascular disease research.

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APPENDICES

SUMMARY
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CURRICULUM VITAE

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SUMMARY OF MAIN FINDINGS

When adipose tissue is present in excessive amounts, as in obesity, it predisposes to a number of pathologies. Obesity is a complex, multifactorial condition as it influences many endogenous genetic, endocrine, and inflammatory pathways. Excess dietary intake is one of the important factors which is responsible for the increasing prevalence of obesity. For the understanding of the reciprocity between consumed diet and excessive amounts of adipose tissue, it is essential to investigate underlying functioning. In this thesis, I have addressed three important aspects that play a role in the development of diet induced obesity and its pathologies with a focus on adipose tissue metabolism.

1. Does a body weight set-point exist?
2. How is the diet-induced metabolic response affected by housing at thermoneutrality?
3. Does oxygen restriction induce inflammation in white adipose tissue?

The first aspect investigated was the existence of a body weight set point. A body weight set point is defined as a pre-determined or preferred level of body weight which is preserved by an internal feedback control mechanism. In **chapter 2,** a dietary intervention with none, one, or two diet alterations of purified diets was performed in C57BL/6J mice to investigate if a long lasting effect on body weight persistence was present. Diets contained equal protein content and source of ingredients but differed in the fat-to-sugar ratio. Therefore, energy content and amount of fat was different for either the low fat diet or the high fat. In the intervention the last consumed diet of the mice determined energy intake, energy expenditure, body weight, body fat stores, circulating hormones and metabolites. These data support the settling point theory as body weight and metabolic parameters 'settle' based on current energetic input and output and do not support the set point theory. Next to that it underlines the importance of diet choice in intervention studies focusing on aspects on the crossroads of nutrition and physiology.

In **chapter 3** adipose tissue physiology and molecular regulation was further investigated by exposure to more metabolic stress in the form of a weight loss challenge with different purified diets. Diet-induced obese C57BL/6J mice were fed a high fat diet restricted to 70% intake of previous *ad libitum* high fat diet intake or they were changed to *ad libitum* low fat diet for 5 weeks. Beneficial effects were seen in both interventions regarding physiological parameters. However, molecular parameters in white adipose tissue differed between the two restriction interventions, with increased activation of mitochondrial carbohydrate and fat metabolism in high fat diet restricted mice. When extrapolated to the human situation this may suggest that a reduction of portion size is the best method for weight loss.

It is standard practice to house mice at ambient temperature during physiological intervention studies. Unfortunately mice are then exposed to a temperature below their thermal neutral zone. This implies that their metabolism is chronically increased which is known to influence study outcomes. In **chapter 4** the second question; "how is the diet-induced metabolic response affected by housing at thermoneutrality?" was investigated. A 14-week dietary intervention with two semi-purified diets, a low fat diet and a moderately high fat diet, was performed at 28°C in C57BL/6J mice. This resulted in a large diet-induced difference in bodyweight, adipose tissue mass, adipocyte size, and serum leptin level. But no differential effects of the diets were seen on serum glucose, free fatty acids, triacylglycerides, insulin, a panel of cardiovascular markers, and a number of (metabolic) parameters in liver and muscle. Although adipose tissue mass and adipocyte size was increased significantly, there was no sign of inflammation or dysfunction in the adipose tissue. This study suggests that diet-induced obesity of C57BL/6J mice at thermoneutrality results in a suitable model for the metabolically 'healthy' obese (people who are significantly overweight but show none of the usual metabolic problems). Next to that, this study emphasizes the importance of consideration and control of housing temperature for mice, as it has profound effects on study outcomes.

The third and last question investigated was if oxygen restriction is able to induce inflammation in white adipose tissue. There is substantial evidence that white adipose tissue becomes hypoxic when excessively enlarged. Due to fast expansion of white adipose tissue the vasculature is not able to keep pace with growth. Next to that, adipocytes are able to increase in size beyond the limit of oxygen diffusion. To investigate if hypoxia was able to induce inflammation in white adipose tissue, the model for healthy obese adipocytes (developed in **chapter 4**) was used and exposed to ambient oxygen restriction (13%) to challenge adipose tissue metabolism. This resulted in the presence of systemic oxygen restriction as shown by increased levels of haemoglobin and haematocrit. Furthermore a switch to glycolytic metabolism, which is indicative for tissue hypoxia, was present. No differences in adipose tissue macrophage infiltration (as marker for inflammation) were found. But, serum branched chain amino acids and adipokines were affected. Branched chain amino acids were increased in mice exposed to oxygen restriction which shows resemblance with findings in humans where increased levels were found in lean versus obese people. The peptide hormone adiponectin was increased in serum, without differences in WAT expression. On the other hand, the peptide hormones CCDC3 and CCK showed decreased transcript levels in white adipose tissue without significant change in serum levels, although for CCDC3 a trend was seen. Together these results suggest that oxygen restriction does not induce inflammation in adipose tissue. However, it does affect adipokine regulation.

After performing these studies it was clear that composition of the diet has a major influence on outcome parameters of physiological studies as shown in **chapter 2**. To compare functional effects of different nutrients, it is important to use standardized

purified diets. Not only the experimental intervention diet is of importance but also the reference control diet can influence outcomes. For example, when an intervention is performed with a high fat purified diet and the reference diet is chow this will lead to a difficult comparison. The content of chow is variable as it is grain or cereal based (ground corn, ground oats alfalfa meal, soybean meal and ground wheat). Nutritional adequacy is ensured by addition of vitamins, minerals, and fat. However, the exact amount of the various ingredients is frequently kept secret by the manufacturer. Next to that, due to the plant based origin of chow it will contain nutritive (protein, carbohydrate, fat) components but also non-nutritive components (phytochemicals). The content of the chow diet will vary from batch to batch as the nutritive and non-nutritive value will change between harvests. When using a chow reference diet in comparison to a purified diet you will never know exactly what you are comparing, i.e. difference in amino acids or effects of phytochemicals etc. Therefore, a reference diet for physiology was designed (**chapter 6**) to improve comparison of study outcomes and to increase efficiency of resources and material. A key feature of the diet is the fixed protein concentration, which allows for an exchange of carbohydrate and fat in a high fat version of the diet.

To conclude, the work presented in this thesis provides clear insight in factors that are of importance for improvement of translatability of mouse studies to the human situation. It was shown that when investigating the weight balance many parameters, i.e. genetics, metabolic rate, environmental factors like ambient housing temperature and light, and cognitive behaviour, besides the diet and its composition are able to influence the outcome parameters. As most mouse experiments are performed in a fixed environment with no choices of food and a standard temperature set to 22°C. This is clearly not reflective of humans under free living conditions. However, these fixed conditions are able to result in experiments that unravel underlying mechanisms of weight balance, which form the basis for discovering a solution to the obesity epidemic.

SAMENVATTING

Verkeerde voeding kan leiden tot overgewicht. Problemen in het overtollige vetweefsel zoals ontstekingen kunnen mogelijk zelfs leiden to hart –en vaat ziekten, diabetes en sommige vormen van kanker. Obesitas is een complexe aandoening die verschillende processen kan beïnvloeden zoals de hormoonhuishouding, ontstekingsprocessen en activatie of inactivatie van verschillende genen. Overconsumptie van voeding is een van de belangrijkste factoren die verantwoordelijk is voor de toenemende prevalentie van obesitas. Om de wisselwerking tussen geconsumeerde voeding en het functioneren van overmatig vetweefsel te doorgronden is het essentieel om het werkings mechanisme te begrijpen. In dit proefschrift heb ik drie belangrijke aspecten onderzocht die een rol spelen in de ontwikkeling van overwicht door verkeerde voeding met een focus op het metabolisme van vetweefsel.

1. Heeft lichaamsgewicht een set-point?
2. Wordt de dieet-geïnduceerde metabole respons beïnvloed door huisvesting bij thermoneutraliteit?
3. Veroorzaakt beperking van zuurstof een ontstekings proces in vetweefsel?

Als eerste hebben we onderzocht of er zoiets als een lichaamsgewicht setpoint bestaat. Een lichaamsgewicht setpoint is een soort thermostaat in je hoofd die regelt dat het gewicht op een constant niveau wordt gehouden. In **hoofdstuk 2** hebben we een voedingsinterventie met geen, een of twee dieet veranderingen uitgevoerd in C57BL/6J-muizen om te onderzoeken of er een lichaamsgewicht setpoint aanwezig is. De gebruikte diëten bevatten een gelijke hoeveelheid eiwit, maar verschilden in de vet - tot- koolhydraat verhouding. Daardoor is de hoeveelheid totale energie (kcal) en de hoeveelheid vet anders voor het vet-arme dan voor het vet-rijke dieet. Tijdens de interventie bepaalde het laatst gegeten dieet de uiteindelijke energie-inname, energie-uitgave, lichaamsgewicht, vetmassa, circulerende hormonen en metabolieten. Deze data ondersteunen een andere theorie dan de setpoint theorie. Hierin passen metabole parameters zich aan naar gelang de omstandigheden. Dit zorgt voor een beweegbaar setpoint in plaats van een vast setpoint. Ook laat deze studie zien dat de keuze voor een specifiek dieet bepalend is voor de uitkomsten van een onderzoek op het kruispunt van voeding en fysiologie. De fysiologie en moleculair regulatie van vetweefsel is verder onderzocht in **hoofdstuk 3**. Gewichtsverlies werd gebruikt om metabole stress te vergroten. Om dit te bereiken werden obese C57BL/6J-muizen beperkt tot 70% inname van het vet-rijke dieet of gewisseld naar een onbeperkt vet-arm dieet gedurende 5 weken. In beide interventies werden gunstige effecten gezien met betrekking tot fysiologische parameters zoals een afname in lichaamsgewicht. Echter, regulatie van moleculaire markers in vetweefsel verschilde tussen de twee interventies. Muizen die het beperkte vetrijke dieet kregen hadden een hoger mitochondriaal (de energiefabriekjes van de

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cel) koolhydraat en vet metabolisme. Wanneer deze data geëxtrapoleerd wordt naar de menselijke situatie betekend dit dat een verkleining van portiegrootte de beste methode is om gewicht te verliezen.

Het is gebruikelijk om muizen te huisvesten bij kamertemperatuur tijdens fysiologische interventiestudies. Helaas worden muizen dan blootgesteld aan een omgevings temperatuur die beneden hun thermisch neutrale zone is. Dit betekent dat het metabolisme van de muizen altijd verhoogd is om zichzelf warm te houden. Hiervan is bekend dat dit studieresultaten kan beïnvloeden. In **hoofdstuk 4** wordt de tweede vraag onderzocht, "of de dieetgeïnduceerde metabole respons beïnvloedt wordt door huisvesting bij thermoneutraliteit". Hiervoor is een 14 weken durende interventie uitgevoerd bij 28°C waarbij C57BL/6J-muizen een vet-arm dieet of een matig hoog vet dieet werden gevoerd. Dit resulteerde in grote dieetgeïnduceerde verschillen in lichaamsgewicht, vetweefselmassa, vetcel grootte, en serum leptine -niveaus. Maar er was geen verschil op serum glucose, vrije vetzuren, triacylglyceriden, insuline, een panel van cardiovasculaire markers, en een aantal (metabole) parameters in de lever en spieren. Hoewel vetmassa en vetcel grootte aanzienlijk waren verhoogd, was er geen teken van ontsteking of dysfunctie in het vetweefsel aanwezig. Deze studie suggereert dat dieet-geïnduceerde zwaarlijvigheid van C57BL/6J-muizen gehuisvest bij thermoneutraliteit resulteert in een geschikt model voor metabool 'gezonde' obese (mensen met aanzienlijk overgewicht die geen van de gebruikelijke stofwisselingsproblemen vertonen). Daarnaast benadrukt deze studie het belang van de huisvestings temperatuur voor muizen, aangezien die een grote invloed op de uitkomst van de studieresultaten heeft.

Met de derde en laatste vraag hebben we onderzocht of zuurstof beperking ontstekingen kan induceren in vetweefsel. Er is substantieel bewijs dat er een te laag zuurstof gehalte in vetweefsel ontstaat wanneer de vetcellen overmatig vergroten. Omdat de uitbreiding van het vetweefsel zeer snel gaat is het vaatstelsel niet in staat om een gelijke groei te vertonen, wat ook kan leiden tot een te laag zuurstofgehalte in het vetweefsel. Daarnaast kunnen de vetcellen zo groot worden dat zuurstof transport over de vetcel niet meer mogelijk is en daardoor een te laag zuurstofgehalte ontstaat. Om te onderzoeken of een zuurstof tekort in vetweefsel in staat is om ontstekingen te starten in vetweefsel hebben we het model voor gezonde obese vetcellen (ontwikkeld in **hoofdstuk 4**) gebruikt en deze muizen blootgesteld aan zuurstof beperking (13% in plaats van 21%) om zo het vetweefselmetabolisme uit te dagen. Dit resulteerde in verhoogde hemoglobine en hematocriet niveaus wat wijst op verlaging van het zuurstof niveau in het gehele lichaam. Daarnaast schakelt het lichaam van aerobe naar anaerobe glycolytische verbanding wat indicatief is voor zuurstof verlaging in het weefsel. Er werd geen verschil gevonden in indringing van macrofagen (marker voor ontstekingen) in vetweefsel. Echter, de niveaus van vertakte keten aminozuren en adipokines in serum waren veranderd. Het niveau van vertakte keten aminozuren was verhoogd in muizen die waren blootgesteld aan zuurstof beperking. Dit vertoont gelijkenis met de bevindingen bij de mens, waar

verhoogde gehaltes gevonden zijn in magere tegenover zwaarlijvige mensen. Het peptide hormoon adiponectine was verhoogd in serum, zonder dat er verschillen waren in gen expressie in vetweefsel. Anderzijds vertoonden de peptide hormonen CCDC3 en CCK juist verlaagde gen expressie niveaus in vetweefsel zonder verandering in serum niveaus, hoewel er voor CCDC3 een trend werd waargenomen. Samen suggereren deze resultaten dat zuurstof beperking niet de onwikkeling van ontstekingen in vetweefsel start. Echter, het beïnvloedt wel adipokine regulatie.

Na het uitvoeren van bovenstaande studies was het duidelijk dat de samenstelling van het voer een grote invloed heeft op de uitkomsten van fysiologische studies, zoals is weergegeven in **hoofdstuk 2**. Om functionele effecten van verschillende voedingsstoffen te vergelijken, is het belangrijk gestandaardiseerde gezuiverde diëten te gebruiken. Niet alleen het experimentele dieet is van belang, maar ook het controle dieet kan de resultaten beïnvloeden. Bijvoorbeeld, wanneer een interventie wordt uitgevoerd met een hoog vet dieet dat gezuiverd is en vergleken wordt met een referentie (chow) dieet. De samenstelling van chow is variabel omdat het op basis is van granen zoals gemalen maïs, gemalen haver, sojameel en gemalen tarwe). Voedzaamheid wordt verzekerd door toevoeging van vitaminen, mineralen en vet. Echter, de exacte samenstelling van de verschillende ingrediënten wordt vaak geheim gehouden door de fabrikant. Daarnaast zorgt de plantaardige oorsprong van het referentie (chow) dieet dat het de nutritieve componenten (eiwitten, koolhydraten, vetten), maar ook niet–nutritieve bestanddelen zoals fotochemicaliën bevat. De samenstelling van het chow dieet variëert van partij tot partij aangezien de nutritieve en niet-nutritieve waarde verandert tussen oogsten. Wanneer er gebruik gemaakt wordt van een chow dieet in vergelijking met een gezuiverd dieet zul je nooit precies weten wat je aan het vergelijken bent, bijvoorbeeld een verschil in aminozuren of de gevolgen van fytochemicaliën etc. Daarom is er een gezuiverd referentie dieet voor fysiologische interventies ontworpen (**hoofdstuk 6**) zodat een vergelijking tussen verschillende studies mogelijk wordt. Een belangrijk kenmerk van het referentie dieet is de vaste eiwitconcentratie. Dit zorgt ervoor dat er alleen een uitwisseling van koolhydraten en vetten plaatsvindt in een vetrijke versie van het dieet.

In conclusie geeft het werk beschreven in dit proefschrift inzicht in factoren die van belang zijn voor de verbetering van de vertaalbaarheid van muis studies naar de menselijke situatie. Er werd aangetoond dat bij het onderzoek van de gewichtsbalans (setpoint) vele factoren zoals, genetica, metabolisme, omgevingsfactoren zoals huisvestingstemperatuur en cognitief gedrag, naast de voeding en de samenstelling van het voer uitkomstparameters kunnen beïnvloeden. De meeste muisexperimenten worden uitgevoerd in een vaste omgeving zonder keuze in voer en een standaard omgevings temperatuur ingesteld op 22°C. Dit is duidelijk geen afspiegeling van de mens bij vrije leefomstandigheden. Echter, deze vaste condities kunnen leiden tot experimenten die de onderliggende mechanismen van de gewichtsbalans ontrafelen. Wat ons uiteindelijk een stap dichter bij de ontdekking van de oplossing voor de obesitas epidemie brengt.

DANKWOORD

Het is af, het is echt een boekje geworden. Iets meer dan 4 jaar geleden had ik geen flauw idee hoe ik dit zou gaan doen, maar het is gelukt! Een ding is zeker, promoveren doe je zeker niet alleen. Omdat dit waarschijnlijk het 'best' gelezen stuk wordt van mijn proefschrift maak ik hier graag gebruik van de gelegenheid om een aantal mensen te bedanken.

Jaap, bedankt voor je onuitputbare enthousiasme voor onderzoek doen en alle verschillende ideeën. Je kritische vragen hebben er voor gezorgd dat ik me ontwikkeld heb tot wetenschapper.

Evert, ontelbare keren ben ik je kamer binnen gelopen om mijn laatste vondsten te bespreken, altijd had je tijd. Ook wist je altijd de positieve kanten naar boven te brengen als ikzelf iets minder overtuigd was.

Elise en **Loes**, ongeveer tegelijkertijd zijn we begonnen aan onze projecten. Zeker het laatste jaar konden we door de diversiteit in onze projecten elkaar helpen en versterken. Buiten dat waren er natuurlijk ook de ontelbare kopjes thee en gezellige momenten.

Lieve **HAP**pers bedankt voor de ontzettend mooie tijd samen! Ik heb veel van jullie mogen leren maar ook veel met jullie gelachen en leuke dingen gedaan, Bedankt!

Andries, **Elise**, **Robin**, **Jenny**, **Lidya**, **Lidwien** and **Hana**, my small army of students. Thank you all very much for your contributions to my project. Unfortunately, not everything is visible in this thesis but all your projects have led to new insights and are appreciated nonetheless!

Hanneke, **Maxence**, en **Lieke** vele avonden hebben we samen aan de tafel gezeten met veel lekker eten en gediscussieerd over van alles en nog wat. Heerlijk ontspannen! En nu nog steeds. Na mijn fijne valpartij had ik niet zonder jullie hulp kunnen opkrabbelen. Dankjewel!

Pap en mam, zonder jullie steun en eeuwige vertrouwen had ik het nooit zover geschopt! Ook al was het misschien niet altijd even duidelijk waar ik mee bezig was interesse was er altijd voor mijn proefjes. En dat geldt natuurlijk ook voor jullie **Hanneke**, **Frank** en **Joep** Dankjewel!

Lieve **Robert**, misschien de laatste in het rijtje maar zeker belangrijk. Bij jou is er voor mij rust, ook al was ik druk of gestrest. Op naar vele nieuwe avonturen samen!

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LIST OF PUBLICATIONS

Hoevenaars FPM, van Schothorst EM, Horakova A, Voigt A, Rossmeisl M, Pico C, Caimari A, Kopecky J, Keijer J. BIOCLAIMS standard diet (BIOsd): a reference diet for nutritional physiology. Genes & Nutrition 2012 Jul;7(3):399-404

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CURRICULUM VITAE

Femke Petronella Maria Hoevenaars was born on June 23 1984 in Valkenswaard, the Netherlands. She graduated from grammar school at SG Were Di in Valkenswaard in 2002. In that same year she started her study Nutrition and Health at Wageningen University, with molecular nutrition as a sub specialism. During her study she conducted her Master internship at the Burnham Institute for Medical Research (La Jolla, CA) under supervision of Prof. Dr. Hudson Freeze and Prof. Dr. Lars Bode on congenital disorders of glycosylation. She completed her MSc degree in 2009 with a thesis at the department of molecular nutrition and pharmacology on the effects of cannabinoids on human mesenchymal stem cells and their differentiation. In October 2009, she started as a PhD student at the department of Human and Animal Physiology at Wageningen University, Wageningen, the Netherlands. The project, under the supervision of Prof. Dr. Jaap Keijer and Dr. Evert M. van Schothorst is presented in this dissertation. From February 2014 onwards, she is employed as a postdoctoral researcher at the department of Physiology of the VU university medical center in Amsterdam.

EDUCATION STATEMENT

WIAS Graduate School Training and Supervision plan 2009-2013

The basic package (3 ECTS)

WIAS introduction Course
Ethics and Philosophy in Life Sciences

Scientific exposure (10 ECTS) *International Conferences, Seminars and Workshops*

Symposium NCMLS Bioenergetics: Live & Let die 2010
Workshop on Obesity and its link to type 2 Diabetes 2010, Munchen
Bioactive Food Components, Energy Metabolism and Health 2011, Wageningen
Recent Advances and Controversies in Measuring Energy Metabolism 2011, Maastricht (poster)
Phenotypic Flexibility Symposium 2013, Madrid (oral)
Symposium: Systems biology of gene transcription 2009
WIAS science day 2010, 2011 (poster), 2012 (oral) and 2013
Nederlandse Associatie voor de Studie van Obesitas symposium 2012

In-Depth studies (6 ECTS)

PhD course Measuring mitochondrial function
Defining Health; from basic science to industrial relevance 9th international masterclass nutrigenomics
Systems biology: 'Statistical analysis of ~Omics data"
Introduction to Echo-MRI
Using R in data analysis
HAP scientific meetings 2009-2013

Statutory courses (4 ECTS)

Use of laboratory Animals, Wageningen University

Professional skills support courses (3 ECTS)

Effective behaviour in your professional surroundings
Course Supervising MSc thesis work
Techniques for writing and presenting a scientific paper
Workshop stress identification and management
Workshop 'Last stretch of the PhD programme'

Research skills training (7 ECTS)

External training period Prague 2 weeks
Writing Marie Curie application FP7-PEOPLE-2013-IEF

Didactic skills training (20 ECTS) *Lecturing, Supervising practical's and Theses, Tutorship*

Oral presentation PhD course Measuring cellular Mitochondrial Function
HAP-30306, partial role in lecturing
Human and animal physiology 2; pf5 blood
Principles of human physiology
Nutritional Physiology
Human and animal physiology 2; pf4 digestion
Supervision of 7 MSc thesis projects
Human and Animal Physiology 1

Total (53 ECTS)

Colophon

Funding

The research described in this thesis was financially supported by Wageningen graduate school WIAS.

Financial support from the Human and Animal Physiology Group of Wageningen University and, TSE systems for printing this thesis is gratefully acknowledged.

Cover design

Thomas van den Berg

Lay out and Printing

Off Page, Amsterdam, The Netherlands

Femke P.M. Hoevenaars, 2014