Metabolic mechanisms behind the type 2 diabetes susceptible phenotype in low birth weight individuals

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Metabolic mechanisms behind the type 2 diabetes susceptible phenotype in low birth weight individuals

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PREFACE

The present PhD thesis represents the current knowledge within foetal programming of type 2 diabetes and the research activities performed in the present PhD project to gain novel insights into this area.

The PhD project comprises three plasma metabolome studies in a population of young, healthy, low and normal birth weight men that have been exposed to a short-term high-fat overfeeding intervention. The overall aim of the studies was to investigate metabolic mechanisms behind the type 2 diabetes susceptible phenotype in low birth weight individuals. The project has been carried out at Department of Biotechnology and Biomedicine, Technical University of Denmark, Kongens Lyngby, Denmark with supervision from Associate Professor Lars I. Hellgren, Associate Professor Susanne Brix Pedersen, and Associate Professor Kristian Fog Nielsen, and in addition at Department of Endocrinology, Diabetes and Metabolism, Rigshospitalet, Copenhagen, Denmark with supervision from Chief Physician and Professor Allan A. Vaag and Post Doc Rasmus Ribel-Madsen. The population of low and normal birth weight men was collected and thoroughly clinically and physiologically characterised by Allan A. Vaag and co-workers prior to the start of the present project. Therefore, the project has involved plasma metabolome analyses on already generated data as regards the first two studies and experimental work for further such analyses as concerns the third study. The findings from the first two studies have been published in original research journal articles, and the results from the third study are submitted for publication in article form as well:

- **Study 1**: Plasma acylcarnitine profiling indicates increased fatty acid oxidation relative to tricarboxylic acid cycle capacity in young, healthy low birth weight men. *Physiological Reports*, September 2016.
- **Study 2**: Plasma amino acid levels are elevated in young, healthy low birth weight men exposed to short-term high-fat overfeeding. *Physiological Reports*, December 2016.
- **Study 3**: Plasma ceramide levels are altered in low and normal birth weight men in response to short-term high-fat overfeeding. Re-submitted to *Scientific Reports*, 22 December 2017.

The articles are enclosed to the thesis in Appendix 1, 2, and 3, respectively.

I was awarded my PhD scholarship from Technical University of Denmark and Rigshospitalet. Furthermore, the third study was supported through obtained grants from the Aase and Ejnar Danielsen Foundation and the Augustinus Foundation.

Amalie Ribel-Madsen

Kongens Lyngby, 27 October 2017

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First of all, I wish to thank my five PhD supervisors:

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Susanne, I am very pleased that I could continue my PhD study together with you and in your group, which I fortunately also have some of my roots in, following the loss of Lasse. I thank you very much for taking good care of me and for our scientific discussions in relation to the third article and the thesis.

Kristian, I especially thank you for the training in the mass spectrometry techniques for the ceramide analyses to the third study. I also thank you for your persistent work prior to and during my PhD study with the development of these fine analyses and additional lipid analyses at the Metabolomics Platform.

Allan, I am very pleased that I had the opportunity to work together with you and Charlotte Brøns with the high-fat overfeeding study population. I followed your research within foetal programming of metabolic diseases with great interest prior to my PhD study, and I was therefore very excited

that I could be involved in such extensive studies. I thank you and Charlotte very much for this and our scientific discussions in connection with the articles.

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Also, I want to thank Christopher B. Newgard, Duke University, for the performance of the plasma acylcarnitine and amino acid analyses for the first two studies and our collaboration with the articles of these studies.

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SUMMARY

Background and aims: Low birth weight (LBW) individuals have an increased risk of developing insulin resistance and type 2 diabetes compared with normal birth weight (NBW) individuals. Accordingly, young, healthy, LBW men of the study population examined in the present plasma metabolome studies show impaired hepatic insulin sensitivity and, in contrast to NBW men, develop impaired peripheral insulin sensitivity in response to a 5-day high-fat overfeeding. However, the metabolic mechanisms behind the type 2 diabetes susceptible phenotype in LBW individuals are not clear. Our primary aim of the present studies was to get novel insights into such mechanisms. LBW men of the present study population have lower pre-adipocyte mRNA expression levels of several differentiation markers, which may potentially lead to an impaired fatty acid storage capacity of these cells and a resulting increased fatty acid load to non-adipose tissue. Also, the LBW men display an increased fatty acid oxidation and a decreased glucose oxidation during both the isocaloric control diet and 5-day high-fat, high-calorie (HFHC) diet. Our specific aims of the present studies were to test the hypotheses that LBW men could have 1) an increased, incomplete fatty acid beta-oxidation in mitochondria, 2) an altered amino acid metabolism to ensure an adequate supply of tricarboxylic acid (TCA) cycle intermediates and thereby enable an efficient acetyl-CoA oxidation, and 3) an increased fatty acid flux into lipogenesis, including de novo ceramide synthesis, in non-adipose tissue.

Methods: Fasting plasma levels of 45 acylcarnitines, 15 amino acids, and 27 ceramides were measured in the young, healthy, LBW (≤ 10th percentile) and NBW (50-90th percentile) men of the above mentioned study population after the isocaloric control diet and 5-day HFHC (60 E % from fat, 50 % extra calories) diet intervention.

Results and interpretations: LBW men had higher plasma C2 and C4-OH acylcarnitine levels after the control diet, compared with NBW men, indicating an increased, incomplete fatty acid beta-oxidation in mitochondria with the limiting step at the acetyl-CoA oxidation via the TCA cycle and an increased ketogenesis, respectively. Furthermore, LBW men had higher plasma C6-DC, C10-OH/C8-DC, and total hydroxyl-/dicarboxyl-acylcarnitine levels after the control diet, compared with NBW men, suggesting an increased fatty acid omega-oxidation in the endoplasmic reticulum of mainly the liver. Interestingly, the total hydroxyl-/dicarboxyl-acylcarnitine level was negatively associated with the fasting serum insulin level and hepatic insulin resistance after this diet. An increased omega-oxidation rate may therefore limit the amount of fatty acid substrates available for

lipogenesis, including the synthesis of lipotoxic lipids such as ceramides and diacylglycerols that impair insulin signalling. In the second study, we demonstrated that LBW men had higher plasma alanine, proline, methionine, citrulline, and total amino acid levels after the HFHC diet compared with NBW men. The alanine level was negatively associated with the plasma C2 acylcarnitine level after this diet. A higher alanine level in the LBW men after the HFHC diet could therefore be accompanied by an increased anaplerotic formation of oxaloacetate to enable an efficient acetyl-CoA oxidation via the TCA cycle. Furthermore, the alanine and total amino acid levels tended to be negatively associated with the insulin-stimulated glucose uptake rate after the HFHC diet. Higher alanine and total amino acid levels in the LBW men after this diet could therefore be a consequence of their reduction in skeletal muscle insulin sensitivity due to high-fat overfeeding with a following increased skeletal muscle proteolysis and/or may potentially contribute to the impaired insulin sensitivity. Moreover, the alanine level was positively associated with the hepatic glucose production after the HFHC diet. A higher alanine level in the LBW men could therefore also be accompanied by an increased gluconeogenesis in the liver. In the third study, we found that LBW men did not show altered plasma ceramide levels after the control or HFHC diet compared with NBW men. An increased fatty acid oxidation rate in the LBW men during both diets may limit the amount of fatty acids available for de novo ceramide synthesis and thereby compensate for a likely increased fatty acid load to non-adipose tissue in these individuals.

Conclusions: LBW men showed alterations in fasting plasma acylcarnitine and amino acid levels after the isocaloric control diet and 5-day HFHC diet, respectively, that have been described to be associated with insulin resistance and type 2 diabetes. Additional plasma and tissue metabolome studies in LBW and NBW individuals, as well as supplementary functional studies, are needed to further explain the metabolic events leading to the altered plasma metabolite profiles in LBW men, and moreover to determine the extent to which these events may be part of the type 2 diabetes susceptible phenotype in LBW individuals.

SAMMENFATNING

Baggrund og formål: Individer med lav fødselsvægt (LBW) har en øget risiko for at udvikle insulinresistens og type 2 diabetes sammenlignet med individer med normal fødselsvægt (NBW). I overensstemmelse hermed udviser unge, raske, LBW-individer fra studiepopulationen, der er blevet nærmere undersøgt i de nærværende metabolomstudier, nedsat hepatisk insulinfølsomhed og, i modsætning til NBW-individer, udvikling af nedsat perifer insulinfølsomhed under en 5-dages høj-fedt overfodring. Mekanismerne bag den type 2 diabetes-følsomme fænotype in LBW-individer er ikke fuldt ud klarlagte. Vores primære formål med metabolomstudierne var at opnå ny indsigt i sådanne mekanismer. LBW-individerne fra den aktuelle studiepopulation har lavere pre-adipocyt mRNA-ekspressionsniveauer af flere differentieringsmarkører, hvilket potentielt kunne føre til en nedsat evne af fedtvævet til at lagre fedtsyrer og således give anledning til et øget fedtsyreload til øvrige væv såsom leveren og skeletmuskel. LBW-individerne udviser endvidere en øget fedtsyreoxidation og en nedsat glukoseoxidation under både kontrol- og høj-fedt, høj-kalorie- (HFHC) kosten. Vores specifikke formål med studierne var at teste hypoteserne, at LBW-individer kunne have 1) en øget, ufuldstændig beta-oxidation af fedtsyrer i mitokondrierne, 2) en ændret aminosyremetabolisme for at kunne sikre en tilstrækkelig mængde TCA-cyklus-intermediater til at opnå en effektiv acetyl-CoA-oxidation og 3) et øget fedtsyreflux til lipogenese, herunder de novo ceramidsyntese, til andre væv end fedtvæv.

Metoder: Faste-plasmaniveauer af 45 acylcarnitiner, 15 aminosyrer og 27 ceramider blev målt i de unge, raske, LBW- (≤ 10. percentil) og NBW- (50.-90. percentil) mænd fra den oven for nævnte studiepopulation efter både den isokaloriske kontrolkost og 5-dages HFHC- (60 E % fra fedt, 50 % ekstra kalorier) kost.

Resultater: LBW-individer havde højere plasma-C2 og C4-OH acylcarnitinniveauer efter kontrolkosten, sammenlignet med NBW-individer, hvilket indikerer en øget, ufuldstændig beta-oxidation af fedtsyrer i mitokondrierne med acetyl-CoA-oxidationen via TCA-cyklus som det begrænsende trin henholdsvis en øget ketogenese. LBW-individerne havde endvidere højere plasma-C6-DC, C10-OH/C8-DC og total hydroxyl-/dicarboxyl-acylcarnitinniveauer efter kontrolkosten, sammenlignet med NBW-individer, hvilket kunne tyde på en øget omega-oxidation af fedtsyrer i det endoplasmatiske retikulum i primært leveren. Interessant var det totale hydroxyl-/dicarboxyl-acylcarnitinniveau negativt associeret med faste-serum-insulinniveauet og hepatisk insulinresistens efter kontrolkosten. En øget omega-oxidation kunne således begrænse mængden af

fedtsyrer, der er tilgængelig for lipogenese, herunder syntesen af lipotoksiske lipider såsom ceramider og diacylglyceroler, der hæmmer insulinsignaleringen. I det andet studie viste vi, at LBW-individer havde højere plasma-alanin-, prolin-, methionin-, citrullin- og total aminosyreniveauer efter HFHC-kosten sammenlignet med NBW-individer. Alaninniveauet var negativt associeret med plasma-C2 acylcarnitinniveauet efter denne kost. Et højere alaninniveau i LBW-individerne efter HFHC-kosten kunne således føre til en øget anaplerotisk dannelse af oxaloacetat for derved at kunne sikre en effektiv acetyl-CoA-oxidation via TCA-cyklus. Alaninniveauet og det totale aminosyreniveau tenderede endvidere til at være negativt associeret med det insulin-stimulerede glukoseoptag efter HFHC-kosten. Højere alanin- og total aminosyreniveauer i LBW-individerne efter denne kost kunne derfor være en følge af deres reduktion i den perifere insulinfølsomhed under høj-fedt overfodringen med en efterfølgende øget proteolyse i skeletmuskel og/eller kunne potentielt give anledning til den nedsatte insulinfølsomhed. Alaninniveauet var desuden positivt associeret med den hepatiske glukoseproduktion efter HFHC-kosten. Et højere alaninniveau i LBW-individerne kunne således også føre til en øget glukoneogenese i leveren. I det tredje studie fandt vi, at LBW-individer ikke havde ændrede plasmaniveauer af ceramider efter kontrol- eller HFHC-kosten sammenlignet med NBW-individer. En øget fedtsyreoxidation i LBW-individerne under begge kostinterventioner kunne således begrænse mængden af fedsyrer, der er tilgængelige for de novo ceramidsyntese, og derved kompensere for et muligt øget fedtsyreload til leveren og skeletmuskel i disse individer.

Konklusioner: LBW-individer udviste ændringer i faste-plasmaniveauer af acylcarnitiner og aminosyrer efter kontrol- henholdsvis 5-dages HFHC-kosten, der har været beskrevet at være associeret med insulinresistens og type 2 diabetes. Yderligere metabolomstudier på både plasma- og vævsniveau i LBW- og NBW-individer, samt supplerende funktionelle studier, vil være nødvendige for nærmere at forstå de metaboliske forandringer, der giver anledning til de ændrerede metabolitprofiler i LBW-individer, og endvidere for at kunne bestemme, hvorvidt disse forandringer er en del af den type 2 diabetes-følsomme fænotype i LBW-individer.

ABBREVIATIONS

AC: Acylcarnitine, **ATP**: Adenosine triphosphate, **BMI**: Body mass index, **BW**: Birth weight, **CHOL**: Cholesterol, **CoA**: Coenzyme A, **CPT**: Carnitine palmitoyl-transferase, **CrAT**: Carnitine acetyl-CoA transferase, **DC**: Dicarboxyl, **DEXA**: Dual-energy X-ray absorptiometry, **DI**: Disposition index, **EDTA**: Ethylenediaminetetraacetic acid, **EE**: Energy expenditure, **ER**: Endoplasmic reticulum, **FFM**: Fat free mass, **FGF**: Fibroblast growth factor, **FOX**: Fatty acid oxidation, **FPIR**: First phase insulin response, **GIP**: Gastric inhibitory polypeptide, **GLUT**: Glucose transporter, **GOX**: Glucose oxidation, **HbA1c**: Haemoglobin A1c, **HBW**: High birth weight, **HDL**: High-density lipoprotein, **HFHC**: High-fat, high-calorie, **HGP**: Hepatic glucose production, **HPLC**: High pressure liquid chromatograph/liquid chromatography, **HRMS**: High-resolution mass spectrometer/mass spectrometry, **IGF**: Insulin-like growth factor, **IR**: Insulin resistance, **IRS**: Insulin receptor substrate, **IS**: Internal standard, **IVGTT**: Intravenous glucose tolerance test, **LBW**: Low birth weight, **LDL**: Low-density lipoprotein, **miR**: Micro RNA, **mRNA**: Messenger RNA, **MRS**: Magnetic resonance spectroscopy, **MS**: Mass spectrometer/mass spectrometry, **NBW**: Normal birth weight, **NEFA**: Non-esterified fatty acid, **NF-KB**: Nuclear factor kappa-light-chain-enhancer of activated B-cells, **OGTT**: Oral glucose tolerance test, **OH**: Hydroxyl, **PK**: Protein kinase, **POX**: Protein oxidation, **RNA**: Ribonucleic acid, **SD**: Standard deviation, **SEM**: Standard error of mean, **SPT**: Serine palmitoyl-transferase, **TCA**: Tricarboxylic acid, **TG**: Triacylglycerol, **TOF**: Time of flight, **VLDL**: Very-low density lipoprotein.

JOURNAL ARTICLES

The findings from the first two studies have been published in the following original research journal articles, and the results from the third study are submitted for publication in article form as well:

- **Ribel-Madsen, Amalie**; Ribel-Madsen, Rasmus; Brøns, Charlotte; Newgard, Christopher B; Vaag, Allan A & Hellgren, Lars I. (2016). Plasma acylcarnitine profiling indicates increased fatty acid oxidation relative to tricarboxylic acid cycle capacity in young, healthy low birth weight men. *Physiological Reports* 4, e12977. DOI: 10.14814/phy2.12977

- **Ribel-Madsen, Amalie**; Hellgren, Lars I; Brøns, Charlotte; Ribel-Madsen, Rasmus; Newgard, Christopher B & Vaag, Allan A. (2016). Plasma amino acid levels are elevated in young, healthy low birth weight men exposed to short-term high-fat overfeeding. *Physiological Reports* 4, e13044. DOI: 10.14814/phy2.13044

- **Ribel-Madsen, Amalie**; Ribel-Madsen, Rasmus; Nielsen, Kristian F; Brix, Susanne; Vaag, Allan A & Brøns, Charlotte. Plasma ceramide levels are altered in low and normal birth weight men in response to short-term high-fat overfeeding. Re-submitted to *Scientific Reports*, 22 December 2017.

CHAPTER 1 – INTRODUCTION

Type 2 diabetes mellitus is a complex metabolic disorder characterised by impaired insulin sensitivity, insufficient insulin secretion, and eventual pancreatic beta-cell failure (1) (Figure 1.1). Insulin regulates the fuel homeostasis through 1) stimulation of glucose uptake into peripheral tissues, including skeletal muscle, cardiac muscle, and adipose tissue, 2) stimulation of glycolysis in the liver, skeletal muscle, and adipose tissue, 3) stimulation of glycogenesis and suppression of glycogenolysis in the liver and skeletal muscle, 4) suppression of gluconeogenesis in the liver, 5) stimulation of fatty acid synthesis in the liver, 6) stimulation of lipogenesis in the liver and adipose tissue and suppression of lipolysis in adipose tissue, 7) stimulation of uptake of certain amino acids into skeletal muscle, and 8) stimulation of protein synthesis in the liver, skeletal muscle, and adipose tissue and suppression of proteolysis in skeletal muscle (2). An impaired insulin secretion and action therefore leads to multiple metabolic abnormalities, including hyperglycaemia and dyslipidaemia. Also, increases in circulating glucose and lipid levels can further impair insulin secretion and sensitivity and cause tissue damage. Accordingly, the diagnostic criteria of diabetes include, as defined by the American Diabetes Association, a haemoglobin A1c (HbA1c) ≥ 6.5 %, a fasting plasma glucose level ≥ 7.0 mmol/L, or a 2 hour plasma glucose level ≥ 11.1 mmol/L during a 75 g oral glucose tolerance test (OGTT) (3).

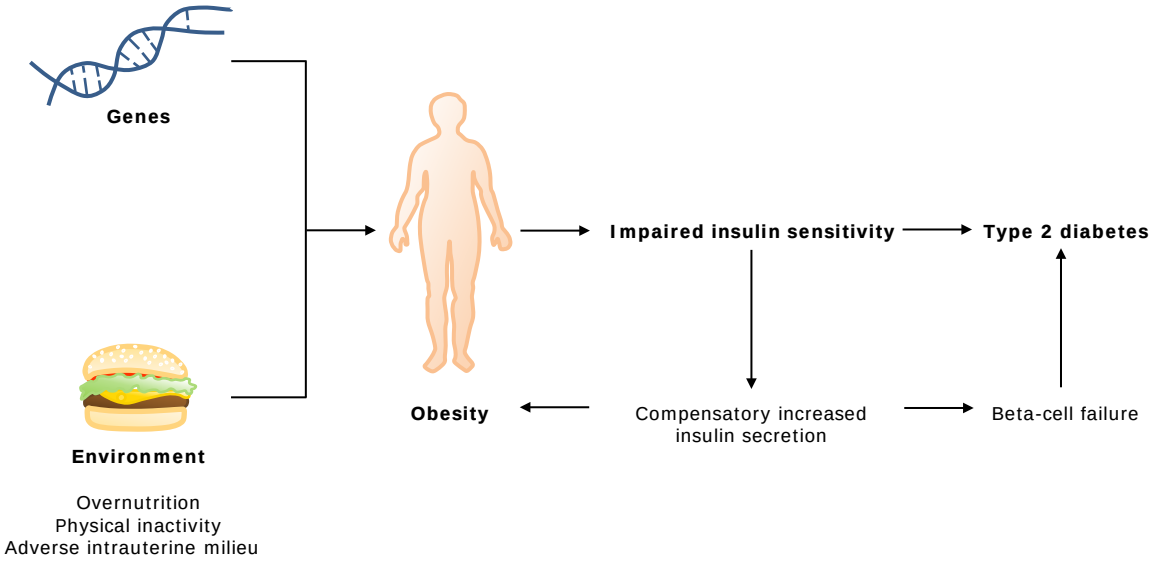


Figure 1.1: Aetiological factors of type 2 diabetes and metabolic events leading to this disease. Obesity is an important risk factor of insulin resistance and type 2 diabetes, and so are a genetic susceptibility to obesity and environmental factors such as overnutrition and physical inactivity important risk factors of these metabolic diseases.

Aetiological factors of type 2 diabetes include genetic and environmental components and, in the interface between these, epigenetic components (Figure 1.1). Obesity is a particularly important risk factor of insulin resistance and type 2 diabetes, and so are the risk factors of obesity, including a genetic susceptibility to this condition as well as overnutrition and physical inactivity, central in the development of these metabolic diseases. Furthermore, among the environmental components, it has become apparent from research by in particular C.N. Hales and D.J.P. Barker with co-workers that an adverse intrauterine milieu, and an associated low birth weight (LBW), is an important risk factor of several metabolic diseases, including obesity, cardiovascular disease, and type 2 diabetes (4-7). Accordingly, studies performed in human LBW subjects, including the high-fat overfeeding study that the present studies are part of, have demonstrated several metabolic abnormalities in these individuals relevant to the pathophysiology of type 2 diabetes. Thus, young (23-27 years of age), apparently healthy, LBW (≤ 10th percentile) men of the present overfeeding study population have higher fasting blood glucose, serum insulin, and serum C-peptide levels, compared with normal birth weight (NBW) (50-90th percentile) men, and moreover an impaired hepatic insulin sensitivity (8). Furthermore, these same LBW men, in contrast to NBW men, develop impaired peripheral insulin sensitivity in response to a 5-day high-fat overfeeding (9). Nevertheless, the underlying or exacerbating metabolic mechanisms behind the type 2 diabetes susceptible phenotype in LBW individuals are not clear.

Our primary overall aim of the present studies included in this thesis was to get novel insights into such mechanisms, as outlined in the section below. Specifically, we performed a series of interrelated plasma metabolome analyses in the young, healthy, LBW and NBW men of the above mentioned overfeeding study population after the isocaloric control diet and 5-day high-fat, high-calorie (HFHC) (60 E % from fat, 50 % extra calories) diet intervention (8; 9).

CHAPTER 2 – AIMS AND HYPOTHESES

LBW men of the present overfeeding study population show, in addition to the metabolic abnormalities mentioned above, an increased fatty acid oxidation rate and a decreased glucose oxidation rate at night time during both the isocaloric control diet and HFHC diet compared with NBW men (10; 11). Also, the LBW men display a higher adjusted total energy expenditure at night during the HFHC diet, compared with NBW men, and a higher relative contribution of fatty acid oxidation to the total energy expenditure during this diet (11).

Our primary specific aims of the first two studies were to test the following hypotheses:

- **Study 1**: LBW men could have an increased, incomplete fatty acid beta-oxidation in mitochondria during the control diet and HFHC diet, expected to be reflected by higher plasma acylcarnitine levels. Also, as acylcarnitines may potentially impair insulin signalling, higher such levels could be associated with their impaired insulin sensitivity.

- **Study 2**: LBW men could, as a consequence of their changes in fatty acid oxidation and glucose oxidation partitioning during both diets, have an altered amino acid metabolism, expected to be reflected in plasma amino acid levels, to ensure an adequate supply of tricarboxylic acid (TCA) cycle intermediates and thereby enable an efficient or enhanced acetyl-CoA oxidation. Furthermore, such alterations in amino acid metabolism could be part of the adverse metabolic events leading to their higher fasting blood glucose level after the control diet and impaired insulin sensitivity.

LBW men of the present study population moreover show lower pre-adipocyte mRNA expression levels of several differentiation markers (12), which may potentially lead to an impaired fatty acid storage capacity of these cells and a resulting increased fatty acid load to non-adipose tissue such as the pancreas, liver, and skeletal muscle. Also, young (20-30 years of age), healthy, LBW men of another study population than the presently examined show an increased whole body (13) and adipose tissue (14) lipolysis. This was, however, not followed by elevated plasma non-esterified fatty acid (NEFA) or triacylglycerol levels (13; 14), indicating an increased uptake of fatty acids from the blood by other organs or tissues and/or an increased clearance or metabolism of fatty acids in the adipose tissue.

Our primary specific aims of the third study were therefore to test the following hypotheses:

- **Study 3**: LBW men could, as a result of a likely increased fatty acid load to non-adipose tissue, have an increased lipogenesis, including de novo ceramide synthesis, in these tissues, expected to be reflected by higher plasma ceramide levels. Also, as ceramides are known to impair insulin signalling and promote inflammation, higher such levels could be associated with their impaired insulin sensitivity, including in particular their reduced hepatic insulin sensitivity after the control diet.

In order to test these hypotheses, we measured fasting plasma levels of 45 acylcarnitines, 15 amino acids, and 27 ceramides (individual or pools of species) in the LBW and NBW men of the overfeeding study population after both the isocaloric control diet and 5-day HFHC diet and associated these plasma levels to measures of, among others, insulin secretion and sensitivity.

CHAPTER 3 – BACKGROUND

The current knowledge on the type 2 diabetes susceptible phenotype in LBW individuals, as obtained from epidemiological studies as well as animal and human experimental studies, are presented in the following sections, and subsequent to this, the background and motivation for the present plasma metabolome studies are presented.

Epidemiological studies on the type 2 diabetes risk in LBW individuals

Several years ago, it was recognised that the early in life environment has long-term effects on health. Some of the first studies describing this concern examinations of geographical variations in mortality rates from cardiovascular disease in Norway (15) and England and Wales (16), respectively. Thus, a higher mortality rate from arteriosclerotic heart disease in particular counties of Norway was found to be linked to a higher past infant mortality rate (15). Therefore, it was suggested that poverty in childhood and adolescence followed by prosperity is a risk factor of arteriosclerotic heart disease (15). A similar relationship between mortality rates from ischaemic heart disease and infant mortality rates was found in a population from England and Wales (16). In connection with these observations, it was proposed that poor nutrition in early life increases the susceptibility to the adverse effects of an affluent diet (16). To further investigate this hypothesis, men born in Hertfordshire, England, for whom data on weight in infancy were recorded, were traced. In one study on this population, it was found that men with a lower weight at birth or at one year had a higher mortality rate from ischaemic heart disease (17). Following, it was sought to investigate if early in life factors could impact on as well subsequent development of glucose intolerance and type 2 diabetes. This idea was based on the knowledge that foetal and neonatal life seemed to be important periods for the development of the pancreatic beta-cells (18). So, 370 men of 64 years of age from Hertfordshire, for whom data on birth weight were available, were subjected to a glucose tolerance test and other clinical examinations (5; 6). From these investigations, it was found that men with a lower weight at birth or at one year were more likely to have an impaired glucose tolerance, defined as a 2 hour plasma glucose level of 7.8-11.0 mmol/L, and type 2 diabetes, defined as a 2 hour plasma glucose level $\geq$ 11.1 mmol/L (5). Among the men with the lowest birth weight (BW $\leq$ 2495 g), 40 % of these had either an impaired glucose tolerance or type 2 diabetes, whereas only 14 % of the men with the highest birth weight (BW > 4309 g) were

affected by one of these diseases (5). These LBW men were thereby 6.6 times more likely to have either an impaired glucose tolerance or type 2 diabetes compared with the men with the highest birth weight (5). Also, these same LBW men were 18 times more likely to be affected by the metabolic syndrome, defined as a 2 hour plasma glucose level > 7.8 mmol/L, a fasting plasma triacylglycerol level ≥ 1.4 mmol/L, and a systolic blood pressure > 160 mmHg (6).

In order to obtain a possible explanation of the observations in the above epidemiological studies, C.N. Hales and D.J.P. Barker formulated in 1992 the "thrifty phenotype" hypothesis (19) (Figure 3.1), as a parallel to the prior "thrifty genotype" hypothesis (20). This latter proposes that when a population goes through longer periods of poor or unstable nutrition, "thrifty genes" are selected to improve the survival of later generations under such conditions (20). The thrifty phenotype hypothesis similarly suggests that a foetus who is exposed to an adverse intrauterine environment, including in particular under- and/or malnutrition, adapt to these settings to increase its chances of pre- and post-natal survival. So, the foetus selectively distributes the sparse nutrients to the different organs to favour the growth and development of the vital organs, including the brain, at the expense of other organs and tissues, including the pancreas, liver, and skeletal muscle (19).

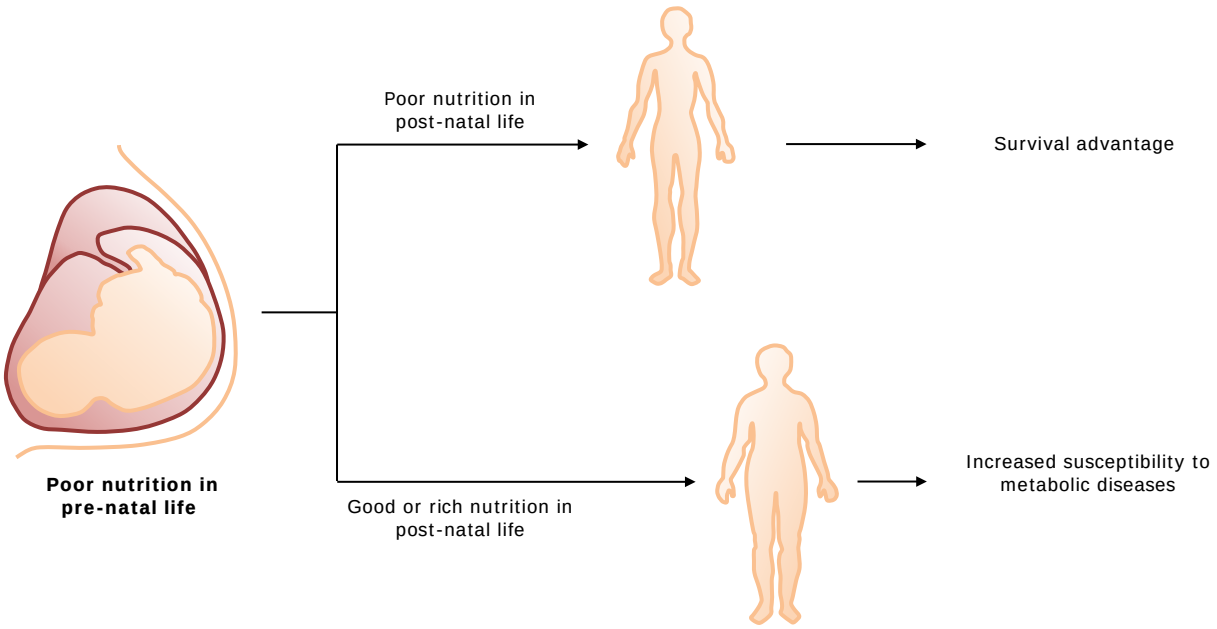


Figure 3.1: Hales and Barker's thrifty phenotype hypothesis. A foetus that is exposed to poor nutrition saves the sparse nutrients to the vital organs, including the brain, to the detriment of other organs and tissues and furthermore changes its metabolism. These adaptions increase its chances of pre- and post-natal survival under persisting poor nutrition, but render the foetus more susceptible to develop metabolic diseases, if it after birth is exposed to good or rich nutrition. Figure modified from C.L. Walker, 2012 (21).

Also, the foetus changes its metabolism to manage poor nutrition. These physiological adaptions, however, render the foetus more susceptible to develop metabolic diseases such as obesity, cardiovascular disease, and type 2 diabetes, if it after birth is exposed to good nutrition (19).

The thrifty phenotype hypothesis extends the concept of "programming" to metabolic diseases, defined by A. Lucas as a "process whereby a stimulus or insult at a critical period of development has lasting or lifelong significance" (22). Since the hypothesis was formulated, it has been extensively investigated in further epidemiological studies and in animal and human models of intrauterine growth restriction. An important epidemiological study is the "Dutch Hunger Winter" study (23). In this study, 702 individuals who were in utero immediately before, during, and after the famine in the Netherlands at the end of World War II (November 1944-May 1945), respectively, were studied. Thereby, it was possible to investigate the effects of maternal undernutrition during specific periods of gestation on metabolic outcomes in the offspring. It was found that individuals exposed to the famine during mid or late gestation had a lower birth weight, were shorter in length, and had a higher head circumference to birth weight ratio compared with individuals exposed to the famine during early gestation or not exposed to this before birth (23). The latter finding was suggested to be due to brain sparing, as consistent with the theories by Hales and Barker (19). Furthermore, individuals exposed to the famine at any stage during gestation had higher 2 hour plasma glucose and insulin levels compared with individuals not exposed to the famine in utero (23). Also, those exposed to the famine during mid or late gestation had the highest 2 hour plasma glucose levels (23). Notably, among these individuals, those with lower birth weights or that became obese as adults had the highest 2 hour plasma glucose levels (23). These findings strongly support the observations by Hales and Barker that LBW is a risk factor of glucose intolerance and type 2 diabetes, and furthermore their suggestion that poor nutrition in pre-natal life followed by good or rich nutrition in later stages of life can explain this association (19). Many other epidemiological studies have replicated and extended the findings by Hales and Barker in several different study populations, including in women and men, different age groups, and different ethnicity groups (7; 24-27). Some of these studies have reported a U-shaped relation between birth weight and risk of type 2 diabetes (7; 28; 29). Thus, in a large meta-analysis, including a total of 132,180 individuals, it has been found that LBW (BW < 2500 g) or high birth weight (HBW) (BW > 4000 g) individuals have a 1.47- and 1.36-fold increased risk, respectively, of developing type 2 diabetes compared with NBW (2500 g $\leq$ BW $\leq$ 4000 g) individuals (7). This odd ratio of type 2 diabetes in LBW individuals is quite lower than that found in the Hertfordshire population (5).

Explanatory studies on the type 2 diabetes susceptible phenotype in LBW individuals

Following the findings of the association between a LBW and an increased susceptibility to metabolic diseases, several studies aimed to provide insights into the causal background of this relation.

Influence of genes versus the intrauterine environment on the type 2 diabetes risk in LBW individuals

A number of studies have first of all sought to determine whether the association between a LBW and type 2 diabetes is mainly due to genes associated with both these traits or an adverse intrauterine environment. The first possibility forms the basis of the "foetal insulin" hypothesis, which proposes that genetically determined insulin resistance results in both an impaired foetal growth and an increased susceptibility to develop type 2 diabetes in adult life (30). In this regard, it is, however, notable that only 2 of the 45 genes known to be associated with type 2 diabetes are also associated with a LBW (31). A study performed in middle-aged monozygotic twin pairs who were discordant for type 2 diabetes has furthermore shown that twins with type 2 diabetes have a lower birth weight compared with their genetically identical co-twins, suggesting that the association between a LBW and type 2 diabetes in twins is at least partly independent of the genotype and may be due to intrauterine malnutrition (32). A couple of studies have, however, demonstrated a link between a LBW and paternal diabetes, which has supported the notion that the association between a LBW and type 2 diabetes could be due to the inheritance of genes associated with both these traits (33). Nevertheless, the thrifty phenotype hypothesis, with its idea that an adverse intrauterine environment has a major influence on the susceptibility to type 2 diabetes (20), is generally well accepted.

Influence of maternal under- and malnutrition on the type 2 diabetes risk in rat offspring

Along with the recognition of the thrifty phenotype hypothesis, several experimental studies have investigated the consequences of different intrauterine conditions, including a limited oxygen or nutrient supply to the foetus, on the growth, development, and metabolic function of the offspring at birth and in later stages of life.

Already prior to the thrifty phenotype hypothesis, a number of studies had described several adverse effects of maternal protein restriction during gestation on the rat offspring, including a lower birth weight and a reduced pancreatic beta-cell proliferation and islet size (34). The thrifty phenotype hypothesis therefore initially focused on an impaired insulin secretion as a key consequence of poor nutrition in pre-natal life (19), and the rat model of maternal protein restriction was identified as an appropriate model to test the validity of this hypothesis. In the standard protein restriction model, rat dams are fed either a low-protein (8 E %), isocaloric diet or a normal-protein (20 E %) control diet during the whole gestation and lactation (0-21 days of age of the offspring) periods (34). After lactation, pups of both groups of rat dams are weaned onto a standard chow containing 20 % of the total energy from protein (34). From studies in this model, it has been found that rats of protein-restricted dams have a higher glucose tolerance in early adult (6 weeks-3 months of age) life compared with control offspring (35-37), but an impaired glucose tolerance at 15 months of age (36). Also, male rats of protein-restricted dams have diabetes at 17 months of age (38). In addition to the studies in the standard protein restriction model, cross-fostering setups have been used to evaluate the separate effects of maternal protein restriction during gestation or lactation, respectively. Thus, it has been shown that rat pups of protein-restricted dams during gestation or lactation only, as for rat pups of protein-restricted dams during both periods, have a higher glucose tolerance at 6 weeks of age compared with control offspring (37). Therefore, it was suggested that maternal protein restriction in each of the gestation and lactation periods have an influence on long-term glucose tolerance in the offspring (37).

Also, a number of studies have focused on the effects of global maternal calorie restriction during gestation on the offspring. In studies concerning severe undernutrition, rat dams were either subjected to nutrient restriction (30 % food intake of the ad libitum intake) or fed ad libitum during the whole gestation period and subsequently fed ad libitum (39-41). It was found that rat pups of nutrient-restricted dams have a lower body weight in late gestation (39) and at birth (39-41) compared with control offspring. Furthermore, nutrient-restricted rat dams have lower plasma insulin-like growth factor 1 (IGF-1) levels during gestation, and their offspring have likewise lower such levels in late gestation and until 9 days of age (39). The latter study therefore proposed that IGF-1 is important in the regulation of foetal growth, and that both maternal and foetal plasma IGF-1 levels are regulated by nutrient availability (39). Moreover, it was shown that rat pups of nutrient-restricted dams have a higher systolic blood pressure (40) and a higher fasting plasma insulin level (41) in adulthood.

Mechanistic studies on the type 2 diabetes susceptible phenotype in LBW individuals

Several of the studies performed in the rat model of maternal protein restriction also aimed to provide insights into the mechanistic background of the increased risk of developing type 2 diabetes in individuals exposed to such protein restriction in pre-natal and/or early post-natal life. Thus, isolated organs or tissues from rats of protein-restricted dams have been extensively analysed to investigate possible alterations in metabolic functions of these that may be linked to later development of insulin resistance and type 2 diabetes. Furthermore, studies performed in human LBW and NBW subjects, including in particular the present high-fat overfeeding study as well as additional studies performed by A.A.Vaag and co-workers, have provided insights into some of the molecular mechanisms behind the type 2 diabetes susceptible phenotype in LBW individuals.

Influence of maternal protein restriction on metabolic functions in rat offspring

First of all, it has been found that rat pups of protein-restricted dams during the whole gestation and lactation (0-21 days of age of the offspring) periods, or during lactation only, have reduced weights of all isolated organs or tissues, including the brain, heart, lung, thymus, pancreas, spleen, liver, kidney, and skeletal muscle, at 21 days of age compared with control offspring, whereas rat pups of protein-restricted dams during gestation only have reduced weights of the liver and skeletal muscle (42). Interestingly, rats exposed to maternal protein restriction in pre-natal and/or early post-natal life show a non-uniform growth restriction of the different organs and tissues, as evaluated from the weights of these relative to the total body weight (42). Thus, rat pups of protein-restricted dams during both gestation and lactation, or during lactation only, have a higher relative weight of the brain and lower relative weights of the pancreas, liver, and skeletal muscle compared with control pups (42). These findings are consistent with the idea in the thrifty phenotype hypothesis that the growth of the brain is sparred at the expense of the growth and development of other organs and tissues in the setting of poor pre-natal nutrition (19). Rats exposed to maternal protein restriction in pre-natal life only have also been shown to have lower relative weights of the liver and skeletal muscle compared with control rats (42). From examinations in adult (11 months of age) rats that have experienced maternal protein restriction, and that after lactation were weaned onto a standard chow, it has been found that both female and male rat offspring of protein-restricted dams during both gestation and lactation still have a higher relative weight of the brain compared with control

offspring (42). Also, these rats, as well as female and male adult (11 months of age) rats exposed to maternal protein restriction in early pre-natal life only, have a lower relative weight of skeletal muscle compared with control rats (42).

In continuation to these studies, it has been found that rat offspring of protein-restricted dams show several alterations in metabolic functions of individual organs and tissues related to the development of insulin resistance and type 2 diabetes. As concerns the pancreas, it has been shown that beta-cells isolated from adult rat offspring of protein-restricted dams during both gestation and lactation do not have an altered insulin release compared with beta-cells from control offspring (43). However, beta-cells isolated from adult rat offspring that have been exposed to maternal protein restriction, and later in life been allowed to access sucrose or fed a high-fat diet, show a reduced glucose-stimulated insulin release (43). These findings support the proposals of the thrifty phenotype hypothesis that poor nutrition in pre-natal and early post-natal life leads to an impaired pancreatic beta-cell function, and furthermore that a shift from poor nutrition in pre-natal life to good or rich nutrition in later stages of life may lead to metabolic diseases such as type 2 diabetes (19). As regards insulin-sensitive tissues, it has been shown that livers isolated from male adult (3 months of age) rat offspring of protein-restricted dams during both gestation and lactation have a decreased glucagon-stimulated hepatic glucose production compared with control rats (44). This was found to be due to a significantly lower expression of glucagon receptors in the liver from the rats of protein-restricted dams (44). Furthermore, livers from these rats show an anomalous response to insulin with this hormone initially stimulating the hepatic glucose production and subsequently, as expected, reducing this glucose production (44). Skeletal muscle and adipocytes isolated from male adult (3 months of age) rat offspring of protein-restricted dams during both gestation and lactation have increased insulin-stimulated glucose uptake rates compared with control offspring, likely due to a higher expression of insulin receptors in these tissues (45; 46). These findings are consistent with the observations of a higher glucose tolerance in young or adult (6 weeks-3 months of age) rats exposed to maternal protein restriction compared with control offspring (35-37; 45). Also, skeletal muscle and adipocytes isolated from older (15 months of age) male rat offspring of protein-restricted dams have decreased insulin-stimulated glucose uptake rates compared with control rats (46; 47) in line with the age-dependent loss of glucose tolerance in rats exposed to maternal protein restriction (36; 38). Moreover, adipocytes isolated from the rat offspring at 15 months of age are less responsive to insulin in terms of its inhibition of lipolysis (46).

Taken together, several of the findings from the rat model of maternal protein restriction have supported the thrifty phenotype hypothesis. Also, studies in the standard protein restriction model have revealed age-dependent changes in organ and tissue functions in rats exposed to maternal protein restriction during both gestation and lactation, compared with control offspring, and corresponding age-dependent changes in glucose tolerance. Furthermore, studies involving cross-fostering setups have proposed that maternal protein restriction during either gestation or lactation only, as for protein restriction during both these periods, have an influence on long-term glucose tolerance in the offspring.

Investigations of metabolic functions in LBW individuals

In the high-fat overfeeding study in young, healthy, LBW (≤ 10th percentile) and NBW (50-90th percentile) men, which the present plasma metabolome studies are part of, it has been found that LBW men have higher fasting blood glucose, serum insulin, and serum C-peptide levels after the isocaloric control diet compared with NBW controls (8). These findings were accompanied by the observation that the LBW men show an impaired hepatic insulin sensitivity after this diet compared with NBW men (8). The LBW men did not show altered peripheral insulin sensitivity after the control diet compared with NBW men. However, when these individuals were subjected to the 5-day HFHC (60 E % from fat, 50 % extra calories) diet intervention, the LBW, but not NBW, men developed impaired peripheral insulin sensitivity (9). In a study performed in another population of young (19 years), healthy, LBW and NBW men by as well A.A.Vaag and co-workers, it was shown that LBW men have lower skeletal muscle expression levels of several key proteins in the insulin signalling cascade, including glucose transporter 4 (GLUT4) and protein kinase C (PKC), compared with NBW men (48). Furthermore, these LBW men show an altered skeletal muscle fiber size and composition (49). These structural and functional changes of skeletal muscle were proposed to contribute to the development of skeletal muscle insulin resistance and type 2 diabetes in LBW individuals (48; 49). LBW men of the latter study population have in addition, as for skeletal muscle, lower adipose tissue expression levels of key insulin signalling-proteins, including GLUT4 and insulin receptor substrate 1 (IRS1) (50). From additional investigations of adipose tissue functions in LBW individuals, it has been found that young (20-30 years of age), healthy, LBW men show an increased adipose tissue lipolysis (14). This was paradoxically accompanied by

decreased adipose tissue mRNA expression levels of several lipases, including triacylglycerol lipase and lipoprotein lipase (14). Furthermore, LBW men of the overfeeding study population have a higher adipose tissue miR-483-3p level (51) and lower pre-adipocyte mRNA expression levels of several differentiation markers (12). Manipulation of adipose tissue miR-483-3p levels in vitro was shown to modulate adipocyte differentiation and fatty acid storage capacity (51). From these findings, it was suggested that LBW individuals could have an impaired pre-adipocyte maturation and that this could result in a lower adipose tissue fatty acid storage capacity (12; 51). A selection of the above described metabolic alterations in young (19-30 years of age), healthy, LBW men, as well as some additional traits demonstrated in these individuals, are summarised in Figure 3.2.

In addition to the investigations of metabolic functions at the organ or tissue level in LBW individuals, it has been found that LBW men of the overfeeding study population show an increased fatty acid oxidation rate and a decreased glucose oxidation rate at night time during both the isocaloric control diet and 5-day HFHC diet compared with NBW men (10; 11). Furthermore, the LBW men display a higher adjusted total energy expenditure at night during the HFHC diet, compared with NBW men, and a higher relative contribution of fatty acid oxidation to the total energy expenditure at night and throughout 24 hours during this diet (11).

Translational value of the rat model of intrauterine growth restriction

In a couple of the above mentioned studies, investigations in the rat model of maternal protein restriction were performed in parallel to the examinations in the human LBW subjects. Interestingly, rat offspring of protein-restricted dams during both gestation and lactation show traits similar to those of the LBW men. Thus, male adult (15 months of age) rats of protein-restricted dams during both gestation and lactation display a skeletal muscle expression profile of insulin signalling-proteins similar to that of the LBW men, including lower GLUT4 and PKC expression levels (48). Furthermore, male rat pups (22 days of age) and adult (3 months of age) rats exposed to maternal protein restriction during both gestation and lactation have a higher adipose tissue miR-483-3p level compared with control offspring (51). These findings support the value of translating results from the rat model of intrauterine growth restriction into human LBW subjects.

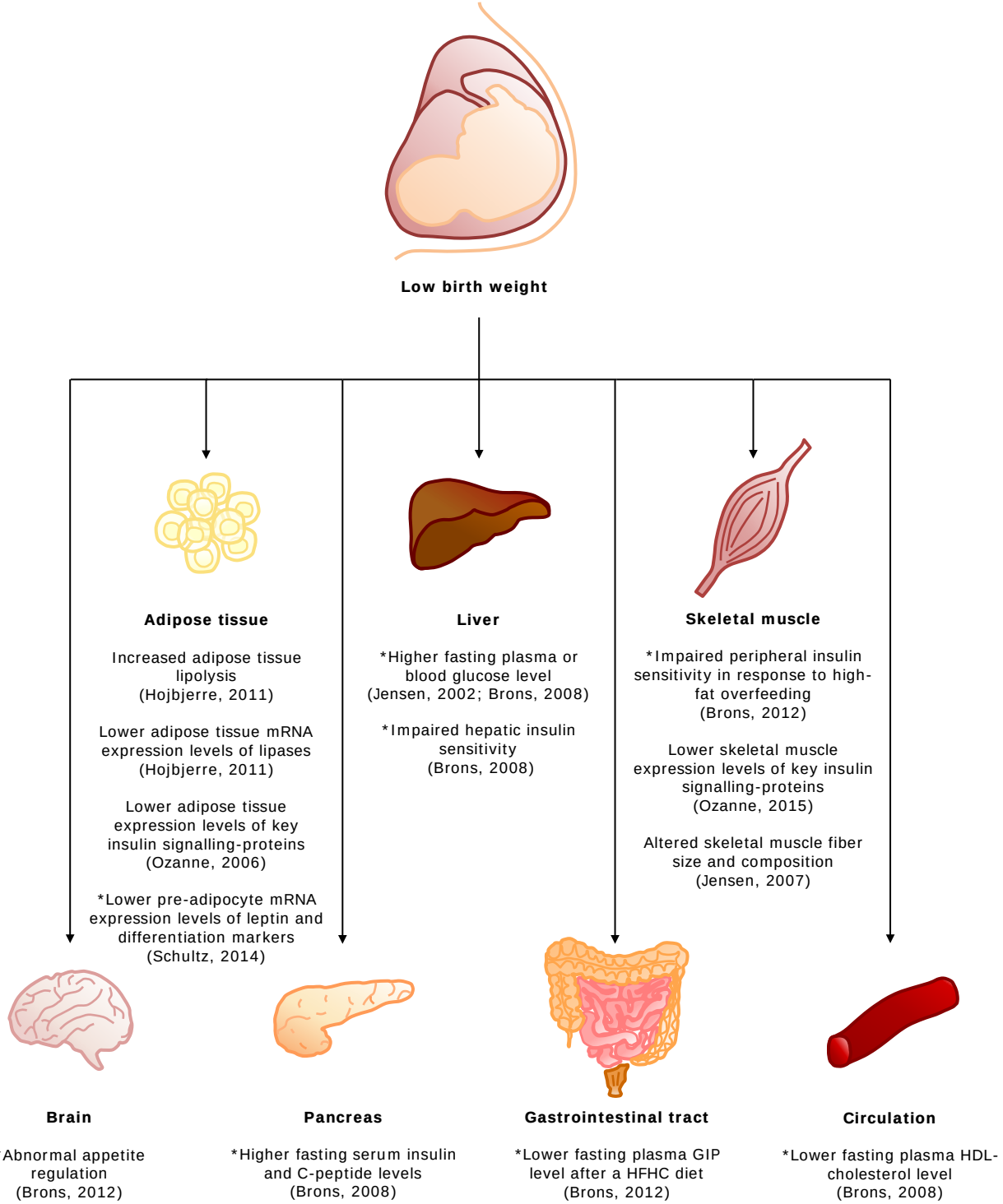


Figure 3.2: Metabolic alterations in young, healthy, LBW men. Findings in LBW men of the overfeeding study population are marked with an asterisk. Figure modified from A.A. Vaag, 2012 (31).

Motivation for the present plasma metabolome studies in LBW individuals

On the basis of the findings of the altered adipose tissue functions and changes in fatty acid oxidation and glucose oxidation partitioning in LBW men, we investigated if these traits were accompanied by 1) an incomplete fatty acid beta-oxidation, 2) changes in amino acid metabolism, and 3) an accumulation of potentially lipotoxic lipids in the blood. The detailed background of the three plasma metabolome studies and the associations between the proposed metabolic changes in LBW individuals and type 2 diabetes are described in brief below and in the individual articles (Appendix 1, 2, and 3, respectively).

Study 1 – Incomplete fatty acid oxidation in the pathogenesis of insulin resistance and type 2 diabetes

High-fat overfeeding and an increased fatty acid load to skeletal muscle leads to an increased expression of genes encoding proteins in the fatty acid beta-oxidation pathway, including carnitine palmitoyl-transferase I (CPT-I) that catalyses the condensation of activated long-chain fatty acids (acyl-CoAs) and carnitine to form acylcarnitines and thereby regulates the entry of these acyl-CoAs into the mitochondrial matrix (52-54). In the state of high-fat overfeeding, an increased beta-oxidation capacity has, however, been suggested to not necessarily be matched by that of the TCA cycle and electron transport chain, which results in an incomplete fatty acid oxidation (1; 52; 53; 55). This leads to an accumulation of acylcarnitines and reactive oxygen species that may contribute to metabolic stress and thereby ultimately impair insulin signalling (56-59). Increased intracellular concentrations of long-chain acyl-CoAs may in addition lead to an increased lipogenesis, including the synthesis of potentially lipotoxic lipids that as well may impair insulin signalling (1). This metabolic fate of long-chain acyl-CoAs has been especially described in the context of high-fat diet-induced hepatic insulin resistance. Actually, high-fat overfeeding has been proposed to lead to a malonyl-CoA-induced inhibition of CPT-I in the liver with a following diversion of long-chain acyl-CoAs away from beta-oxidation and towards other metabolic fates in the cytosol, including lipogenesis (1; 60). Some studies, however, indicate that an increased fatty acid load to the liver leads to a simultaneously increased beta-oxidation and incorporation of long-chain acyl-CoAs into lipids (61).

Incomplete fatty acid beta-oxidation downstream of CPT-I is reflected by elevated plasma acylcarnitine levels (55), as acyl-CoAs in the mitochondrial matrix can be converted to acylcarnitines that subsequently are transported through the mitochondrial membranes and then from the cytosol to the blood (55; 62; 63). Interestingly, higher plasma acylcarnitine levels have been demonstrated in adults with pre-diabetes and type 2 diabetes (56; 64-66). Acylcarnitine profiles may reveal defects in specific steps of beta-oxidation (67-69) as well as the involvement of other metabolic pathways upstream of beta-oxidation (55). Thus, an incomplete beta-oxidation give rise to even-chain C4-C22 acylcarnitines, and amino acid catabolism is a source for C3, C4, and C5 acylcarnitines (55). These pathways are, together with glucose oxidation, in addition sources of C2 acylcarnitine (acetylcarnitine), when acetyl-CoA is generated in excess in the mitochondrial matrix relative to its flux into the TCA cycle (55; 70) (Figure 3.3). In this situation, carnitine acetyl-CoA transferase (CrAT) catalyses the transfer of acetyl-CoA to carnitine to form acetylcarnitine, which is subsequently transported to the cytosol (70; 71).

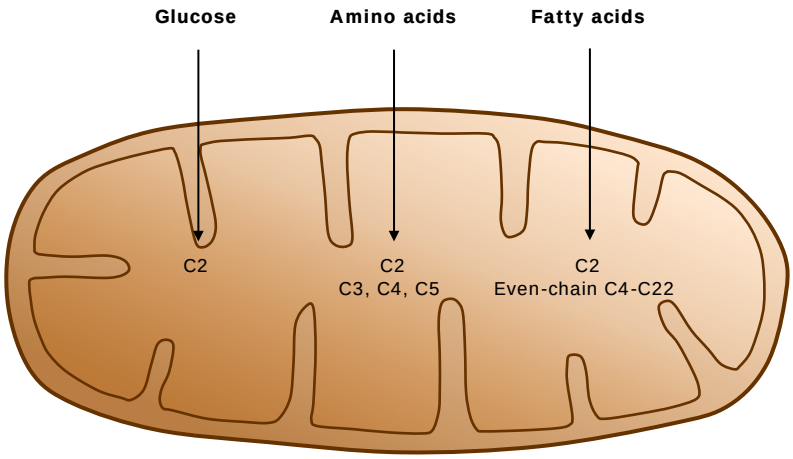


Figure 3.3: Acylcarnitine species generated from glucose, amino acid, and fatty acid metabolism. C2 acylcarnitine is generated from both glucose, amino acid, and fatty acid oxidation. Other even-chain C4-C22 acylcarnitines are formed during incomplete fatty acid beta-oxidation, and C4 is also generated from amino acid catabolism. Odd-chain C3-C5 acylcarnitines are formed from metabolism of branched-chain amino acids, including valine, leucine, and isoleucine. Hydroxyl- and dicarboxyl-acylcarnitines (not shown) are formed from hydroxyl- and dicarboxyl-fatty acids, respectively. These fatty acids are intermediates in fatty acid omega-oxidation in the endoplasmic reticulum of mainly the liver. Hydroxyl-fatty acids are in addition generated in fatty acid beta-oxidation in mitochondria and peroxisomes.

Increased intracellular concentrations of acyl-CoAs, as a consequence of an increased fatty acid load to the cells and/or an incomplete beta-oxidation, may also lead to an increased fatty acid omega-oxidation in the endoplasmic reticulum of mainly the liver (72-74). Omega-oxidation is a minor pathway for oxidation of fatty acids under normal physiological conditions (73; 74), but an important supplementary pathway to beta-oxidation when the intracellular NEFA levels are high such as following high-fat feeding or under fasting and starvation (74; 75). Fatty acid metabolism via omega-oxidation generates hydroxyl- and dicarboxyl-fatty acids that are further oxidized in beta-oxidation in mitochondria and peroxisomes (73; 76). An increased fatty acid flux into omega-oxidation is therefore expected to be reflected in higher plasma hydroxyl-/dicarboxyl-acylcarnitine levels (73; 76). Hydroxyl-fatty acids are, however, also intermediates in beta-oxidation (73; 77).

Study 2 – Amino acid metabolism and type 2 diabetes

Incomplete fatty acid beta-oxidation, with the limiting step at the acetyl-CoA entry into the TCA cycle, may result from a depletion of TCA cycle intermediates (78; 79). Amino acids are precursors of pyruvate and several of the TCA cycle intermediates, including oxaloacetate, alpha-ketoglutarate, succinyl-CoA, and fumarate (80). LBW men of the present study population display an increased fatty acid oxidation rate and a decreased glucose oxidation rate at night time during both the control diet and HFHC diet compared with NBW men (10; 11). Furthermore, the LBW men have a higher adjusted total energy expenditure at night during the HFHC diet compared with NBW men (11), suggesting that the TCA cycle is likely to be upregulated in the LBW men at night. The changes in fatty acid oxidation and glucose oxidation partitioning in the LBW men during both diets, and in addition in the total energy expenditure during the HFHC diet, could be expected to be accompanied by changes in amino acid metabolism to ensure an adequate supply of TCA cycle intermediates and thereby enable an efficient acetyl-CoA oxidation. This may in turn lead to changes in tissue and plasma amino acid levels. Altered plasma amino acid levels have repeatedly been demonstrated in individuals with pre-diabetes and type 2 diabetes and found to associate with insulin resistance (81-86). In particular, plasma levels of branched-chain amino acids, aromatic amino acids, and alanine have been shown to be predictive of type 2 diabetes several years before its onset (87-90). An overview of amino acid anaplerotic and cataplerotic pathways that replenish or deplete TCA cycle intermediates, respectively, is shown in Figure 5.2 in the result section.

Study 3 – Lipotoxicity in the pathogenesis of insulin resistance and type 2 diabetes

Increased fatty acid exposures to tissues such as the pancreas, liver, and skeletal muscle may, as described in the section above on incomplete fatty acid beta-oxidation, lead to an increased lipogenesis, including the synthesis of lipotoxic lipids such as ceramides and diacylglycerols that may impair insulin signalling (1). Young (20-30 years of age), healthy, LBW men of another study population than the presently examined display an increased whole body (13) and adipose tissue (14) lipolysis. These traits were, however, not accompanied by elevated fasting plasma NEFA or triacylglycerol levels (13; 14), suggesting that the LBW men could have an increased uptake of fatty acids from the blood into tissues and/or an increased clearance or metabolism of fatty acids in adipose tissue. From the acylcarnitine profiling in the LBW and NBW men of the overfeeding study population, we found that LBW men had higher fasting plasma hydroxyl-/dicarboxyl-acylcarnitine levels, including 3-hydroxy-butyrylcarnitine, indicating an increased hepatic fatty acid oxidation rate, involving omega-oxidation, and an increased ketogenesis (91). The LBW men could therefore likely have an increased fatty acid load to non-adipose tissue, including in particular the liver, and a resulting increased lipogenesis and ectopic fat deposition.

Ectopic fat comprises potentially lipotoxic lipids such as long-chain acyl-CoAs, ceramides, and diacylglycerols (92), and the extent of it has been linked to pancreatic beta-cell dysfunction and insulin resistance (93; 94). Furthermore, individuals with pre-diabetes and type 2 diabetes have elevated plasma ceramide levels (95-97). Ceramides are synthesised de novo in the endoplasmic reticulum or from salvage pathways of other sphingolipids (98). The de novo synthesis involves the condensation of L-serine and palmitoyl-CoA, catalysed by serine palmitoyl-transferase (SPT), to form 3-ketosphinganine that is subsequently reduced to sphinganine. Then, a variable acyl-CoA is attached to sphinganine, catalysed by one of six different ceramide synthases (CerS1-6), to form a dihydroceramide (d18:0- species) that is finally converted to a ceramide (d18:1- species) by a dihydroceramide desaturase (98). Ceramide synthases have different selectivity for acyl-CoAs based on the carbon chain length of these activated fatty acids. CerS2, for instance, has highest selectivity for very-long chain acyl-CoAs (C20-C26), and CerS6 for long chain acyl-CoAs (C14-C18) (99). SPT is the rate-limiting enzyme of de novo ceramide synthesis, and an increased availability of palmitic acid enhances this synthesis (100-102). An increased uptake of fatty acids from the blood into non-adipose tissue is therefore expected to increase ceramide synthesis.

Ceramides are potentially lipotoxic to the cells in several ways (101; 103). Thus, these lipids inhibit the phosphorylation and thereby activation of Akt/protein kinase B (PKB) of the insulin signalling cascade, which is a central regulator of glucose and amino acid uptake, anabolic processes, and cell survival (104; 105). Moreover, ceramides activate Jun and NF-KB transcription factors and may thereby enhance inflammatory responses that as well may interfere with insulin signalling (101). A number of studies have focused on chain length specific actions of ceramides, including dihydroceramides, on different cellular processes (99), and ceramide synthase knock-out mouse models have been used to further explain these. Thus, CerS6-deficient mice have lower adipose tissue and liver d18:1-16:0 ceramide contents compared with wild-type mice (106). Also, these CerS6-deficient mice are protected from high-fat diet-induced obesity and glucose intolerance (106). In contrast, CerS2-deficient mice have lower liver d18:1-22:0 and d18:1-24:0 ceramide contents, a higher liver d18:1-16:0 ceramide content, and unaltered liver total ceramide content compared with control mice (107). These mice are more susceptible to develop diet-induced steatohepatitis and insulin resistance (107). Alterations in liver ceramide composition as a result of changes in the expression and/or activities of the different ceramide synthases could therefore be an important mechanism behind the development of insulin resistance and type 2 diabetes.

CHAPTER 4 – MATERIALS AND METHODS

The population of LBW and NBW men was collected and clinically and physiologically characterised prior to the present project. Therefore, the project has involved plasma metabolome analyses on already generated data as regards the first two studies and experimental work for further such analyses on the sample material as concerns the third study.

Study population

Forty-six young (23-27 years of age), healthy men were recruited from the Danish National Birth Registry according to birth weight. Among these, 20 men had a LBW (2717 ± 268 g) (≤ 10th percentile) and 26 men a NBW (3901 ± 207 g) (50-90th percentile). All men were born at term (39-41 weeks of gestation) in Copenhagen, Denmark in the period 1979-1980. Furthermore, all men were non-obese (BMI < 30 kg/m^2) and ensured to not perform strenuous physical activity > 10 hours per week, not take pharmaceuticals that affect metabolism, not have an abuse of alcohol or drugs, and not have a family history of diabetes in two generations.

Study design

Diet interventions

All men were, in a randomised crossover study setup, standardised with regard to diet and physical activity and subsequently subjected to a 3-day control diet and a 5-day HFHC diet separated by a 6-8 week wash out-period (Figure 4.1). The control diet was composed to reflect a habitual, weight-maintaining diet (2,819 ± 238 kcal/11,800 ± 1,000 kJ) with 15, 50, and 35 E % from protein, carbohydrate, and fat, respectively, and the HFHC diet was prepared to contain 50 % extra calories (4,228 ± 334 kcal/17,700 ± 1,400 kJ) above the requirements with 7.5, 32.5, and 60 E % from protein, carbohydrate, and fat, respectively. Both diets were provided as five daily servings with identical meals from day to day. Energy requirements of the individual participants were calculated from a World Health Organization equation for men < 30 years of age with a physical activity level of 1.4 corresponding to a low physical activity (108), and dietary calculations were made in Dankost Pro (The National Food Agency, Copenhagen, Denmark).

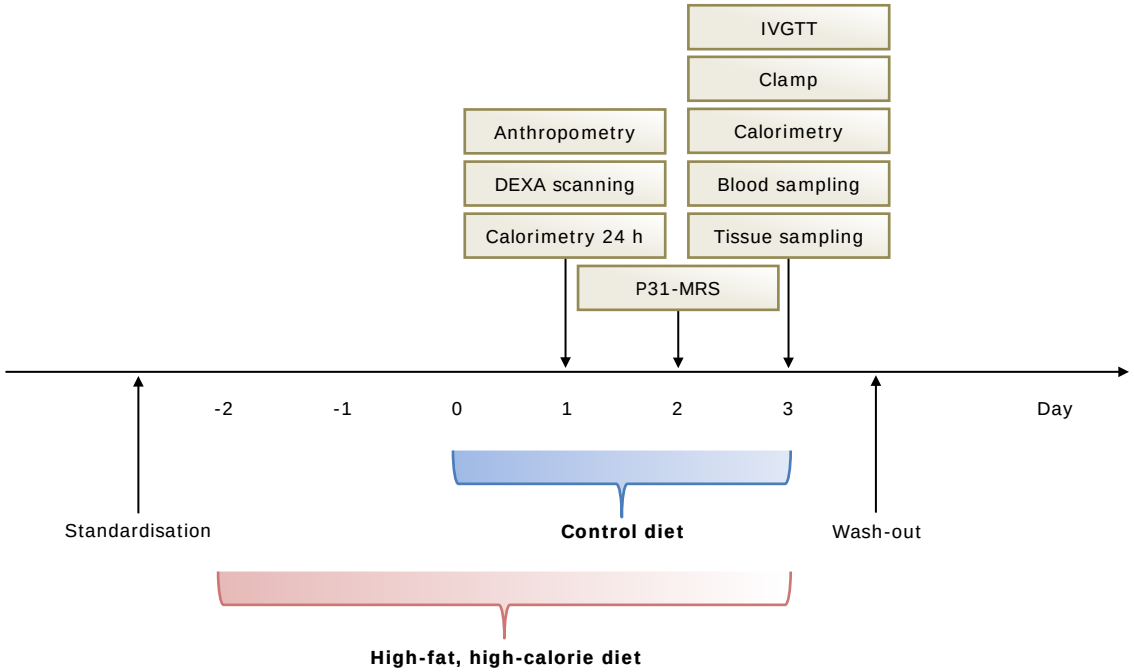


Figure 4.1: Time line of diet interventions and clinical examinations in young, healthy, LBW and NBW men. All participants were subjected to both a 3-day isocaloric control diet and a 5-day HFHC diet separated by a 6-8 week wash-out period. Metabolome analyses were performed on plasma obtained from blood samples collected after each of the diet interventions and following an overnight fast.

Clinical examinations

Study activities were carried out over three days, with the first of these days being placed one or three days after the start of the control and HFHC diet intervention, respectively (Figure 4.1). Anthropometry and a dual-energy X-ray absorptiometry (DEXA) scanning were performed on the first study day to evaluate body composition. Furthermore, phosphorous 31 magnetic resonance spectroscopy (P31-MRS) was performed on the second study day to evaluate skeletal muscle ATP synthesis rates, as described in previously published work on the study population (8). An intravenous glucose tolerance test (IVGTT) and a hyperinsulinaemic-euglycaemic clamp were carried out in the morning on the third study day to assess insulin secretion and sensitivity, as also described previously (8; 9). Moreover, indirect calorimetry was performed throughout 24 hours from the first to second study day by use of a respiratory chamber and in addition in the basal and insulin-stimulated steady-state periods of the clamp to evaluate substrate utilisation rates and total energy expenditures, as also described previously (9-11). Blood samples and skeletal muscle and adipose tissue biopsies were collected prior to and during the clamp examination.

Laboratory measurements

All plasma metabolome analyses, including acylcarnitine, amino acid, and ceramide profiling, were performed on EDTA-plasma obtained from blood samples collected following an overnight fast (10.00 PM-7.00 AM) and immediately prior to the clamp examination. Eighteen LBW and 25 NBW men of the recruited participants completed the diet interventions and physiological tests and were included in the present plasma metabolome study part.

Study 1 – Plasma acylcarnitine analyses

Acylcarnitine analyses included a semi-quantitative determination of 45 individual or pools of species, designated in this text by their acyl group in accordance to its carbon chain length (e.g. C16), double bonds (e.g. C16:1), and possible hydroxyl- or a second carboxyl-group (e.g. C16-OH or C16-DC, respectively). These analyses were performed by flow injection-MS/MS, as described in details in the method section of article 1 (Appendix 1) (91) and supplementary material to this article (Appendix 4).

Study 2 – Plasma amino acid analyses

Amino acid analyses included a quantitative determination of 15 individual or pools of types. These analyses were performed, as for the acylcarnitine analyses, by flow injection-MS/MS, as described in the method section of article 2 (Appendix 2) (109).

Study 3 – Plasma ceramide analyses

Ceramide analyses included a semi-quantitative determination of 27 individual or pools of species, designated in this text by their sphingoid base in accordance to its carbon chain length and double bonds (i.e. d18:0-, d18:1-, or d18:2-) and their acyl group in accordance to as well its carbon chain length (e.g. -24:0) and double bonds (e.g. -24:1). These analyses were performed by HPLC-HRMS, as described in details in the method section of article 3 manuscript (Appendix 3) and supplementary material to this manuscript (also included in Appendix 3).

Ethical approval

All study procedures were in accordance with the principles of The Declaration of Helsinki and approved by The Regional Research Ethics Committee of Copenhagen, Denmark. Also, all participants were provided with written information on the study purpose and procedures and signed an informed consent form prior to their participation.

Statistical analyses

Plasma metabolite levels and their relation to other lipid levels and physiological measures

Statistically significant differences in plasma acylcarnitine, amino acid, and ceramide levels between NBW and LBW men within each diet or between the control and HFHC diets within each birth weight group were assessed by Student's unpaired or paired t-tests (for normally distributed values), respectively, or Wilcoxon ranked-sum or signed-rank tests (for non-normally distributed values), respectively, as described in details in the method section of the individual articles (Appendix 1, 2, and 3, respectively).

Associations between plasma metabolite levels and other lipid levels or physiological measures were assessed from linear regression analyses. These analyses were performed on the pooled data set of LBW and NBW men and were adjusted for age, BMI, and birth weight group, as also described in the individual articles.

CHAPTER 5 – RESULTS

The most important findings from the plasma metabolome studies are presented below with focus on differences in metabolite levels between LBW and NBW men. Also, the overall interpretations of the results are included in this section and collected in schemes of metabolic pathways.

Study 1 – Plasma acylcarnitine profiling

We hypothesised in the first study that the increased fatty acid oxidation rate and decreased glucose oxidation rate in LBW men during both the isocaloric control diet and HFHC diet (10; 11) could be accompanied by an incomplete fatty acid beta-oxidation in mitochondria with a resulting accumulation of specific acylcarnitines in tissues and the blood. Furthermore, higher such levels could contribute to the impaired insulin sensitivity in these individuals.

We demonstrated that LBW men had a higher plasma C2 acylcarnitine (acetylcarnitine) level after the control diet, compared with NBW men, and showed a tendency to this same after the HFHC diet, but otherwise had unaltered plasma acylcarnitine levels with exception of some hydroxyl-/dicarboxyl-species, as described below. LBW men therefore seem to have an increased, incomplete fatty acid beta-oxidation in mitochondria, with the limiting step at the acetyl-CoA conversion. This could likely be due to a mismatch between fatty acid fluxes through beta-oxidation and the acetyl-CoA flux into the TCA cycle, resulting in an accumulation of acetyl-CoA in the mitochondrial matrix and subsequently of C2 acylcarnitine in the cytosol and blood. The plasma C2 acylcarnitine level did not associate with measures of insulin secretion or sensitivity after the control or HFHC diet. Furthermore, we found that LBW men had a higher plasma C4-OH acylcarnitine (3-hydroxy-butyrylcarnitine) level after the control diet compared with NBW men. This indicates an increased ketogenesis in the liver, involving the conversion of acetyl-CoA in the mitochondrial matrix to 3-hydroxy-butyrate. LBW men moreover had higher plasma C6-DC, C10-OH/C8-DC, and total hydroxyl-/dicarboxyl-acylcarnitine levels after the control diet compared with NBW men. This could be a result of increased fatty acid fluxes through omega-oxidation in the endoplasmic reticulum of mainly the liver along with increased dicarboxyl-fatty acid fluxes through beta-oxidation in peroxisomes, resulting in an accumulation of hydroxyl- and dicarboxyl-acylcarnitines in the cytosol. Plasma C10-OH/C8-DC and total hydroxyl-/dicarboxyl-acylcarnitine levels were negatively associated with the fasting serum insulin level after the control diet, independent of birth

weight group, and the total hydroxyl-/dicarboxyl-acylcarnitine level was in addition negatively associated with hepatic insulin resistance after this diet. This suggests that omega-oxidation may be a scavenger pathway for oxidation of fatty acids that, if not oxidised, would be available for lipogenesis, including the synthesis of lipotoxic lipids such as ceramides and diacylglycerols that impair insulin signalling in the liver. The proposed alterations in fatty acid fluxes through beta- and omega-oxidation in LBW men after the isocaloric control diet are illustrated in Figure 5.1.

Furthermore, we demonstrated that both LBW and NBW men decreased plasma levels of several acylcarnitines in response to high-fat overfeeding. Also, we found from the 24 hour indirect calorimetry that the LBW and NBW men markedly increased fatty acid oxidation rates and total energy expenditures due to this challenge. The decreases in plasma levels of several acylcarnitines in LBW and NBW men in response to overfeeding could therefore likely be a result of their increases in fatty acid oxidation rates and total energy expenditures due to this intervention.

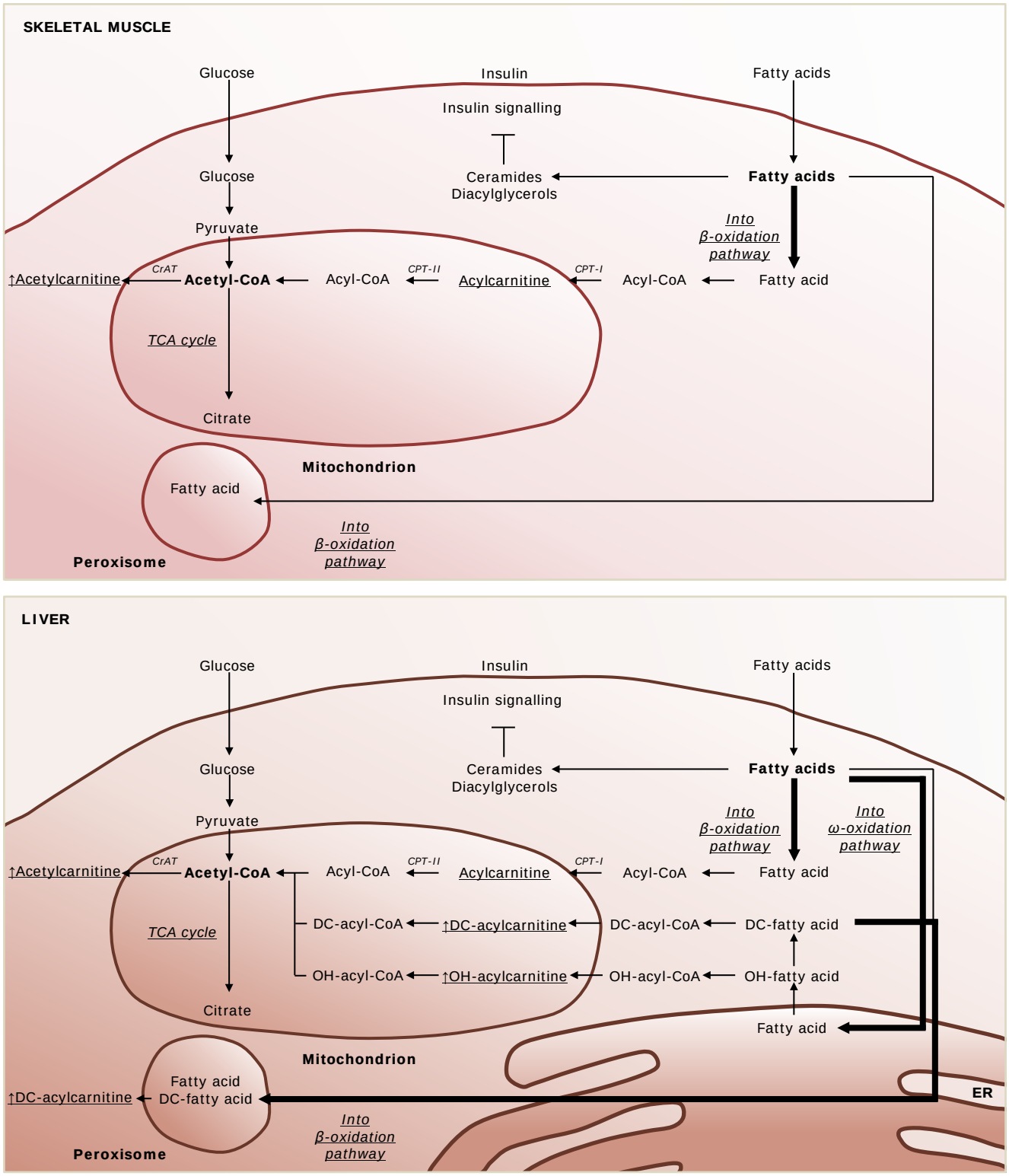


Figure 5.1: Model of possible alterations in fatty acid fluxes through beta- and omega-oxidation pathways in LBW men after the isocaloric control diet. LBW men have a higher plasma C2 acylcarnitine (acetylcarnitine) level after the control diet, compared with NBW men, but otherwise unaltered plasma acylcarnitine levels with exception of some hydroxyl-/dicarboxyl-species, indicating an incomplete fatty acid beta-oxidation in mitochondria of skeletal muscle (top) and liver (bottom). This could be due to increased fatty acid fluxes through beta-oxidation relative to the acetyl-CoA flux into the TCA cycle. LBW men furthermore have higher plasma C6-DC, C10-OH/C8-DC, and total

hydroxyl-/dicarboxyl-acylcarnitine levels after the control diet, compared with NBW men, suggesting increased fatty acid fluxes through omega-oxidation in the endoplasmic reticulum of mainly the liver along with increased dicarboxyl-fatty acid fluxes through beta-oxidation in peroxisomes. The plasma total hydroxyl-/dicarboxyl-acylcarnitine level was negatively associated with hepatic insulin resistance after the control diet. This suggests that omega-oxidation may be a scavenger pathway for oxidation of fatty acids that, if not oxidised, would be available for lipogenesis of lipotoxic lipids such as ceramides and diacylglycerols that impair insulin signalling in the liver.

Study 2 – Plasma amino acid profiling

We hypothesised in the second study that the changes in fatty acid oxidation and glucose oxidation partitioning in LBW men during both diets could be followed by alterations in amino acid metabolism. Furthermore, such alterations could be part of the adverse metabolic events leading to the impaired insulin sensitivity in these individuals.

We demonstrated that LBW men had higher plasma alanine, proline, methionine, citrulline, and total amino acid levels after the HFHC diet compared with NBW men, whereas LBW and NBW men did not show differences in plasma amino acid levels after the control diet. The plasma alanine level was negatively associated with the fasting plasma C2 acylcarnitine level after the HFHC diet. When combined with the findings of an increased, incomplete fatty acid oxidation, a decreased glucose oxidation rate, and a higher adjusted total energy expenditure in the LBW men during the HFHC diet (11; 91), the higher plasma alanine level in these individuals after this diet could be accompanied by an increased anaplerotic formation of oxaloacetate to enable an efficient acetyl-CoA oxidation via the TCA cycle. Furthermore, plasma alanine and total amino acid levels tended to be negatively associated with the insulin-stimulated glucose uptake rate after the HFHC diet. The higher plasma alanine and total amino acid levels in LBW men after this diet could therefore be a consequence of their reduction in skeletal muscle insulin sensitivity due to the overfeeding challenge (9) with a possible increased skeletal muscle proteolysis and/or could potentially contribute to the impaired peripheral insulin sensitivity in these individuals. Moreover, the plasma alanine level was positively associated with the hepatic glucose production after the HFHC diet. A higher plasma alanine level in LBW men could therefore also be accompanied by an increased gluconeogenesis in the liver. The proposed changes in amino acid fluxes into the TCA cycle in LBW men after the HFHC diet are illustrated in Figure 5.2.

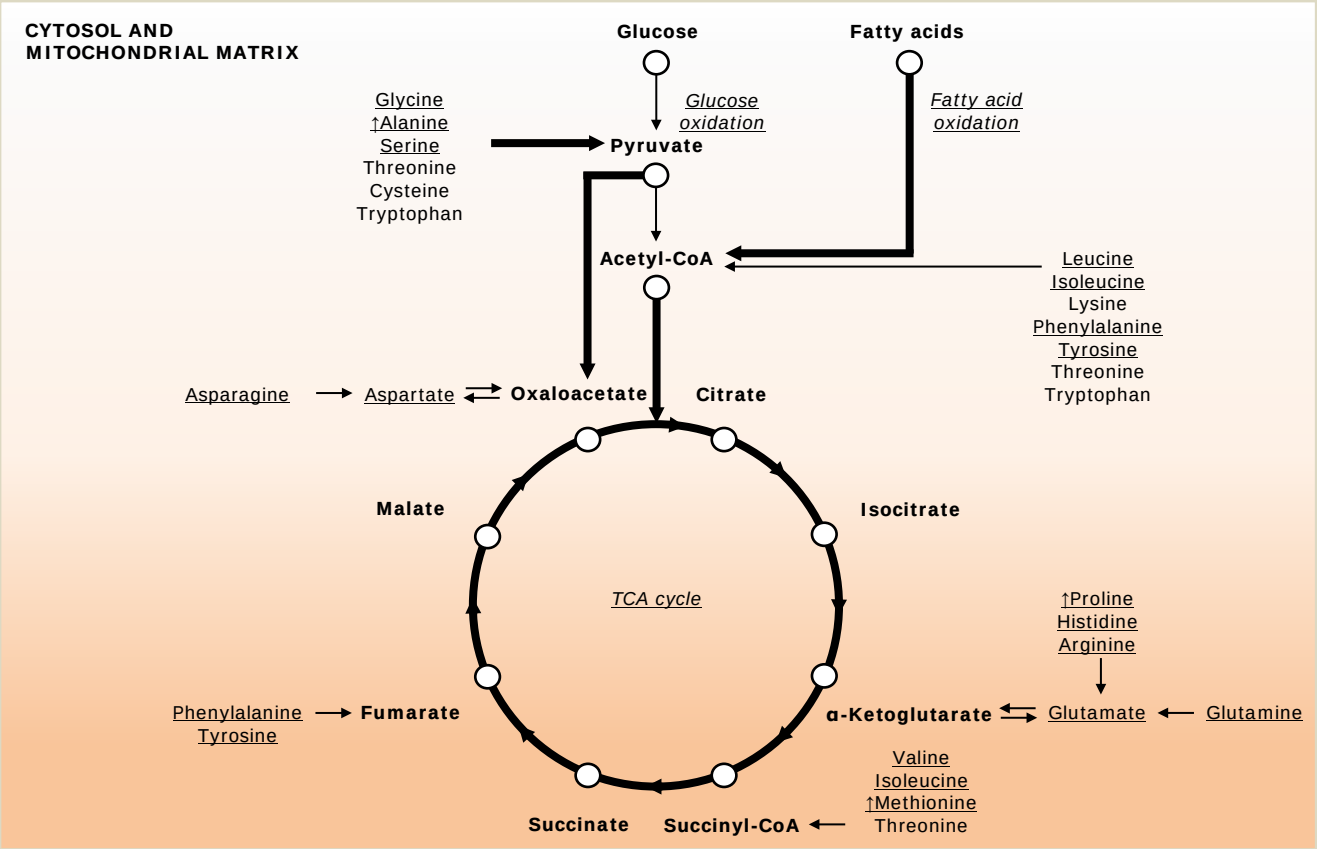


Figure 5.2: Model of possible alterations in amino acid fluxes into the TCA cycle in LBW men after the HFHC diet. LBW men have higher plasma alanine and total amino acid levels after the HFHC diet compared with NBW men. Also, the plasma alanine level was negatively associated with the plasma C2 acylcarnitine level after this diet. Combined with the findings of an increased, incomplete fatty acid oxidation, a decreased glucose oxidation rate, and a higher adjusted total energy expenditure in LBW men during the HFHC diet (11; 91), the higher plasma alanine level in these individuals after this diet could be accompanied by an increased anaplerotic formation of oxaloacetate to enable an efficient acetyl-CoA oxidation via the TCA cycle. Plasma amino acids measured in the second study are underlined.

Moreover, we demonstrated that both LBW and NBW men decreased plasma valine and leucine/isoleucine levels and increased plasma alanine levels in response to high-fat overfeeding. Also, an increase in the plasma alanine level was associated with a decrease in the plasma C2 acylcarnitine level. An increase in the plasma alanine level due to overfeeding could therefore be accompanied by an increased formation of oxaloacetate and a subsequent enhanced acetyl-CoA oxidation via the TCA cycle. Furthermore, as valine, leucine, and isoleucine are the major nitrogen sources for de novo alanine synthesis in skeletal muscle (110; 111), the decreases in plasma valine and leucine/isoleucine levels in LBW and NBW men in response to overfeeding could be due to an increased metabolism of these amino acids to alanine in skeletal muscle.

Study 3 – Plasma ceramide profiling

We hypothesised in the third study that the likely increased fatty acid load to non-adipose tissue, including in particular the liver, in LBW men during both diets could be followed by higher tissue and plasma ceramide levels. Furthermore, higher such levels and subsequent lipotoxicity in insulin-sensitive tissues could contribute to the impaired insulin sensitivity in these individuals.

In contrast to expected, we did not demonstrate altered plasma ceramide levels in LBW men after the control or HFHC diet compared with NBW men. This may be explained by the increased fatty acid oxidation rate in the LBW men during both diets (10; 11). An increased fatty acid oxidation rate may thus limit the amount of fatty acid substrates available for lipogenesis, including de novo ceramide synthesis, and thereby compensate for a possible increased fatty acid load to non-adipose tissue. LBW and NBW men decreased plasma levels of several ceramides, including for both birth weight groups d18:0-18:1/d18:1-18:0 and d18:1-24:2/d18:2-24:1, and increased d18:0-24:1a levels in response to high-fat overfeeding. The decreases in plasma levels of several ceramides in LBW and NBW men could likely be a consequence of their markedly increases in fatty acid oxidation rates and total energy expenditures due to the overfeeding (91) with a resulting depletion of fatty acid substrates for de novo ceramide synthesis. This interpretation is supported by the findings of decreases in fasting plasma levels of several other lipids, including total NEFA, total cholesterol, and total triacylglycerol, in the LBW and NBW men in response to overfeeding (9). LBW and NBW men of the present study population increased fasting serum fibroblast growth factor 21 (FGF-21) (112) and plasma adiponectin (9) levels in response to high-fat overfeeding. The decreases in plasma levels of several ceramides in LBW and NBW men due to this challenge could therefore also be a result of a FGF-21 and adiponectin mediated enhancement of ceramidase activities in the liver with a subsequent increase in the ceramide degradation. Moreover, we found that plasma levels of several ceramides and total ceramide were positively associated with the fasting blood glucose level after the control diet, and d18:0-18:1/d18:1-18:0, d18:0-24:1a, and d18:0-26:1/d18:1-26:0 levels were in addition positively associated with the hepatic glucose production after this diet. Also, d18:1-22:0 and total ceramide levels were positively associated with hepatic insulin resistance after the control diet. An increase in fatty acid oxidation rates in response to high-fat overfeeding may thus prevent an accumulation of ceramides that impair insulin signalling in the liver. The proposed alterations in fatty acid fluxes into oxidation pathways and lipogenesis in LBW and NBW men in response to high-fat overfeeding are shown in Figure 5.3.

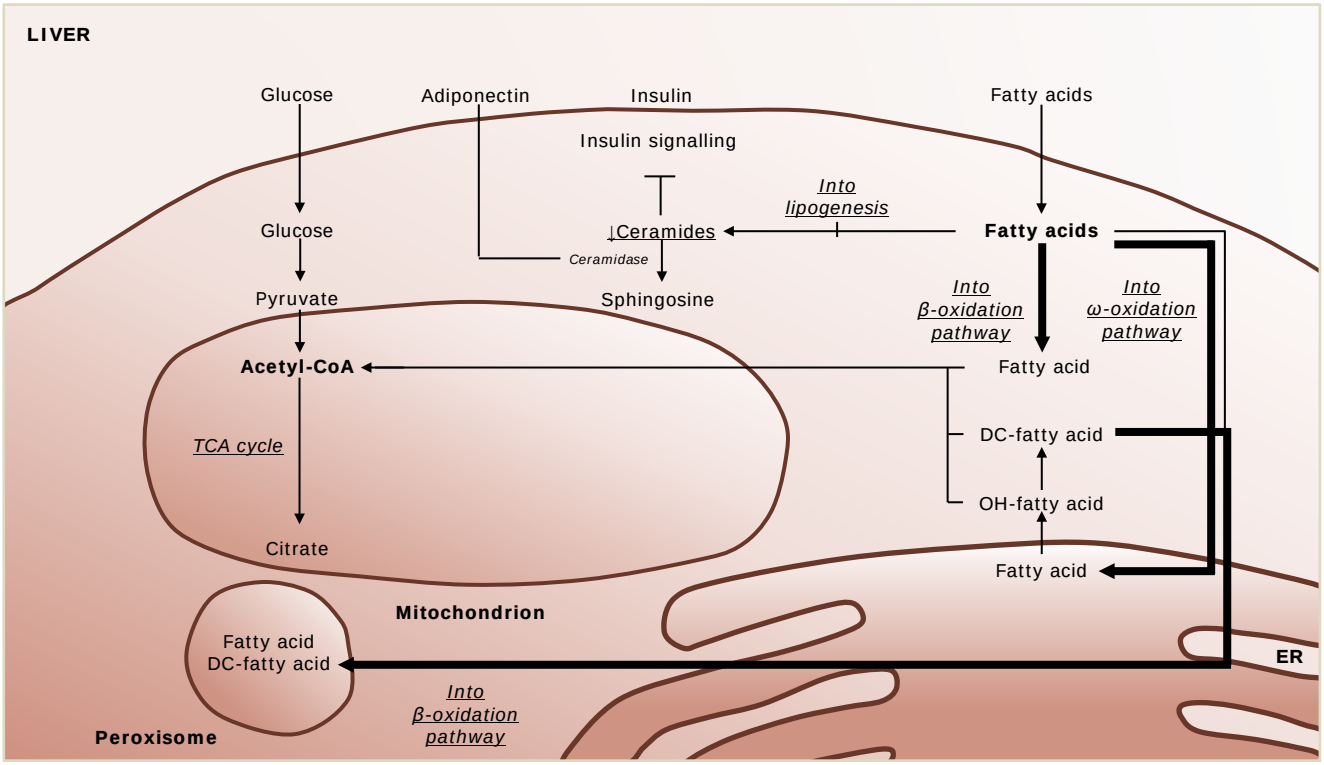


Figure 5.3: Model of possible alterations in fatty acid fluxes through beta- and omega-oxidation pathways and into de novo ceramide synthesis in LBW and NBW men in response to 5-day high-fat overfeeding. LBW and NBW men both decrease plasma levels of several ceramides in response to high-fat overfeeding. This could likely be a consequence of their increases in fatty acid oxidation rates and total energy expenditures due to overfeeding (91) with a resulting depletion of fatty acid substrates for lipogenesis, including de novo ceramide synthesis in the endoplasmic reticulum (not illustrated in this organelle, but in the cytosol). Furthermore, the decreases in plasma levels of several ceramides in LBW and NBW men could be a result of a FGF-21 and adiponectin mediated enhancement of ceramide degradation in the liver (shown here as the only tissue). The plasma total ceramide level was positively associated with hepatic insulin resistance. This suggests that increases in fatty acid oxidation rates in response to high-fat overfeeding may prevent an accumulation of ceramides that impair insulin signalling in the liver.

CHAPTER 6 – DISCUSSION

A series of plasma metabolome analyses, including acylcarnitine, amino acid, and ceramide profiling, were performed in young, healthy, LBW and NBW men after an isocaloric control diet and a 5-day HFHC diet to improve the understanding of the metabolic mechanisms behind the type 2 diabetes susceptible phenotype in LBW individuals and pathogenesis in general.

Insights into the type 2 diabetes susceptible phenotype in LBW individuals obtained from the plasma metabolome studies

LBW men had a higher plasma C2 acylcarnitine level after the control diet compared with NBW men, and a tendency to this same after the HFHC diet, indicating, together with the findings of an increased fatty acid oxidation rate in these individuals during both diets (10; 11), an increased, but incomplete fatty acid beta-oxidation with the limiting step at the acetyl-CoA entry into the TCA cycle. An incomplete fatty acid oxidation with a resulting accumulation of acylcarnitines and reactive oxygen species in tissues has been proposed to contribute to the development of insulin resistance and type 2 diabetes (1; 55). Specifically, acylcarnitines have been shown to activate pro-inflammatory pathways in a mouse monocyte/macrophage cell line (57) and furthermore to inhibit the insulin response in both mouse and human muscle cells at the level of Akt phosphorylation and/or glucose uptake (58). However, we did not demonstrate an association between the plasma C2 acylcarnitine level and insulin secretion or sensitivity. Interestingly, LBW men had higher plasma C4-OH, C6-DC, C10-OH/C8-DC, and total hydroxyl-/dicarboxyl-acylcarnitine levels after the control diet, compared with NBW men, suggesting an increased fatty acid omega-oxidation rate. The higher plasma C4-OH level in LBW men may in addition reflect an increased ketogenesis. This interpretation is supported by the finding of the higher plasma C2 acylcarnitine level in these individuals, as the C2 acylcarnitine level reflects the intracellular acetyl-CoA pool (56). Hydroxyl-fatty acids are intermediates in both fatty acid beta-oxidation and omega-oxidation (73; 76; 77), whereas dicarboxyl-fatty acids are formed in the omega-oxidation pathway. A limitation of the mass spectrometry method used for the acylcarnitine analyses is therefore that isobaric hydroxyl- and dicarboxyl-acylcarnitines are not distinguished. Thus, the higher plasma hydroxyl-/dicarboxyl-acylcarnitine levels in LBW men could reflect an increased omega-oxidation rate, if these acylcarnitines are in fact both hydroxyl- and dicarboxyl-species, or, alternatively, an accumulation

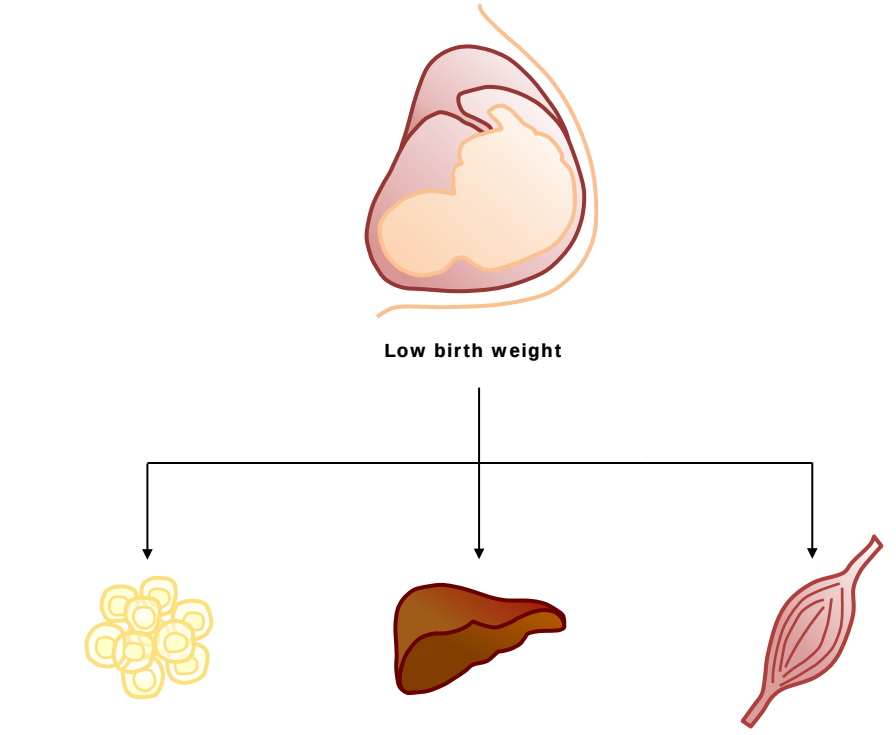
of intermediates in beta-oxidation pathways, if the pooled species are comprised solely or predominantly of the hydroxyl-species. The finding of a higher plasma C4-OH level in LBW men, however, supports that these individuals could have an increased fatty acid load to the liver and a subsequently increased omega-oxidation rate, as the liver is the primary site of ketogenesis. Notably, plasma C10-OH/C8-DC and total hydroxyl-/dicarboxyl-acylcarnitine levels were negatively associated with the fasting serum insulin level after the control diet, and the total hydroxyl-/dicarboxyl-acylcarnitine level was in addition negatively associated with hepatic insulin resistance after this diet. Omega-oxidation may therefore be a scavenger pathway for oxidation of fatty acids that, if not oxidised, would be available for lipogenesis, including the synthesis of lipotoxic lipids such as ceramides and diacylglycerols that may impair insulin signalling. Importantly, omega-oxidation has been shown to be upregulated in experimental models of diabetes (113; 114) and in individuals with diabetes (115). An increased omega-oxidation rate could therefore be part of the metabolic phenotype of pre-diabetes and diabetes.

In the second study, we demonstrated that LBW men had higher plasma alanine, proline, methionine, citrulline, and total amino acid levels after the HFHC diet compared with NBW men. The plasma alanine level was negatively associated with the plasma C2 acylcarnitine level after this diet. In the state of a high intracellular acetyl-CoA level, which seems to be the case in LBW men after both diets compared with NBW men (91), pyruvate dehydrogenase is inhibited and pyruvate carboxylase is activated, favouring pyruvate carboxylation to oxaloacetate as well as pyruvate transamination to alanine (116). This promotes anaplerosis and gluconeogenesis, respectively, (116). The higher plasma alanine level in LBW men after the HFHC diet could therefore reflect an increased pyruvate transamination to alanine in tissues, and the negative association between the plasma alanine and C2 acylcarnitine levels may be due to an increased anaplerotic formation of oxaloacetate. Alternatively, the higher plasma alanine level in LBW men could be a result of an increased skeletal muscle proteolysis, as discussed below. The LBW men show a higher adjusted total energy expenditure at night during the HFHC diet compared with NBW men (11), suggesting that the TCA cycle is likely to be upregulated in the LBW men during high-fat overfeeding. The higher plasma alanine level in LBW men after the HFHC diet could reflect an increased availability of alanine and pyruvate in tissues for anaplerotic formation of oxaloacetate, which may enhance the acetyl-CoA oxidation. Notably, the TCA cycle activity has been shown to be upregulated in the liver of mice progressing to hepatic insulin resistance during high-fat feeding (117). These mice also show an increased fatty acid oxidation rate (117). Furthermore, we demonstrated that plasma

alanine and total amino acid levels tended to be negatively associated with the insulin-stimulated glucose uptake rate after the HFHC diet. The LBW men decrease this rate in response to high-fat overfeeding (9). Whether the higher plasma alanine and total amino acid levels in LBW men could be a consequence of and/or contribute to impaired skeletal muscle insulin sensitivity in these individuals is uncertain. Insulin suppresses tissue proteolysis, and so an impaired skeletal muscle insulin sensitivity may increase the amino acid release from muscle (118). The amino acid release from skeletal muscle following an overnight fast does not reflect the amino acid composition of this tissue (119). Thus, relatively more alanine and glutamine, which are the main amino acid gluconeogenic precursors in the liver and kidney, respectively, are released from muscle (111; 119). This has been suggested to be due to an in situ amino acid metabolism that leads to de novo synthesis of non-essential amino acids, including in particular alanine and glutamine (111; 119). Interestingly, LBW men showed a tendency to a larger relative contribution of alanine and non-essential amino acids to the total plasma amino acid level after the HFHC diet compared with NBW men (Appendix 2). The higher plasma alanine level in LBW men after this diet could therefore be a result of both an increased skeletal muscle proteolysis and an increased de novo alanine synthesis from other amino acids (119; 120). Moreover, we found that plasma alanine and total amino acid levels were positively associated with the hepatic glucose production after the HFHC diet. Amino acids may enhance the endogenous glucose production via indirect and/or direct mechanisms. Thus, certain amino acids could stimulate insulin and glucagon secretion (121-123), and changes in the portal vein concentration of these hormones may increase the hepatic glucose production (124). Also, amino acids could by acting as substrates induce gluconeogenesis and thereby enhance the endogenous glucose production (125). Interestingly, higher fasting serum levels of gluconeogenic precursors, including alanine, lactate, and pyruvate, have been shown to be predictive of an increased 2 hour post-challenge blood glucose level after a 6.5 year follow-up (88). Gluconeogenic precursors were therefore proposed to be potential markers of long-term impaired insulin sensitivity (88). A higher plasma alanine level in LBW men after the HFHC diet may thus reveal additional metabolic abnormalities in these individuals associated with the development of type 2 diabetes.

In the third study, we did not demonstrate altered plasma ceramide levels in LBW men after the control or HFHC diet compared with NBW men. This may be explained by the increased fatty acid oxidation rates in the LBW men during both diets (10; 11). An increased fatty acid oxidation rate may limit the amount of fatty acids available for lipogenesis, including de novo ceramide synthesis, and thereby compensate for a likely increased fatty acid load to non-adipose tissue in LBW men.

An overview of the findings from the plasma metabolome studies on the birth weight dimension is shown in Figure 6.1 together with the proposed metabolic events leading to the respective changes in plasma metabolite levels in LBW men.



Plasma metabolites		
*Higher plasma C2 acylcarnitine level after the control diet	Increased, incomplete fatty acid beta-oxidation	Increased, incomplete fatty acid beta-oxidation
*Higher plasma hydroxyl-/dicarboxyl-acylcarnitine levels after the control diet	Increased fatty acid omega-oxidation	
*Higher plasma C4-OH acylcarnitine level after the control diet	Increased ketogenesis	
*Higher plasma alanine and total amino acid levels after the HFHC diet		Increased proteolysis and de novo alanine synthesis from other amino acids
*Unaltered plasma ceramide levels after both the control and HFHC diet	Compensatory increased fatty acid oxidation, including omega-oxidation	Compensatory increased fatty acid oxidation

Figure 6.1: Metabolic alterations in young, healthy, LBW men, as proposed from the plasma metabolome analyses. Findings in LBW men of the overfeeding study population are marked with an asterisk. Metabolic events expected to cause the changes in plasma metabolite levels are listed to the right of the individual findings.

Insights into metabolic responses to 5-day high-fat overfeeding obtained from the plasma metabolome studies

LBW and NBW men displayed changes in plasma levels of all the three groups of metabolites in response to high-fat overfeeding. In the first study, we found that both birth weight groups decreased plasma levels of several acylcarnitine species and total acylcarnitine in response to overfeeding and increased plasma levels of a few acylcarnitines due to this challenge. Both LBW and NBW men showed markedly increases in fatty acid oxidation rates and total energy expenditures in response to high-fat overfeeding (91). The decreases in plasma levels of several acylcarnitines, including C2 acylcarnitine, in LBW and NBW men in reaction to overfeeding could therefore be a result of an upregulation of fatty acid beta-oxidation and the TCA cycle due to this challenge. This interpretation is strongly supported by the findings of decreases in fasting plasma levels of several other lipid classes, including total NEFA, VLDL-cholesterol, and total triacylglycerol, in the LBW and NBW men in response to high-fat overfeeding (9). A study has reported that mice fed a 12-week high-fat (45 E %) diet have higher serum levels of several medium- and long-chain acylcarnitines, compared with mice fed a standard chow, when studied in the fed state (55). This was suggested to be due to an incomplete fatty acid beta-oxidation in mitochondria (55). Important differences between this and the present overfeeding study in LBW and NBW men are the duration of the high-fat diet interventions and the blood sampling in the fed or fasted state, respectively. An upregulation of fatty acid oxidation pathways and the TCA cycle in the LBW and NBW men in response to the 5-day overfeeding is probably only a transient, compensatory mechanism and may not persist for long-term high-fat overfeeding exposures. This hypothesis, however, requires further investigations.

In the second study, we demonstrated that LBW and NBW men decreased plasma valine and leucine/isoleucine levels and increased plasma alanine levels in response to high-fat overfeeding. An increase in the plasma alanine level was associated with a decrease in the plasma C2 acylcarnitine level. Thus, an increase in the alanine level due to overfeeding could be accompanied by an increase in the formation of oxaloacetate and following in the acetyl-CoA oxidation via the TCA cycle. Interestingly, LBW men showed a tendency to a larger increase in the plasma alanine level in response to overfeeding, compared with NBW men, and a tendency to a larger increase in the relative contribution of alanine to the total plasma amino acid level (Appendix 2). This could be due to an increased skeletal muscle alanine release in LBW men as a consequence of their decline

in skeletal muscle insulin sensitivity during high-fat overfeeding (9). Valine, leucine, and isoleucine are the major nitrogen sources for de novo alanine synthesis in skeletal muscle (110; 111). Thus, the decreases in plasma valine and leucine/isoleucine levels in LBW and NBW men in response to overfeeding may indicate an increased metabolism of these amino acids to alanine in muscle. A decrease in the plasma levels of these amino acids could also be a result of the lower protein content in the HFHC diet compared with the control diet (Appendix 5). In this regard, it is, however, remarkable that an increase in the relative plasma alanine level was strongly significantly associated with decreases in both relative plasma valine and leucine/isoleucine levels (Appendix 2), indicating that an increased metabolism of these amino acids to alanine could take place.

In the third study, we found that both LBW and NBW men decreased plasma levels of several ceramide species, including for both birth weight groups d18:0-18:1/d18:1-18:0 and d18:1-24:2/d18:2-24:1, and increased d18:0-24:1a levels in response to high-fat overfeeding. The decreases in plasma levels of several ceramides in both LBW and NBW men could, as for the decreases in acylcarnitine levels, likely be a result of their increases in fatty acid oxidation rates and total energy expenditures due to the overfeeding challenge (91). Several studies have reported altered plasma and/or tissue ceramide levels in mice exposed to high-fat feeding (126-129), and a recent study has described changes in plasma ceramide levels in human subjects exposed to high-fat overfeeding (130). Thus, sedentary women and men decrease fasting serum d18:0-18:0, d18:1-18:0, and d18:1-24:1 levels and increase d18:0-22:0, d18:0-24:0, d18:1-22:0, d18:1-24:0, and total d18:1-ceramide levels in response to a 28-day high-fat overfeeding (45 E % from fat, 1,250 extra kcal) (130). Important differences between this and the present overfeeding study are the duration and fat content of the HFHC diet interventions. An upregulation of fatty acid oxidation pathways in the LBW and NBW men in response to the 5-day overfeeding is probably a transient, compensatory mechanism. Among the studies performed in mice, it has been found that mice fed a 16-week high-fat (60 E % from fat) diet have higher plasma levels of all the measured ceramides, compared with mice fed a low-fat (10 E % from fat) diet, and also a higher plasma total ceramide level and adipose tissue total ceramide content (126). Furthermore, mice fed an 8-week high-fat (60 E % from fat) diet have higher liver total ceramide and total diacylglycerol contents compared with mice fed a standard chow (127). Plasma ceramide levels were not measured in these mice (127). Interestingly, adiponectin administration to the mice fed the 8-week high-fat diet, and in addition to leptin deficient (ob/ob) mice, rapidly normalises liver ceramide, but not diacylglycerol, contents, regardless of ceramide species (d18:0- or d18:1-) (127). Also, adiponectin administration to the

ob/ob mice leads to a reduction of the hepatic glucose production and an improvement in hepatic, but not skeletal muscle, insulin sensitivity (127). Adiponectin exerted these effects through lowering of the liver ceramide content via receptor-mediated enhancement of ceramidase activities (127). A recent study has shown that FGF-21 stimulates adiponectin secretion and reduces serum ceramide levels in mice fed a high-fat (60 E % from fat) diet, and that adiponectin-knockout mice are refractory to FGF-21 effects, including lowering of ceramide levels (131). Therefore, it was concluded that FGF-21 is dependent on adiponectin to exert its insulin-sensitising effects (131). LBW and NBW men of the overfeeding study population increases fasting plasma adiponectin levels in response to the high-fat overfeeding (9). Furthermore, both birth weight groups increases serum FGF-21 levels due to this challenge, likely as a result of an increased FGF-21 secretion from the liver (112). Taken together, the decreases in plasma levels of several ceramides in LBW and NBW men in response to high-fat overfeeding could be due to their increases in fatty acid oxidation rates, potentially evoked by the increases in serum FGF-21 levels, and/or a FGF-21 and adiponectin mediated activation of ceramidases in the liver with a subsequent increase in the ceramide degradation. Moreover, we demonstrated that plasma levels of several ceramides and total ceramide were positively associated with the fasting blood glucose level and hepatic insulin resistance after the control diet. These findings are consistent with the observed link between a higher liver ceramide content and hepatic insulin sensitivity in the mice models used to investigate adiponectin effects (127). NBW men of the overfeeding study population develop impaired hepatic insulin sensitivity and increase the hepatic glucose production in response to high-fat overfeeding, while the LBW men show an impaired hepatic insulin sensitivity already after the control diet and do not reduce this sensitivity further due to the overfeeding (8; 9). The decreases in plasma levels of several ceramides in response to overfeeding in the NBW men do not support a possible role of ceramides in promoting hepatic insulin resistance in these individuals. However, it is remarkable that some dihydroceramides (d18:0- species) were only or predominantly detected in plasma from the LBW and NBW men after the HFHC diet (Appendix 3). Furthermore, LBW and NBW men increased, as the only of the measured ceramides, d18:0-24:1a levels in response to overfeeding. The d18:0-24:1a level was strongly significantly positively associated with the hepatic glucose production after the control diet. Interestingly, a newly study has reported that individuals who progress to type 2 diabetes have elevated plasma levels of specific long-chain fatty acid-containing d18:0- species up to 9 years before its onset (132). Plasma dihydroceramides were therefore suggested to be potential biomarkers of pre-diabetes (132).

An overview of the findings from the plasma metabolome studies on the diet dimension is shown in Figure 6.2 together with the proposed metabolic events leading to the respective changes in plasma metabolite levels in LBW and NBW men in response to 5-day high-fat overfeeding.

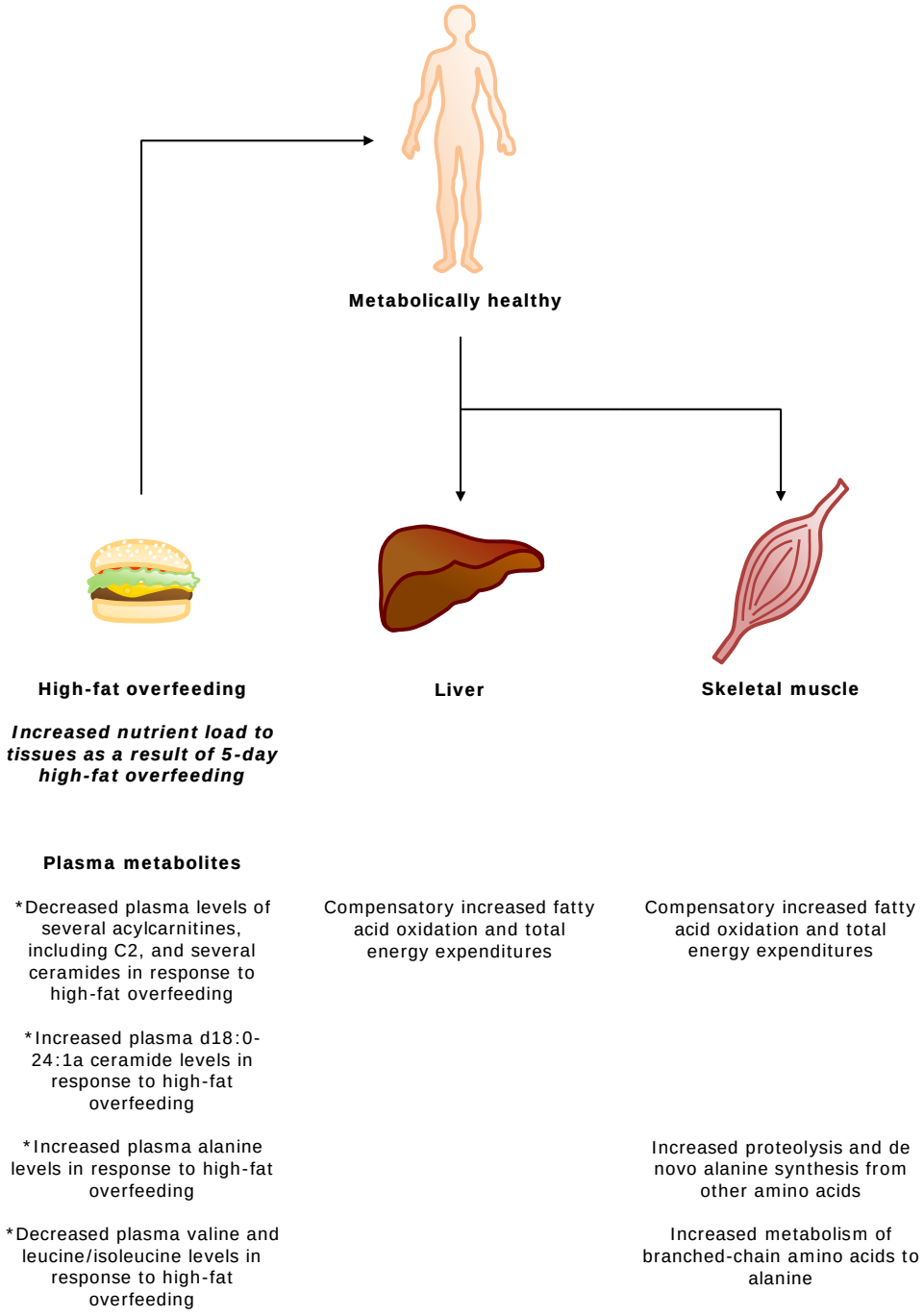


Figure 6.2: Metabolic alterations in young, healthy, LBW and NBW men in response to 5-day high-fat overfeeding, as proposed from the plasma metabolome analyses. Findings in both LBW and NBW men of the overfeeding study population are marked with an asterisk. Metabolic events expected to cause the changes in plasma metabolite levels are listed to the right of the individual findings.

CHAPTER 7 – CONCLUSIONS

Young, healthy, LBW men showed alterations in fasting plasma metabolite levels that have been described to be associated with insulin resistance and type 2 diabetes. Thus, LBW men had higher plasma C2 and C4-OH acylcarnitine levels after the isocaloric control diet, compared with NBW controls, indicating an increased, incomplete fatty acid beta-oxidation and an increased ketogenesis, respectively. Furthermore, LBW men had higher plasma C6-DC, C10-OH/C8-DC, and total hydroxyl-/dicarboxyl-acylcarnitine levels after as well the control diet, suggesting an increased fatty acid omega-oxidation in the liver. The plasma total hydroxyl-/dicarboxyl-acylcarnitine level was negatively associated with hepatic insulin resistance after this diet, suggesting that an increased fatty acid omega-oxidation rate may prevent an accumulation of lipotoxic lipids that impair insulin signalling. LBW men moreover had higher plasma alanine and total amino acid levels after the 5-day HFHC diet, compared with NBW men, possible as a result of an increased skeletal muscle proteolysis and de novo alanine synthesis from other amino acids. The observed alterations in plasma metabolite levels in LBW men could be a result of an impaired maturation of the subcutaneous adipose tissue. This would be in agreement with the thrifty phenotype hypothesis with its idea of saving nutrients to the vital organs at the expense of the growth and development of other organs and tissues in the setting of poor pre-natal nutrition. Thus, the LBW men show lower pre-adipocyte mRNA expression levels of several differentiation markers, which may potentially lead to an impaired fatty acid storage capacity of these cells and a resulting increased fatty acid load to non-adipose tissue. The higher plasma C2 acylcarnitine level in LBW men after the control diet, and the increased fatty acid oxidation rates in these individuals during both diets, could likely be due to an increased fatty acid load to non-adipose tissue. Also, the higher C4-OH and total hydroxyl-/dicarboxyl-acylcarnitine levels in LBW men may reflect an increased fatty acid load to the liver, as this organ is the primary site of ketogenesis and fatty acid omega-oxidation. LBW men did not show elevated plasma ceramide levels after the control or HFHC diet compared with NBW men. The increased fatty acid oxidation rate in LBW men during both diets may limit the amount of fatty acid substrates available for lipogenesis, including de novo ceramide synthesis, and thereby compensate for a likely increased fatty acid load to non-adipose tissue. Additional plasma and tissue metabolome studies in LBW and NBW individuals, as well as supplementary functional studies, are needed to further explain the metabolic events leading to the altered plasma metabolite profiles in LBW men, and moreover to determine the extent to which these events may be part of the type 2 diabetes susceptible phenotype in LBW individuals.

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APPENDICES

Appendix 1 – Article 1

Physiological Reports

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ORIGINAL RESEARCH

Plasma acylcarnitine profiling indicates increased fatty acid oxidation relative to tricarboxylic acid cycle capacity in young, healthy low birth weight men

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Keywords
Acylcarnitines, high-fat overfeeding, low birth weight, type 2 diabetes.

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Abstract

We hypothesized that an increased, incomplete fatty acid beta-oxidation in mitochondria could be part of the metabolic events leading to insulin resistance and thereby an increased type 2 diabetes risk in low birth weight (LBW) compared with normal birth weight (NBW) individuals. Therefore, we measured fasting plasma levels of 45 acylcarnitine species in 18 LBW and 25 NBW men after an isocaloric control diet and a 5-day high-fat, high-calorie diet. We demonstrated that LBW men had higher C2 and C4-OH levels after the control diet compared with NBW men, indicating an increased fatty acid beta-oxidation relative to the tricarboxylic acid cycle flux. Also, they had higher C6-DC, C10-OH/C8-DC, and total hydroxyl-/dicarboxyl-acylcarnitine levels, which may suggest an increased fatty acid omega-oxidation in the liver. Furthermore, LBW and NBW men decreased several acylcarnitine levels in response to overfeeding, which is likely a result of an upregulation of fatty acid oxidation due to the dietary challenge. Moreover, C10-OH/C8-DC and total hydroxyl-/dicarboxyl-acylcarnitine levels tended to be negatively associated with the serum insulin level, and the total hydroxyl-/dicarboxyl-acylcarnitine level additionally tended to be negatively associated with the hepatic insulin resistance index. This indicates that an increased fatty acid omega-oxidation could be a compensatory mechanism to prevent an accumulation of lipid species that impair insulin signaling.

Introduction

Low birth weight (LBW) individuals have an increased risk of developing obesity, cardiovascular disease, and type 2 diabetes, compared with normal birth weight (NBW) individuals, when exposed to an affluent life style such as overfeeding (Ravelli et al. 1976; Hales et al. 1991; Barker et al. 1993; Harder et al. 2007). In a short-term

high-fat overfeeding study in young, healthy LBW and NBW men, we have shown that LBW men display a number of metabolic abnormalities relevant to the pathophysiology of type 2 diabetes, including a decreased hepatic insulin sensitivity (Brons et al. 2008) prior to, and development of a decreased peripheral insulin sensitivity in response to a high-fat, high-calorie diet intervention (Brons et al. 2012). Furthermore, we have shown that LBW men exhibit an increased fatty acid oxidation rate, but an unaltered total energy expenditure, during night time compared with NBW men (Brons et al. 2013). However, the extent to which the disproportionately increased fatty acid oxidation rate could contribute to the decreased insulin sensitivity in LBW men as a result of a differential and potentially incomplete fatty acid oxidation remains to be studied.

High-fat overfeeding and an increased lipid exposure to skeletal muscle has been shown to lead to an increased expression of genes in the fatty acid beta-oxidation pathway, including the gene encoding carnitine palmitoyltransferase I (CPT-I) that catalyzes the condensation of activated long-chain fatty acids (acyl-CoAs) to carnitine to form acylcarnitines and thereby regulates the entry of these acyl-CoAs into the mitochondrial matrix (Koves et al. 2005; Muoio and Newgard 2006; Turner et al. 2007). Also, in the state of high-fat overfeeding, an increased beta-oxidation has been suggested to not necessarily be matched by increased tricarboxylic acid (TCA) cycle and electron transport chain fluxes, which results in an incomplete fatty acid oxidation (Koves et al. 2005, 2008; Muoio and Newgard 2006, 2008b). This leads to an accumulation of acylcarnitines and reactive oxygen species that may contribute to metabolic stress and thereby ultimately impair insulin signaling (Bloch-Damti and Bashan 2005; Adams et al. 2009; Rutkowsky et al. 2014; Aguer et al. 2015). In addition to an incomplete beta-oxidation, increased intracellular concentrations of long-chain acyl-CoAs may lead to an increased lipogenesis, hereunder the synthesis of lipid species that impair insulin signaling (Muoio and Newgard 2008b). This metabolic fate of long-chain acyl-CoAs has been especially described in the context of high-fat diet-induced hepatic insulin resistance. Actually, high-fat overfeeding has been proposed to lead to malonyl-CoA induced inhibition of CPT-I activity in the liver, and a following diversion of long-chain acyl-CoAs away from beta-oxidation and toward other metabolic fates in the cytosol, including lipogenesis (Muoio and Newgard 2008a,b). However, studies also point to that an increased lipid exposure to the liver leads to a simultaneously increased beta-oxidation and incorporation of long-chain acyl-CoAs into lipids (Ciapaite et al. 2011).

Incomplete fatty acid beta-oxidation downstream of CPT-I is reflected by elevated plasma acylcarnitine levels (Koves et al. 2008), as acyl-CoAs in the mitochondrial matrix can be converted into acylcarnitines that subsequently are transported through the mitochondrial membranes and thereafter from the cytosol to the blood (Koves et al. 2008; Millington and Stevens 2011; Violante et al. 2013). Actually, higher plasma acylcarnitine levels have been found in adults with prediabetes and type 2 diabetes (Adams et al. 2009; Mihalik et al. 2010; Ha et al. 2012; Mai et al. 2013). Also, defects in specific steps of beta-oxidation can be revealed by altered acylcarnitine levels, as made use of in the diagnosis of inborn errors in fatty acid metabolism (Millington et al. 1990; Chace et al. 2001, 2003). Moreover, the involvement of other metabolic pathways upstream of beta-oxidation may be reflected in the composition and concentrations of acylcarnitine species (Koves et al. 2008). Thus, an incomplete beta-oxidation give rise to even-chain C4–C22 acylcarnitine species, and amino acid catabolism is a source for C3, C4, and C5 species (Koves et al. 2008). These pathways are, together with glucose oxidation, in addition sources of acetylcarnitine, C2, when acetyl-CoA is generated in excess in the mitochondrial matrix relative to the flux into the TCA cycle (Zammit 1999; Koves et al. 2008). In this situation, carnitine acetyl-CoA transferase (CrAT) catalyzes the transfer of acetyl-CoA to carnitine to form acetylcarnitine, which is subsequently transported to the cytosol (Zammit 1999; Muoio Deborah et al. 2012). An accumulation of acyl-CoAs in the cytosol due to an incomplete beta-oxidation may lead to an increased fatty acid omega-oxidation in the endoplasmic reticulum of mainly the liver (Bjorkhem 1976; Reddy and Hashimoto 2001; Patsouris et al. 2006). This latter is expected to be reflected in higher plasma hydroxyl-/dicarboxyl-acylcarnitine levels (Reddy and Hashimoto 2001; Houten et al. 2012).

We hypothesized that an increased, incomplete fatty acid beta-oxidation could contribute to the impaired insulin sensitivity in LBW individuals, reflected by elevated plasma acylcarnitine levels. Accordingly, we analyzed fasting plasma levels of 45 acylcarnitine species, including even-chain C2–C22 species, odd-chain C3–C7 species, and hydroxyl-/dicarboxyl-species, in LBW and NBW men following an isocaloric control diet and a 5-day high-fat, high-calorie diet.

Materials and Methods

Study population

Forty-six young (23–27 years of age), healthy men were recruited from the Danish National Birth Registry according to birth weight. All individuals were born at term (39–41 weeks of gestation) and in Copenhagen in the period 1979–1980. LBW was defined as a birth weight below the

10th percentile, as earlier studies have shown that individuals within this range have an increased risk of developing type 2 diabetes (Jaquet et al. 2000; Jensen et al. 2002), and NBW was defined as a birth weight within the 50th–90th percentile range. Among the recruited men, 20 had LBW (2717 ± 268 g) and 26 had NBW (3901 ± 207 g). Furthermore, all participants were ensured to not have a family history of diabetes in two generations, not have a body mass index (BMI) greater than 30 kg/m^2, not perform strenuous physical activity more than 10 h per week, not take pharmaceuticals that affect metabolism, and not have an abuse of alcohol or drugs.

Study design

Diet interventions

All individuals were in a randomized crossover setup standardized with respect to diet and physical activity and following given a 3-day control diet and a 5-day high-fat, high-calorie diet separated by a 6–8 weeks wash-out period. Energy requirements of the individual subjects were calculated from a World Health Organization equation for men less than 30 years of age and a physical activity level of 1.4 corresponding to a low physical activity (WHO, 2001). The control diet was composed to reflect a habitual, weight-maintaining diet (2819 ± 238 kcal/11,800 ± 1000 kJ) with 15% of the total energy from protein, 50% from carbohydrate, and 35% from fat, and the high-fat, high-calorie diet was prepared to contain 50% extra calories (4228 ± 334 kcal/17,700 ± 1,400 kJ) with 7.5% of the total energy from protein, 32.5% from carbohydrate, and 60% from fat (Table S1). Both diets were provided as five daily servings with 25% of the total energy from breakfast, 10% from morning snack, 25% from lunch, 10% from afternoon snack, and 30% from dinner, and the meals were identical from day to day. Dietary calculations were made in Dankost Pro (http://dankost.dk/english) (The National Food Agency, Copenhagen, Denmark).

Clinical examinations

Study activities were carried out over 3 days, with the first of these days being placed 1 or 3 days after the start of the control and high-fat, high-calorie diet intervention, respectively. Anthropometry was performed on the first study day. An intravenous glucose tolerance test (IVGTT) and a hyperinsulinemic-euglycemic clamp were carried out in the morning on the third study day following an overnight fast to assess insulin secretion and sensitivity, as previously described (Brons et al. 2008, 2012). Furthermore, calorimetry was performed throughout 24 h from the first to second study day by use of a respiratory chamber and in the basal and insulin-stimulated steady-state periods of the clamp to evaluate substrate utilization rates and energy expenditures, as previously described (Brons et al. 2012, 2013, 2015). Blood samples were collected prior to and during the clamp.

Laboratory measurements

Acylcarnitine analyses

Acylcarnitine analyses were performed on EDTA-plasma samples collected following an overnight fast (10.00 PM–7.00 AM) and immediately prior to the clamp examination. These analyses included a semi-quantitative determination of 45 a priori selected acylcarnitine species or sets of species (ions with equal mass), noted in this text by their acyl group in accordance to its carbon chain length (e.g., C16), possible double bonds (e.g., C16:1), and possible hydroxyl- or a second carboxyl-group (e.g., C16-OH or C16-DC, respectively) (Table 3, Table S2), and were performed by use of sample preparation procedures and flow injection-tandem mass spectrometry (MS/MS), as previously described (An et al. 2004; Ferrara et al. 2008; Millington and Stevens 2011). In brief, plasma samples were spiked with a selection of deuterium-labeled acylcarnitine standards, including D3-C2, D3-C3, D3-C4, D9-C5, D3-C8, and D3-C16 carnitines (Cambridge Isotope Laboratories, Andover, MA). Following, proteins were removed by precipitation with methanol, and the supernatants were then transferred to a 96-well plate, evaporated to dryness under nitrogen gas, and incubated with either acidified methanol or butanol to form methyl and butyl ester derivatives of the acylcarnitines, respectively. After this, the reagents were evaporated to dryness under nitrogen gas, and the residues were redissolved in 85:15 (v/v) methanol:water. Subsequently, the samples were introduced into a Quattro Micro MS/MS system (Waters, Milford, MA) equipped with a model HTS-PAL autosampler (Leap Technologies, Carrboro, NC) and a model 1100 HPLC solvent delivery system (Agilent Technologies, Santa Clara, CA). Mass spectra of the acylcarnitine esters were obtained by positive precursor ion scanning of m/z 99 and m/z 85 for methyl or butyl esters, respectively. Following, acylcarnitines were identified from the peaks of these derivatives and quantified from the ratio of their molecular signals to respective internal standards (Table S2). Some acylcarnitine species shared the same internal standard due to the limited number of commercially available analytical standards. Addition of more internal

standards, however, does not appear to significantly improve the analytical precision (Millington and Stevens 2011). Mass spectra were analyzed by use of MassLynx 4.0 (Waters). Acylcarnitine analyses were performed in The Sarah W. Stedman Nutrition and Metabolism Center Metabolomics/Biomarker Core Laboratory, Duke University, Durham, NC. The laboratory was blinded to the birth weight of the individuals.

Ethical approval

All study procedures were in accordance with the principles of The Declaration of Helsinki and were approved by The Regional Research Ethics Committee of Copenhagen, Denmark. Also, all participants were provided with written information on the study purpose and procedures and signed an informed consent prior to their participation.

Table 1. Clinical characteristics of low (LBW) and normal birth weight (NBW) men following the control (C) and high-fat, high-calorie (O) diets.

	NBW (n = 25)			LBW (n = 18)			LBW versus NBW (n = 18, n = 25)		
	C (Mean ± SD)	O (Mean ± SD)	P_{NBW}	C (Mean ± SD)	O (Mean ± SD)	P_{LBW}	P_C	P_O	P_Δ
Anthropometry									
Birth weight (g)	3901 ± 207	–	–	2717 ± 268	–	–	**≤0.001**	–	–
Weight (kg)	78.4 ± 9.3	78.6 ± 9.7	n.s.	77.1 ± 11.3	77.1 ± 11.4	n.s.	n.s.	n.s.	n.s.
Height (m)	1.83 ± 0.07	–	–	1.77 ± 0.05	–	–	**≤0.05**	–	–
Body mass index (kg/m^2)	23.3 ± 2.4	23.3 ± 2.5	n.s.	24.6 ± 3.8	24.6 ± 3.8	n.s.	n.s.	n.s.	n.s.
Lipid profiling									
P-TG (mmol/L)	0.92 ± 0.35	0.73 ± 0.35	**≤0.05**	1.07 ± 0.37	0.72 ± 0.24	**≤0.01**	n.s.	n.s.	n.s.
P-CHOL (mmol/L)	4.36 ± 0.83	4.18 ± 0.82	n.s.	4.36 ± 0.78	4.27 ± 0.79	n.s.	n.s.	n.s.	n.s.
P-VLDL-CHOL (mmol/L)	0.42 ± 0.16	0.33 ± 0.16	**≤0.05**	0.49 ± 0.18	0.32 ± 0.12	**≤0.01**	n.s.	n.s.	n.s.
P-LDL-CHOL (mmol/L)	2.51 ± 0.72	2.28 ± 0.78	**≤0.05**	2.69 ± 0.76	2.57 ± 0.80	n.s.	n.s.	n.s.	n.s.
P-HDL-CHOL (mmol/L)	1.40 ± 0.22	1.56 ± 0.25	**≤0.01**	1.19 ± 0.23	1.38 ± 0.28	**≤0.01**	**≤0.01**	**≤0.05**	n.s.
Clamp									
Basal									
B-Glucose (mmol/L)	4.59 ± 0.47	5.05 ± 0.40	**≤0.001**	4.97 ± 0.48	5.18 ± 0.34	**≤0.05**	**≤0.01**	n.s.	n.s.
S-Insulin (pmol/L)	30.2 ± 14.7	43.4 ± 29.2	**≤0.05**	41.7 ± 14.6	44.7 ± 21.9	n.s.	**≤0.01**	n.s.	n.s.
S-C-peptide (pmol/L)	408 ± 146	529 ± 260	**≤0.01**	492 ± 116	539 ± 172	n.s.	**≤0.05**	n.s.	n.s.
P-NEFA (μmol/L)	334 ± 136	205 ± 82	**≤0.001**	406 ± 200	188 ± 91	**≤0.001**	n.s.	n.s.	n.s.
HGP (mg/kg·FFM/min)	2.21 ± 0.48	2.85 ± 0.99	**≤0.01**	2.40 ± 0.5	2.48 ± 0.5	n.s.	n.s.	n.s.	**≤0.05**
Hepatic IR (mg/kg·FFM/min·pmol/L)	68.7 ± 34.1	113.7 ± 61.5	**≤0.001**	102.3 ± 50.8	108.7 ± 55.5	n.s.	**≤0.05**	n.s.	**≤0.05**
GOX (mg/kg·FFM/min)	2.34 ± 0.76	2.43 ± 0.71	n.s.	1.95 ± 0.78	2.20 ± 0.56	n.s.	n.s.	n.s.	n.s.
FOX (mg/kg·FFM/min)	1.00 ± 0.38	1.02 ± 0.33	n.s.	1.11 ± 0.53	1.17 ± 0.33	n.s.	n.s.	n.s.	n.s.
Insulin-stimulated									
P-NEFA (μmol/L)	9.29 ± 4.39	12.42 ± 6.43	**≤0.01**	9.56 ± 5.03	14.39 ± 7.76	**≤0.01**	n.s.	n.s.	n.s.
M-value (mg/kg·FFM/min)	13.73 ± 2.32	13.29 ± 3.32	n.s.	13.47 ± 3.14	11.89 ± 3.57	**≤0.05**	n.s.	n.s.	n.s.
GOX (mg/kg·FFM/min)	5.18 ± 0.82	5.04 ± 0.98	n.s.	4.95 ± 0.92	4.78 ± 0.82	n.s.	n.s.	n.s.	n.s.
FOX (mg/kg·FFM/min)	0.01 ± 0.25	0.17 ± 0.33	n.s.	0.13 ± 0.46	0.37 ± 0.35	**≤0.05**	n.s.	**≤0.05**	n.s.
IVGTT									
FPIR (pmol/L)	1894 ± 1431	2604 ± 1793	**≤0.001**	2135 ± 1034	2750 ± 1509	**≤0.01**	n.s.	n.s.	n.s.
Hepatic DI	0.38 ± 0.63	0.25 ± 0.21	n.s.	0.21 ± 0.11	0.24 ± 0.13	n.s.	n.s.	n.s.	n.s.
Peripheral DI	0.29 ± 0.19	0.35 ± 0.20	**≤0.05**	0.33 ± 0.13	0.32 ± 0.17	n.s.	n.s.	n.s.	n.s.

Data are presented as mean ± SD. P-values from Student's t-tests are presented unadjusted for multiple comparisons, and P-values ≤0.5 are considered statistically significant. P_{NBW} and P_{LBW}: O versus C diet within each birth weight group, P_C and P_O: LBW versus NBW individuals within each diet, P_Δ: LBW versus NBW individuals on response values. n.s.: Not significant. P-values ≤0.05 are marked in bold. Abbreviations: B, Blood; CHOL, Cholesterol; DI, Disposition index; FFM, Fat free mass; FOX, Fatty acid oxidation; FPIR, First-phase insulin response; GOX, Glucose oxidation; HDL, High-density lipoprotein; HGP, Hepatic glucose production; IR, Insulin resistance; IVGTT, Intravenous glucose tolerance test; LDL, Low-density lipoprotein; NEFA, Nonesterified fatty acid; P, Plasma; S, Serum; TG, Triacylglycerol; VLDL, Very low-density lipoprotein.

Statistical analyses

Acylcarnitine levels and their relation to physiological measures

Differences in plasma acylcarnitine levels between NBW and LBW individuals within each diet or between the control and high-fat, high-calorie diets within each birth weight group were assessed from Student's unpaired or paired *t*-test (for normally distributed values), respectively, or Wilcoxon ranked-sum or signed-rank test (for not normally distributed values), respectively. Prior to these tests, statistical outliers (1.5 interquartile range) were removed from the dataset. Also, values below the lower limit of detection were replaced by 0.5 times this limit, which was defined as the minimum value for the actual metabolite. Following, outliers were replaced by the mean value within the group. Normal distribution of values (variables or differences between variables, respectively) was evaluated from Shapiro–Wilk test. Finally, after *P*-values were calculated, adjustment for multiple testing was done by calculating false discovery rates, *Q*-values, by the Benjamini and Hochberg method (Benjamini and Hochberg 1995). Data in Table 3 are presented as mean value plus or minus standard deviation (SD) together with *P*- and *Q*-values. *P*-values ≤0.05 were considered statistically significant if their corresponding *Q*-values were ≤0.1. Student's *t*-tests and Wilcoxon tests were performed in SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC), and Benjamini and Hochberg corrections were performed in R 3.1.0 (https://www.r-project.org/).

Associations between plasma acylcarnitine levels and physiological measures were assessed from linear regression analyses. These analyses were performed on the pooled dataset of LBW and NBW individuals and were adjusted for age, BMI, and birth weight group. Only acylcarnitine levels that significantly differed between NBW

Table 2. Glucose, fatty acid, and protein oxidation rates and total energy expenditures in low (LBW) and normal birth weight (NBW) men during the control (C) and high-fat, high-calorie (O) diets.

	LBW versus NBW (n = 26)			LBW versus NBW (C: n = 20, O: n = 18)			LBW versus NBW (n = 20/n = 18, n = 26)		
(kJ/min)	C (Mean ± SEM)	O (Mean ± SEM)	P_{NBW}	C (Mean ± SEM)	O (Mean ± SEM)	P_{LBW}	P_C	P_O	P_Δ
Calorimetry 24 h									
GOX									
Day	3.85 ± 0.17	3.50 ± 0.08	**0.0297**	3.69 ± 0.16	3.30 ± 0.14	0.0609	0.52	0.19	0.94
Night	1.97 ± 0.10	2.07 ± 0.07	0.3126	1.78 ± 0.09	1.84 ± 0.10	0.3391	0.18	0.06	0.97
Sleep	1.91 ± 0.12	1.89 ± 0.08	0.9131	1.58 ± 0.10	1.77 ± 0.11	0.0836	**0.05**	0.37	0.21
24 h	3.10 ± 0.13	2.93 ± 0.07	0.1510	2.92 ± 0.13	2.73 ± 0.09	0.2620	0.34	0.09	0.97
FOX									
Day	3.34 ± 0.16	4.23 ± 0.14	**<0.0001**	3.46 ± 0.14	4.52 ± 0.21	**<0.0001**	0.60	0.23	0.60
Night	2.34 ± 0.10	2.80 ± 0.10	**0.0005**	2.60 ± 0.08	3.06 ± 0.12	**0.0023**	0.07	0.10	0.93
Sleep	2.14 ± 0.14	2.72 ± 0.12	**0.0001**	2.50 ± 0.09	2.87 ± 0.13	**0.0221**	**0.05**	0.38	0.40
24 h	2.92 ± 0.12	3.63 ± 0.12	**<0.0001**	3.11 ± 0.11	3.91 ± 0.14	**<0.0001**	0.24	0.14	0.76
POX									
Day	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
Night	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
Sleep	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
24 h	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
EE									
Day	8.32 ± 0.15	8.52 ± 0.13	**0.0142**	8.24 ± 0.16	8.56 ± 0.18	**0.0021**	0.71	0.86	0.39
Night	5.43 ± 0.09	5.65 ± 0.10	**0.0001**	5.46 ± 0.11	5.66 ± 0.13	**0.0017**	0.82	0.97	0.99
Sleep	5.17 ± 0.09	5.39 ± 0.09	**0.0010**	5.16 ± 0.11	5.30 ± 0.13	**0.0009**	0.96	0.93	0.82
24 h	7.14 ± 0.12	7.36 ± 0.12	**0.0005**	7.12 ± 0.14	7.38 ± 0.15	**0.0008**	0.88	0.90	0.55

Data are presented as mean ± SEM. *P*-values from Student's *t*-tests are presented unadjusted for multiple comparisons, and *P*-values ≤0.5 are considered statistically significant. P_{NBW} and P_{LBW}: O versus C diet within each birth weight group, P_C and P_O: LBW versus NBW individuals within each diet, P_Δ: LBW versus NBW individuals on response values. *P*-values ≤0.05 are marked in bold. *P*-values on intervention effects are presented here for the first time. Details on the measurements have been described in previous articles (Brons et al. 2013, 2015). Abbreviations: EE, Energy expenditure; FOX, Fatty acid oxidation; GOX, Glucose oxidation; POX, Protein oxidation.

Table 3. Plasma acylcarnitine levels in low (LBW) and normal birth weight (NBW) men following the control (C) and high-fat, high-calorie (O) diets.

	NBW (n = 25)			LBW (n = 18)			LBW versus NBW (n = 18, n = 25)		
(μmol/L)	C (Mean ± SD)	O (Mean ± SD)	P_{NBW} Q_{NBW}	C (Mean ± SD)	O (Mean ± SD)	P_{LBW} Q_{LBW}	P_C Q_C	P_O Q_O	P_Δ Q_Δ
Lipid profiling									
Acylcarnitines									
C2	4.771 ± 0.797	3.985 ± 0.738	**0.0015** **0.0095**	5.985 ± 1.587	4.393 ± 0.784	**0.0007** **0.0046**	**0.0066** **0.0660**	0.0886	0.0595
C3	0.317 ± 0.102	0.273 ± 0.075	**0.0487** 0.1131	0.298 ± 0.103	0.259 ± 0.054	0.1582	0.5694	0.5058	0.8998
C4/Ci4	0.146 ± 0.057	0.126 ± 0.037	0.1054	0.162 ± 0.047	0.123 ± 0.049	**0.0082** **0.0291**	0.3243	0.7823	0.2661
C5:1	0.088 ± 0.040	0.073 ± 0.030	0.1275	0.084 ± 0.026	0.088 ± 0.039	0.5944	0.7246	0.1369	0.2252
C5's	0.122 ± 0.032	0.116 ± 0.030	0.5296	0.129 ± 0.029	0.128 ± 0.048	0.9683	0.4736	0.3526	0.7215
C4-OH	0.038 ± 0.016	0.034 ± 0.010	0.3581	0.054 ± 0.019	0.030 ± 0.012	**<0.0001** **0.0020**	**0.0039** **0.0520**	0.2229	**0.0006** **0.0222**
C6	n.d.	n.d.	–	n.d.	n.d.	–	–	–	–
C5-OH/C3-DC	0.118 ± 0.047	n.d.	–	0.114 ± 0.054	n.d.	–	0.8051	–	–
C4-DC/Ci4-DC	0.033 ± 0.013	0.036 ± 0.017	0.5226	0.042 ± 0.013	0.044 ± 0.009	0.5642	**0.0328** 0.1966	**0.0431** 0.6667	0.9323
C8:1	0.100 ± 0.032	0.190 ± 0.054	**<0.0001** **0.0013**	0.116 ± 0.032	0.222 ± 0.067	**<0.0001** **0.0020**	0.1354	0.0957	0.3314
C8	0.122 ± 0.049	0.105 ± 0.030	0.1010	0.127 ± 0.042	0.111 ± 0.030	0.1193	0.6921	0.5241	0.9872
C5-DC	0.048 ± 0.012	0.047 ± 0.015	0.7495	0.050 ± 0.013	0.050 ± 0.020	0.9169	0.7073	0.5795	0.7856
C8:1-OH/C6:1-DC	0.037 ± 0.009	0.062 ± 0.023	**<0.0001** **0.0013**	0.034 ± 0.014	0.060 ± 0.023	**0.0013** **0.0072**	0.3884	0.5660	0.5679
C6-DC	0.064 ± 0.022	0.080 ± 0.022	**0.0039** **0.0165**	0.084 ± 0.017	0.089 ± 0.019	0.4862	**0.0032** **0.0520**	0.1926	0.1504
C10:3	n.d.	0.088 ± 0.031	–	0.053 ± 0.034	0.102 ± 0.035	**0.0005** **0.0039**	–	0.1965	–
C10:2	n.d.	n.d.	–	n.d.	n.d.	–	–	–	–
C10:1	0.136 ± 0.053	0.121 ± 0.037	0.2216	0.138 ± 0.039	0.131 ± 0.023	0.5697	0.9120	0.3127	0.5908
C10	0.347 ± 0.160	0.311 ± 0.117	0.3218	0.297 ± 0.125	0.287 ± 0.102	0.8378	0.2736	0.4908	0.6401
C7-DC	n.d.	n.d.	–	n.d.	n.d.	–	–	–	–
C8:1-DC	0.024 ± 0.009	0.028 ± 0.012	0.1054	0.028 ± 0.012	0.026 ± 0.007	0.5224	0.1744	0.4497	0.1216
C10-OH/C8-DC	0.033 ± 0.014	0.032 ± 0.010	0.6502	0.048 ± 0.015	0.036 ± 0.010	**0.0048** **0.0208**	**0.0025** **0.0520**	0.1459	0.0585
C12:1	0.093 ± 0.040	0.067 ± 0.017	**0.0074** **0.0263**	0.109 ± 0.039	0.072 ± 0.016	**0.0005** **0.0039**	0.1226	0.3820	0.3802
C12	0.082 ± 0.037	0.083 ± 0.031	0.9887	0.089 ± 0.028	0.084 ± 0.024	0.5806	0.5344	0.8875	0.6768
C12-OH/C10-DC	0.008 ± 0.004	0.006 ± 0.003	**0.0327** **0.0866**	0.007 ± 0.002	0.006 ± 0.002	0.2281	0.5988	0.4714	0.3471
C14:2	0.026 ± 0.015	0.018 ± 0.009	**0.0076** **0.0263**	0.031 ± 0.012	n.d.	–	0.3231	–	–
C14:1	0.056 ± 0.018	0.049 ± 0.020	0.1900	0.071 ± 0.025	0.049 ± 0.016	**0.0054** **0.0211**	**0.0308** 0.1966	0.8905	0.1259
C14	n.d.	n.d.	–	n.d.	0.026 ± 0.011	–	–	–	–
C14:1-OH/C12:1-DC	0.012 ± 0.006	0.011 ± 0.005	0.3108	0.014 ± 0.006	0.012 ± 0.005	0.2743	0.2065	0.2887	0.7372
C14-OH/C12-DC	0.008 ± 0.004	0.007 ± 0.003	0.1782	0.009 ± 0.002	0.008 ± 0.004	0.7148	0.4479	0.1429	0.5903
C16:2	0.006 ± 0.003	0.005 ± 0.003	**0.0199** **0.0630**	0.006 ± 0.002	0.004 ± 0.003	**0.0251** **0.0699**	0.9316	0.2900	0.3964
C16:1	0.019 ± 0.009	0.010 ± 0.005	**<0.0001** **0.0013**	0.020 ± 0.009	0.011 ± 0.007	**0.0121** **0.0393**	0.6717	0.5271	0.9987
C16	0.077 ± 0.012	0.066 ± 0.008	**0.0006** **0.0057**	0.083 ± 0.016	0.072 ± 0.015	**0.0133** **0.0399**	0.1681	0.1796	0.9050
C16:1-OH/C14:1-DC	0.008 ± 0.003	0.006 ± 0.003	**0.0022**	0.008 ± 0.003	0.006 ± 0.003	0.0912	0.7204	0.8956	0.8272

(*Continued*)

Table 3. Continued.

(μmol/L)	NBW (n = 25) C (Mean ± SD)	NBW (n = 25) O (Mean ± SD)	NBW P_{NBW} Q_{NBW}	LBW (n = 18) C (Mean ± SD)	LBW (n = 18) O (Mean ± SD)	LBW P_{LBW} Q_{LBW}	LBW versus NBW (n = 18, n = 25) P_C Q_C	P_O Q_O	P_Δ Q_Δ
			0.0119						
C16-OH/C14-DC	0.005 ± 0.002	0.004 ± 0.002	0.2545	0.004 ± 0.002	0.005 ± 0.003	0.3113	0.2796	0.3719	0.1247
C18:2	0.032 ± 0.006	0.033 ± 0.008	0.6651	0.036 ± 0.013	0.036 ± 0.007	0.9486	0.3153	0.2563	0.8531
C18:1	0.096 ± 0.021	0.076 ± 0.016	**0.0012**	0.110 ± 0.024	0.083 ± 0.020	**0.0002**	**0.0488**	0.1802	0.4145
			0.0091			**0.0026**	0.2440		
C18	0.049 ± 0.008	0.042 ± 0.011	**0.0342**	0.051 ± 0.011	0.045 ± 0.012	**0.0307**	0.4460	0.3463	0.7884
			0.0866			**0.0798**			
C18:2-OH	0.005 ± 0.004	n.d.	–	0.005 ± 0.005	0.004 ± 0.003	0.2252	0.7582	–	–
C18:1-OH/C16:1-DC	0.006 ± 0.002	0.004 ± 0.002	**0.0028**	0.007 ± 0.004	0.003 ± 0.002	**0.0015**	0.3136	0.3860	0.1537
			0.0133			**0.0073**			
C18-OH/C16-DC	0.006 ± 0.003	0.005 ± 0.003	0.6185	0.007 ± 0.003	0.006 ± 0.002	0.2424	**0.0344**	0.2525	0.4401
							0.1966		
C20:4	0.006 ± 0.003	0.006 ± 0.004	0.6264	0.007 ± 0.003	0.006 ± 0.004	0.5415	0.5908	0.8081	0.8447
C20	0.004 ± 0.002	0.005 ± 0.002	0.4190	0.006 ± 0.003	0.005 ± 0.003	0.3517	0.1378	0.6414	0.1991
C20:1-OH/C18:1-DC	0.008 ± 0.004	0.006 ± 0.002	0.0506	0.007 ± 0.003	0.007 ± 0.003	0.8446	0.6730	0.1213	0.1343
C20-OH/C18-DC	0.008 ± 0.003	0.009 ± 0.004	0.4546	0.009 ± 0.003	0.010 ± 0.002	0.6724	0.2669	0.5315	0.7204
C22	0.004 ± 0.002	0.005 ± 0.002	**0.0243**	0.005 ± 0.003	0.006 ± 0.002	0.2680	0.2850	0.5532	0.6343
			0.0710						
Total levels									
OH/DC	0.345 ± 0.061	0.377 ± 0.075	0.0511	0.412 ± 0.057	0.397 ± 0.041	0.3833	**0.0007**	0.2679	**0.0488**
Non-OH/DC	6.674 ± 0.913	5.746 ± 0.865	**0.0006**	7.928 ± 1.822	6.215 ± 0.888	**0.0008**	**0.0049**	0.0901	0.0901
All	7.019 ± 0.945	6.122 ± 0.905	**0.0011**	8.340 ± 1.854	6.614 ± 0.885	**0.0009**	**0.0039**	0.0836	0.0790

Data are presented as mean ± SD, unless the acylcarnitine was not detected (n.d.) in more than 25% of the samples. P-values ≤0.05 are presented together with Q-values, and P-values ≤0.05 with corresponding Q-values ≤0.1 are considered statistically significant. P_{NBW} and P_{LBW}, O versus C diet within each birth weight group; P_C and P_O, LBW versus NBW individuals within each diet; P_Δ, LBW versus NBW individuals on response values. P-values ≤0.05 and Q-values ≤0.1 are marked in bold.

and LBW individuals after the control or high-fat, high-calorie diet were included in the analyses. Data in Tables 4, 5, and 6 are presented as slope plus or minus SD together with P- and Q-values. P-values were considered statistically significant as described above. Linear regression analyses were performed in R.

Results

Eighteen LBW and 25 NBW men were included in this study. Two LBW individuals of the recruited participants failed to consume all the food provided during the high-fat, high-calorie diet, and a NBW subject felt discomfort in connection with the clamp after the control diet and therefore did not further participate in this test in either the control or high-fat, high-calorie diet study part.

Clinical characteristics

Low birth weight and NBW men displayed differences in body composition and glucose and lipid metabolism after the control and high-fat, high-calorie diets, and both birth weight groups showed changes in metabolism in response to the dietary challenge, as previously reported (Brons et al. 2008, 2012, 2013, 2015). A selection of variables that provide background for the current findings is shown in Tables 1 and 2 and also presented here.

Low birth weight men had higher fasting blood glucose and serum insulin levels after the control diet compared with NBW men (Table 1). Also, LBW and NBW men both increased the fasting blood glucose level and decreased the fasting plasma nonesterified fatty acid level in response to overfeeding (Table 1). NBW men additionally increased the fasting serum insulin level due to this challenge. LBW and NBW men did not show differences in basal glucose or fatty acid oxidation rates after the control or high-fat, high-calorie diet when evaluated from the indirect calorimetry examination in connection with the clamp on the last study day, and they also did not change these rates in response to overfeeding (Table 1). However, when studied during the 24 h calorimetry during the interventions, LBW men had a higher fatty acid

Table 4. Associations between C2 or C4-OH level and physiological measures following the control (C) and high-fat, high-calorie (O) diets and between response values (Δ).

	C2 (Slope, SD, *P*, *Q*)			C4-OH (Slope, SD, *P*, *Q*)		
	C	O	Δ	C	O	Δ
Lipid profiling						
P-TG	0.050	0.103	0.057	−1.540	−1.123	0.926
	0.039	0.054	0.045	2.817	3.623	3.132
	0.2140	0.0683	0.2079	0.5880	0.7580	0.7691
Clamp						
Basal						
B-Glucose	−0.009	−0.023	−0.053	−0.881	−5.257	−0.954
	0.059	0.078	0.047	4.160	4.786	3.577
	0.8855	0.7649	0.2696	0.8335	0.2790	0.7912
S-Insulin	−0.246	−2.109	−1.451	99.78	358.9	165.8
	1.389	2.893	1.368	96.49	178.0	100.4
	0.8604	0.4713	0.2970	0.3080	0.0522	0.1080
P-NEFA	52.92	29.97	43.43	650.3	521.6	2038
	18.70	17.25	17.79	1335	1136	1274
	0.0075	0.0908	**0.0197**	0.6290	0.6490	0.1183
	0.1050		0.2352			
HGP	0.000	−0.078	−0.051	−0.222	−0.453	−1.593
	0.068	0.111	0.082	4.804	7.012	6.030
	0.9990	0.4875	0.5339	0.9630	0.9490	0.7931
Hepatic IR	1.222	−0.865	0.262	154.2	527.8	565.4
	4.012	10.31	5.149	283.6	653.4	387.0
	0.7625	0.9336	0.9600	0.5902	0.4247	0.1532
GOX	−0.115	−0.085	−0.129	−0.371	−4.375	−13.61
	0.111	0.159	0.099	7.973	10.82	7.284
	0.3070	0.5973	0.1990	0.9630	0.6883	0.0704
FOX	0.048	0.130	0.057	−0.678	−4.477	5.303
	0.065	0.068	0.051	4.604	4.455	3.854
	0.4640	0.0634	0.2790	0.8840	0.3219	0.1780
Insulin-stimulated						
M-value	0.306	0.974	0.016	2.371	69.74	−7.566
	0.286	0.547	0.289	21.08	34.32	21.17
	0.2920	0.0842	0.9560	0.9111	**0.0500**	0.7230
					0.1827	
GOX	−0.032	0.191	−0.166	−1.444	30.09	7.168
	0.122	0.196	0.128	9.430	10.84	9.759
	0.7930	0.3350	0.2040	0.8790	**0.0088**	0.4680
					0.0616	
FOX	0.017	−0.012	0.041	−2.894	−12.19	−7.563
	0.053	0.068	0.050	4.043	3.781	3.517
	0.7430	0.8619	0.4210	0.4790	**0.0027**	**0.0394**
					0.0378	0.2760
IVGTT						
FPIR	−100.5	−202.2	−181.9	4261	−8831	−1.0 E^4
	113.5	309.3	94.15	8032	−2.1 E^4	7553
	0.3819	0.5175	0.0618	0.5992	0.6700	0.1880

(*Continued*)

Table 4. Continued.

	C2 (Slope, SD, *P*, *Q*)			C4-OH (Slope, SD, *P*, *Q*)		
	C	O	Δ	C	O	Δ
Hepatic DI	−0.012	−0.011	−0.029	−0.953	−1.588	−2.153
	0.016	0.032	0.013	1.062	2.107	1.044
	0.4430	0.7230	**0.0336**	0.3760	0.4560	**0.0470**
			0.2352			0.2760
Peripheral DI	−0.021	−0.031	−0.026	−1.774	0.2400	−2.457
	0.016	0.037	0.018	1.081	2.423	1.358
	0.1950	0.4080	0.1480	0.1104	0.9220	0.0795

Data are presented as slope, SD, and *P*-value. *P*-values ≤0.05 are presented together with *Q*-values, and *P*-values ≤0.05 with corresponding *Q*-values ≤0.1 are considered statistically significant. *P*-values ≤0.05 and *Q*-values ≤0.1 are marked in bold. Regression analyses were performed on the pooled data set of LBW and NBW individuals and were adjusted for age, BMI, and birth weight group. Abbreviations: See Table 1.

oxidation rate and a lower glucose oxidation rate during sleep on the control diet compared with NBW men (Table 2). Also, LBW and NBW men both increased fatty acid oxidation rates and decreased protein oxidation rates in all measured time intervals during the 24 h calorimetry in response to overfeeding, and NBW men decreased the glucose oxidation rate during day time due this challenge (Table 2). Furthermore, LBW and NBW men both increased total energy expenditures in response to overfeeding (Table 2). LBW men had a higher hepatic insulin resistance index after the control diet compared with NBW men, but LBW and NBW men did not have a different insulin-stimulated glucose infusion rate, M-value, after this diet (Table 1). Furthermore, NBW men increased the hepatic insulin resistance index in response to overfeeding, whereas LBW men decreased the M-value in reaction to overfeeding. LBW and NBW men did not show a different first-phase insulin response (FPIR) and nor different hepatic or peripheral disposition indices (DI) following the control or high-fat, high-calorie diet (Table 1). However, LBW and NBW men both increased the FPIR in response to overfeeding, and NBW men additionally increased the peripheral DI due to this challenge.

Acylcarnitine levels and their relation to physiological measures

Low birth weight and NBW men showed differences in plasma acylcarnitine levels after the control diet, but not after the high-fat, high-calorie diet, and both birth weight groups furthermore showed changes in acylcarnitine levels in response to overfeeding (Table 3).

Low birth weight men had higher C2, C4-OH, C6-DC, and C10-OH/C8-DC levels after the control diet compared with NBW men, and they also displayed higher total hydroxyl-/dicarboxyl-acylcarnitine and total acylcarnitine levels after this diet. However, LBW men did not have a higher total acylcarnitine level when C2 was excluded from the sum (data not shown). Furthermore, LBW and NBW men both decreased C2, C12:1, C16:2, C16:1, C16, C18:1, C18, C18:1-OH/C16:1-DC, and total acylcarnitine levels in response to overfeeding. LBW men additionally decreased C4/C4i, C4-OH, C10-OH/C8-DC, and C14:1 levels when exposed to overfeeding, whereas NBW men decreased C12-OH/C10-DC, C14:2, and C16:1-OH/C14:1-DC levels due to this challenge. Moreover, LBW and NBW men both increased C8:1 and C8:1-OH/C6:1-DC levels in response to overfeeding. Also, LBW men increased the C10:3 level, whereas NBW men increased C6-DC and C22 levels due to overfeeding.

C10-OH/C8-DC, total hydroxyl-/dicarboxyl-acylcarnitine, and total acylcarnitine levels were positively associated with the plasma nonesterified fatty acid level after the control diet (Tables 5 and 6). Also, C10-OH/C8-DC and total hydroxyl-/dicarboxyl-acylcarnitine levels tended to be negatively associated with the serum insulin level after the control diet. In addition, the C10-OH/C8-DC level tended to be positively associated with the M-value after the control diet, and the total hydroxyl-/dicarboxyl-acylcarnitine level tended to be negatively associated with the hepatic insulin resistance index after this diet. Furthermore, the C4-OH level was positively associated with the insulin-stimulated glucose oxidation rate and negatively associated with the insulin-stimulated fatty acid oxidation rate after the high-fat, high-calorie diet, and tended to be positively associated with the M-value after this diet (Table 4). Moreover, a decrease in total hydroxyl-/dicarboxyl-acylcarnitine and total acylcarnitine levels in response to overfeeding was associated with a decrease in the plasma nonesterified fatty acid level, and a decrease in the total hydroxyl-/dicarboxyl-acylcarnitine

Table 5. Associations between C6-DC or C10-OH/C8-DC level and physiological measures following the control (C) and high-fat, high-calorie (O) diets and between response values (Δ).

	C6-DC (Slope, SD, *P*, *Q*)			C10-OH/C8-DC (Slope, SD, *P*, *Q*)		
	C	O	Δ	C	O	Δ
Lipid profiling						
P-TG	0.893	1.170	5.201	−1.976	0.774	5.816
	2.298	1.847	2.184	3.209	3.828	3.574
	0.6999	0.5302	**0.0227**	0.5420	0.8410	0.1124
			0.1589			
Clamp						
Basal						
B-Glucose	−1.683	−5.724	−2.958	−7.441	−9.371	−4.710
	3.409	2.404	2.522	4.689	4.895	3.395
	0.6245	**0.0226**	0.2487	0.1211	0.0635	0.2392
		0.1848				
S-Insulin	−113.9	−45.45	−16.15	−257.4	64.69	47.87
	77.68	96.33	70.67	102.1	225.3	114.1
	0.1512	0.6402	0.8210	**0.0162**	0.7760	0.6780
				0.1134		
P-NEFA	2775	138.1	801.9	5505	894.5	3793
	992.8	602.5	978.8	1338	1169	1423
	0.0082	0.8200	0.4180	**0.0002**	0.4490	**0.0114**
	0.1148			**0.0028**		0.1596
HGP	0.662	3.164	1.891	−2.140	6.143	8.350
	3.900	3.712	4.235	5.419	7.286	6.613
	0.8660	0.3994	0.6578	0.6950	0.4050	0.2140
Hepatic IR	−296.3	−3561	−125.2	−397.3	−353.2	681.8
	224.6	336.6	268.9	315.9	760.8	416.6
	0.1956	0.2973	0.6440	0.2168	0.6454	0.1110
GOX	−11.80	−3.977	−10.08	−14.41	−0.277	−12.05
	6.210	5.189	5.197	8.846	10.78	8.477
	0.0654	0.4486	0.0608	0.1120	0.9796	0.1640
FOX	7.191	5.105	6.744	6.254	7.978	7.003
	3.567	2.203	2.627	5.190	4.642	4.322
	0.0513	**0.0264**	**0.0148**	0.2360	0.0945	0.1140
		0.1848	0.1589			
Insulin-stimulated						
M-value	33.49	20.55	5.711	46.84	23.39	1.585
	17.21	18.70	15.25	21.59	37.19	23.35
	0.0595	0.2793	0.7104	**0.0367**	0.5336	0.9463
				0.1713		
GOX	−1.617	5.470	1.158	−2.194	23.63	12.30
	7.505	6.240	6.853	11.41	12.31	11.49
	0.8310	0.3867	0.8670	0.8490	0.0630	0.2930
FOX	2.189	1.103	0.904	−1.085	−0.947	−1.110
	3.222	2.237	2.642	4.930	4.599	4.451
	0.5020	0.6249	0.7350	0.8270	0.8380	0.8047
IVGTT						
FPIR	−4457	−1.4 E^4	−8198	−1.0 E^4	−1.8 E^4	−1.3 E^4
	6603	1.0 E^4	5370	9035	2.0 E^4	8439
	0.5043	0.1780	0.1361	0.2615	0.3572	0.1193

(*Continued*)

Table 5. Continued.

	C6-DC (Slope, SD, *P*, *Q*)			C10-OH/C8-DC (Slope, SD, *P*, *Q*)		
	C	O	Δ	C	O	Δ
Hepatic DI	−0.350	0.433	−0.131	0.483	−0.794	−0.566
	0.926	1.029	0.730	1.277	2.111	1.160
	0.7080	0.6770	0.8590	0.7080	0.7090	0.6290
Peripheral DI	0.2370	−0.119	0.267	0.192	−3.438	−1.464
	0.9630	1.193	1.024	1.329	2.437	1.585
	0.8070	0.9210	0.7960	0.8860	0.1672	0.3620

Data are presented as slope, SD, and *P*-value. *P*-values ≤0.05 are presented together with *Q*-values, and *P*-values ≤0.05 with corresponding *Q*-values ≤0.1 are considered statistically significant. *P*-values ≤0.05 and *Q*-values ≤0.1 are marked in bold. Regression analyses were performed on the pooled data set of LBW and NBW individuals and were adjusted for age, BMI, and birth weight group. Abbreviations: See Table 1.

level was additionally associated with a decrease in the plasma triacylglycerol level and an increase in FPIR.

Discussion

In order to investigate a possible differential and potentially incomplete fatty acid oxidation in LBW individuals, we measured fasting plasma levels of 45 acylcarnitine species or sets of species in 18 LBW and 25 NBW men following an isocaloric control diet and a 5-day high-fat, high-calorie diet.

We demonstrated that LBW men had higher C2, C4-OH, C6-DC, and C10-OH/C8-DC levels after the control diet compared with NBW men, and also a higher total hydroxyl-/dicarboxyl-acylcarnitine level after this diet. Moreover, C10-OH/C8-DC and total hydroxyl-/dicarboxyl-acylcarnitine levels tended to be negatively associated with the serum insulin level after the control diet, and the total hydroxyl-/dicarboxyl-acylcarnitine level additionally tended to be negatively associated with the hepatic insulin resistance index after this diet.

Low birth weight individuals' higher C2 level after the control diet is reflective of an excess of acetyl-CoA in the mitochondrial matrix. This indicates increased fatty acid, glucose, and/or amino acid oxidation rates relative to the TCA cycle flux (Fig. 1). Also, their unaltered total acylcarnitine level when excluding C2 suggests that they do not have limitations in fatty acid beta-oxidation. In previous studies, we have found that LBW men have an increased fatty acid oxidation rate and a decreased glucose oxidation rate at night time during the control diet compared with NBW men, whereas they do not have a different protein oxidation rate (Brons et al. 2013). Thus, an accumulation of acetyl-CoA in LBW men is likely to be due to an increased fatty acid beta-oxidation. Furthermore, we have shown that LBW and NBW men do not show differences in expression levels of genes involved in oxidative phosphorylation or differences in ATP synthesis in skeletal muscle (Brons et al. 2008). Thus, at least in skeletal muscle, available data indicate that LBW individuals could have an increased beta-oxidation rate and an unchanged TCA cycle flux, which would cause an accumulation of acetyl-CoA in the mitochondrial matrix (Fig. 1). Their higher 3-hydroxy-butyrylcarnitine, C4-OH, level is thought to reflect ketogenesis (McGarry and Foster 1972), consistent with an excess pool of acetyl-CoA. As the liver is a primary site of ketogenesis, these findings may suggest increased rates of hepatic fatty acid oxidation in LBW men (Fig. 1). Hydroxyl- and dicarboxyl-fatty acids are products of fatty acid omega-oxidation in the endoplasmic reticulum of mainly the liver (Reddy and Hashimoto 2001), and with regards to medium-chain dicarboxyl-fatty acids and their acylcarnitine esters, in addition of beta-oxidation in peroxisomes, as long-chain dicarboxyl-fatty acids derived from omega-oxidation are oxidized in peroxisomes (Houten et al. 2012). Omega-oxidation is a minor route for oxidation of fatty acids under normal physiological conditions (Reddy and Hashimoto 2001; Patsouris et al. 2006). However, the flux of fatty acids through this pathway is increased when intracellular levels of nonesterified fatty acids are high such as following high-fat feeding (Patsouris et al. 2006) or under fasting (Patsouris et al. 2006) and starvation (Bjorkhem 1976; Kroetz et al. 1998). In addition to the omega-oxidation pathway, hydroxyl-fatty acids are intermediates in beta-oxidation in mitochondria and peroxisomes (Reddy and Hashimoto 2001; Jones and Bennett 2010). Therefore, the higher C6-DC, C10-OH/C8-DC, and total hydroxyl-/dicarboxyl-acylcarnitine levels in LBW men could reflect an increased omega-oxidation (Mortensen and Gregersen 1981) along with an increased beta-oxidation of dicarboxyl-fatty acids (Houten et al. 2012) (Fig. 1), if these acylcarnitines are in fact both hydroxyl- and dicarboxyl-species, or, alternatively, an accumulation of intermediates

Table 6. Associations between total OH-/DC-acylcarnitine, total non-OH-/DC-acylcarnitine, or total acylcarnitine level and physiological measures following the control (C) and high-fat, high-calorie (O) diets and between response values (Δ).

	OH/DC-acylcarnitines (Slope, SD, *P*, *Q*)			Non-OH/DC-acylcarnitines (Slope, SD, *P*, *Q*)			All acylcarnitines (Slope, SD, *P*, *Q*)		
	C	O	Δ	C	O	Δ	C	O	Δ
Lipid profiling									
P-TG	−0.210	0.714	2.119	0.045	0.101	0.071	0.042	0.099	0.074
	0.787	0.584	0.696	0.033	0.045	0.041	0.033	0.043	0.039
	0.7910	0.2294	**0.0043**	0.1888	**0.0308**	0.0893	0.2040	**0.0285**	0.0697
			0.0315		0.2793			0.2772	
Clamp									
Basal									
B-Glucose	−1.605	−1.190	−0.246	−0.014	−0.665	−0.047	−0.016	−0.068	−0.045
	1.142	0.786	0.920	0.050	0.064	0.043	0.049	0.061	0.043
	0.1681	0.1387	0.7912	0.7778	0.3181	0.2898	0.7393	0.2781	0.2944
S-Insulin	−54.46	−0.031	−5.130	−0.307	−1.698	−1.171	−0.389	−1.607	−1.131
	25.91	32.60	25.37	1.184	2.446	1.240	1.156	2.381	1.212
	0.0427	0.9920	0.8410	0.7970	0.4930	0.3520	0.7382	0.5050	0.3580
	0.1993								
P-NEFA	1044	153.9	887.4	49.78	30.20	48.20	49.68	29.34	48.66
	339.9	188.6	292.7	15.62	14.16	15.81	15.18	13.74	15.34
	0.0040	0.4200	**0.0045**	**0.0029**	**0.0399**	**0.0043**	**0.0023**	**0.0396**	**0.0031**
	0.0560		**0.0315**	**0.0406**	0.2793	**0.0602**	**0.0322**	0.2772	**0.0434**
HGP	−0.402	0.212	0.191	0.009	−0.052	−0.042	0.008	−0.047	−0.039
	1.326	1.155	1.507	0.058	0.093	0.077	0.057	0.090	0.075
	0.7640	0.8551	0.8999	0.8710	0.5819	0.5910	0.8840	0.6040	0.6041
Hepatic IR	−160.9	−22.89	20.38	1.182	−0.209	1.137	0.835	−0.324	1.131
	73.23	114.1	95.36	3.400	8.697	4.678	3.323	8.461	4.568
	0.0347	0.8422	0.8320	0.7302	0.9810	0.8090	0.8030	0.9697	0.8060
	0.1993								
GOX	−2.652	0.924	−4.280	−0.125	−0.062	−0.147	−0.124	−0.053	−0.150
	2.156	1.759	2.032	0.094	0.136	0.091	0.092	0.131	0.089
	0.2270	0.6025	**0.0426**	0.1920	0.6514	0.1160	0.1840	0.6906	0.1020
			0.1491						
FOX	1.159	0.930	1.899	0.056	0.105	0.071	0.056	0.104	0.073
	1.256	0.772	1.017	0.055	0.057	0.048	0.053	0.055	0.047
	0.3620	0.2365	0.0704	0.3120	0.0736	0.1470	0.3040	0.0673	0.1310
Insulin-stimulated									
M-value	10.20	4.765	−2.950	0.332	0.799	0.012	0.334	0.776	0.004
	5.593	5.715	5.392	0.241	0.450	0.276	0.235	0.435	0.271
	0.0764	0.4102	0.5879	0.1770	0.0848	0.9669	0.1640	0.0832	0.9890
GOX	0.568	5.344	2.610	−0.030	0.245	−0.136	−0.028	0.259	−0.126
	2.590	1.917	2.603	0.105	0.160	0.123	0.102	0.153	0.121
	0.8280	**0.0085**	0.3240	0.7750	0.1340	0.2750	0.7870	0.0994	0.3050
		0.1190							
FOX	−0.509	−0.772	−1.380	0.019	−0.034	0.032	0.017	−0.036	0.028
	1.116	0.739	0.993	0.045	0.056	0.047	0.044	0.055	0.047
	0.6510	0.3035	0.1743	0.6810	0.5513	0.4987	0.7020	0.5124	0.5466
IVGTT									
FPIR	−1437	−4583	−4802	−1037	−232.8	−198.0	−101.4	−246.1	−201.0
	2214	3368	1810	95.87	260.0	85.64	93.56	252.7	83.68
	0.5206	0.1820	**0.0120**	0.2871	0.3765	**0.0270**	0.2862	0.3368	**0.0219**

(*Continued*)

Table 6. Continued.

	OH/DC-acylcarnitines (Slope, SD, *P*, *Q*)			Non-OH/DC-acylcarnitines (Slope, SD, *P*, *Q*)			All acylcarnitines (Slope, SD, *P*, *Q*)		
	C	O	Δ	C	O	Δ	C	O	Δ
			0.0560			0.1549			0.1533
Hepatic DI	−0.043	−0.136	−0.055	−0.011	−0.012	−0.027	−0.010	−0.013	−0.026
	0.308	0.347	0.269	0.013	0.027	0.012	0.013	0.026	0.012
	0.8890	0.6970	0.8380	0.4320	0.6470	**0.0332**	0.4390	0.6350	**0.0357**
						0.1549			0.1666
Peripheral DI	−0.185	−0.451	−0.329	−0.016	−0.030	−0.023	−0.016	−0.031	−0.023
	0.323	0.393	0.363	0.014	0.031	0.017	0.014	0.030	0.016
	0.5710	0.2590	0.3720	0.2490	0.3450	0.1700	0.2500	0.3150	0.1640

Data are presented as slope, SD, and *P*-value. *P*-values ≤0.05 are presented together with *Q*-values, and *P*-values ≤0.05 with corresponding *Q*-values ≤0.1 are considered statistically significant. *P*-values ≤0.05 and *Q*-values ≤0.1 are marked in bold. Regression analyses were performed on the pooled dataset of LBW and NBW individuals and were adjusted for age, body mass index, and birth weight group. Abbreviations: See Table 1.

in beta-oxidation pathways, if the pooled species are comprised solely or predominantly of the hydroxyl-species. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses will be required to resolve this issue, as opposed to the flow injection-MS/MS analyses conducted herein. White adipose tissue lipolysis is increased during deficient adipose tissue insulin signaling, and we have previously described this trait in LBW individuals (Alibegovic et al. 2010). Furthermore, our recent studies of adipose tissue cells suggest that LBW is associated with an impaired development of subcutaneous adipose tissue (Ferland-McCollough et al. 2012; Schultz et al. 2014). An increased lipolysis results in a shift in the equilibrium of fat storage from adipose tissue toward an increased storage in nonadipose tissue such as the liver (Samuel and Shulman 2016). Also, an increased hepatic fatty acid load is expected to induce omega-oxidation (Patsouris et al. 2006). Interestingly, omega-oxidation has been shown to be upregulated in experimental models of diabetes (Yoshioka et al. 1994; Miura 2013) as well as in patients with diabetes (Lippe et al. 1987). This, together with the present findings of a possible increase in omega-oxidation in LBW individuals, suggests that an increased omega-oxidation could be part of the metabolic phenotype of prediabetes and diabetes.

Acetylcarnitine, C2, has been reported to be a marker of prediabetes (Wang-Sattler et al. 2012), and an elevated fasting plasma C2 level has been found in adults with type 2 diabetes (Adams et al. 2009; Villarreal-Perez et al. 2014). Also, the fasting plasma C2 level has been shown to positively associate with the fasting plasma HbA1c level in women with or without diabetes (Adams et al. 2009). However, in this study, the C2 level did not associate with measures of insulin secretion or sensitivity. In addition, the fasting plasma C4-OH level has been found to be elevated in obese women with type 2 diabetes (Fiehn et al. 2010). Furthermore, the fatty acid moiety of this acylcarnitine has been shown to interfere with insulin signaling (Tardif et al. 2001), and its levels in skeletal muscle have been associated with muscle insulin resistance in diet-induced obesity rodent models (An et al. 2004). In this study, we did not observe any associations between the plasma C4-OH level and insulin secretion or sensitivity. However, C10-OH/C8-DC and total hydroxyl-/dicarboxyl-acylcarnitine levels tended to be negatively associated with the serum insulin level, and the total hydroxyl-/dicarboxyl-acylcarnitine level additionally tended to be negatively associated with the hepatic insulin resistance index. This suggests that omega-oxidation could act as a scavenger pathway for oxidation of fatty acids when intracellular acyl-CoA levels are high to thereby reduce the availability of these precursors for the synthesis of lipotoxic lipid species such as ceramides and diacylglycerols that impair insulin signaling (Chavez and Summers 2012; Jornayvaz and Shulman 2012) (Fig. 1).

We furthermore demonstrated that LBW and NBW men decreased levels of 12 and 11 of the measured acylcarnitine species, respectively, as well as the total acylcarnitine level in response to overfeeding. In addition, they increased levels of three and four species, respectively, due to this challenge.

Low birth weight and NBW individuals' decrease in several short-, medium-, and long-chain acylcarnitine species, including C2, in response to overfeeding could be due to an increased fatty acid beta-oxidation and TCA cycle flux following the high-fat, high-calorie diet compared to the control diet. This interpretation is strongly supported by the findings that LBW and NBW men increased both fatty acid oxidation rates and total energy

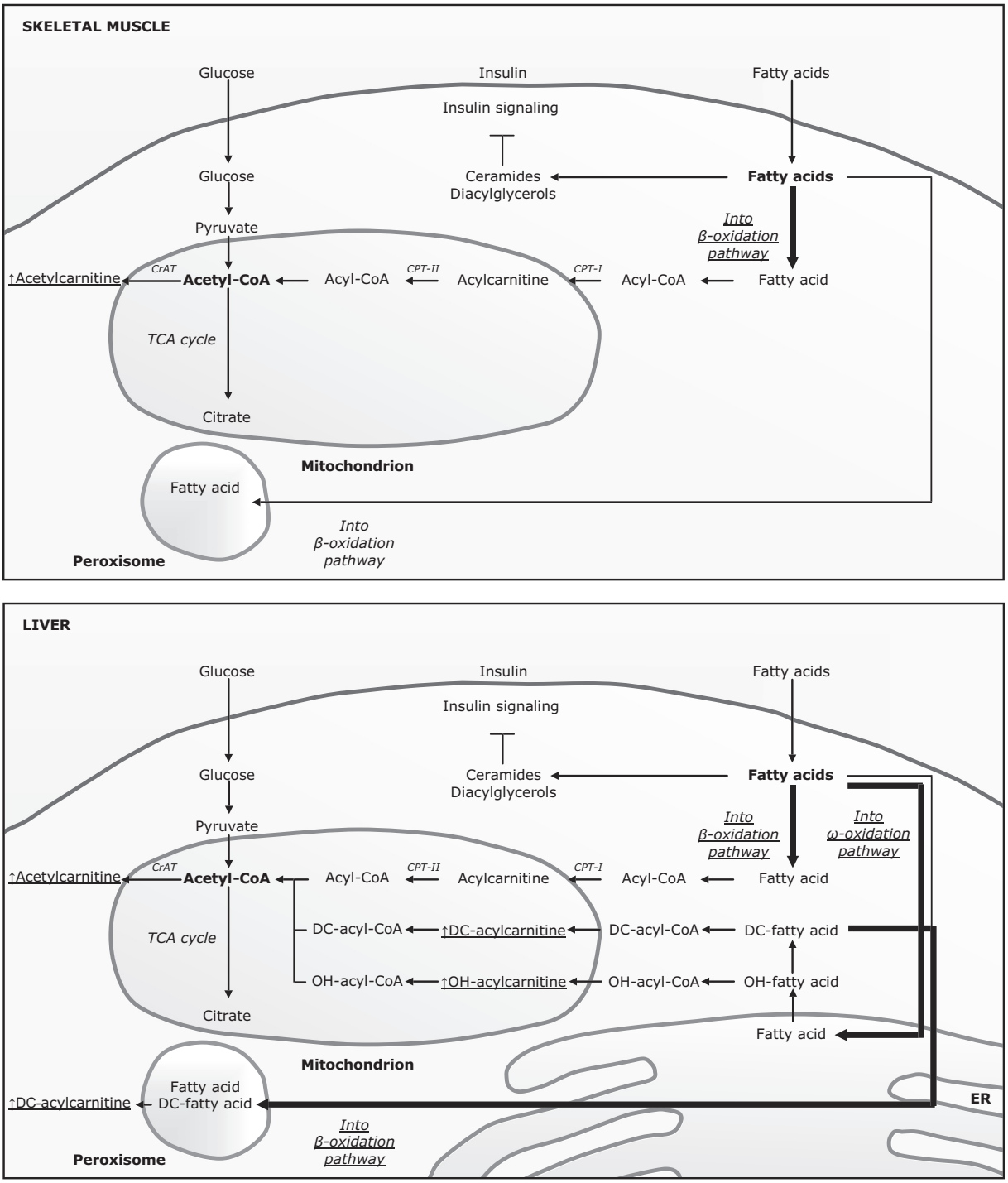

Figure 1. Proposed differences in fatty acid oxidation pathways between low (LBW) and normal birth weight (NBW) individuals. LBW individuals may have an increased beta-oxidation in mitochondria of skeletal muscle (upper panel) and liver (bottom panel) that is not matched by an equivalently increased TCA cycle flux, resulting in an accumulation of acetyl-CoA in the mitochondrial matrix and following of acetylcarnitine in the cytosol. Also, an excess of acetyl-CoA could lead to an increased ketogenesis in the liver and following an accumulation of 3-hydroxy-butyrylcarnitine (not shown). Furthermore, LBW individuals may have an increased omega-oxidation in the endoplasmic reticulum of the liver along with an increased beta-oxidation of dicarboxyl-fatty acids in peroxisomes, leading to an accumulation of hydroxyl- and dicarboxyl-acylcarnitine species. Omega-oxidation may be a scavenger pathway for oxidation of fatty acids that otherwise could be available for synthesis of lipotoxic lipid species such as ceramides and diacylglycerols that impair insulin signaling.

expenditures during all time intervals of the 24 h calorimetry in response to overfeeding, and furthermore from the finding that they reduced the plasma nonesterified fatty acid level due to this challenge (Brons et al. 2012). In a prior rodent study, mice fed a high-fat diet had higher serum levels of several medium- and long-chain acylcarnitines compared with mice fed a standard diet (Koves et al. 2008). It was suggested that the high-fat feeding resulted in an incomplete fatty acid beta-oxidation (Koves et al. 2008). However, in this study, the high-fat overfeeding was for only 5 days and the blood samples were collected after an overnight fast, as opposite to the rodent study in which the intervention was for 12 weeks, and the samples were collected in the fed state (Koves et al. 2008). Thus, an increased beta-oxidation and TCA cycle flux in response to short-term high-fat overfeeding could be a compensatory mechanism to prevent an accumulation of lipids in nonadipose tissue. Such a mechanism is probably only transient and may not persist for long-term high-fat overfeeding exposure. This hypothesis, however, requires further studies. Their increase in C8:1 and C8:1-OH/C6:1-DC levels in response to overfeeding may be explained by the markedly higher n-3 fatty acid content in the high-fat, high-calorie diet compared to the control diet (Table S1), as C8:1 and C6:1 n-3 fatty acids are oxidation products of several n-3 fatty acids, including alpha-linolenic acid, C18:3 n-3 fatty acid.

Our study is the first to describe fasting plasma acylcarnitine levels in LBW individuals with an increased risk of developing type 2 diabetes. It has its strengths in the careful selection of LBW and NBW men, highly standardized study setup, and in depth physiological and metabolic characterization of the individuals. In relation to the biological interpretation of the results, however, it has its limitations in the acylcarnitine profiling being on the plasma level, as plasma acylcarnitines represent the sum of contributions of acylcarnitines from various tissues, mainly skeletal muscle and liver, that may respond differently to a given metabolic challenge (Schooneman et al. 2013, 2014). Also, plasma and these tissues may have different turnover rates of acylcarnitines (Schooneman et al. 2014). In summary, we demonstrated that LBW men had higher C2 and C4-OH levels after the control diet compared with NBW men, suggestive of an increased fatty acid beta-oxidation in mitochondria relative to the TCA cycle flux. Furthermore, we showed that LBW men had higher C6-DC, C10-OH/C8-DC, and total hydroxyl-/dicarboxyl-acylcarnitine levels, which may suggest an increased fatty acid omega-oxidation in the endoplasmic reticulum of the liver concomitant with an increased beta-oxidation in peroxisomes of omega-oxidation-derived dicarboxyl-fatty acids. Interestingly, a cluster of short-chain, dicarboxyl-acylcarnitine species, including C6-DC, has recently been shown to be prognostic for myocardial infarction and all-cause cardiac mortality (Shah et al. 2010, 2012). Moreover, we found that C10-OH/C8-DC and total hydroxyl-/dicarboxyl-acylcarnitine levels tended to be negatively associated with the serum insulin level, and the total hydroxyl-/dicarboxyl-acylcarnitine level additionally tended to be negatively associated with the hepatic insulin resistance index. Therefore, we proposed that an increased fatty acid omega-oxidation could prevent an accumulation of lipotoxic lipid species that impair insulin signaling in the liver. Intervention studies that aim to increase the efficiency of fatty acid oxidation, including hepatic omega-oxidation, and TCA cycle flux to thereby potentially reduce an accumulation of lipids and improve insulin action in LBW individuals are needed.

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Conflicts of Interest

We all declare no financial or otherwise conflicts of interest in the study.

Prior Publication

Preliminary results from this study has been published in abstract form in connection with The 51st European Association for the Study of Diabetes Annual Meeting, Stockholm, Sweden, 14–18 September 2015 (Ribel-Madsen et al. 2015). The abstract can be found in the online version of Diabetologia and furthermore at: http://www.easdvirtualmeeting.org/users/31861.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Protein, carbohydrate, and fat contents of the control (C) and high-fat, high-calorie (O) diets.

Table S2. Acylcarnitine names, molecular formulas, methyl and butyl ester ion mass to charge ratios, and internal standards (IS) used for quantifications.

Appendix 2 – Article 2

Physiological Reports

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ORIGINAL RESEARCH

Plasma amino acid levels are elevated in young, healthy low birth weight men exposed to short-term high-fat overfeeding

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Keywords
Amino acids, high-fat overfeeding, insulin resistance, low birth weight, type 2 diabetes.

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Abstract

Low birth weight (LBW) individuals exhibit a disproportionately increased, incomplete fatty acid oxidation and a decreased glucose oxidation, compared with normal birth weight (NBW) individuals, and furthermore have an increased risk of developing insulin resistance and type 2 diabetes. We hypothesized that changes in amino acid metabolism may occur parallel to alterations in fatty acid and glucose oxidation, and could contribute to insulin resistance. Therefore, we measured fasting plasma levels of 15 individual or pools of amino acids in 18 LBW and 25 NBW men after an isocaloric control diet and after a 5-day high-fat, high-calorie diet. We demonstrated that LBW and NBW men increased plasma alanine levels and decreased valine and leucine/isoleucine levels in response to overfeeding. Also, LBW men had higher alanine, proline, methionine, citrulline, and total amino acid levels after overfeeding compared with NBW men. Alanine and total amino acid levels tended to be negatively associated with the insulin-stimulated glucose uptake after overfeeding. Therefore, the higher amino acid levels in LBW men could be a consequence of their reduction in skeletal muscle insulin sensitivity due to overfeeding with a possible increased skeletal muscle proteolysis and/or could potentially contribute to an impaired insulin sensitivity. Furthermore, the alanine level was negatively associated with the plasma acetylcarnitine level and positively associated with the hepatic glucose production after overfeeding. Thus, the higher alanine level in LBW men could be accompanied by an increased anaplerotic formation of oxaloacetate and thereby an enhanced tricarboxylic acid cycle activity and as well an increased gluconeogenesis.

Introduction

Low birth weight (LBW) individuals have an increased risk of developing insulin resistance and type 2 diabetes later in life, compared with normal birth weight (NBW) individuals, when exposed to an affluent life style such as overfeeding (Hales et al. 1991; Barker et al. 1993; Hofman et al. 2004; Vaag et al. 2006). Indeed, otherwise healthy

LBW men display several prediabetic metabolic abnormalities, including higher fasting blood glucose and serum insulin levels and a decreased hepatic insulin sensitivity compared with NBW men (Brons et al. 2008). Also, LBW in contrast to NBW men develop a decreased peripheral insulin sensitivity in response to a short-term high-fat, high-calorie diet (Brons et al. 2012). Nevertheless, the underlying mechanisms behind the LBW prediabetic phenotype are not clear. Previously, we have found that LBW men exhibit an increased fatty acid oxidation along with a decreased glucose oxidation at night as determined during an isocaloric control diet and a high-fat, high-calorie diet compared with NBW men (Brons et al. 2013, 2015). Furthermore, LBW men have a higher relative contribution of fatty acid oxidation to the total energy expenditure at night and throughout 24 h when studied on the high-fat, high-calorie diet (Brons et al. 2015). Recently, we have extended our understanding of the LBW prediabetic phenotype with the finding of elevated fasting plasma acetylcarnitine levels in LBW men, pointing toward an increased acetyl-CoA generation relative to its oxidation in the tricarboxylic acid (TCA) cycle (Ribel-Madsen et al. 2016). An accumulation of acetyl-CoA may theoretically lead to mitochondrial stress responses and the activation of serine kinases, which in turn may impair skeletal muscle insulin signaling and glucose transporter 4 (GLUT4) translocation (Muoio and Newgard 2008).

In the present extension study, we hypothesized that the changes in fatty acid and glucose oxidation partitioning in LBW individuals would be associated with changes in plasma amino acid levels, reflecting the need for an adequate supply of TCA cycle intermediates to allow an efficient acetyl-CoA oxidation in the TCA cycle (Fig. 1). Furthermore, such changes could be parts of the adverse metabolic events leading to insulin resistance in LBW individuals. In order to test our hypotheses, we measured fasting plasma levels of 15 individual or pools of amino acids in LBW and NBW men after an isocaloric control diet and after a 5-day high-fat, high-calorie diet, and associated these levels to the plasma acetylcarnitine level, as a measure of the intracellular acetyl-CoA level, and to measures of hepatic and peripheral insulin sensitivity.

Materials and Methods

Study population

Forty-six young (23–27 years of age), healthy men were recruited from the Danish National Birth Registry according to birth weight. Among these, 20 individuals had

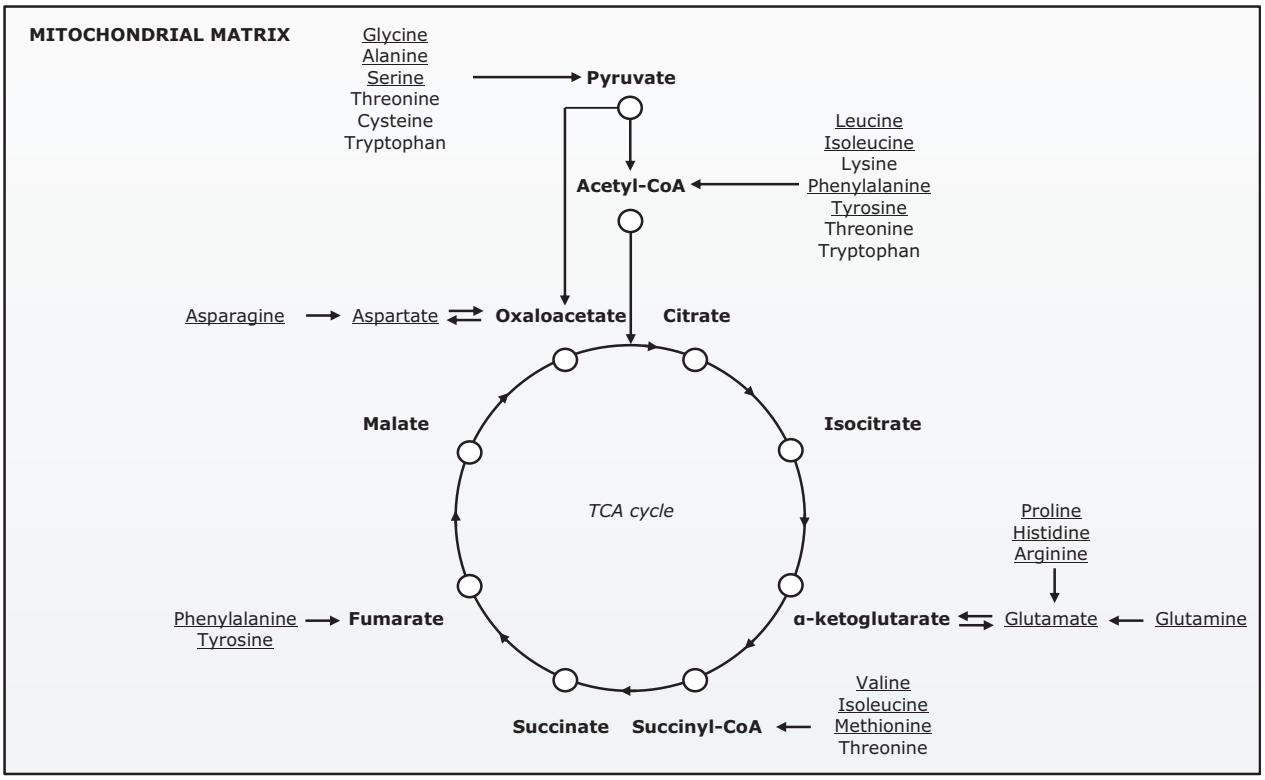

Figure 1. Amino acid anaplerotic and cataplerotic pathways that replenish or deplete tricarboxylic acid cycle intermediates, respectively. Plasma amino acid levels measured in this study are underlined.

LBW, which was defined as a birth weight within the 0–10th percentile range (2717 ± 268 g), as in a prior study (Jensen et al. 2002), and 26 individuals had NBW, which was defined as a birth weight within the 50–90th percentile range (3901 ± 207 g). All individuals were born at term (39–41 weeks of gestation) in Copenhagen in the period 1979–1980. Also, all participants were ensured to not have a family history of diabetes in two generations, not have a body mass index (BMI) greater than 30 kg/m^2, not perform strenuous physical activity more than 10 h per week, not take pharmaceuticals that affect metabolism, and not have an abuse of alcohol or drugs.

Study design

Diet interventions

All individuals were in a randomized crossover setup standardized with respect to diet and physical activity and following given a 3-day control diet and a 5-day high-fat, high-calorie diet separated by a 6–8 weeks washout period (Fig. 2). Energy requirements of the individual subjects were calculated from a World Health Organization equation for men less than 30 years of age with a physical activity level of 1.4 corresponding to a low physical activity (WHO, 2001). The control diet was composed to replicate a habitual, weight-maintaining diet (2819 ± 238 kcal/11,800 ± 1000 kJ) with 15% of the total energy from protein, 50% from carbohydrate, and 35% from fat, whereas the high-fat, high-calorie diet was prepared to contain 50% extra calories above the requirements (4228 ± 334 kcal/17,700 ± 1400 kJ) with 7.5% of the total energy from protein, 32.5% from carbohydrate, and 60% from fat (Table S1). Also, the meals contained in each intervention were identical from day to day. Dietary calculations were made in Dankost Pro (http://dankost.dk/english) (The National Food Agency, Copenhagen, Denmark).

Clinical examinations

Study activities were carried out over 3 days, with the first of these days being placed 1 or 3 days after the start of the control and high-fat, high-calorie diet intervention, respectively (Fig. 2). Anthropometry was performed on the first study day. An intravenous glucose tolerance test (IVGTT) and a hyperinsulinemic–euglycemic clamp were carried out in the morning on the third study day following an overnight fast to assess insulin secretion and sensitivity, as described previously (Brons et al. 2008, 2012). Furthermore, calorimetry was performed throughout 24 h from the first to second study day by use of a whole-body respiratory chamber to evaluate substrate oxidation rates and energy expenditures, as described previously (Brons et al. 2013, 2015). Blood and tissue samples were collected prior to and during the clamp.

Laboratory measurements

Amino acid analyses

Amino acid analyses were performed on EDTA-plasma samples collected following an overnight fast (10.00 PM–7.00 AM) and immediately prior to the clamp examination

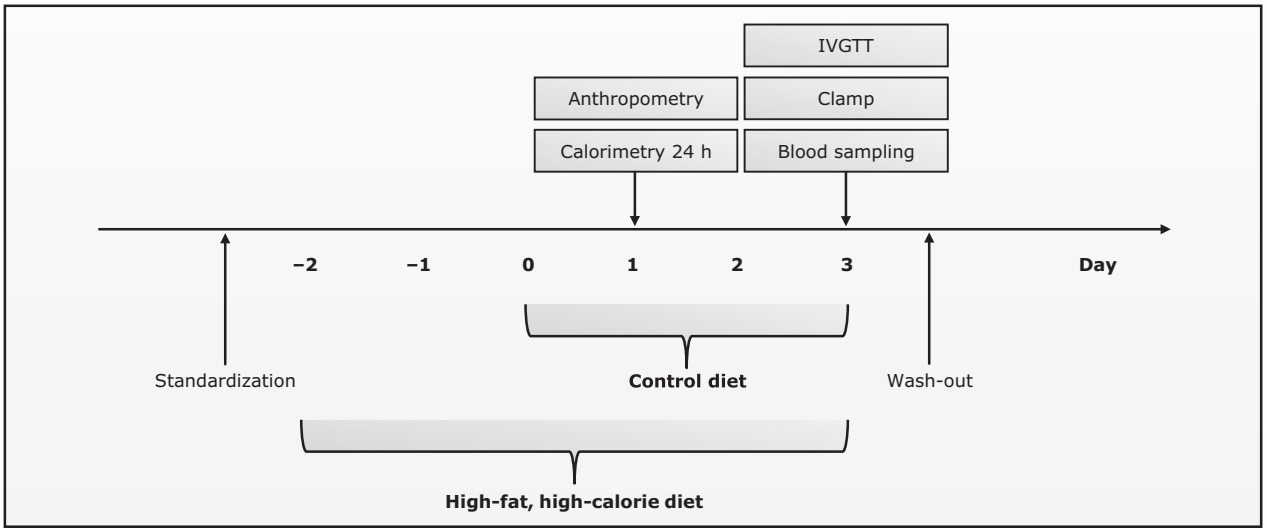

Figure 2. Study setup. Plasma amino acid levels were measured from blood samples collected following an overnight fast and immediately prior to the clamp examination on the last study day.

(Fig. 2). These analyses included a quantitative determination of 15 individual or pools of amino acids, noted in the tables by their three letter code, and were performed by use of sample preparation procedures and flow injection–tandem mass spectrometry, as described previously (Chace et al. 1995; Ferrara et al. 2008). In brief, plasma samples were spiked with known quantities of stable isotope-labeled amino acid standards. Following, proteins were removed by precipitation with methanol and the supernatants were then evaporated to dryness under nitrogen gas and incubated with acidified butanol to form butyl ester derivatives of the amino acids. After this, the reagents were evaporated to dryness under nitrogen gas, and the samples were reconstituted in 1:1 (v/v) methanol: glycerol. Subsequently, the samples were introduced into a Quattro Micro MS/MS system (Waters, Milford, MA) equipped with a data system running MassLynx 4.0 (Waters). Mass spectra of the amino acid butyl esters were obtained by precursor ion and neutral loss scanning of $[M+H]^+$ and $[M+H–102]^+$ ions, respectively, the latter ion corresponding to a loss of butyl formate of 102 Da from the original $[M+H]^+$ ion. Finally, amino acids were quantified from the ratio of their molecular signals to respective internal standards. Leucine and isoleucine were not resolved by the method, and therefore these amino acids are reported as a single analyte. Also, the quantification of these amino acids includes contributions from allo-isoleucine and hydroxy-proline, but these isobaric amino acids generally contribute little to the signal assigned to leucine and isoleucine (Chace et al. 1995). Furthermore, partial hydrolysis of asparagine to aspartic acid and of glutamine to glutamic acid occurs under the acidic conditions used to form butyl esters, and so the amounts of these amino acids plus their hydrolysis products are noted as Asx and Glx, respectively. Amino acid measurements were performed in The Sarah W. Stedman Nutrition and Metabolism Center Metabolomics/Biomarker Core Laboratory, Duke University, Durham, NC. The laboratory was blinded to the birth weight of the individuals.

Acylcarnitine analyses

Acylcarnitine analyses were performed on the same plasma samples as for the amino acid analyses. These analyses included a semiquantitative determination of 45 acylcarnitine species or sets of species and were performed as described previously (Ribel-Madsen et al. 2016).

Ethical approval

All study procedures were in accordance with the principles of the Declaration of Helsinki and were approved by the Regional Research Ethics Committee of Copenhagen, Denmark. Also, all participants were provided with written information on the study purpose and procedures and signed an informed consent prior to their participation.

Statistical analyses

Amino acid levels and their relation to physiological measures

Differences in plasma amino acid levels between NBW and LBW individuals within each diet or between the control and high-fat, high-calorie diets within each birth weight group were assessed from Student's unpaired or paired *t*-test (for normally distributed values), respectively, or Wilcoxon ranked-sum or signed-rank test (for not normally distributed values), respectively. Prior to these tests, statistical outliers (1.5 interquartile range) were removed from the dataset and replaced by the mean value within the given group. Normal distribution of the values (variables or differences between variables, respectively) was evaluated from Shapiro–Wilk test. Finally, adjustment for multiple testing was done by calculating false discovery rates, *Q*-values, by the Benjamini and Hochberg method (Benjamini and Hochberg 1995). Data in Tables 2 and 4 are presented as mean value plus or minus standard deviation (SD) together with *P*- and *Q*-values. $P \leq 0.05$ were considered statistically significant if $Q \leq 0.2$. Student's *t*-test and Wilcoxon tests were performed in SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC), and Benjamini and Hochberg corrections were performed in R 3.1.0 (https://www.r-project.org/).

Associations between individual plasma amino acid levels or between these levels and other variables were obtained from linear regression analyses. These analyses were performed on the pooled dataset of LBW and NBW individuals and were adjusted for age, BMI, and birth weight group. Data in Table 3 are presented as slope (plus or minus signs for a positive or negative association, respectively) and *P*-value (number of plus or minus signs indicates the significance level) for those values meeting the false discovery rate criteria. *P*-values were considered statistically significant as described above. Linear regression analyses and selected plots from these were performed in R.

Results

Eighteen LBW and 25 NBW men were included in the present study. Two LBW men of the recruited participants failed to consume all the food during the high-fat, high-calorie diet, and a NBW subject felt discomfort during the clamp after the control diet and therefore did not

further participate in this test in either the control or high-fat, high-calorie diet study part.

Clinical characteristics

LBW and NBW men displayed differences in body composition and glucose and lipid metabolism after the control diet and high-fat, high-calorie diet, and both birth weight groups showed several changes in these parameters in response to the overfeeding challenge, as published previously (Brons et al. 2008, 2012, 2013, 2015; Ribel-Madsen et al. 2016). A selection of these findings is presented in Tables 1 and S2.

Amino acid levels and their relation to physiological measures

LBW and NBW men only displayed tendencies to differences in plasma amino acid levels after the control diet when accounted for multiple testing, but significant differences after the high-fat, high-calorie diet, and both groups showed several changes in amino acid levels in response to overfeeding (Table 2).

LBW men tended to have higher proline and tyrosine levels and a lower serine level after the control diet compared with NBW men. Furthermore, LBW and NBW men both increased alanine levels and decreased valine, leucine/isoleucine, and essential amino acid levels in response to overfeeding. In addition, LBW men increased citrulline and nonessential amino acid levels and decreased the phenylalanine level due to overfeeding, whereas NBW men increased tyrosine and glutamine/glutamic acid levels and decreased proline, methionine, and histidine levels in response to this challenge. Also, LBW men had higher alanine, proline, methionine, and citrulline levels after the high-fat, high-calorie diet compared with NBW men, and as well higher essential, nonessential, and total amino acid levels after this diet.

Table 1. Clinical characteristics of low (LBW) and normal birth weight (NBW) men following the control (C) and high-fat, high-calorie (O) diets.

	NBW (n = 25)			LBW (n = 18)			LBW versus NBW (n = 18, n = 25)		
	C (mean ± SD)	O (mean ± SD)	P_{NBW}	C (mean ± SD)	O (mean ± SD)	P_{LBW}	P_C	P_O	P_Δ
Anthropometry									
Birth weight (g)	3901 ± 207	—	—	2717 ± 268	—	—	**≤0.001**	—	—
Weight (kg)	78.4 ± 9.3	78.6 ± 9.7	n.s.	77.1 ± 11.3	77.1 ± 11.4	n.s.	n.s.	n.s.	n.s.
Height (m)	1.83 ± 0.07	—	—	1.77 ± 0.05	—	—	**≤0.05**	—	—
Body mass index (kg/m^2)	23.3 ± 2.4	23.3 ± 2.5	n.s.	24.6 ± 3.8	24.6 ± 3.8	n.s.	n.s.	n.s.	n.s.
Clamp									
Basal									
B-Glucose (mmol/L)	4.59 ± 0.47	5.05 ± 0.40	**≤0.001**	4.97 ± 0.48	5.18 ± 0.34	**≤0.05**	**≤0.01**	n.s.	n.s.
S-Insulin (pmol/L)	30.2 ± 14.7	43.4 ± 29.2	**≤0.05**	41.7 ± 14.6	44.7 ± 21.9	n.s.	**≤0.01**	n.s.	n.s.
P-NEFA (μmol/L)	334 ± 136	205 ± 82	**≤0.001**	406 ± 200	188 ± 91	**≤0.001**	n.s.	n.s.	n.s.
P-Acetylcarnitine (μmol/L)	4.771 ± 0.797	3.985 ± 0.738	**≤0.01**	5.985 ± 1.587	4.393 ± 0.784	**≤0.001**	**≤0.01**	n.s.	n.s.
HGP (mg/kg·FFM/min)	2.21 ± 0.48	2.85 ± 0.99	**≤0.01**	2.40 ± 0.5	2.48 ± 0.5	n.s.	n.s.	n.s.	**≤0.05**
Hepatic IR (mg/kg·FFM/min·pmol/L)	68.7 ± 34.1	113.7 ± 61.5	**≤0.001**	102.3 ± 50.8	108.7 ± 55.5	n.s.	**≤0.05**	n.s.	**≤0.05**
Insulin-stimulated									
P-NEFA (μmol/L)	9.29 ± 4.39	12.42 ± 6.43	**≤0.01**	9.56 ± 5.03	14.39 ± 7.76	**≤0.01**	n.s.	n.s.	n.s.
M-value (mg/kg·FFM/min)	13.73 ± 2.32	13.29 ± 3.32	n.s.	13.47 ± 3.14	11.89 ± 3.57	**≤0.05**	n.s.	n.s.	n.s.
IVGTT									
FPIR (pmol/L)	1894 ± 1431	2604 ± 1793	**≤0.001**	2135 ± 1034	2750 ± 1509	**≤0.01**	n.s.	n.s.	n.s.
Hepatic DI	0.38 ± 0.63	0.25 ± 0.21	n.s.	0.21 ± 0.11	0.24 ± 0.13	n.s.	n.s.	n.s.	n.s.
Peripheral DI	0.29 ± 0.19	0.35 ± 0.20	**≤0.05**	0.33 ± 0.13	0.32 ± 0.17	n.s.	n.s.	n.s.	n.s.

Data are presented as mean ± SD. *P*-values from Student's *t*-test are presented unadjusted for multiple comparisons, and $P \leq 0.05$ are considered statistically significant. P_{NBW} and P_{LBW}: O versus C diet within each birth weight group, P_C and P_O: LBW versus NBW individuals within each diet, P_Δ: LBW versus NBW individuals on response values. n.s.: Not significant. $P \leq 0.05$ are marked in bold.

Abbreviations: B, blood; DI, disposition index; FFM, fat-free mass; FPIR, first-phase insulin response; HGP, hepatic glucose production; IR, insulin resistance; IVGTT, intravenous glucose tolerance test; NEFA, nonesterified fatty acid; P, plasma; S, serum.

Table 2. Plasma amino acid levels in low (LBW) and normal birth weight (NBW) men following the control (C) and high-fat, high-calorie (O) diets.

	NBW (n = 25)			LBW (n = 18)			LBW versus NBW (n = 18, n = 25)		
(μmol/L)	C (mean ± SD)	O (mean ± SD)	P_{NBW} Q_{NBW}	C (mean ± SD)	O (mean ± SD)	P_{LBW} Q_{LBW}	P_C Q_C	P_O Q_O	P_Δ Q_Δ
Amino acid profiling									
Gly	320.6 ± 36.0	319.1 ± 35.0	0.8304	319.6 ± 30.4	320.2 ± 38.4	0.9531	0.9205	0.9512	0.8618
Ala	286.5 ± 58.3	345.0 ± 53.3	**0.0007** **0.0021**	288.6 ± 78.0	403.9 ± 87.5	**<0.0001** **0.0008**	0.8929	**0.0174** **0.1305**	**0.0134** 0.2010
Ser	109.9 ± 16.2	104.3 ± 14.6	0.1590	100.2 ± 13.5	106.7 ± 10.9	0.0673	**0.0445** 0.2455	0.5590	**0.0291** 0.2110
Pro	157.2 ± 19.2	145.0 ± 24.6	**0.0007** **0.0021**	180.0 ± 42.8	169.2 ± 42.4	0.1183	**0.0462** 0.2455	**0.0396** **0.1485**	0.8532
Val	234.4 ± 30.2	205.9 ± 11.0	**<0.0001** **0.0005**	238.0 ± 33.4	216.4 ± 25.9	**0.0258** **0.0774**	0.7138	0.1185	0.4983
Leu/Ile	178.6 ± 24.5	146.0 ± 16.6	**<0.0001** **0.0005**	181.0 ± 19.5	152.9 ± 13.4	**<0.0001** **0.0008**	0.7359	0.1527	0.4522
Met	27.1 ± 3.7	25.5 ± 3.2	**0.0358** **0.0761**	27.8 ± 2.4	28.2 ± 3.7	0.5893	0.4757	**0.0135** **0.1305**	0.0625
His	65.1 ± 6.6	58.4 ± 5.6	**<0.0001** **0.0005**	62.3 ± 7.9	58.8 ± 5.7	0.1042	0.2157	0.8456	0.1920
Phe	60.3 ± 7.8	58.1 ± 7.8	0.1171	60.9 ± 5.2	57.2 ± 6.6	**0.0065** **0.0244**	0.3364	0.8929	0.7413
Tyr	53.7 ± 7.0	57.5 ± 9.3	**0.0406** **0.0761**	58.1 ± 7.2	61.9 ± 10.7	0.1622	**0.0491** 0.2455	0.1583	0.9917
Asx	144.0 ± 20.8	136.1 ± 24.0	0.2760	148.2 ± 23.7	149.4 ± 24.7	0.9006	0.5486	0.0853	0.4351
Glx	56.1 ± 11.0	63.6 ± 15.8	**0.0035** **0.0088**	63.1 ± 13.6	71.5 ± 19.9	0.1118	0.0687	0.1513	0.6688
Orn	58.4 ± 6.6	61.5 ± 12.1	0.2189	59.3 ± 10.6	58.6 ± 8.6	0.8457	0.7437	0.3972	0.3657
Cit	28.1 ± 4.0	28.8 ± 5.5	0.5057	29.6 ± 3.5	32.6 ± 5.2	**0.0065** **0.0244**	0.1997	**0.0284** **0.1420**	0.1463
Arg	90.0 ± 14.9	85.2 ± 13.1	0.0875	90.4 ± 12.3	92.7 ± 13.5	0.4444	0.9296	0.0763	0.0601
Total levels									
Essential	655.5 ± 67.8	579.2 ± 33.2	**<0.0001**	660.4 ± 53.8	606.2 ± 43.7	**0.0035**	0.8006	**0.0260**	0.2603
Nonessential	1128 ± 101.1	1171 ± 107.5	0.0980	1158 ± 98.4	1283 ± 131.8	**0.0004**	0.3418	**0.0038**	**0.0353**
All	1870 ± 157.1	1840 ± 131.9	0.3896	1907 ± 132.7	1980 ± 159.4	0.0805	0.4205	**0.0030**	0.0556

Data are presented as mean ± SD. $P \leq 0.05$ are presented together with Q-values, and $P \leq 0.05$ with corresponding $Q \leq 0.2$ are considered statistically significant. P_{NBW} and P_{LBW}: O versus C diet within each birth weight group, P_C and P_O: LBW versus NBW individuals within each diet, P_Δ: LBW versus NBW individuals on response values. $P \leq 0.05$ and $Q \leq 0.2$ are marked in bold. Essential amino acids: Val, Leu/Ile, Met, His, Phe, and Arg; nonessential amino acids: Gly, Ala, Ser, Pro, Tyr, Asx, and Glx.

Plasma alanine levels were negatively associated with the fasting plasma acetylcarnitine level after both the control diet and high-fat, high-calorie diet (Fig. 3). Furthermore, an increase in the alanine level due to overfeeding was associated with a decrease in the acetylcarnitine level (Fig. 3). In addition, both alanine and total amino acid levels tended to be negatively associated with the insulin-stimulated glucose uptake rate, M-value, (P = 0.0911, Q = 0.1707; P = 0.0846, Q = 0.2115, respectively) after the high-fat, high-calorie diet (Table 3). Also, an increase in the proline level was associated with a decrease in the M-value (P = 0.0271, Q = 0.1350) (Table 3). Furthermore, alanine and total amino acid levels were positively associated with the hepatic glucose production (P = 0.0022, Q = 0.0110; P = 0.0114, Q = 0.0570, respectively) after the high-fat, high-calorie diet (Table 3).

Discussion

We demonstrated that LBW men had higher fasting plasma alanine, proline, methionine, citrulline, and total amino acid levels after the high-fat, high-calorie diet

Table 3. Associations between plasma amino acid levels and physiological measures following the control (C) and high-fat, high-calorie (O) diets and between response values (Δ).

		Gly	Ala	Ser	Pro	Val	Leu/Ile	Met	His	Phe	Tyr	Asx	Glx	Orn	Cit	Arg	All
Clamp																	
Basal																	
B-Glucose	C															+	
	O																
	Δ			−−													
S-Insulin	C												(+)				
	O							−−								−	
	Δ																
HGP	C												+ +				
	O		+ +			(+)											+
	Δ									+							
Hepatic IR	C												+				
	O							(−)									
	Δ																
Insulin-stimulated																	
M-value	C	+														(−)	
	O		(−)			(−)											
	Δ				−												

Data are presented as slope (+/− for a positive or negative association, respectively) and *P*-value (+/−: $P \leq 0.05$, + +/− −: $P \leq 0.01$, and (+)/(−): $P \leq 0.1$) for those values meeting the false discovery rate criteria. $P \leq 0.05$ with corresponding $Q \leq 0.2$ are considered statistically significant. Regression analyses were performed on the pooled dataset of low (LBW) and normal birth weight (NBW) individuals and were adjusted for age, BMI, and birth weight group.
Abbreviations: See Table 1.

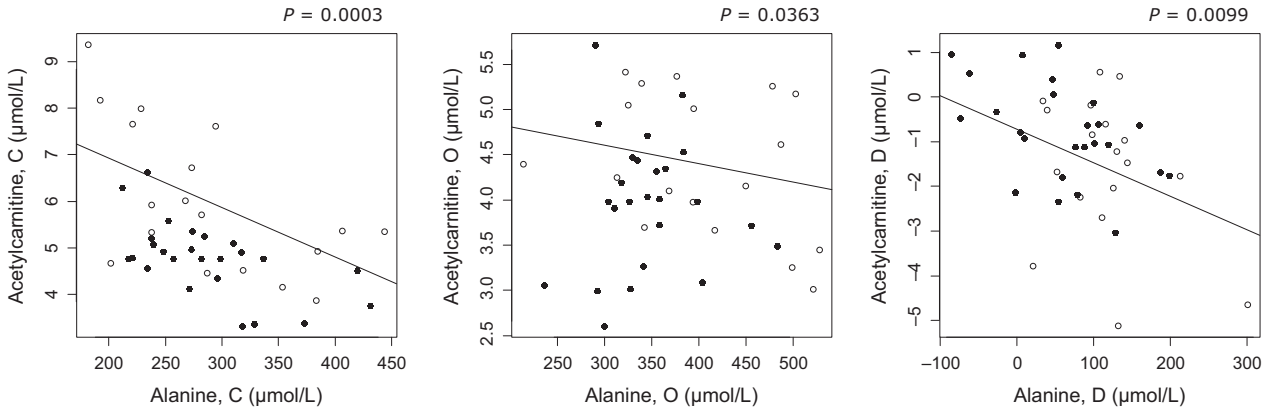

Figure 3. Associations between plasma alanine and acetylcarnitine levels following the control (C) and high-fat, high-calorie (O) diets and between response values (D). Open circles: Low birth weight (LBW) individuals, filled circles: Normal birth weight (NBW) individuals.

compared with NBW men. Furthermore, the plasma alanine level was negatively associated with the fasting plasma acetylcarnitine level, which reflects the intracellular acetyl-CoA pool (Adams et al. 2009), after both the control diet and high-fat, high-calorie diet. Previously, we have shown that LBW men have a higher plasma acetylcarnitine level after the control diet and a tendency to this after the high-fat, high-calorie diet compared with NBW men (Ribel-Madsen et al. 2016), and in addition, an increased fatty acid oxidation and a decreased glucose

oxidation at night during intake of both diets (Brons et al. 2013, 2015). Taken together, we proposed that the acetyl-CoA generation from in particular the increased fatty acid oxidation exceeds its oxidation in the TCA cycle in LBW individuals (Ribel-Madsen et al. 2016), possibly due to a depletion of TCA cycle intermediates. In the state of a high intracellular acetyl-CoA concentration, as seems to be the case in LBW individuals (Ribel-Madsen et al. 2016), pyruvate dehydrogenase is inhibited and pyruvate carboxylase is activated, favoring pyruvate carboxylation to oxaloacetate, the four-carbon unit TCA cycle intermediate that condenses with acyl-CoA to form citrate, and as well favoring pyruvate transamination to alanine (Hue and Taegtmeyer 2009). This promotes anaplerosis and gluconeogenesis, respectively (Hue and Taegtmeyer 2009). Therefore, the higher plasma alanine level in LBW men could reflect an increased pyruvate transamination to alanine in tissues, and the negative association between the plasma alanine and acetylcarnitine levels may be due to an increased anaplerotic formation of oxaloacetate. A recent study has reported that induced pluripotent stem cells from patients with genetic insulin resistance have a reduced mitochondrial oxidative function, including a decreased citrate synthase activity (Burkart et al. 2016). Also, addition of exogenous oxaloacetate to the cell culture system could resolve citrate synthase activity, and it was therefore proposed that substrate availability could be a limiting factor of TCA cycle activity in insulin resistance (Burkart et al. 2016). Alternatively, the higher plasma alanine level in LBW men could be a result of an increased skeletal muscle proteolysis and de novo alanine synthesis, as further discussed below. Indeed, LBW men display a higher adjusted total energy expenditure at night during the high-fat, high-calorie diet compared with NBW men (Brons et al. 2015). Therefore, the TCA cycle is likely to be upregulated in LBW individuals at night, and we propose that a higher plasma alanine level in these individuals could reflect an increased availability of alanine and pyruvate in tissues for anaplerotic formation of oxaloacetate and furthermore that this may enhance the acetyl-CoA oxidation in the TCA cycle, which in turn may contribute to development of hepatic insulin resistance (Satapati et al. 2012).

We furthermore demonstrated that plasma alanine and total amino acid levels tended to be negatively associated with the insulin-stimulated glucose uptake rate, M-value, after the high-fat, high-calorie diet. Also, we have previously demonstrated that LBW men decreased the M-value in response to this diet (Brons et al. 2012). Whether the higher plasma alanine and total amino acid levels in LBW individuals could be a consequence of and/or contribute to impaired skeletal muscle insulin sensitivity is uncertain. Insulin has a suppressive effect on tissue proteolysis, and so an impaired skeletal muscle insulin sensitivity may increase the amino acid release from skeletal muscle (Magkos et al. 2010). Also, the amino acid release from skeletal muscle following an overnight fast does not reflect its amino acid composition (Ruderman 1975), and so relatively more alanine and glutamine, which represent the main amino acid gluconeogenic precursors in liver and kidney, respectively, (Felig 1973; Felig et al. 1977; Stumvoll et al. 1999), are released (Ruderman 1975; Felig et al. 1977). Actually, alanine and glutamine together accounts for approximately two thirds of the amino acids released from skeletal muscle (Ruderman 1975; Garber et al. 1976). This has been suggested to be due to an in situ amino acid metabolism that results in de novo synthesis of nonessential amino acids, primarily alanine and glutamine (Ruderman 1975; Felig et al. 1977). Alanine is generated through pyruvate transamination (Ruderman 1975), and it has been suggested that pyruvate to this synthesis may be derived from other amino acids, as skeletal muscle theoretically could generate pyruvate from glutamate, aspartate, and other amino acids that are metabolized in the TCA cycle (Ruderman 1975). Notably, it has been demonstrated that more alanine relative to glutamine is released from skeletal muscle in subjects with diabetes (Jungas et al. 1992). It is therefore interesting that LBW men showed a tendency to a larger relative contribution of alanine and nonessential amino acids to the total plasma amino acid level after the high-fat, high-calorie diet compared with NBW men (Table 4). Accordingly, the higher plasma alanine level in LBW individuals could be a result of both an increased skeletal muscle proteolysis due to an impaired skeletal muscle insulin sensitivity and of an increased de novo alanine synthesis from other amino acids, including in particular glutamate, aspartate, valine, leucine, and isoleucine, and pyruvate (Ruderman 1975; Snell 1980). Although this is expected to cause reductions in the relative contributions of these amino acids to the total plasma amino acid level in LBW men, we did not observe this (Table 4). As concerns, the possibility that the higher plasma amino acid levels in LBW individuals could contribute to an impaired skeletal muscle insulin sensitivity, it has been shown that short-term elevation of plasma amino acid levels to postprandial concentrations causes insulin resistance by direct inhibition of muscle glucose transport and/or phosphorylation with a subsequent reduction in glycogen synthesis rates (Krebs et al. 2002). In addition to the potential effects of amino acids on skeletal muscle insulin sensitivity, we demonstrated that plasma alanine and total amino acid levels were positively associated with the hepatic glucose production after the high-fat, high-calorie diet. Higher plasma amino acid levels could induce this effect

Table 4. Relative plasma amino acid levels compared to the total plasma amino acid level in low (LBW) and normal birth weight (NBW) men following the control (C) and high-fat, high-calorie (O) diets.

	NBW (n = 25)			LBW (n = 18)			LBW versus NBW (n = 18, n = 25)		
(Fraction)	C (mean ± SD)	O (mean ± SD)	P_{NBW} Q_{NBW}	C (mean ± SD)	O (mean ± SD)	P_{LBW} Q_{LBW}	P_C Q_C	P_O Q_O	P_Δ Q_Δ
Amino acid profiling									
Rel Gly	0.172 ± 0.017	0.174 ± 0.016	0.6024	0.168 ± 0.017	0.162 ± 0.018	0.1692	0.4775	**0.0306** **0.1759**	0.1473
Rel Ala	0.152 ± 0.021	0.187 ± 0.019	**<0.0001** **0.0005**	0.150 ± 0.035	0.202 ± 0.033	**<0.0001** **0.0005**	0.8284	0.0607	0.0569
Rel Ser	0.059 ± 0.008	0.057 ± 0.006	0.2302	0.053 ± 0.007	0.054 ± 0.005	0.3978	**0.0105** **0.1575**	0.1345	0.1614
Rel Pro	0.084 ± 0.009	0.079 ± 0.011	**0.0007** **0.0021**	0.094 ± 0.021	0.085 ± 0.018	**0.0019** **0.0057**	**0.0385** 0.2888	0.1500	0.2023
Rel Val	0.125 ± 0.012	0.112 ± 0.010	**<0.0001** **0.0005**	0.125 ± 0.014	0.109 ± 0.011	**0.0003** **0.0011**	0.8817	0.3412	0.5603
Rel Leu/Ile	0.095 ± 0.008	0.079 ± 0.008	**<0.0001** **0.0005**	0.095 ± 0.008	0.077 ± 0.005	**<0.0001** **0.0005**	0.8682	0.3504	0.5328
Rel Met	0.014 ± 0.002	0.014 ± 0.001	**0.0489** **0.0917**	0.015 ± 0.001	0.014 ± 0.002	0.3376	0.8000	0.4082	0.5281
Rel His	0.035 ± 0.004	0.032 ± 0.002	**0.0002** **0.0008**	0.033 ± 0.005	0.030 ± 0.004	**0.0186** **0.0465**	0.0957	**0.0428** **0.1759**	0.8346
Rel Phe	0.032 ± 0.003	0.032 ± 0.003	0.1942	0.032 ± 0.003	0.029 ± 0.003	**<0.0001** **0.0005**	0.8210	**0.0100** **0.1500**	**0.0031** **0.0465**
Rel Tyr	0.029 ± 0.003	0.031 ± 0.004	**0.0052** **0.0130**	0.030 ± 0.003	0.031 ± 0.004	0.4836	0.0861	0.9512	0.1441
Rel Asx	0.077 ± 0.011	0.074 ± 0.013	0.3956	0.078 ± 0.012	0.076 ± 0.013	0.6593	0.8953	0.6937	0.8523
Rel Glx	0.030 ± 0.005	0.034 ± 0.008	**0.0064** **0.0137**	0.033 ± 0.007	0.037 ± 0.011	0.1960	0.1126	0.4800	0.6973
Rel Orn	0.031 ± 0.004	0.033 ± 0.006	0.1522	0.031 ± 0.006	0.030 ± 0.005	0.4918	0.8797	**0.0469** **0.1759**	0.1479
Rel Cit	0.015 ± 0.002	0.016 ± 0.003	0.1384	0.016 ± 0.002	0.017 ± 0.003	0.0790	0.3957	0.3707	0.6564
Rel Arg	0.048 ± 0.007	0.046 ± 0.007	0.1866	0.048 ± 0.007	0.047 ± 0.007	0.6541	0.7784	0.7904	0.5188
Total levels									
Rel Essential	0.350 ± 0.018	0.315 ± 0.017	**<0.0001**	0.347 ± 0.021	0.307 ± 0.018	**<0.0001**	0.5284	0.1148	0.4509
Rel Nonessential	0.603 ± 0.018	0.635 ± 0.017	**<0.0001**	0.607 ± 0.020	0.647 ± 0.021	**<0.0001**	0.5464	0.0576	0.2379

Data are presented as mean ± SD. $P \leq 0.05$ are presented together with Q-values, and $P \leq 0.05$ with corresponding $Q \leq 0.2$ are considered statistically significant. P_{NBW} and P_{LBW}: O versus C diet within each birth weight group, P_C and P_O: LBW versus NBW individuals within each diet, P_Δ: LBW versus NBW individuals on response values. $P \leq 0.05$ and $Q \leq 0.2$ are marked in bold. Essential amino acids: Val, Leu/Ile, Met, His, Phe, and Arg; nonessential amino acids: Gly, Ala, Ser, Pro, Tyr, Asx, and Glx.

on the liver via indirect and/or direct mechanisms. Thus, amino acids can stimulate insulin and glucagon secretion (Floyd et al. 1966; Ohneda et al. 1968; Newsholme et al. 2006), and changes in the portal vein concentration of these hormones could increase the endogenous glucose production (Roden et al. 1996). On the other hand, amino acids could by acting as substrates induce gluconeogenesis and thereby enhance the endogenous glucose production (Rui 2014). It is notable that the plasma glutamine/glutamic acid and alanine levels were strongly positively associated with the hepatic glucose production after the control or high-fat, high-calorie diet, respectively, (Table 3), keeping in mind that these amino acids are the main amino acid gluconeogenic precursors (Felig 1973; Felig et al. 1977; Stumvoll et al. 1999). Previously, we have shown that LBW men have a higher fasting blood glucose level after the control diet compared with NBW men (Brons et al. 2008), and we propose that an increased gluconeogenesis, occurring parallel to an increased hepatic fatty acid oxidation, may contribute to

this. Alanine and glutamine metabolism in the liver for gluconeogenesis results in the production of ammonia. Therefore, the higher plasma citrulline level in LBW men after the high-fat, high-calorie diet could reflect an increased metabolism of these amino acids with a subsequent increased urea cycle activity due to a greater demand of ammonia removal.

In similarity to our study, higher plasma amino acid levels have repeatedly been reported to associate with insulin resistance (Newgard et al. 2009; Tai et al. 2010; Wurtz et al. 2012a; Nakamura et al. 2014; Seibert et al. 2015; Pedersen et al. 2016). To this end, plasma amino acid levels, in particular of the branched chain amino acids valine, leucine, and isoleucine, aromatic amino acids tyrosine and phenylalanine, and alanine, are predictive of development of type 2 diabetes even several years before its onset (Wang et al. 2011; Wurtz et al. 2012b, 2013; Yamakado et al. 2015). Importantly, higher fasting serum valine, leucine, isoleucine, and phenylalanine levels have been shown to predict increased fasting and 2 h postchallenge blood glucose levels after a 6.5-year follow-up (Wurtz et al. 2012b), whereas higher levels of gluconeogenic precursors, including alanine, lactate, and pyruvate, predict increased 2 h postchallenge, but not fasting blood glucose levels, after this follow-up (Wurtz et al. 2012b). Therefore, it was concluded that gluconeogenic precursors could be potential markers of long-term impaired insulin sensitivity that may relate to attenuated glucose tolerance later in life (Wurtz et al. 2012b). Another study performed in rodent has described that an increased plasma citrulline level may predict development of the metabolic syndrome (Sailer et al. 2013). Thus, our findings of elevated plasma amino acid levels, including alanine and citrulline levels, in LBW men exposed to a high-fat, high-calorie diet challenge reveal additional metabolic abnormalities in these individuals associated to early stages of development of insulin resistance and type 2 diabetes.

We, moreover, demonstrated that LBW and NBW men both increased the plasma alanine level and decreased valine and leucine/isoleucine levels in response to overfeeding. Furthermore, an increase in the plasma alanine

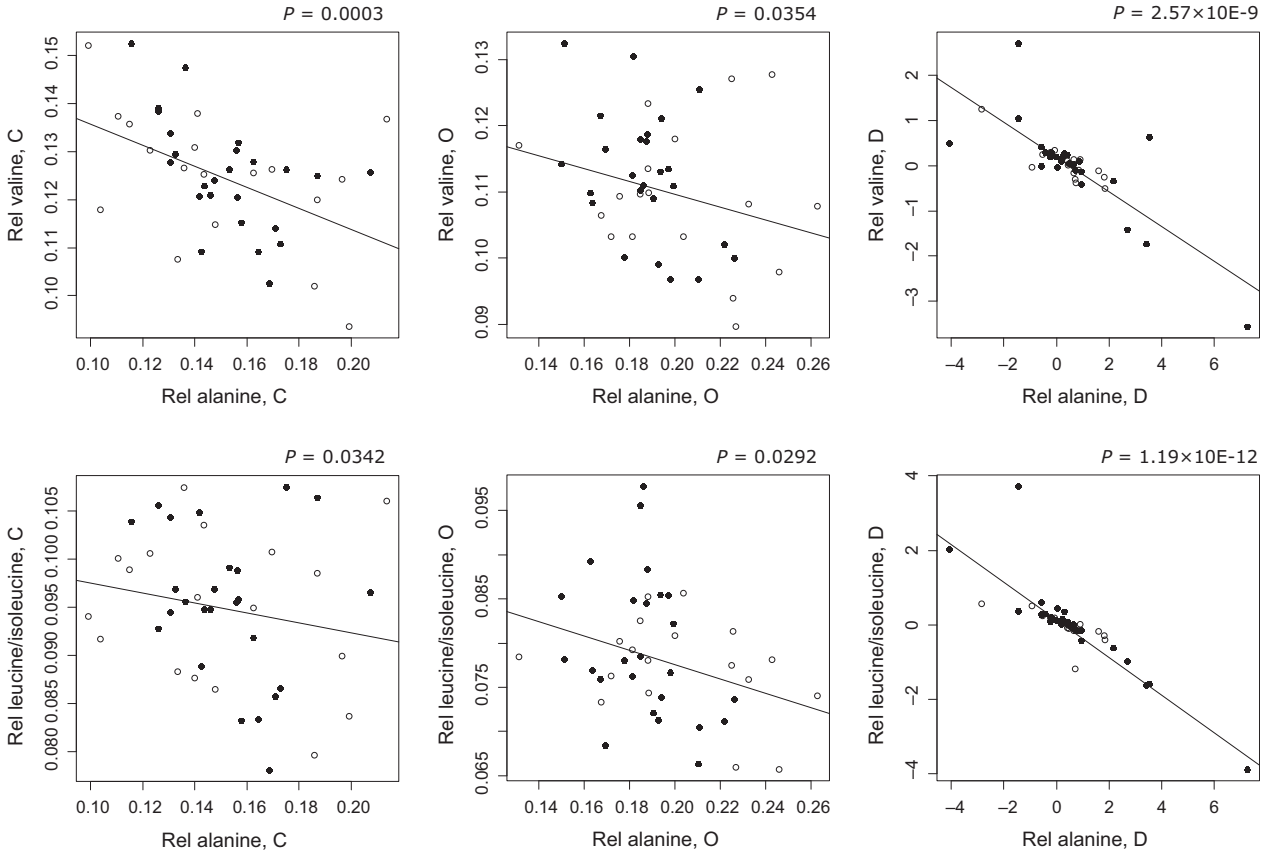

Figure 4. Associations between relative plasma alanine and valine or leucine/isoleucine levels, respectively, following the control (C) and high-fat, high-calorie (O) diets and between response values (D). Open circles: Low birth weight (LBW) individuals, filled circles: Normal birth weight (NBW) individuals.

level was associated with a decrease in the fasting plasma acetylcarnitine level. Therefore, an increase in the plasma alanine level due to overfeeding could be accompanied by an increase in the formation of oxaloacetate that may enhance the acetyl-CoA oxidation. Interestingly, LBW men showed a tendency to a larger increase in the plasma alanine level in response to overfeeding compared with NBW men (Table 2), and in addition, a tendency to a larger increase in the relative contribution of alanine to the total plasma amino acid level (Table 4). This could be due to an increased skeletal muscle alanine release as a consequence of their decline in skeletal muscle insulin sensitivity during overfeeding (Brons et al. 2012). Valine, leucine, and isoleucine are the major nitrogen sources for de novo alanine synthesis in skeletal muscle (Felig et al. 1977; Haymond and Miles 1982). Thus, the decrease in plasma valine and leucine/isoleucine levels in LBW and NBW men in response to overfeeding could indicate an increased metabolism of these amino acids to alanine in skeletal muscle. A decrease in these amino acid levels as well as in other essential amino acid levels could also be a result of the lower protein content in the high-fat, high-calorie diet compared to the control diet (Table S1). However, it is remarkable that an increase in the relative plasma alanine level was strongly significantly associated with decreases in both relative plasma valine and leucine/isoleucine levels (Fig. 4), indicating that an increased metabolism of these amino acids to alanine could take place. LBW men additionally showed a tendency to a larger increase in the serine level in response to overfeeding compared with NBW men (Table 2). Serine is, together with alanine, a precursor to pyruvate, and so an increased availability of this amino acid may contribute to an increased anaplerosis and thus an enhanced TCA cycle activity as well as to an increased gluconeogenesis.

Our study is the first to investigate fasting plasma amino acid levels in LBW individuals susceptible of developing type 2 diabetes after a control diet and after a high-fat, high-calorie diet. It has its strengths in the careful selection of LBW and NBW subjects, highly standardized study setup, and thorough physiological and metabolic characterization of the individuals. However, in relation to the biological interpretation of the results, and especially of the association between the amino acid availability and acetyl-CoA oxidation in the TCA cycle, it has its limitations in the metabolite profiling being restricted to plasma amino acids and acylcarnitines, and thereby not having pyruvate and TCA cycle intermediates in its range. Furthermore, skeletal muscle biopsies from the LBW and NBW men collected prior to and during the clamp examination are snap frozen, which limits the possibilities of performing supplementary functional studies, including for instance measurements of the cellular localization of GLUT4 (Ploug et al. 1998; Lauritzen et al. 2008). In conclusion, our study describes elevated fasting plasma amino acid levels in LBW men after a 5-day high-fat, high-calorie diet, including alanine, proline, methionine, citrulline, and total amino acid levels. Furthermore, these elevated plasma amino acid levels, and in particular of alanine, may be a result of an impaired glucose oxidation and/or an increased skeletal muscle proteolysis, and could be part of the adverse metabolic changes leading to skeletal muscle and hepatic insulin resistance in LBW individuals.

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Conflicts of Interest

All authors declare no financial or otherwise conflicts of interest in this study.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Protein, carbohydrate, and fat contents of the control (C) and high-fat, high-calorie (O) diets.

Table S2. Glucose, fatty acid, and protein oxidation rates and total energy expenditures in low (LBW) and normal birth weight (NBW) men during the control (C) and high-fat, high-calorie (O) diets.

Appendix 3 – Article 3 manuscript

ARTICLE MANUSCRIPT

Revised 22 December 2017

TITLE

Plasma ceramide levels are altered in low and normal birth weight men in response to short-term high-fat overfeeding.

AUTHORS

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Methods: 1730 words (max 1500 words)

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Figures: 1 (max 8 including tables)

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ABSTRACT

Low birth weight (LBW) individuals have an increased risk of developing insulin resistance and type 2 diabetes compared with normal birth weight (NBW) individuals. We hypothesised that LBW individuals exhibit an increased fatty acid flux into lipogenesis in non-adipose tissue with a resulting accumulation of lipotoxic lipids, including ceramides, in the blood. Therefore, we measured fasting plasma levels of 27 ceramides in 18 young, healthy, LBW men and 25 NBW controls after an isocaloric control diet and a 5-day high-fat, high-calorie diet by HPLC-HRMS. LBW men did not show elevated plasma ceramide levels after the control or high-fat, high-calorie diet. An increased fatty acid oxidation rate in these individuals during both diets may limit ceramide synthesis and thereby compensate for a likely increased fatty acid load to non-adipose tissue. Interestingly, LBW and NBW men decreased d18:0-18:1/d18:1-18:0 and d18:1-24:2/d18:2-24:1 levels and increased the d18:0-24:1a level in response to overfeeding. Plasma d18:0-24:1a and total ceramide levels were positively associated with the fasting blood glucose level and endogenous glucose production after the control diet, and the total ceramide level was in addition positively associated with hepatic insulin resistance. Further studies are needed to determine if lipotoxicity contributes to insulin resistance in LBW individuals.

INTRODUCTION

Low birth weight (LBW) individuals have an increased risk of developing insulin resistance and type 2 diabetes later in life compared with normal birth weight (NBW) individuals [1-4]. Accordingly, we have shown that young, healthy, LBW men have higher fasting blood glucose and serum insulin levels [5], display impaired hepatic insulin sensitivity [5], and, in contrast to NBW men, develop impaired peripheral insulin sensitivity in response to 5-day high-fat overfeeding [6]. However, the metabolic mechanisms behind the type 2 diabetes susceptible phenotype in LBW individuals are not clear. In the present study, our primary aim was to investigate if lipotoxicity induced by ceramides could contribute to impaired hepatic insulin sensitivity in LBW men. Moreover, as we have shown that NBW men of the above mentioned study population develop impaired hepatic insulin sensitivity in response to 5-day high-fat overfeeding [6], our secondary aim was to investigate if lipotoxicity could be part of the adverse events leading to this. Therefore, we studied fasting plasma ceramide levels, including their precursor dihydroceramides, in the LBW and NBW men of the above study population after an isocaloric control diet and after a 5-day high-fat, high-calorie diet intervention.

We have previously found that young, healthy, LBW men of another study population than the present herein display an increased whole body and adipose tissue lipolysis [7,8]. This was, however, not linked to elevated plasma non-esterified fatty acid (NEFA) or triacylglycerol levels, suggesting that an increased uptake of fatty acids from the blood into tissues and/or an increased clearance or metabolism of fatty acids in adipose tissue could be in play. Furthermore, we have demonstrated that LBW men of the present study population have a higher adipose tissue miR-483-3p level, and that manipulation of this level in vitro modulates adipocyte differentiation and fatty acid storage capacity [9]. Pre-adipocytes isolated from the LBW men have lower mRNA expression levels of several differentiation markers, supporting a notion of an impaired pre-adipocyte maturation in LBW men [10]. Moreover, the LBW men show an increased fatty acid oxidation rate [11] and a higher fasting plasma acetylcarnitine level [12], indicative of an increased fatty acid flux through beta-oxidation in mitochondria being non-matched by the acetyl-CoA flux through the tricarboxylic acid cycle. Also notably, the LBW men have higher plasma hydroxyl-/dicarboxyl-acylcarnitine levels, including 3-hydroxy-butyrylcarnitine, suggestive of an increased hepatic fatty acid oxidation rate, involving omega-oxidation in the endoplasmic reticulum, and an increased ketogenesis [12]. Omega-oxidation is a minor pathway for oxidation of fatty acids under normal physiological conditions, but

an important supplementary pathway to beta-oxidation when the intracellular NEFA levels are high [13]. Taken together, we speculated that LBW individuals might have an increased fatty acid flux into non-adipose tissue, including in particular the liver, and further into lipogenesis with a resulting increased ectopic fat deposition.

Ectopic fat comprises potentially lipotoxic lipids such as long-chain acyl-CoAs, ceramides, and diacylglycerols [14], and the extent of it has been linked to pancreatic beta-cell dysfunction and insulin resistance [15,16]. Also, individuals with type 2 diabetes have elevated plasma ceramide levels [17-19]. Importantly, obese individuals with type 2 diabetes have a higher plasma LDL-ceramide level compared with obese, insulin-sensitive individuals [19], indicating that an increased ceramide synthesis in the liver may contribute to the development of insulin resistance [20]. Ceramides are synthesised de novo in the endoplasmic reticulum from L-serine, palmitoyl-CoA, and variable acyl-CoAs or generated in salvage pathways of other sphingolipids [21-23]. Serine palmitoyl-transferase, catalysing the condensation of L-serine and palmitoyl-CoA, is the rate-limiting enzyme of ceramide synthesis, and an increased availability of palmitic acid enhances this synthesis [24-26]. Therefore, an increased uptake of fatty acids from the blood into non-adipose tissue is expected to increase ceramide synthesis. Ceramides are potentially lipotoxic to cells in several ways [25,27]. Thus, these lipids inhibit the phosphorylation and thereby activation of Akt/protein kinase B (PKB) of the insulin signalling cascade, which is a central regulator of glucose and amino acid uptake, anabolic processes, and cell survival [28,29]. Furthermore, ceramides activate Jun and NF-KB transcription factors and may thereby enhance inflammatory responses that may interfere with insulin signalling as well [25]. A number of studies have focused on specific actions of individual ceramides, including dihydroceramides, on different cellular processes [22]. In this regard, d18:1-16:0 ceramide has been suggested to be the principal mediator of diet-induced insulin resistance [30,31]. In the present study, we hypothesised that LBW men have higher fasting plasma ceramide levels, including specific ceramide species and/or total ceramide, and that such changes are associated with their impaired insulin sensitivity.

RESULTS

Eighteen LBW and 25 NBW men were included in the present study. Two LBW men of the recruited participants failed to consume all the food provided during the 5-day high-fat, high-calorie (60 E% from fat, 50 % extra calories) diet, and a NBW subject felt discomfort during the clamp after the control diet and therefore did not further participate in this test in the control or high-fat, high-calorie diet study part.

Clinical characteristics

LBW and NBW men displayed several differences in glucose, lipid, and protein metabolism after the isocaloric control diet and high-fat, high-calorie diet and also differential changes in metabolism in response to the overfeeding, as published previously [5,6,11,12,32,33] . Furthermore, both birth weight groups displayed several changes in metabolism in response to the overfeeding challenge, as also published previously [6,12,33]. A selection of these findings is presented in brief below to provide background of the current findings and in Table 1 and Supplementary Table S3 online.

LBW men had higher fasting blood glucose ($P \leq 0.01$) and serum insulin ($P \leq 0.01$) levels after the control diet compared with NBW men [5] (Table 1). Also, both LBW and NBW men increased the fasting blood glucose level ($P \leq 0.05$ and $P \leq 0.001$, respectively) in response to overfeeding, and NBW men additionally increased the fasting serum insulin level ($P \leq 0.05$) due to this challenge [6]. LBW men had a higher hepatic insulin resistance index ($P \leq 0.05$) after the control diet compared with NBW men [5], and NBW men, but not LBW men, showed an increase in the hepatic glucose production ($P \leq 0.01$) and in the hepatic insulin resistance index ($P \leq 0.001$) in response to overfeeding [6] (Table 1). LBW and NBW men did not show a different insulin-stimulated glucose infusion rate (M-value) after the control diet [5] (Table 1). LBW men, however, in contrast to NBW men, decreased this rate ($P \leq 0.05$) in reaction to overfeeding [6]. In terms of plasma lipid profiles, LBW men had a lower fasting plasma HDL-cholesterol level ($P \leq 0.01$ and $P \leq 0.05$, respectively) after both the control and high-fat, high-calorie diets compared with NBW men [6] (Table 1). Furthermore, both LBW and NBW men decreased fasting plasma total NEFA (both $P \leq 0.001$), VLDL-cholesterol ($P \leq 0.01$ and $P \leq 0.05$, respectively), and total triacylglycerol ($P \leq 0.01$ and $P \leq 0.05$, respectively) levels and increased the HDL-cholesterol level (both $P \leq 0.01$) in response to overfeeding [6] (Table 1). LBW and NBW men did not show differences in basal glucose or fatty acid

oxidation rates after the control or high-fat, high-calorie diet when evaluated from the indirect calorimetry in connection with the clamp, and they also did not change these rates in response to overfeeding [6] (Table 1). However, LBW men had higher fatty acid oxidation rates (P=0.05/0.07 and P=0.10, respectively) and a lower glucose oxidation rate (P=0.05 and P=0.06, respectively) at night or during sleep during both the control and high-fat, high-calorie diets, compared with NBW men, when studied during the 24-hour indirect calorimetry [11,32] (Supplementary Table S3 online). Also, both LBW and NBW men increased the fatty acid oxidation rate in all four reported time intervals during the 24-hour indirect calorimetry in reaction to overfeeding (both P<0.0001 for the 24-hour period) [12]. Moreover, both LBW and NBW men increased the total energy expenditure in all four time intervals due to this challenge (P=0.0008 and P=0.0005, respectively, for the 24-hour period) [12] (Supplementary Table S3 online).

Ceramide levels and their relation to other lipid levels and physiological measures

LBW and NBW men did not show any differences in fasting plasma ceramide levels after the control or high-fat, high-calorie diet intervention, but both birth weight groups changed plasma levels of several ceramide species in response to overfeeding (Table 2). Thus, both LBW and NBW men significantly decreased d18:0-18:1/d18:1-18:0 (P=0.0004 and P<0.0001, respectively) and d18:1-24:2/d18:2-24:1 (P=0.0002 and P=0.0003, respectively) levels and increased the d18:0-24:1a level (P=0.0062 and P=0.0147, respectively) in response to overfeeding. NBW men furthermore decreased d18:0-16:1/d18:1-16:0 (P=0.0093), d18:1-22:1/d18:2-22:0 (P=0.0072), and d18:1-23:1/d18:2-23:0 (P=0.0129) levels and showed a tendency to decrease the d18:0-20:1/d18:1-20:0 level (P=0.0511) in response to overfeeding, while LBW men showed a tendency to decrease d18:0-16:1/d18:1-16:0 (P=0.0553) and d18:1-22:1/d18:2-22:0 (P=0.0850) levels and increase the d18:0-25:2/d18:1-25:1/d18:2-25:0 level (P=0.0649) due to this challenge.

Plasma levels of many of the detected ceramides as well as of total ceramide were positively associated with fasting plasma VLDL-cholesterol, LDL-cholesterol, total cholesterol, and total triacylglycerol levels after both the control and high-fat, high-calorie diets (Supplementary Table S4 online). Furthermore, d18:0-16:1/d18:1-16:0 (P=0.0298), d18:0-18:1/d18:1-18:0 (P=0.0076), d18:1-22:0 (P=0.0819), d18:0-24:1a (P=0.0144), d18:0-26:1/d18:1-26:0 (P=0.0054), d18:1-24:1 (P=0.0049), d18:1-24:2/d18:2-24:1 (P=0.0109), and total ceramide (P=0.0349) levels were or

tended to be positively associated with the fasting blood glucose level after the control diet (Fig. 1). Among these ceramides, d18:0-18:1/d18:1-18:0 (P=0.0192), d18:0-24:1a (P=0.0010), d18:0-26:1/d18:1-26:0 (P=0.0362), and d18:1-24:2/d18:2-24:1 (P=0.0879) levels were or tended to be positively associated with the hepatic glucose production, and d18:1-22:0 (P=0.0205), d18:1-24:1 (P=0.0561), and total ceramide (P=0.0252) levels were or tended to be positively associated with the hepatic insulin resistance index (Fig. 1). Also, d18:0-16:1/d18:1-16:0 (P=0.0263), d18:0-18:1/d18:1-18:0 (P=0.0214), d18:1-22:0 (P=0.0279), d18:0-26:1/d18:1-26:0 (P=0.0912), d18:1-24:1 (P=0.0927), d18:1-24:2/d18:2-24:1 (P=0.0376), and total ceramide (P=0.0326) levels were or tended to be positively associated with the fatty acid oxidation rate determined in connection with the clamp examination after the control diet (Fig. 1).

An increase in the d18:0-25:2/d18:1-25:1/d18:2-25:0 level (P=0.0578) in response to overfeeding tended to associate with an increase in the hepatic insulin resistance index (Fig. 1). Furthermore, decreases in d18:0-18:1/d18:1-18:0 (P=0.0607), d18:1-24:0 (P=0.0793), d18:1-22:1/d18:2-22:0 (P=0.0487), d18:1-24:1 (P=0.0437), and total ceramide (P=0.0336) levels due to overfeeding were or tended to be associated with a decrease in the insulin-stimulated glucose uptake rate (M-value) (Fig. 1).

DISCUSSION

In contrast to expected, LBW men did not show altered fasting plasma ceramide levels after the control or high-fat, high-calorie diet intervention compared with NBW men. The increased fatty acid oxidation rate in the LBW men during both diets [11,32] may limit the amount of fatty acid substrates available for de novo ceramide synthesis and thereby compensate for a possible increased fatty acid load to non-adipose tissue in these individuals. Both LBW and NBW men decreased plasma levels of several ceramide species in response to high-fat overfeeding, all of these d18:0-/d18:1- or d18:1-/d18:2- levels with the d18:1- species expected to be the dominant isomer in the pools, and increased the d18:0-24:1a level. Our findings of decreases in plasma levels of several ceramides in response to overfeeding are consistent with decreases in fasting plasma levels of several other lipid classes in the LBW and NBW men due to this challenge, including total NEFA, VLDL-cholesterol, total triacylglycerol, and total acylcarnitine levels [6,12]. The decreases in fasting plasma levels of several lipids, including a number of ceramide species, in the LBW and NBW men in response to overfeeding could very like be a result of their markedly increases in fatty acid oxidation rates and total energy expenditures due to this challenge [12].

Several studies have reported altered plasma and/or tissue ceramide levels in mice exposed to high-fat feeding [34-37], and a recent study has described changes in serum ceramide levels in human subjects exposed to high-fat overfeeding [38]. Thus, sedentary women and men decrease fasting serum d18:0-18:0, d18:1-18:0, and d18:1-24:1 levels and increase d18:0-22:0, d18:0-24:0, d18:1-22:0, d18:1-24:0, and total d18:1- ceramide levels in response to 28-day high-fat overfeeding (45 E% from fat, 1,250 extra kcal) [38]. Furthermore, these individuals increase fasting serum HDL-cholesterol and total cholesterol levels in response to overfeeding, while they do not change LDL-cholesterol, total NEFA, and total triacylglycerol levels [38]. Important differences between this and the present overfeeding study are the duration and fat contents of the high-fat, high-calorie diet interventions. The increases in fatty acid oxidation rates and total energy expenditures in the LBW and NBW men in response to the 5-day high-fat overfeeding could be a transient, compensatory mechanism to prevent an accumulation of lipids in non-adipose tissue and may not persist for long-term high-fat overfeeding exposures. This hypothesis, however, requires further investigations. Also, both genders are examined in the reported 28-day overfeeding study and these individuals are somewhat older (21-65 years of age) (37±2 years of age) [38] than the presently examined men (23-27 years of age) (24±1 years of age). Among the studies performed in mice, several different

experimental setups in regard to the high-fat diet intervention have been used [34-37]. Thus, wild-type mice fed a 16-week high-fat (60 E% from fat) diet have higher plasma levels of all measured ceramides, including seven d18:1- species, compared with mice fed a low-fat (10 E% from fat) diet, and also higher plasma total ceramide and adipose tissue total ceramide levels [34]. In addition, mice fed an 8-week high-fat (60 E% from fat) diet have higher liver total ceramide, including seven d18:1- species, and total diacylglycerol contents compared with mice fed a standard chow [35]. Plasma ceramide levels were not measured in these mice [35]. Interestingly, adiponectin administration to the mice fed the 8-week high-fat diet, and in addition to leptin deficient (ob/ob) mice, rapidly normalises liver ceramide, but not diacylglycerol, contents, regardless of ceramide species (d18:0- or d18:1-) [35]. Also, adiponectin administration to the ob/ob mice results in a reduction of the hepatic glucose production and an improvement in hepatic, but not skeletal muscle, insulin sensitivity [35]. Notably, adiponectin exerted these effects through lowering of the liver ceramide content via receptor-mediated enhancement of ceramidase activities [35]. Previously, we have found that both LBW and NBW men of the present study population increase the fasting plasma adiponectin level in response to overfeeding [6]. This, besides to their increases in fatty acid oxidation rates, may prevent an accumulation of ceramides in the liver. Furthermore, the LBW and NBW men examined herein increase the fasting serum fibroblast growth factor 21 (FGF-21) level in response to the overfeeding challenge, apparently due to an increased FGF-21 secretion from the liver [39]. FGF-21 administration to mice fed a high-fat (40 E% from fat) diet, and in addition to ob/ob mice, increases fatty acid oxidation rates and total energy expenditures and reduces hepatosteatosis [40]. A recent study has shown that FGF-21 stimulates adiponectin secretion and reduces serum ceramide levels in mice fed a high-fat (60 E% from fat) diet [41]. Also, adiponectin-knockout mice are refractory to FGF-21 effects, including lowering of ceramide levels [41]. Therefore, it was concluded that FGF-21 depends on adiponectin to exert its insulin-sensitising effects [41].

We furthermore showed that higher plasma levels of several ceramides as well as of total ceramide were associated with a higher fasting blood glucose level and a higher degree of hepatic insulin resistance after the control diet. These findings are consistent with the observed link between liver ceramide contents and hepatic insulin sensitivity in the mice models used to investigate adiponectin effects [35]. A recent study has moreover shown that cultured rat hepatocytes exposed to high concentrations of palmitic acid have higher intracellular ceramide concentrations and are less responsive to insulin [26]. The latter was caused by an inhibition of the insulin-stimulated

phosphorylation of Akt/PKB and glycogen synthase kinase [26]. The NBW men develop impaired hepatic insulin sensitivity and increase the hepatic glucose production in response to high-fat overfeeding, while the LBW men show impaired hepatic insulin sensitivity already after the control diet and do not reduce this sensitivity further in response to the overfeeding challenge [5,6]. Our findings of decreases in plasma levels of several ceramides in response to overfeeding in the NBW men do not support a possible role of ceramides in promoting hepatic insulin resistance in these individuals. Nevertheless, it is remarkable that some d18:0- species, or dihydroceramides, were only or predominantly detected in plasma from the LBW and NBW men after the high-fat, high-calorie diet (Table 2). Furthermore, the LBW and NBW men solely increased the d18:0-24:1a level in response to overfeeding. A higher d18:0-24:1a level was strongly significantly associated with an increased hepatic glucose production after the control diet. Interestingly, a newly study has reported that individuals who progress to type 2 diabetes have elevated plasma levels of specific long-chain fatty acid-containing d18:0- species several years before disease onset [42]. Moreover, we found that decreases in plasma levels of several ceramides and total ceramide in response to overfeeding were associated with a decrease in peripheral insulin sensitivity. The LBW men, but not NBW men, develop impaired peripheral insulin sensitivity in response to the high-fat overfeeding challenge [6]. Several studies have reported negative correlations between skeletal muscle total ceramide contents and insulin sensitivity [43-45]. One of these studies additionally reports a positive correlation between skeletal muscle levels of some ceramide species and insulin sensitivity in older individuals [45]. Interestingly, it has been shown that circulating LDL-ceramide in mice specifically targets skeletal muscle and induces insulin resistance [19], emphasising the important role of ceramide metabolism in the liver. Here, we demonstrated that higher plasma levels of most of the detected ceramide species as well as of total ceramide were strongly significantly associated with higher fasting plasma VLDL- and LDL-cholesterol levels after both the control and high-fat, high-calorie diets. However, it is unknown to what extent circulating VLDL- or LDL-ceramides formed in the liver versus ceramides synthesised in skeletal muscle interfere with insulin signalling in this tissue.

Our study is the first to investigate plasma ceramide profiles in LBW men at risk of developing type 2 diabetes, compared with NBW men, and among a few studies to examine plasma ceramide profiles in human subjects exposed to short-term high-fat overfeeding. The ceramide profiles reported herein are moreover very detailed and related to measures of both hepatic and peripheral insulin sensitivity. In conclusion, LBW men did not show altered fasting plasma ceramide levels after the control or high-fat, high-calorie diet intervention compared with NBW men. We suggest

that the increased fatty acid oxidation rate in the LBW men during both diets [11,32] may limit the amount of fatty acid substrates available for lipogenesis, including de novo ceramide synthesis, and thereby may compensate for a likely increased fatty acid load to non-adipose tissue in these individuals. Both LBW and NBW men decreased plasma levels of several ceramide species in response to overfeeding. This could very likely be a result of their increases in fatty acid oxidation rates and total energy expenditures due to the overfeeding challenge [12], potentially evoked by their increases in fasting serum FGF-21 [39] and plasma adiponectin [6] levels. Alternatively, it might be a result of a FGF-21 and adiponectin mediated activation of ceramidases in the liver with a following increase in the ceramide degradation. A higher plasma total ceramide level was associated with a higher degree of hepatic insulin resistance after the control diet. Further studies are needed to determine if an accumulation of potentially lipotoxic lipids in tissue and plasma is part of the adverse metabolic events leading to insulin resistance in LBW individuals. Also, additional investigations are required to determine possible specific roles of individual ceramides, including dihydroceramides, in interfering with insulin signalling in the liver and skeletal muscle.

METHODS

Ceramide analyses were performed on plasma samples from LBW and NBW men subjected to dietary interventions and physiological tests prior to the present study, as described in short below and in earlier publications [5,6,11,12,32].

Study population

Forty-six young (23-27 years of age), healthy men were recruited from the Danish National Birth Registry according to birth weight. Among these, 20 men had a LBW (2717±268 g) (≤10th percentile) and 26 men a NBW (3901±207 g) (50-90th percentile). All men were born at term (39-41 weeks of gestation) in Copenhagen from 1979-1980. Also, all men were non-obese (BMI <30 kg/m^2), did not perform strenuous physical activity >10 h/week, and did not have a family history of diabetes in two generations.

Study design

Diet interventions

All men were, in a randomised crossover study setup, standardised with regard to diet and physical activity and following subjected to a 3-day control diet and a 5-day high-fat, high-calorie diet intervention separated by a 6-8 week wash out-period. The control diet was prepared to reflect a habitual, weight-maintaining diet (2,819±238 kcal/day) with 15, 50, and 35 E% from protein, carbohydrate, and fat, respectively, and the high-fat, high-calorie diet was composed to contain 50 % extra calories (4,228±334 kcal/day) with 7.5, 32.5, and 60 E% from protein, carbohydrate, and fat, respectively (Supplementary Table S1 online). Both diets were provided as five daily servings, and the meals were identical from day to day. Energy requirements of the individual participants were calculated from a WHO equation for men <30 years of age with a low physical activity level [46], and dietary calculations were made in Dankost Pro (The National Food Agency, Copenhagen, Denmark).

Clinical examinations

Study activities were performed over 3 days with the first of these days being placed 1 or 3 days following the start of the control and high-fat, high-calorie diet intervention, respectively. Anthropometry measures were recorded on day 1. An intravenous glucose tolerance test (IVGTT) and a hyperinsulinaemic-euglycaemic clamp were carried out in the morning on day 3 to assess insulin secretion and sensitivity, as described previously [5,6]. Furthermore, indirect calorimetry was conducted throughout 24 hours from day 1-2 as well as in the basal and insulin-stimulated steady-state periods of the clamp to determine substrate oxidation rates and total energy expenditures, as also described previously [6,11,32]. Blood samples were collected prior to and during the clamp.

Laboratory measurements

Ceramide analyses

Ceramide analyses were performed on EDTA-plasma samples collected following an overnight fast (10.00 PM-7.00 AM) and immediately prior to the clamp examination. These analyses included a semi-quantitative determination of 27 ceramide species (individual or pools), denoted by the sphingoid base (i.e. d18:0-, d18:1-, or d18:2-) and acyl group (e.g. -24:0) in accordance to their carbon chain length and number of double bonds (Table 2, Supplementary Table S2 online).

Aliquots of 200 µL plasma were spiked with 100 µL d18:1-16:0-$^{2}H_{31}$ internal standard (Avanti Polar Lipids, Alabaster, AL, USA) solution (0.4 nmol/100 µL 2:1 chloroform:methanol), and lipids were then extracted with 1.5 mL ice-cold methanol, 3.0 ice-cold mL chloroform, and 960 µL ice-cold 0.73 % sodium chloride solution according to the Bligh and Dyer method [47]. After centrifugation, the lower organic phases were transferred to new glass tubes, evaporated to dryness under nitrogen gas, and re-dissolved in 200 µL chloroform. Following, the lipid extracts were applied to n-hexane washed Strata NH_2-columns (55 µm, 500 mg, 3 mL) (Phenomenex, Torrance, CA, USA). Cholesterolesters and triacylglycerols were first eluted with 3.0 mL 100:5:5 hexane:chloroform:ethylacetate, and cholesterol, diacylglycerols, and ceramides were subsequently eluted with 6.0 mL 23:1 chloroform:methanol into separate glass tubes. Ceramide fractions were evaporated to dryness under nitrogen gas, re-dissolved in 50 µL chloroform and 100 µL iso-propanol, and transferred to HPLC vials.

For HPLC-HRMS analyses, 1.0 µL sample was injected into an UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) and separated through a Kinetex C_8-column (2.1×100 mm, 2.7 µm) (Phenomenex) at 60 ºC by elution with a water with 20 mM formic acid (A)-1:4 iso-propanol:acetonitrile (B) gradient. An eluent flow rate of 0.4 mL/min was applied, and the gradient started at 35 % B, increased linearly from 35-70 % B (0.0-1.5 min) and further from 70-100 % B (1.5-9.5 min), remained at 100 % B (9.5-11.0 min), decreased linearly from 100-35 % B (11.0-11.1 min), and finally remained at 35 % B (11.1-13.0 min). Following, the effluent was introduced into a maXis HD quadrupole TOF-MS (Bruker, Bremen, Germany) equipped with an ESI ion source operated in positive ion mode with the collision energy alternating between 0 and 25 eV every 500 ms (BBCid fragmentation), thereby generating the $[M+H]^+$-ion of the ceramides and $[M+H–H_2O]^+$-fragment ion as well as a fragment ion only containing the sphingoid base moiety of the ceramides. Using an aggressive dereplication approach [48] and Bruker Target Analysis 1.3 (Bruker), ceramide species were then identified from HPLC retention times and accurate masses of the $[M+H]^+$-ion (quantifier ion), $[M+H–H_2O]^+$-fragment ion (qualifier ion 1), and sphingoid base moiety fragment ion (qualifier ion 2) (for all ions with a m/z tolerance of ±10 ppm). This enables the distinction between different position isomers (e.g. d18:0-24:1 and d18:1-24:0).

A total of 90 ceramide species were searched, including ceramides with a d18:0, d18:1, or d18:2 sphingoid base in all possible combinations with a 14:0-26:0, 14:1-26:1, 14:2-26:2, 24:3-26:3, or 26:4 acyl group, listed in Supplementary Table S2 online together with m/z-values of their respective $[M+H]^+$-ions, $[M+H–H_2O]^+$-fragment ions, and sphingoid base moiety fragment ions. Among these, 57 ceramide species were identified in the total set of plasma samples. Some species were, however, only detected in a few samples and therefore not included in Table 2. Furthermore, the peak areas of several position isomers (e.g. d18:0-16:1 and d18:1-16:0) were combined in the HPLC chromatograms due to similar retention times of these isomers. These were therefore pooled. Also, a few ceramide species with identical molecular formulas (e.g. d18:0-24:1) were identified at two different retention times, presumably corresponding to different structural isomers of the unsaturated acyl group. These isomers are denoted a and b, respectively. Taken together, 27 ceramide species (individual or pools) were selected for quantifications and included in Table 2.

For quantifications, external standard samples, comprising a dilution row of d18:1-14:0, d18:1-24:0, d18:1-18:1, d18:1-24:1, and d18:1-16:0-$^2H_{31}$ standard (Avanti Polar Lipids, Alabaster, AL, USA) solutions (1:2 chloroform:iso-propanol), were analysed by HPLC-HRMS parallel to the plasma

samples to determine the area-mass relationships of these species. For all standards, this was linear within the relevant concentration ranges. Ceramide peak areas in the plasma samples were adjusted for the recovery of the d18:1-16:0-$^{2}H_{31}$ internal standard (79.0±3.6 %) and following converted to masses from the response of their respective external standard or an estimated response. Calculations of estimated responses were based on the assumption of an equal change in response per carbon atom and per double bond in the acyl group. These changes were determined from the responses of d18:1-14:0 compared with d18:1-24:0 and d18:1-24:0 compared with d18:1-24:1, respectively. d18:0-, d18:1-, and d18:2- species with identical acyl groups were assigned the same response. Also, ceramides that were pooled were assigned the response of the d18:1- position isomer that was expected to be the dominant isomer. Total d18:0-, or dihydroceramide, levels were calculated as the sum of the d18:0-16:0, d18:0-23:0, d18:0-22:1, d18:0-24:1a, d18:0-24:1b, and d18:0-25:1a levels, and total ceramide levels were calculated as the sum of the levels of the 27 ceramide species (individual or pools) included in Table 2.

Ceramide analyses were performed at the Metabolomics Platform, Department of Biotechnology and Biomedicine, Technical University of Denmark, Kongens Lyngby, Denmark.

Ethical approval

All study procedures were in accordance with the principles of The Declaration of Helsinki and approved by The Regional Research Ethics Committee of Copenhagen, Denmark. Furthermore, all participants were provided with written information on the study purpose and procedures and signed an informed consent prior to their participation.

Statistical analyses

Ceramide levels and their relation to other lipid levels and physiological measures

Ceramide data presented in Table 2, including plasma levels of individual species within each combination of birth weight group and diet intervention and differences in these levels between the two diets (response values) within each birth weight group, were evaluated for normality by Shapiro-Wilk tests with a significance level of 0.05. Plasma levels of individual ceramide species

within each diet and response values were furthermore evaluated for equality of variances between the birth weight groups by F-tests with a significance level of 0.05 as well. Statistically significant differences in plasma ceramide levels between NBW and LBW men within each diet and in response values between the birth weight groups were subsequently assessed by Student's two-tailed, unpaired t-tests (for normally distributed values) or Wilcoxon ranked-sum tests (for non-normally distributed values). Furthermore, significant differences in plasma ceramide levels between the control and high-fat, high-calorie diets within each birth weight group were assessed by Student's two-tailed, paired t-tests (for normally distributed values) or Wilcoxon signed-rank tests (for non-normally distributed values). P-values from Student's t-tests and Wilcoxon tests were evaluated in context with false discovery rates (Q-values) to account for multiple testing within each diet or birth weight group. Q-values were calculated by the Benjamini and Hochberg method. P-values $\leq$0.05 with Q-values $\leq$0.05 were considered statistically significant. Data in Table 2 are presented as mean values with 95 % confidence intervals together with the number of observations within each group. Moreover, P- and Q-values are indicated. Student's t-tests and Wilcoxon tests were not performed, if more than 25 % of the values within one of the two groups to be compared were missing. Total d18:0- levels were calculated as the sum of the d18:0-16:0, d18:0-23:0, d18:0-22:1, d18:0-24:1a, d18:0-24:1b, and d18:0-25:1a levels, and total ceramide levels were calculated as the sum of the levels of all 27 ceramides (individual or pools) included in Table 2.

Associations between plasma ceramide levels and other lipid levels or physiological measures within each diet and furthermore between response values, as presented in Figure 1 and Supplementary Table S4 online, were assessed from linear regression analyses. These analyses were performed on the pooled data set of LBW and NBW men and were adjusted for age, BMI, and birth weight group. P-values $\leq$0.05 were considered statistically significant. Data in Figure 1 and Supplementary Table S4 online are presented as Spearman correlation coefficients (r-values) and P-values.

Data availability

Plasma ceramide data generated in the present study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

A.R-M. contributed to the hypotheses, performed the ceramide analyses, performed the data analyses, interpreted the results, and wrote and submitted the manuscript, R.R-M. contributed to the hypotheses and discussion of the results, K.F.N. developed and contributed to the ceramide analyses, S.B. contributed to the discussion of the results, A.A.V. designed the study, and C.B. performed the diet interventions and clinical tests. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

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Competing interests

All authors declare no financial or otherwise conflicts of interest in this study.

FIGURE AND TABLE LEGENDS

Figure 1: Heat-map of associations between plasma ceramide levels and physiological measures following the control (C) and high-fat, high-calorie (O) diets and between response values (D). Data are presented as r-values (red or blue colour variations for positive or negative values, respectively) and P-values (+/–: P≤0.05, + +/– –: P≤0.01, + + +/– – –: P≤0.001, (+)/(–): P≤0.1 for positive or negative associations, respectively). r-values are in the range -0.4 to 0.4. P-values are presented unadjusted for multiple comparisons, and P-values ≤0.05 are considered statistically significant. Regression analyses were performed on the pooled data set of LBW and NBW men and were adjusted for age, BMI, and birth weight group. B or I indicated in parentheses after clamp measures specify basal or insulin-stimulated state, respectively. Other abbreviations: See Table 1.

Table 1: Clinical characteristics of low (LBW) and normal birth weight (NBW) men following the control (C) and high-fat, high-calorie (O) diets. Data are presented as mean values ± standard deviations (SD). P-values are presented unadjusted for multiple comparisons, and P-values ≤0.05 are considered statistically significant. P_{NBW} and P_{LBW}: O vs. C diet within each birth weight group, P_C and P_O: LBW vs. NBW men within each diet, P_Δ: LBW vs. NBW men on response values. P-values ≤0.05 are marked in bold. n.s.: Not significant. Other abbreviations: B: Blood, CHOL: Cholesterol, DI: Disposition index, FFM: Fat free mass, FOX: Fatty acid oxidation, FPIR: First phase insulin response, GOX: Glucose oxidation, HGP: Hepatic glucose production, IR: Insulin resistance, P: Plasma, S: Serum, TG: Triacylglycerol.

Table 2: Plasma ceramide levels in low (LBW) and normal birth weight (NBW) men following the control (C) and high-fat, high-calorie (O) diets. Data are presented as mean values with 95 % confidence intervals (CI). P-values ≤0.05 are presented together with Q-values, and P-values ≤0.05 with corresponding Q-values ≤0.05 are considered statistically significant. P-values were not calculated, if more than 25 % of the values within one of the two groups to be compared were missing. P_{NBW} and P_{LBW}: O vs. C diet within each birth weight group, P_C and P_O: LBW vs. NBW men within each diet, P_Δ: LBW vs. NBW men on response values. P-values ≤0.05 and Q-values ≤0.05 are marked in bold. Total d18:0- levels: d18:0-16:0, d18:0-23:0, d18:0-22:1, d18:0-24:1a, d18:0-24:1b, and d18:0-25:1a, Total ceramide levels: All 27 ceramide species (individual or pools).

FIGURES AND TABLES

Figure 1:

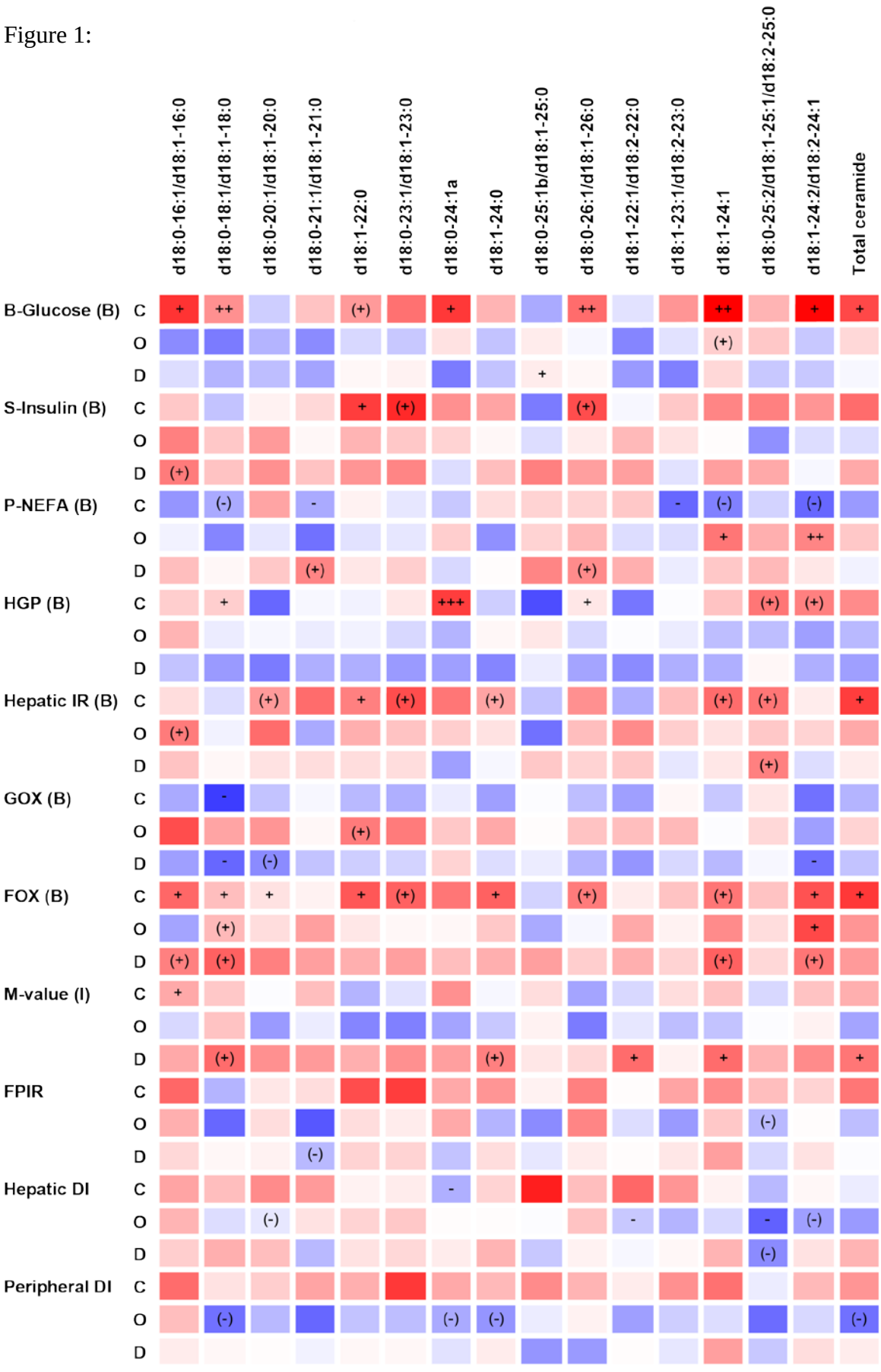

Table 1:

	NBW (n = 25)			**LBW** (n = 18)			**LBW vs. NBW** (n = 18, n = 25)		
	C (Mean ± SD)	**O** (Mean ± SD)	**P_{NBW}**	**C** (Mean ± SD)	**O** (Mean ± SD)	**P_{LBW}**	**P_C**	**P_O**	**P_Δ**
Anthropometry									
Birth weight (g)	3901 ± 207	-	-	2717 ± 268	-	-	**≤0.001**	-	-
Weight (kg)	78.4 ± 9.3	78.6 ± 9.7	n.s.	77.1 ± 11.3	77.1 ± 11.4	n.s.	n.s.	n.s.	n.s.
Height (m)	1.83 ± 0.07	-	-	1.77 ± 0.05	-	-	**≤0.05**	-	-
BMI (kg/m^2)	23.3 ± 2.4	23.3 ± 2.5	n.s.	24.6 ± 3.8	24.6 ± 3.8	n.s.	n.s.	n.s.	n.s.
Lipid profiling									
P-VLDL-CHOL (mM)	0.42 ± 0.16	0.33 ± 0.16	**≤0.05**	0.49 ± 0.18	0.32 ± 0.12	**≤0.01**	n.s.	n.s.	n.s.
P-LDL-CHOL (mM)	2.51 ± 0.72	2.28 ± 0.78	**≤0.05**	2.69 ± 0.76	2.57 ± 0.80	n.s.	n.s.	n.s.	n.s.
P-HDL-CHOL (mM)	1.40 ± 0.22	1.56 ± 0.25	**≤0.01**	1.19 ± 0.23	1.38 ± 0.28	**≤0.01**	**≤0.01**	**≤0.05**	n.s.
P-CHOL (mM)	4.36 ± 0.83	4.18 ± 0.82	n.s.	4.36 ± 0.78	4.27 ± 0.79	n.s.	n.s.	n.s.	n.s.
P-TG (mM)	0.92 ± 0.35	0.73 ± 0.35	**≤0.05**	1.07 ± 0.37	0.72 ± 0.24	**≤0.01**	n.s.	n.s.	n.s.
Clamp									
Basal									
B-Glucose (mM)	4.59 ± 0.47	5.05 ± 0.40	**≤0.001**	4.97 ± 0.48	5.18 ± 0.34	**≤0.05**	**≤0.01**	n.s.	n.s.
S-Insulin (pM)	30.2 ± 14.7	43.4 ± 29.2	**≤0.05**	41.7 ± 14.6	44.7 ± 21.9	n.s.	**≤0.01**	n.s.	n.s.
P-NEFA (µM)	334 ± 136	205 ± 82	**≤0.001**	406 ± 200	188 ± 91	**≤0.001**	n.s.	n.s.	n.s.
HGP (mg/kg·FFM/min)	2.21 ± 0.48	2.85 ± 0.99	**≤0.01**	2.40 ± 0.5	2.48 ± 0.5	n.s.	n.s.	n.s.	**≤0.05**
Hepatic IR (mg/kg·FFM/min ·pM)	68.7 ± 34.1	113.7 ± 61.5	**≤0.001**	102.3 ± 50.8	108.7 ± 55.5	n.s.	**≤0.05**	n.s.	**≤0.05**
GOX (mg/kg·FFM/min)	2.34 ± 0.76	2.43 ± 0.71	n.s.	1.95 ± 0.78	2.20 ± 0.56	n.s.	n.s.	n.s.	n.s.
FOX (mg/kg·FFM/min)	1.00 ± 0.38	1.02 ± 0.33	n.s.	1.11 ± 0.53	1.17 ± 0.33	n.s.	n.s.	n.s.	n.s.
Insulin-stimulated									
P-NEFA (µM)	9.29 ± 4.39	12.42 ± 6.43	**≤0.01**	9.56 ± 5.03	14.39 ± 7.76	**≤0.01**	n.s.	n.s.	n.s.
M-value (mg/kg·FFM/min)	13.73 ± 2.32	13.29 ± 3.32	n.s.	13.47 ± 3.14	11.89 ± 3.57	**≤0.05**	n.s.	n.s.	n.s.
GOX (mg/kg·FFM/min)	5.18 ± 0.82	5.04 ± 0.98	n.s.	4.95 ± 0.92	4.78 ± 0.82	n.s.	n.s.	n.s.	n.s.
FOX (mg/kg·FFM/min)	0.01 ± 0.25	0.17 ± 0.33	n.s.	0.13 ± 0.46	0.37 ± 0.35	**≤0.05**	n.s.	**≤0.05**	n.s.
IVGTT									
FPIR (pM)	1894 ± 1431	2604 ± 1793	**≤0.001**	2135 ± 1034	2750 ± 1509	**≤0.01**	n.s.	n.s.	n.s.
Hepatic DI	0.38 ± 0.63	0.25 ± 0.21	n.s.	0.21 ± 0.11	0.24 ± 0.13	n.s.	n.s.	n.s.	n.s.
Peripheral DI	0.29 ± 0.19	0.35 ± 0.20	**≤0.05**	0.33 ± 0.13	0.32 ± 0.17	n.s.	n.s.	n.s.	n.s.

Table 2:

	NBW (n = 25)			**LBW** (n = 18)			**LBW vs. NBW** (n = 18, n = 25)		
(µM)	**C** (Mean, CI)	**O** (Mean, CI)	**P_{NBW}** **Q_{NBW}**	**C** (Mean, CI)	**O** (Mean, CI)	**P_{LBW}** **Q_{LBW}**	**P_C** **Q_C**	**P_O** **Q_O**	**P_Δ** **Q_Δ**
Lipid profiling									
Ceramides									
d18:0-16:0	0.39 (0.25, 0.54) (n = 2)	0.39 (0.38, 0.41) (n = 8)	- (n = 1)	- - (n = 0)	0.39 (0.38, 0.39) (n = 9)	- (n = 0)	-	-	-
d18:0-23:0	0.31 (0.29, 0.33) (n = 5)	0.30 (0.11, 0.50) (n = 2)	- (n = 0)	0.30 (0.24, 0.36) (n = 3)	0.34 (0.24, 0.45) (n = 3)	- (n = 0)	-	-	-
d18:1-14:0	0.42 (0.42, 0.42) (n = 3)	0.41 (0.40, 0.42) (n = 6)	- (n = 1)	0.42 (0.37, 0.48) (n = 3)	0.41 (0.40, 0.42) (n = 7)	- (n = 3)	-	-	-
d18:0-16:1/ d18:1-16:0	0.68 (0.65, 0.72) (n = 25)	0.64 (0.60, 0.67) (n = 25)	**0.0093** **0.0349** (n = 25)	0.68 (0.64, 0.72) (n = 18)	0.64 (0.61, 0.68) (n = 18)	0.0553 (n = 18)	0.9317	0.7598	0.7528
d18:0-18:1/ d18:1-18:0	0.54 (0.51, 0.58) (n = 25)	0.47 (0.45, 0.50) (n = 23)	**<0.0001** **0.0005** (n = 23)	0.55 (0.52, 0.59) (n = 18)	0.49 (0.46, 0.52) (n = 18)	**0.0004** **0.0030** (n = 18)	0.4717	0.4904	0.6504
d18:0-20:1/ d18:1-20:0	0.56 (0.52, 0.60) (n = 25)	0.53 (0.49, 0.57) (n = 25)	0.0511 (n = 25)	0.56 (0.51, 0.60) (n = 18)	0.55 (0.50, 0.59) (n = 18)	0.5866 (n = 18)	0.3612	0.5177	0.4032
d18:0-21:1/ d18:1-21:0	0.39 (0.37, 0.41) (n = 24)	0.39 (0.37, 0.41) (n = 22)	0.9066 (n = 21)	0.40 (0.37, 0.43) (n = 16)	0.40 (0.37, 0.42) (n = 18)	0.7817 (n = 16)	0.4361	0.4592	0.8432
d18:0-22:1	- - (n = 0)	0.31 (0.29, 0.32) (n = 7)	- (n = 0)	- - (n = 0)	0.31 (0.28, 0.34) (n = 3)	- (n = 0)	-	-	-
d18:1-22:0	0.93 (0.86, 1.01) (n = 25)	0.96 (0.89, 1.04) (n = 25)	0.3741 (n = 25)	0.93 (0.85, 1.01) (n = 18)	0.98 (0.91, 1.06) (n = 18)	0.1514 (n = 18)	0.8353	0.6164	0.5904
d18:0-23:1/ d18:1-23:0	1.03 (0.93, 1.13) (n = 25)	1.11 (1.02, 1.20) (n = 25)	0.1032 (n = 25)	1.05 (0.94, 1.15) (n = 18)	1.12 (1.02, 1.23) (n = 18)	0.1684 (n = 18)	0.7634	0.8162	0.9146
d18:0-24:1a	0.34 (0.32, 0.37) (n = 24)	0.39 (0.35, 0.42) (n = 24)	**0.0147** **0.0368** (n = 23)	0.35 (0.32, 0.38) (n = 18)	0.40 (0.37, 0.43) (n = 18)	**0.0062** **0.0310** (n = 18)	0.7144	0.4813	0.7271
d18:0-24:1b	0.27 (0.26, 0.28) (n = 7)	0.27 (0.22, 0.33) (n = 2)	- (n = 2)	0.27 (0.25, 0.28) (n = 6)	0.26 (0.14, 0.37) (n = 2)	- (n = 1)	-	-	-
d18:1-24:0	2.46 (2.24, 2.69) (n = 25)	2.50 (2.23, 2.76) (n = 25)	0.7670 (n = 25)	2.36 (2.09, 2.63) (n = 18)	2.60 (2.37, 2.83) (n = 18)	0.1415 (n = 18)	0.8736	0.5577	0.5826

d18:0-25:1a	0.23 (0.21, 0.25) (n = 3)	0.24 (0.22, 0.27) (n = 7)	- (n = 3)	0.24 (0.14, 0.34) (n = 2)	0.25 (0.10, 0.40) (n = 2)	- (n = 1)	-	-	-
d18:0-25:1b/ d18:1-25:0	0.83 (0.76, 0.90) (n = 23)	0.84 (0.75, 0.93) (n = 21)	0.4492 (n = 20)	0.76 (0.65, 0.86) (n = 17)	0.81 (0.72, 0.89) (n = 14)	0.3151 (n = 14)	0.1708	0.4947	0.9980
d18:0-26:1/ d18:1-26:0	0.35 (0.32, 0.38) (n = 25)	0.35 (0.32, 0.38) (n = 24)	0.9815 (n = 24)	0.33 (0.30, 0.36) (n = 18)	0.35 (0.32, 0.38) (n = 18)	0.1540 (n = 18)	0.4142	0.8835	0.2877
d18:0-16:2/ d18:1-16:1/ d18:2-16:0	0.40 (0.39, 0.41) (n = 12)	0.40 (0.37, 0.44) (n = 3)	- (n = 3)	0.40 (0.39, 0.40) (n = 8)	0.40 (0.38, 0.42) (n = 3)	- (n = 2)	-	-	-
d18:0-18:2/ d18:1-18:1/ d18:2-18:0	0.38 (0.37, 0.40) (n = 10)	0.37 - (n = 1)	- (n = 1)	0.37 (0.35, 0.39) (n = 4)	0.37 (0.36, 0.37) (n = 3)	- (n = 1)	-	-	-
d18:0-20:2/ d18:1-20:1/ d18:2-20:0	0.35 (0.33, 0.37) (n = 6)	0.35 (0.33, 0.37) (n = 2)	- (n = 2)	0.36 (0.30, 0.41) (n = 2)	0.34 - (n = 1)	- (n = 0)	-	-	-
d18:1-22:1/ d18:2-22:0	0.61 (0.55, 0.67) (n = 25)	0.55 (0.49, 0.61) (n = 24)	**0.0072** **0.0349** (n = 24)	0.60 (0.53, 0.66) (n = 18)	0.55 (0.50, 0.60) (n = 18)	0.0850 (n = 18)	0.7229	0.9095	0.6341
d18:1-23:1/ d18:2-23:0	0.43 (0.39, 0.47) (n = 24)	0.39 (0.36, 0.42) (n = 25)	**0.0129** **0.0368** (n = 24)	0.41 (0.37, 0.44) (n = 16)	0.40 (0.37, 0.43) (n = 16)	0.8063 (n = 14)	0.3331	0.3484	0.4277
d18:1-24:1	1.80 (1.61, 1.98) (n = 25)	1.67 (1.49, 1.85) (n = 25)	0.1996 (n = 25)	1.74 (1.54, 1.95) (n = 18)	1.68 (1.52, 1.85) (n = 18)	0.4831 (n = 18)	0.7598	0.5826	0.6266
d18:0-25:2/ d18:1-25:1/ d18:2-25:0	0.42 (0.35, 0.48) (n = 25)	0.46 (0.41, 0.51) (n = 25)	0.1854 (n = 25)	0.42 (0.33, 0.50) (n = 16)	0.44 (0.38, 0.51) (n = 18)	0.0649 (n = 16)	0.8421	0.6268	0.9093
d18:0-26:2/ d18:1-26:1/ d18:2-26:0	0.25 (0.23, 0.28) (n = 15)	0.23 (0.22, 0.24) (n = 20)	- (n = 12)	0.23 (0.21, 0.25) (n = 12)	0.24 (0.23, 0.26) (n = 13)	- (n = 10)	-	-	-
d18:1-24:2/ d18:2-24:1	0.87 (0.77, 0.97) (n = 25)	0.66 (0.55, 0.76) (n = 24)	**0.0003** **0.0023** (n = 24)	0.84 (0.73, 0.95) (n = 18)	0.62 (0.52, 0.73) (n = 18)	**0.0002** **0.0030** (n = 18)	0.4659	0.8498	0.9681
d18:0-26:3/ d18:1-26:2	0.27 (0.24, 0.31) (n = 20)	0.31 (0.17, 0.45) (n = 5)	- (n = 5)	0.29 (0.25, 0.32) (n = 13)	0.37 (0.32, 0.42) (n = 7)	- (n = 4)	-	-	-
d18:0-26:4b/ d18:1-26:3/ d18:2-26:2	0.15 (0.13, 0.17) (n = 5)	0.16 (0.00, 0.32) (n = 2)	- (n = 0)	0.16 (0.13, 0.19) (n = 3)	0.13 (0.08, 0.18) (n = 3)	- (n = 1)	-	-	-
Total levels									
d18:0-	0.53 (0.39, 0.66) (n = 25)	0.70 (0.53, 0.87) (n = 25)	0.0778 (n = 25)	0.52 (0.41, 0.62) (n = 18)	0.76 (0.56, 0.97) (n = 18)	**0.0197** (n = 18)	0.9244	0.6176	0.6015
All	13.21	12.39	0.1549	12.71	12.82	0.8709	0.7785	0.4569	0.2853

	(11.93, 14.49) (n = 25)	(11.22, 13.55) (n = 25)	(n = 25)	(11.30, 14.12) (n = 18)	(11.61, 14.02) (n = 18)	(n = 18)			

SUPPLEMENTARY INFORMATION

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TITLE

Plasma ceramide levels are altered in low and normal birth weight men in response to short-term high-fat overfeeding.

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Table S1: Protein, carbohydrate, and fat contents of the control (C) and high-fat, high-calorie (O) diets.

	C		O		O/C	
	Total (Mean)	**Per 100 g** (Mean)	**Total** (Mean)	**Per 100 g** (Mean)	**Total** (Ratio)	**Per 100 g** (Ratio)
Energy (kJ)						
Total	9664	698	14848	1135	1.54	1.63
Energy (E%)						
Protein	15	15	8	8	0.53	0.53
Carbohydrate	49	49	33	33	0.67	0.67
Fat	35	35	60	60	1.71	1.71
Energy (g)						
Protein	88	6.4	67.1	5.1	0.76	0.80
Carbohydrate	266.7	19.2	277.6	21.2	1.04	1.10
Fat	92.1	6.6	239	18.3	2.60	2.77
Fat (g)						
Saturated fatty acids	35.6	2.6	109.8	8.4	3.08	3.23
Monounsaturated fatty acids	31.5	2.3	85.4	6.5	2.71	2.83
Polyunsaturated fatty acids	8.6	0.6	28.5	2.2	3.31	3.67
n-3 fatty acids	0.9	0.1	5.8	0.4	6.44	4.00
n-6 fatty acids	7.2	0.5	21.8	1.7	3.03	3.40

Table S2: Ceramides searched in the plasma samples and mass to charge ratios (m/z-values) of their respective $[M+H]^+$-ions, $[M+H–H_2O]^+$-fragment ions, and sphingoid base moiety fragment ions. Ceramide position isomers are listed in the same rows according to the total number of double bonds in the structures with indication in parentheses of m/z-values of the $[M+H]^+$-ion (quantifier ion) and $[M+H–H_2O]^+$-fragment ion (qualifier 1 ion). d18:0-, d18:1-, and d18:2- position isomers have constant m/z-values of the sphingoid base moiety fragment ion (qualifier 2 ion) of 266.2842, 264.2686, or 262.2529 Da, respectively, and variable m/z-values of the $[M+H]^+$-ion and $[M+H–H_2O]^+$-fragment ion dependent on the acyl group in the structures. Ceramides detected in the plasma samples are marked in bold.

	d18:0- species	**d18:1- species**	**d18:2- species**
Ceramides (Formula)	**Quantifier ion: $[M+H]^+$** **Qualifier 1 ion: $[M+H–H_2O]^+$** **Qualifier 2 ion: 266.2842 Da** (Da)	**Quantifier ion: $[M+H]^+$** **Qualifier 1 ion: $[M+H–H_2O]^+$** **Qualifier 2 ion: 264.2686 Da** (Da)	**Quantifier ion: $[M+H]^+$** **Qualifier 1 ion: $[M+H–H_2O]^+$** **Qualifier 2 ion: 262.2529 Da** (Da)
Double bonds			
0			
$C_{32}H_{65}NO_3$	d18:0-14:0 (512.5038, 494.4932)	-	-
$C_{33}H_{67}NO_3$	d18:0-15:0 (526.5194, 508.5088)	-	-
$C_{34}H_{69}NO_3$	**d18:0-16:0** (540.5351, 522.5245)	-	-
$C_{35}H_{71}NO_3$	d18:0-17:0 (554.5507, 536.5401)	-	-
$C_{36}H_{73}NO_3$	d18:0-18:0 (568.5664, 550.5558)	-	-
$C_{37}H_{75}NO_3$	d18:0-19:0 (582.5820, 564.5714)	-	-
$C_{38}H_{77}NO_3$	d18:0-20:0 (596.5977, 578.5871)	-	-
$C_{39}H_{79}NO_3$	**d18:0-21:0** (610.6133, 592.6027)	-	-
$C_{40}H_{81}NO_3$	**d18:0-22:0** (624.6290, 606.6184)	-	-
$C_{41}H_{83}NO_3$	**d18:0-23:0** (638.6446, 620.6340)	-	-
$C_{42}H_{85}NO_3$	**d18:0-24:0** (652.6603, 634.6497)	-	-
$C_{43}H_{87}NO_3$	d18:0-25:0 (666.6759, 648.6653)	-	-
$C_{44}H_{89}NO_3$	d18:0-26:0 (680.6916, 662.6810)	-	-
1			
$C_{32}H_{63}NO_3$	d18:0-14:1 (510.4881, 492.4775)	**d18:1-14:0** (510.4881, 492.4775)	-
$C_{33}H_{65}NO_3$	**d18:0-15:1** (524.5038, 506.4932)	**d18:1-15:0** (524.5038, 506.4932)	-
$C_{34}H_{67}NO_3$	**d18:0-16:1** (538.5194, 520.5088)	**d18:1-16:0** (538.5194, 520.5088)	-
$C_{35}H_{69}NO_3$	**d18:0-17:1** (552.5351, 534.5245)	d18:1-17:0 (552.5351, 534.5245)	-
$C_{36}H_{71}NO_3$	**d18:0-18:1** (566.5507, 548.5401)	**d18:1-18:0** (566.5507, 548.5401)	-
$C_{37}H_{73}NO_3$	**d18:0-19:1** (580.5664, 562.5558)	d18:1-19:0 (580.5664, 562.5558)	-
$C_{38}H_{75}NO_3$	**d18:0-20:1** (594.5820, 576.5714)	**d18:1-20:0** (594.5820, 576.5714)	-
$C_{39}H_{77}NO_3$	**d18:0-21:1** (608.5977, 590.5871)	**d18:1-21:0** (608.5977, 590.5871)	-
$C_{40}H_{79}NO_3$	**d18:0-22:1** (622.6133, 604.6027)	**d18:1-22:0** (622.6133, 604.6027)	-
$C_{41}H_{81}NO_3$	**d18:0-23:1** (636.6290, 618.6184)	**d18:1-23:0** (636.6290, 618.6184)	-
$C_{42}H_{83}NO_3$	**d18:0-24:1** (650.6446, 632.6340)	**d18:1-24:0** (650.6446, 632.6340)	-

$C_{43}H_{85}NO_3$	**d18:0-25:1** (664.6603, 646.6497)	**d18:1-25:0** (664.6603, 646.6497)	-
$C_{44}H_{87}NO_3$	**d18:0-26:1** (678.6759, 660.6653)	**d18:1-26:0** (678.6759, 660.6653)	-
2			
$C_{32}H_{61}NO_3$	d18:0-14:2 (508.4725, 490.4619)	d18:1-14:1 (508.4725, 490.4619)	d18:2-14:0 (508.4725, 490.4619)
$C_{33}H_{63}NO_3$	d18:0-15:2 (522.4881, 504.4775)	d18:1-15:1 (522.4881, 504.4775)	d18:2-15:0 (522.4881, 504.4775)
$C_{34}H_{65}NO_3$	**d18:0-16:2** (536.5038, 518.4932)	**d18:1-16:1** (536.5038, 518.4932)	**d18:2-16:0** (536.5038, 518.4932)
$C_{35}H_{67}NO_3$	d18:0-17:2 (550.5194, 532.5088)	d18:1-17:1 (550.5194, 532.5088)	d18:2-17:0 (550.5194, 532.5088)
$C_{36}H_{69}NO_3$	**d18:0-18:2** (564.5351, 546.5245)	**d18:1-18:1** (564.5351, 546.5245)	**d18:2-18:0** (564.5351, 546.5245)
$C_{37}H_{71}NO_3$	d18:0-19:2 (578.5507, 560.5401)	d18:1-19:1 (578.5507, 560.5401)	d18:2-19:0 (578.5507, 560.5401)
$C_{38}H_{73}NO_3$	**d18:0-20:2** (592.5664, 574.5558)	**d18:1-20:1** (592.5664, 574.5558)	**d18:2-20:0** (592.5664, 574.5558)
$C_{39}H_{75}NO_3$	d18:0-21:2 (606.5820, 588.5714)	d18:1-21:1 (606.5820, 588.5714)	d18:2-21:0 (606.5820, 588.5714)
$C_{40}H_{77}NO_3$	d18:0-22:2 (620.5977, 602.5871)	**d18:1-22:1** (620.5977, 602.5871)	**d18:2-22:0** (620.5977, 602.5871)
$C_{41}H_{79}NO_3$	d18:0-23:2 (634.6133, 616.6027)	**d18:1-23:1** (634.6133, 616.6027)	**d18:2-23:0** (634.6133, 616.6027)
$C_{42}H_{81}NO_3$	d18:0-24:2 (648.6290, 630.6184)	**d18:1-24:1** (648.6290, 630.6184)	d18:2-24:0 (648.6290, 630.6184)
$C_{43}H_{83}NO_3$	**d18:0-25:2** (662.6446, 644.6340)	**d18:1-25:1** (662.6446, 644.6340)	**d18:2-25:0** (662.6446, 644.6340)
$C_{44}H_{85}NO_3$	**d18:0-26:2** (676.6603, 658.6497)	**d18:1-26:1** (676.6603, 658.6497)	**d18:2-26:0** (676.6603, 658.6497)
3			
$C_{42}H_{79}NO_3$	d18:0-24:3 (646.6133, 628.6027)	**d18:1-24:2** (646.6133, 628.6027)	**d18:2-24:1** (646.6133, 628.6027)
$C_{43}H_{81}NO_3$	d18:0-25:3 (660.6290, 642.6184)	**d18:1-25:2** (660.6290, 642.6184)	**d18:2-25:1** (660.6290, 642.6184)
$C_{44}H_{83}NO_3$	**d18:0-26:3** (674.6446, 656.6340)	**d18:1-26:2** (674.6446, 656.6340)	d18:2-26:1 (674.6446, 656.6340)
4			
$C_{44}H_{81}NO_3$	**d18:0-26:4** (672.6290, 654.6184)	**d18:1-26:3** (672.6290, 654.6184)	**d18:2-26:2** (672.6290, 654.6184)

Table S3: Glucose, fatty acid, and protein oxidation rates and total energy expenditures in low (LBW) and normal birth weight (NBW) men during the control (C) and high-fat, high-calorie (O) diets. Data are presented as mean values ± standard errors of means (SEM). P-values are presented unadjusted for multiple comparisons, and P-values ≤0.05 are considered statistically significant. P_{NBW} and P_{LBW}: O vs. C diet within each birth weight group, P_C and P_O: LBW vs. NBW men within each diet, P_Δ: LBW vs. NBW men on response values. P-values ≤0.05 are marked in bold. Day: 9 am-11 pm, Night: 11 pm-8 am, Sleep: 1 am-6 am, 24 hours: 9 am-9 am.
Abbreviations: EE: Energy expenditure, FOX: Fatty acid oxidation, GOX: Glucose oxidation, POX: Protein oxidation.

	NBW (n = 26)			**LBW** (C: n = 20, O: n = 18)			**LBW vs. NBW** (n = 20/n = 18, n = 26)		
(kJ/min)	**C** (Mean ± SEM)	**O** (Mean ± SEM)	**P_{NBW}**	**C** (Mean ± SEM)	**O** (Mean ± SEM)	**P_{LBW}**	**P_C**	**P_O**	**P_Δ**
Calorimetry 24 h									
GOX									
Day	3.85 ± 0.17	3.50 ± 0.08	**0.0297**	3.69 ± 0.16	3.30 ± 0.14	0.0609	0.52	0.19	0.94
Night	1.97 ± 0.10	2.07 ± 0.07	0.3126	1.78 ± 0.09	1.84 ± 0.10	0.3391	0.18	0.06	0.97
Sleep	1.91 ± 0.12	1.89 ± 0.08	0.9131	1.58 ± 0.10	1.77 ± 0.11	0.0836	**0.05**	0.37	0.21
24 h	3.10 ± 0.13	2.93 ± 0.07	0.1510	2.92 ± 0.13	2.73 ± 0.09	0.2620	0.34	0.09	0.97
FOX									
Day	3.34 ± 0.16	4.23 ± 0.14	**<0.0001**	3.46 ± 0.14	4.52 ± 0.21	**<0.0001**	0.60	0.23	0.60
Night	2.34 ± 0.10	2.80 ± 0.10	**0.0005**	2.60 ± 0.08	3.06 ± 0.12	**0.0023**	0.07	0.10	0.93
Sleep	2.14 ± 0.14	2.72 ± 0.12	**0.0001**	2.50 ± 0.09	2.87 ± 0.13	**0.0221**	**0.05**	0.38	0.40
24 h	2.92 ± 0.12	3.63 ± 0.12	**<0.0001**	3.11 ± 0.11	3.91 ± 0.14	**<0.0001**	0.24	0.14	0.76
POX									
Day	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
Night	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
Sleep	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
24 h	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
EE									
Day	8.32 ± 0.15	8.52 ± 0.13	**0.0142**	8.24 ± 0.16	8.56 ± 0.18	**0.0021**	0.71	0.86	0.39
Night	5.43 ± 0.09	5.65 ± 0.10	**0.0001**	5.46 ± 0.11	5.66 ± 0.13	**0.0017**	0.82	0.97	0.99
Sleep	5.17 ± 0.09	5.39 ± 0.09	**0.0010**	5.16 ± 0.11	5.30 ± 0.13	**0.0009**	0.96	0.93	0.82
24 h	7.14 ± 0.12	7.36 ± 0.12	**0.0005**	7.12 ± 0.14	7.38 ± 0.15	**0.0008**	0.88	0.90	0.55

Table S4: Associations between plasma ceramide levels and other lipid levels or physiological measures following the control (C) and high-fat, high-calorie (O) diets and between response values (Δ). Data are presented as r-values (+/– for positive or negative values, respectively) and P-values (+/–: P≤0.05, + +/– –: P≤0.01, + + +/– – –: P≤0.001, (+)/(–): P≤0.1 for positive or negative associations, respectively). P-values are presented unadjusted for multiple comparisons, and P-values ≤0.05 are considered statistically significant. Regression analyses were performed on the pooled data set of LBW and NBW men and were adjusted for age, BMI, and birth weight group. Abbreviations: See Table 1.

		d18:0-16:1/d18:1-16:0	d18:0-18:1/d18:1-18:0	d18:0-20:1/d18:1-20:0	d18:0-21:1/d18:1-21:0	d18:1-22:0	d18:0-23:1/d18:1-23:0	d18:0-24:1a	d18:1-24:0	d18:0-25:1 b/d18:1-25:0	d18:0-26:1/d18:1-26:0	d18:1-22:1/d18:2-22:0	d18:1-23:1/d18:2-23:0	d18:1-24:1	d18:0-25:2/d18:1-25:1/d18:2-25:0	d18:1-24:2/d18:2-24:1	Total ceramide
Lipid profiling																	
P-VLDL-CHOL	**C**	+	++		+	++	++	(+)	+		(+)		+	++		+++	++
	O									++	(+)			++		+++	+
	Δ																
P-LDL-CHOL	**C**	+++				++	+	+++						++	+	+++	++
	O		+++	+	+	+++	+++	+	+++	+++	+++	++	+	++	+	++	+++
	Δ		(+)			(+)	(+)	(+)	+		+					(+)	
P-HDL-CHOL	**C**																
	O														(–)		
	Δ	+		(+)		+	+		+	++	(+)			++			+
P-CHOL	**C**	+++				+++	++	++						++	(+)	+++	++
	O		+++	++	++	+++	+++	+	+++	+++	+++	++	++	+++	+	+++	+++
	Δ	(+)	(+)			+	+	(+)	+		+			+		+	(+)
P-TG	**C**	+	+++		+	++	++		(+)		+		+	++		+++	++
	O		++	++	+++	++	++	(+)	++	++	+	+++	+	+++	(+)	+++	++
	Δ																
Clamp																	
Basal																	
B-Glucose	**C**	+	++			(+)		+			++			++		+	+
	O													(+)			
	Δ									+							
S-Insulin	**C**					+	(+)				(+)						
	O																
	Δ	(+)															
P-NEFA	**C**		(–)		–								–	(–)		(–)	

	O													+		++	
	Δ				(+)						(+)						
HGP	**C**		+					+++			+				(+)	(+)	
	O																
	Δ																
Hepatic IR	**C**			(+)		+	(+)		(+)					(+)	(+)		+
	O	(+)															
	Δ														(+)		
GOX	**C**		–														
	O					(+)											
	Δ		–	(–)												–	
FOX	**C**	+	+	+		+	(+)		+		(+)			(+)		+	+
	O		(+)													+	
	Δ	(+)	(+)											(+)		(+)	
Insulin-stimulated																	
M-value	**C**	+															
	O																
	Δ		(+)						(+)			+		+			+
IVGTT																	
FPIR	**C**																
	O														(–)		
	Δ				(–)												
Hepatic DI	**C**							–									
	O			(–)								–			–	(–)	
	Δ														(–)		
Peripheral DI	**C**																
	O		(–)					(–)	(–)								(–)
	Δ																

Appendix 4 – Supplementary material to article 1

Table S1: **Protein, carbohydrate, and fat contents of the control (C) and high-fat, high-calorie (O) diets.**

	C		**O**		**O/C**	
	Total (Mean)	**Per 100 g** (Mean)	**Total** (Mean)	**Per 100 g** (Mean)	**Total** (Ratio)	**Per 100 g** (Ratio)
Energy (kJ)						
Total	9664	698	14848	1135	1.54	1.63
Energy (E%)						
Protein	15	15	8	8	0.53	0.53
Carbohydrate	49	49	33	33	0.67	0.67
Fat	35	35	60	60	1.71	1.71
Energy (g)						
Protein	88	6.4	67.1	5.1	0.76	0.80
Carbohydrate	266.7	19.2	277.6	21.2	1.04	1.10
Fat	92.1	6.6	239	18.3	2.60	2.77
Fat (g)						
Saturated fatty acids	35.6	2.6	109.8	8.4	3.08	3.23
Monounsaturated fatty acids	31.5	2.3	85.4	6.5	2.71	2.83
Polyunsaturated fatty acids	8.6	0.6	28.5	2.2	3.31	3.67
n-3 fatty acids	0.9	0.1	5.8	0.4	6.44	4.00
n-6 fatty acids	7.2	0.5	21.8	1.7	3.03	3.40

Table S2: Acylcarnitine names, molecular formulas, methyl and butyl ester ion mass to charge ratios, and internal standards (IS) used for quantifications.

	Names	Formulas	Methyl esters	Butyl esters	IS
			$[M+H]^+$	$[M+H]^+$	
Lipid profiling					
Acylcarnitines					
C2	Ethanoylcarnitine (Acetylcarnitine)	$C_9H_{17}NO_4$	218	260	C2-IS
C2-IS	D_3-Ethanoylcarnitine	$C_9D_3H_{14}NO_4$	221	263	-
C3	Propanoylcarnitine (Propionylcarnitine)	$C_{10}H_{19}NO_4$	232	274	C3-IS
C3-IS	D_3-Propanoylcarnitine	$C_{10}D_3H_{16}NO_4$	235	277	-
C4/ Ci4	Butanoylcarnitine (Butyrylcarnitine)/ Isobutanoylcarnitine (Isobutyrylcarnitine)	$C_{11}H_{21}NO_4$	246	288	C4-IS
C4-IS	D_3-Butanoylcarnitine	$C_{11}D_3H_{18}NO_4$	249	291	-
C5:1	Pentenoylcarnitine (Tiglylcarnitine)	$C_{12}H_{21}NO_4$	258	300	C5-IS
C5's	Pentanoylcarnitine (Valerylcarnitine)/ Isopentanoylcarnitine (Isovalerylcarnitine)	$C_{12}H_{23}NO_4$	260	302	C5-IS
C4-OH	3-Hydroxybutanoylcarnitine	$C_{11}H_{21}NO_5$	262	304	C4-IS
C5-IS	D_9-Isopentanoylcarnitine	$C_{12}D_3H_{20}NO_4$	269	311	-
C6	Hexanoylcarnitine (Caproylcarnitine)	$C_{13}H_{25}NO_4$	274	316	C8-IS
C5-OH/ C3-DC	3-Hydroxypentanoylcarnitine/ Propanedioylcarnitine (Malonylcarnitine)	$C_{12}H_{23}NO_5$/ $C_{10}H_{17}NO_6$	276/ 276	318/ 360	C8-IS
C4-DC/ Ci4-DC	Butanedioylcarnitine (Succinylcarnitine)/ Isobutanedioylcarnitine	$C_{11}H_{19}NO_6$	290	374	C4-IS
C8:1	Octenoylcarnitine	$C_{15}H_{27}NO_4$	300	342	C8-IS
C8	Octanoylcarnitine (Capryloylcarnitine)	$C_{15}H_{29}NO_4$	302	344	C8-IS
C5-DC	Pentanedioylcarnitine (Glutarylcarnitine)	$C_{12}H_{21}NO_6$	304	388	C8-IS
C8-IS	D_3-Octanoylcarnitine	$C_{15}D_3H_{26}NO_4$	305	347	-
C8:1-OH/ C6:1-DC	3-Hydroxyoctenoylcarnitine/ Hexenedioylcarnitine	$C_{15}H_{27}NO_5$/ $C_{13}H_{21}NO_6$	316/ 316	358/ 400	C8-IS
C6-DC	Hexanedioylcarnitine (Adipoylcarnitine)	$C_{13}H_{23}NO_6$	318	402	C8-IS
C10:3	Decatrienoylcarnitine	$C_{17}H_{27}NO_4$	324	366	C8-IS

C10:2	Decadienoylcarnitine	$C_{17}H_{29}NO_4$	326	368	C8-IS
C10:1	Decenoylcarnitine	$C_{17}H_{31}NO_4$	328	370	C8-IS
C10	Decanoylcarnitine (Caprylcarnitine)	$C_{17}H_{33}NO_4$	330	372	C8-IS
C7-DC	Heptanedioylcarnitine (Pimeloylcarnitine)	$C_{14}H_{25}NO_6$	332	416	C8-IS
C8:1-DC	Octenedioylcarnitine	$C_{15}H_{25}NO_6$	344	428	C8-IS
C10-OH/ C8-DC	3-Hydroxydecanoylcarnitine/ Octanedioylcarnitine (Suberoylcarnitine)	$C_{17}H_{33}NO_5$/ $C_{15}H_{27}NO_6$	346/ 346	388/ 430	C8-IS
C12:1	Dodecenoylcarnitine (Lauroleoylcarnitine)	$C_{19}H_{35}NO_4$	356	398	C8-IS
C12	Dodecanoylcarnitine (Lauroylcarnitine)	$C_{19}H_{37}NO_4$	358	400	C8-IS
C12-OH/ C10-DC	3-Hydroxydodecanoylcarnitine/ Decanedioylcarnitine (Sebacoylcarnitine)	$C_{19}H_{37}NO_5$/ $C_{17}H_{31}NO_6$	374/ 374	416/ 458	C16-IS
C14:2	Tetradecadienoylcarnitine	$C_{21}H_{37}NO_4$	382	424	C16-IS
C14:1	Tetradecenoylcarnitine (Myristoleoylcarnitine)	$C_{21}H_{39}NO_4$	384	426	C16-IS
C14	Tetradecanoylcarnitine (Myristoylcarnitine)	$C_{21}H_{41}NO_4$	386	428	C16-IS
C14:1-OH/ C12:1-DC	3-Hydroxytetradecenoylcarnitine/ Dodecenedioylcarnitine	$C_{21}H_{39}NO_5$/ $C_{19}H_{33}NO_6$	400/ 400	442/ 484	C16-IS
C14-OH/ C12-DC	3-Hydroxytetradecanoylcarnitine/ Dodecanedioylcarnitine	$C_{21}H_{41}NO_5$/ $C_{19}H_{35}NO_6$	402/ 402	444/ 486	C16-IS
C16:2	Hexadecadienoylcarnitine (Palmitolinoleoylcarnitine)	$C_{23}H_{41}NO_4$	410	452	C16-IS
C16:1	Hexadecenoylcarnitine (Palmitoleoylcarnitine)	$C_{23}H_{43}NO_4$	412	454	C16-IS
C16	Hexadecanoylcarnitine (Palmitoylcarnitine)	$C_{23}H_{45}NO_4$	414	456	C16-IS
C16-IS	D_3-Hexadecanoylcarnitine	$C_{23}D_3H_{42}NO_4$	417	459	-
C16:1-OH/ C14:1-DC	3-Hydroxyhexadecenoylcarnitine/ Tetradecenedioylcarnitine	$C_{23}H_{43}NO_5$/ $C_{21}H_{37}NO_6$	428/ 428	470/ 512	C16-IS
C16-OH/ C14-DC	3-Hydroxyhexadecanoylcarnitine/ Tetradecanedioylcarnitine	$C_{23}H_{45}NO_5$/ $C_{21}H_{39}NO_6$	430/ 430	472/ 514	C16-IS
C18:2	Octadecadienoylcarnitine (Linoleoylcarnitine)	$C_{25}H_{45}NO_4$	438	480	C16-IS
C18:1	Octadecenoylcarnitine (Oleylcarnitine)	$C_{25}H_{47}NO_4$	440	482	C16-IS
C18	Octadecanoylcarnitine (Stearoylcarnitine)	$C_{25}H_{49}NO_4$	442	484	C16-IS
C18:2-OH	3-Hydroxyoctadecadienoylcarnitine	$C_{25}H_{45}NO_5$	454	496	C16-IS
C18:1-OH/ C16:1-DC	3-Hydroxyoctadecenoylcarnitine/ Hexadecenedioylcarnitine	$C_{25}H_{47}NO_5$/ $C_{23}H_{41}NO_6$	456/ 456	498/ 540	C16-IS

C18-OH/ C16-DC	3-Hydroxyoctadecanoylcarnitine/ Hexadecanedioylcarnitine	$C_{25}H_{49}NO_5$/ $C_{23}H_{43}NO_6$	458/ 458	500/ 542	C16-IS
C20:4	Eicosatetraenoylcarnitine (Arachidonoylcarnitine)	$C_{27}H_{45}NO_4$	462	504	C16-IS
C20	Eicosanoylcarnitine (Arachidoylcarnitine)	$C_{27}H_{53}NO_4$	470	512	C16-IS
C20:1-OH/ C18:1-DC	3-Hydroxyeicosenoylcarnitine/ Octadecenedioylcarnitine	$C_{27}H_{51}NO_5$/ $C_{25}H_{45}NO_6$	484/ 484	526/ 568	C16-IS
C20-OH/ C18-DC	3-Hydroxyeicosanoylcarnitine/ Octadecanedioylcarnitine	$C_{27}H_{53}NO_5$/ $C_{25}H_{47}NO_6$	486/ 486	528/ 570	C16-IS
C22	Docosanoylcarnitine (Behenoylcarnitine)	$C_{29}H_{57}NO_4$	498	540	C16-IS

Appendix 5 – Supplementary material to article 2

Table S1: **Protein, carbohydrate, and fat contents of the control (C) and high-fat, high-calorie (O) diets.**

	C		**O**		**O/C**	
	Total (Mean)	**Per 100 g** (Mean)	**Total** (Mean)	**Per 100 g** (Mean)	**Total** (Ratio)	**Per 100 g** (Ratio)
Energy (kJ)						
Total	9664	698	14848	1135	1.54	1.63
Energy (E%)						
Protein	15	15	8	8	0.53	0.53
Carbohydrate	49	49	33	33	0.67	0.67
Fat	35	35	60	60	1.71	1.71
Energy (g)						
Protein	88	6.4	67.1	5.1	0.76	0.80
Carbohydrate	266.7	19.2	277.6	21.2	1.04	1.10
Fat	92.1	6.6	239	18.3	2.60	2.77
Fat (g)						
Saturated fatty acids	35.6	2.6	109.8	8.4	3.08	3.23
Monounsaturated fatty acids	31.5	2.3	85.4	6.5	2.71	2.83
Polyunsaturated fatty acids	8.6	0.6	28.5	2.2	3.31	3.67
n-3 fatty acids	0.9	0.1	5.8	0.4	6.44	4.00
n-6 fatty acids	7.2	0.5	21.8	1.7	3.03	3.40

Table S2: Glucose, fatty acid, and protein oxidation rates and total energy expenditures in low (LBW) and normal birth weight (NBW) men during the control (C) and high-fat, high-calorie (O) diets. Data are presented as mean ± SEM. *P*-values from Student's *t*-tests are presented unadjusted for multiple comparisons, and *P*-values ≤ 0.05 are considered statistically significant. P_{NBW} and P_{LBW}: O versus C diet within each birth weight group, P_C and P_O: LBW versus NBW individuals within each diet, P_Δ: LBW versus NBW individuals on response values. *P*-values ≤ 0.05 are marked in bold. Details on the measurements have been described in previous articles (10; 11). Abbreviations: EE, Energy expenditure; FOX, Fatty acid oxidation; GOX, Glucose oxidation; POX, Protein oxidation.

	NBW (*n* = 26)			**LBW** (C: *n* = 20, O: *n* = 18)			**LBW versus NBW** (*n* = 20/*n* = 18, *n* = 26)		
(kJ/min)	**C** (Mean ± SEM)	**O** (Mean ± SEM)	P_{NBW}	**C** (Mean ± SEM)	**O** (Mean ± SEM)	P_{LBW}	P_C	P_O	P_Δ
Calorimetry 24 h									
GOX									
Day	3.85 ± 0.17	3.50 ± 0.08	**0.0297**	3.69 ± 0.16	3.30 ± 0.14	0.0609	0.52	0.19	0.94
Night	1.97 ± 0.10	2.07 ± 0.07	0.3126	1.78 ± 0.09	1.84 ± 0.10	0.3391	0.18	0.06	0.97
Sleep	1.91 ± 0.12	1.89 ± 0.08	0.9131	1.58 ± 0.10	1.77 ± 0.11	0.0836	**0.05**	0.37	0.21
24 h	3.10 ± 0.13	2.93 ± 0.07	0.1510	2.92 ± 0.13	2.73 ± 0.09	0.2620	0.34	0.09	0.97
FOX									
Day	3.34 ± 0.16	4.23 ± 0.14	**<0.0001**	3.46 ± 0.14	4.52 ± 0.21	**<0.0001**	0.60	0.23	0.60
Night	2.34 ± 0.10	2.80 ± 0.10	**0.0005**	2.60 ± 0.08	3.06 ± 0.12	**0.0023**	0.07	0.10	0.93
Sleep	2.14 ± 0.14	2.72 ± 0.12	**0.0001**	2.50 ± 0.09	2.87 ± 0.13	**0.0221**	**0.05**	0.38	0.40
24 h	2.92 ± 0.12	3.63 ± 0.12	**<0.0001**	3.11 ± 0.11	3.91 ± 0.14	**<0.0001**	0.24	0.14	0.76
POX									
Day	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
Night	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
Sleep	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
24 h	1.13 ± 0.04	0.79 ± 0.03	**<0.0001**	1.08 ± 0.04	0.74 ± 0.04	**<0.0001**	0.48	0.32	0.71
EE									
Day	8.32 ± 0.15	8.52 ± 0.13	**0.0142**	8.24 ± 0.16	8.56 ± 0.18	**0.0021**	0.71	0.86	0.39
Night	5.43 ± 0.09	5.65 ± 0.10	**0.0001**	5.46 ± 0.11	5.66 ± 0.13	**0.0017**	0.82	0.97	0.99
Sleep	5.17 ± 0.09	5.39 ± 0.09	**0.0010**	5.16 ± 0.11	5.30 ± 0.13	**0.0009**	0.96	0.93	0.82
24 h	7.14 ± 0.12	7.36 ± 0.12	**0.0005**	7.12 ± 0.14	7.38 ± 0.15	**0.0008**	0.88	0.90	0.55