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

Amalie Ribel-Madsen1,2, Rasmus Ribel-Madsen2,3, Kristian Fog Nielsen4, Susanne Brix5,6, Allan A. Vaag2 & Charlotte Brøns2

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.

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) individuals1-4. Accordingly, we have shown that young, healthy, LBW men have higher fasting blood glucose and serum insulin levels5, display impaired hepatic insulin sensitivity6, and, in contrast to NBW men, develop impaired peripheral insulin sensitivity in response to 5-day high-fat overfeeding8. 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 overfeeding8, 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 lipolysis8-9. 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 capacity10. 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 men10. Moreover,

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the LBW men show an increased fatty acid oxidation rate and a higher fasting plasma acetyl carnitine level, 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- and dicarboxyl-acetyl carnitine 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. 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. 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, and the extent of it has been linked to pancreatic beta-cell dysfunction and insulin resistance. Also, individuals with type 2 diabetes have elevated plasma ceramide levels. Importantly, obese individuals with type 2 diabetes have a higher plasma LDL-ceramide level compared with obese, insulin-sensitive individuals, indicating that an increased ceramide synthesis in the liver may contribute to the development of insulin resistance. 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. 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. 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. 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. Furthermore, ceramides activate Jun and NF-KB transcription factors and may thereby enhance inflammatory responses that may interfere with insulin signalling as well. A number of studies have focused on specific actions of individual ceramides, including dihydroceramides, on different cellular processes. In this regard, d18:1–16:0 ceramide has been suggested to be the principal mediator of diet-induced insulin resistance. 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. Furthermore, both birth weight groups displayed several changes in metabolism in response to the overfeeding challenge, as also published previously. 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. LBW men had higher fasting blood glucose (P ≤ 0.01) and serum insulin (P ≤ 0.01) levels after the control diet compared with NBW men. Also, both LBW and NBW men increased the fasting blood glucose level (P ≤ 0.05 and P ≤ 0.001, respectively) in response to overfeeding, and NBW men additionally increased the fasting serum insulin level (P ≤ 0.05) due to this challenge. LBW men had a higher hepatic insulin resistance index (P ≤ 0.05) after the control diet compared with NBW men, and NBW men, but not LBW men, showed an increase in the hepatic glucose production (P ≤ 0.01) and in the hepatic insulin resistance index (P ≤ 0.001) in response to overfeeding. LBW and NBW men did not show a different insulin-stimulated glucose infusion rate (M-value) after the control diet. LBW men, however, in contrast to NBW men, decreased this rate (P ≤ 0.05) in reaction to overfeeding. In terms of plasma lipid profiles, LBW men had a lower fasting plasma HDL-cholesterol level (P ≤ 0.01 and P ≤ 0.05, respectively) after both the control and high-fat, high-calorie diets compared with NBW men. Furthermore, both LBW and NBW men decreased fasting plasma total NEFA (both P ≤ 0.001), VLDL-cholesterol (P ≤ 0.01 and P ≤ 0.05, respectively), and total triacylglycerol (P ≤ 0.01 and P ≤ 0.05, respectively) levels and increased the HDL-cholesterol level (both P ≤ 0.01) in response to overfeeding. 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. 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 (Supplementary Table S3). 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). 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) (Supplementary Table S3).

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
<table>
<thead>
<tr>
<th></th>
<th>NBW (n=25)</th>
<th>O (Mean ± SD)</th>
<th>F_PBW</th>
<th>LBW (n=18)</th>
<th>O (Mean ± SD)</th>
<th>F_PBW</th>
<th>LBW vs. NBW (n=18, n=25)</th>
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<tbody>
<tr>
<td><strong>Anthropometry</strong></td>
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<tr>
<td>Birth weight (g)</td>
<td>3901 ± 207</td>
<td>2717 ± 268</td>
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<td>≤0.001</td>
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<td>Weight (kg)</td>
<td>78.4 ± 9.3</td>
<td>77.1 ± 11.3</td>
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<td></td>
<td>n.s.</td>
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<td>Height (m)</td>
<td>1.83 ± 0.07</td>
<td>1.77 ± 0.05</td>
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<td>≤0.05</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>23.3 ± 2.4</td>
<td>24.6 ± 3.8</td>
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<td><strong>Lipid profiling</strong></td>
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<tr>
<td>P-VLDL-CHOL (mM)</td>
<td>0.42 ± 0.16</td>
<td>0.49 ± 0.18</td>
<td>≤0.05</td>
<td>0.32 ± 0.12</td>
<td>≤0.01</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>P-LDL-CHOL (mM)</td>
<td>2.51 ± 0.72</td>
<td>2.69 ± 0.76</td>
<td>≤0.05</td>
<td>2.57 ± 0.80</td>
<td>≤0.05</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>P-HDL-CHOL (mM)</td>
<td>1.40 ± 0.22</td>
<td>1.91 ± 0.23</td>
<td>≤0.01</td>
<td>1.38 ± 0.28</td>
<td>≤0.01</td>
<td>≤0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>M-NEFA (µM)</td>
<td>3.34 ± 136</td>
<td>406 ± 200</td>
<td>≥0.001</td>
<td>188 ± 91</td>
<td>≤0.001</td>
<td>n.s.</td>
<td>≤0.05</td>
</tr>
<tr>
<td>HGP (mg/kg·FFM/min)</td>
<td>2.21 ± 0.48</td>
<td>2.40 ± 0.50</td>
<td>≤0.01</td>
<td>2.48 ± 0.50</td>
<td>≤0.05</td>
<td>n.s.</td>
<td>≤0.05</td>
</tr>
<tr>
<td>Hepatic IR (mg/kg·FFM/min)</td>
<td>68.7 ± 34.1</td>
<td>102.3 ± 50.8</td>
<td>≤0.001</td>
<td>108.7 ± 55.5</td>
<td>≤0.05</td>
<td>n.s.</td>
<td>≤0.05</td>
</tr>
<tr>
<td>GOX (mg/kg·FFM/min)</td>
<td>2.34 ± 0.76</td>
<td>1.95 ± 0.78</td>
<td>n.s.</td>
<td>2.20 ± 0.56</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>FOX (mg/kg·FFM/min)</td>
<td>1.00 ± 0.38</td>
<td>1.11 ± 0.53</td>
<td>n.s.</td>
<td>1.17 ± 0.33</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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<td><strong>Clamp</strong></td>
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<td><strong>Basal</strong></td>
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<tr>
<td>B-Glucose (mM)</td>
<td>4.59 ± 0.47</td>
<td>4.97 ± 0.48</td>
<td>≤0.001</td>
<td>5.18 ± 0.34</td>
<td>≤0.05</td>
<td>n.s.</td>
<td>n.s.</td>
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<tr>
<td>S-Insulin (pM)</td>
<td>30.2 ± 14.7</td>
<td>41.7 ± 14.6</td>
<td>≤0.05</td>
<td>44.7 ± 21.9</td>
<td>≤0.05</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>P-NEFA (µM)</td>
<td>3.34 ± 136</td>
<td>406 ± 200</td>
<td>≥0.001</td>
<td>188 ± 91</td>
<td>≤0.001</td>
<td>n.s.</td>
<td>≤0.05</td>
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<td><strong>Insulin-stimulated</strong></td>
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<tr>
<td>M-value (mg/kg·FFM/min)</td>
<td>13.73 ± 2.32</td>
<td>13.47 ± 3.14</td>
<td>≤0.05</td>
<td>11.89 ± 3.57</td>
<td>≤0.05</td>
<td>n.s.</td>
<td>≤0.05</td>
</tr>
<tr>
<td>GOX (mg/kg·FFM/min)</td>
<td>5.18 ± 0.82</td>
<td>4.95 ± 0.92</td>
<td>n.s.</td>
<td>4.78 ± 0.82</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>FOX (mg/kg·FFM/min)</td>
<td>0.01 ± 0.25</td>
<td>0.13 ± 0.46</td>
<td>n.s.</td>
<td>0.37 ± 0.35</td>
<td>≤0.05</td>
<td>≤0.05</td>
<td>n.s.</td>
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<tr>
<td><strong>IVGTT</strong></td>
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<tr>
<td>FPIR (pM)</td>
<td>1894 ± 1431</td>
<td>2135 ± 1034</td>
<td>≤0.001</td>
<td>2750 ± 1509</td>
<td>≤0.01</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Hepatic DI</td>
<td>0.38 ± 0.63</td>
<td>0.21 ± 0.11</td>
<td>n.s.</td>
<td>0.24 ± 0.13</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>Peripheral DI</td>
<td>0.29 ± 0.19</td>
<td>0.33 ± 0.13</td>
<td>≤0.05</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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</table>

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_PBW and P_O vs. C diet within each birth weight group, P_C and P_O: LBW vs. NBW men within each diet, P_C: 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.

(P = 0.0004 and P < 0.0001, respectively) and d18:1–22:1/d18:2–22:0 (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 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). 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 = 0.0019), 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),...
<table>
<thead>
<tr>
<th>Lipid profiling</th>
<th>Ceramides</th>
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<tbody>
<tr>
<td>NBW (n = 25)</td>
<td>LBW (n = 18)</td>
</tr>
<tr>
<td>(μM)</td>
<td>C (Mean, CI)</td>
</tr>
<tr>
<td>0.39 (0.25, 0.54)</td>
<td>0.39 (0.38, 0.41)</td>
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<tr>
<td>0.31 (0.29, 0.33)</td>
<td>0.30 (0.11, 0.50)</td>
</tr>
<tr>
<td>0.42 (0.42, 0.42)</td>
<td>0.41 (0.40, 0.42)</td>
</tr>
<tr>
<td>0.68 (0.65, 0.72)</td>
<td>0.64 (0.60, 0.67)</td>
</tr>
<tr>
<td>0.54 (0.51, 0.58)</td>
<td>0.47 (0.45, 0.50)</td>
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<tr>
<td>0.26 (0.25, 0.27)</td>
<td>0.27 (0.22, 0.33)</td>
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<tr>
<td>0.40 (0.39, 0.43)</td>
<td>0.37 (0.37, 0.40)</td>
</tr>
<tr>
<td>0.93 (0.86, 1.01)</td>
<td>0.96 (0.89, 1.04)</td>
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<tr>
<td>0.34 (0.32, 0.37)</td>
<td>0.39 (0.35, 0.42)</td>
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<tr>
<td>0.7598 0.5826 0.6266</td>
<td>0.8432 0.6164 0.5904</td>
</tr>
</tbody>
</table>

**Continued**
amid (P = 0.0487), d18:1–24:1 (P = 0.0343), 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 d18:0-1/28:0, d18:1-1/24:0, and d18:1-24:1 levels 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 likely be a result of their markedly increases in fatty acid oxidation rates and total energy expenditures due to this challenge\(^ {15}\).

Several studies have reported altered plasma and/or tissue ceramide levels in mice exposed to high-fat feeding\(^ {6–11}\), and a recent study has described changes in serum ceramide levels in human subjects exposed to high-fat overfeeding\(^ {28}\). 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.

<table>
<thead>
<tr>
<th>(µM)</th>
<th>NBW (n = 25)</th>
<th>LBW (n = 18)</th>
<th>LBW vs. NBW (n = 18, n = 25)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>C (Mean, CI)</td>
<td>O (Mean, CI)</td>
<td>P(<em>{\text{ANW}}) Q(</em>{\text{ANW}})</td>
</tr>
<tr>
<td>d18:0–26:2/ d18:1–26:1/ d18:2–26:0</td>
<td>0.25 (0.23, 0.28)</td>
<td>0.23 (0.22, 0.24)</td>
<td>—</td>
</tr>
<tr>
<td>d18:1–24:2/ d18:2–24:1</td>
<td>0.87 (0.77, 0.97)</td>
<td>0.66 (0.55, 0.76)</td>
<td>0.0003 (n = 24)</td>
</tr>
<tr>
<td>d18:0–26:3/ d18:1–26:2</td>
<td>0.27 (0.24, 0.31)</td>
<td>0.31 (0.17, 0.45)</td>
<td>—</td>
</tr>
<tr>
<td>d18:0–26:4/ d18:1–26:3/ d18:2–26:2</td>
<td>0.15 (0.13, 0.17)</td>
<td>0.16 (0.09, 0.32)</td>
<td>—</td>
</tr>
<tr>
<td>Total levels</td>
<td>0.53 (0.39, 0.66)</td>
<td>0.70 (0.53, 0.87)</td>
<td>0.0778 (n = 25)</td>
</tr>
</tbody>
</table>

**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\(_{\text{ANW}}\) and P\(_{\text{QANW}}\) O vs. C diet within each birth weight group, P\(_{\text{O}}\) and P\(_{\text{N}}\) 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).
### 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 ($+/-$: $P \leq 0.05$, $++/-$: $P \leq 0.01$, $+++/-$: $P \leq 0.001$, $(+)/(−)$: $P \leq 0.1$ 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 $\leq 0.05$ 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.
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. 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. 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. Also, adiponectin knockout mice are refractory to FGF-21 effects, including lowering of ceramide levels. Therefore, it was concluded that FGF-21 depends on adiponectin to exert its insulin-sensitising effects.

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. 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. The latter was caused by an inhibition of the insulin-stimulated phosphorylation of Akt/PKB and glycogen synthase kinase. 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. 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-intervention. 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. 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. 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. Also, adiponectin knockout mice are refractory to FGF-21 effects, including lowering of ceramide levels. Therefore, it was concluded that FGF-21 depends on adiponectin to exert its insulin-sensitising effects.

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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 publications5,6,11,12,32.

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/m²), 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). 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 level60, and dietary calculations were made in Dankost Pro (The National Food Agency, Copenhagen, Denmark).

Clinical activities. 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 previously60. 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 previously5,6,11,32. 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).

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 C₈-column (2.1 × 100 mm, 2.7 µm) (Phenomenex, Torrance, CA, USA). Cholesterol esters 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, and 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 C₈-column (2.1 × 100 mm, 2.7 µm) (Phenomenex) at 60 °C by elution with a water with 20 mM formic acid (A)-1:4 iso-propanol:acetonitrile (B) gradient. An elution of (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 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 previously5,6,11,32. Blood samples were collected prior to and during the clamp.
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–H31, standard (Avanti Polar Lipids, Alabaster, AL, USA) solutions (1:2 chloroform:niso-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–H31 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. 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 ≤ 0.05 with Q-values ≤ 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 Fig. 1 and Supplementary Table S4, 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 ≤ 0.05 were considered statistically significant. Data in Fig. 1 and Supplementary Table S4 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. contributed to the ceramide analyses, S.B. contributed to the discussion of the results, A.A.V. contributed to the ceramide analyses, and C.B. performed the diet interventions and clinical tests. All authors reviewed the manuscript.

Additional Information
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