Plasma Metabolomics Reveal Alterations of Sphingo- and Glycerophospholipid Levels in Non-Diabetic Carriers of the Transcription Factor 7-Like 2 Polymorphism rs7903146

Cornelia Then1,2,6, Simone Wahl16, Anna Kirchhofer1, Harald Grallert2,3, Susanne Krug4,5, Gabi Kastenmüller6, Werner Römisch-Margl1, Melina Claussnitzer4,5, Thomas Illig7, Margit Heier8, Christa Meisinger8, Jerzy Adamski9, Barbara Thorand8,9, Cornelia Huth8,9, Annette Peters3,8, Cornelia Prehn10, Ina Heukamp1, Helmut Laumen4,5, Andreas Lechner1,2, Hans Hauner4,5, Jochen Seissler1,2

1 Medizinische Klinik und Poliklinik IV, Diabetes Zentrum - Campus Innenstadt, Klinikum der Universität München, Munich, Germany, 2 Clinical Cooperation Group Diabetes, Ludwig-Maximilians-Universität München and Helmholtz Zentrum München, Munich, Germany, 3 Research Unit of Molecular Epidemiology, German Research Center for Environmental Health, Neuherberg, Germany, 4 Else-Kröner-Fresenius-Centre for Nutritional Medicine, ZIEL - Research Centre for Nutrition and Food Sciences, Technical University München, Freising-Weihenstephan, Germany, 5 Clinical Cooperation Group Nutrigenomics and Type 2 Diabetes, Technical University München and Helmholtz Zentrum München, Munich, Germany, 6 Institute of Bioinformatics and Systems Biology, Helmholtz Zentrum München, Neuherberg, Germany, 7 Hannover Unified Biobank, Hannover Medical School, Hannover, Germany, 8 Institute of Epidemiology II, Helmholtz Zentrum München – German Research Center for Environmental Health (GmbH), Neuherberg, Germany, 9 German Center for Diabetes Research (DZD), Neuherberg, Germany, 10 Institute of Experimental Genetics, Genome Analysis Center, Helmholtz Zentrum München, Neuherberg, Germany.

Abstract

**Aims/Hypothesis:** Polymorphisms in the transcription factor 7-like 2 (TCF7L2) gene have been shown to display a powerful association with type 2 diabetes. The aim of the present study was to evaluate metabolic alterations in carriers of a common TCF7L2 risk variant.

**Methods:** Seventeen non-diabetic subjects carrying the T risk allele at the rs7903146 TCF7L2 locus and 24 subjects carrying no risk allele were submitted to intravenous glucose tolerance test and euglycemic-hyperinsulinemic clamp. Plasma samples were analysed for concentrations of 163 metabolites through targeted mass spectrometry.

**Results:** TCF7L2 risk allele carriers had a reduced first-phase insulin response and normal insulin sensitivity. Under fasting conditions, carriers of TCF7L2 rs7903146 exhibited a non-significant increase of plasma sphingomyelins (SMs), phosphatidylylcholines (PCs) and lysophosphatidylylcholines (lysoPCs) species. A significant genotype effect was detected in response to challenge tests in 6 SMs (C16:0, C16:1, C18:0, C18:1, C24:0, C24:1), 5 hydroxy-SMs (C14:1, C16:1, C22:1, C22:2, C24:1), 4 lysoPCs (C14:0, C16:0, C16:1, C17:0), 3 diacyl-PCs (C28:1, C36:6, C40:4) and 4 long-chain acyl-alkyl-PCs (C40:2, C40:5, C44:5, C44:6).

**Discussion:** Plasma metabolomic profiling identified alterations of phospholipid metabolism in response to challenge tests in subjects with TCF7L2 rs7903146 genotype. This may reflect a genotype-mediated link to early metabolic abnormalities prior to the development of disturbed glucose tolerance.

Citation: Then C, Wahl S, Kirchhofer A, Grallert H, Krug S, et al. (2013) Plasma Metabolomics Reveal Alterations of Sphingo- and Glycerophospholipid Levels in Non-Diabetic Carriers of the Transcription Factor 7-Like 2 Polymorphism rs7903146. PLoS ONE 8(10): e78430. doi:10.1371/journal.pone.0078430

Copyright: © 2013 Then et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The KORA research platform studies were initiated and financed by the Helmholtz Zentrum München—National Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Science, Research and Technology and by the State of Bavaria. The study was supported by a research grant from the Virtual Diabetes Institute (Helmholtz Zentrum München), the Clinical Cooperation Group LMU (Ludwig-Maximilians-Universität München - Helmholtz Zentrum Muenchen), the Clinical Cooperation Group TÜ (Else Kröner-Fresenius-Center for Nutritional Medicine, Technische Universität München - Helmholtz Zentrum München) and in part by the German Center for Diabetes Research (DZD e.V.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

* E-mail: jochen.seissler@med.uni-muenchen.de

☯ These authors contributed equally to this work.
**Introduction**

Type 2 diabetes mellitus (T2D) is a multifactorial disease resulting from a complex interaction between environment, adverse health behaviour and genetic risk factors, which may affect β-cell function and/or insulin resistance. Genome-wide association studies including thousands of cases and controls described an association of 56 single nucleotide polymorphisms (SNPs) with T2D susceptibility [1]. The contribution of most gene polymorphisms appears to be small. However, certain loci have substantial impact on the risk of T2D. Variants in the transcription factor 7-like 2 (TCF7L2) locus at 10q25.2 have been identified as the strongest common genetic risk factors for T2D in Caucasians [1,2]. TCF7L2 SNPs primarily affect insulin secretion and hepatic insulin sensitivity [3,4]. A reduced GLP-1-induced insulin secretion in response to oral glucose was detected in TCF7L2 risk allele carriers [3,5–7]. TCF7L2 SNPs were shown to affect GLP-1 responsiveness of β-cells [3,7], indicating an impaired incretin effect as one of the factors contributing to decreased insulin secretion in TCF7L2 risk allele carriers. Other studies demonstrated that TCF7L2 has an important role for vital functions in islet cells [8]. Recently, it has been shown that tissue-specific alternative splicing patterns of TCF7L2 mRNA variants determine insulin sensitivity of adipose tissue and are involved in the regulation of hepatic gluconeogenesis [9–11].

However, the underlying molecular and cellular mechanisms of progression of insulin secretion deficiency and other potential metabolic alterations related to TCF7L2 SNPs are incompletely understood.

Metabolomics approaches have been successfully used to distinguish normal glucose tolerant probands and patients with impaired glucose tolerance or T2D [12–15]. Previous work focused mainly on the relationship of metabolomic alterations and insulin resistance and results are consistent with effects explained by impaired insulin action, such as diminished plasma amino acids due to increased amino acid oxidation [12] or inadequate suppression of lipolysis [16].

Prediction and prevention of T2D would benefit from a better understanding of the pathophysiological mechanisms involved in prediabetes and the identification of metabolic alterations associated with diabetes risk in a state when glucose homeostasis is still normal. Therefore, in the present study, we recruited non-diabetic probands carrying the TCF7L2 polymorphism rs7903146 and compared fasting plasma metabolite levels and changes in defined metabolite concentrations in response to challenge tests.

**Methods**

**Study participants**

Forty-one male subjects of western European descent were recruited from the population-based KORA (Cooperative Health Research in the Region of Augsburg, southern Germany) cohort [17]. All study participants gave written informed consent and the study was approved by the Ethics Committee of the Bavarian Medical Association. Eight participants were homozygous (TT) and nine heterozygous (CT) carriers of the TCF7L2 rs7903146 allele, and 24 participants carrying no risk allele (CC) with similar age and BMI served as control subjects. Genotyping of TCF7L2 was performed as described previously [18].

39 subjects had normal glucose tolerance (75 g oral glucose tolerance test [oGTT]), one control subject had disturbed glucose tolerance and in one carrier of the TCF7L2 rs7903146 allele with normal fasting glucose and normal HbA1c, the 2 h-glucose level was missing. Plasma samples from oGTT were not available in many probands. Probands did not take any medication known to affect insulin sensitivity or secretion. Participants underwent standard procedures including physical examination, assessment of medical history, measurement of blood pressure, and anthropometric measurements of weight, height and waist circumference. Neither the participant nor any of the attending physicians or assistants conducting the intravenous glucose tolerance test (ivGTT) and the euglycemic-hyperinsulinemic (EH) clamp knew the genotype of the probands at the time of the test procedure.

**Metabolic challenge tests**

All metabolic challenge tests were carried out between 8:00 and 9:00 a.m. after a 10 to 12 hour fasting period and after baseline blood samples were obtained. Participants underwent an intravenous glucose tolerance test (ivGTT) receiving an i.v. bolus of 0.33 g glucose/kg body weight of a 50 % (vol/vol) glucose solution within two minutes. Venous blood samples from the opposite arm were obtained at 1, 3, 5, 10, 15, 25 and 35 minutes for determination of insulin and proinsulin concentrations. The sample taken at 35 minutes (t35) was also kept for metabolomics measurement. Subsequently, the EH clamp was initiated by an insulin bolus followed by a continuous insulin infusion (1.05 mIU/kg/hour of short-acting human insulin) and a variable infusion of a 20 % glucose infusion to maintain the plasma glucose concentration at 80 mg/dl for 120 minutes. Blood samples for determination of plasma glucose were obtained at 6 minute intervals throughout the clamp and measured using a bedside glucose analyser (Super-GL Ambulance, HITADO, Möhnesee, Germany). 45 min after steady state conditions were reached (about 3-4 hours after the start of the ivGTT), venous blood samples were drawn for metabolomics measurement (t240).

**Laboratory measurements**

All blood samples were immediately cooled to 4°C and centrifuged at 3000 g for 10 min. Aliquots of plasma samples were stored at -80°C until assayed. Plasma glucose levels were assessed using the hexokinase method (GLU Flex, Dade Behring, Marburg, Germany). Total cholesterol concentrations, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels were measured with enzymatic methods (CHOD-PAP, Dade Behring). Triglycerides were measured by an enzymatic color test (GPO-PAP-method, TGL Flex, Dade Behring). Serum creatinine concentrations were assessed with a modified Jaffe test (KREA Flex, Dade Behring). High sensitive C-reactive protein (hsCRP) was determined by IRMA (Dade Behring). HbA1c was measured using the HPLC method. Plasma proinsulin and insulin concentrations were quantified via a chemiluminescence immunoassay (Proinsulin Flex, Dade Behring, Marburg, Germany).
with ELISA Kits (LINCO research, St. Charles, USA) as described recently [19,20]. Serum C-peptide was determined with the radioimmunoassay from Radim Diagnostics (Pomezia, Italy). Total levels of non-esterified free fatty acids (NEFAs) were measured using the Wako NEFA-HR(2) test (Wako Chemicals GmbH, Neuss, Germany).

**Targeted Metabolomics**

Plasma samples from three different time points were subjected to metabolomics measurement (t0, fasting sample; t35, post-ivGTT; t240, clamp-steady state). Metabolite concentrations were determined using the targeted metabolomics kit Absolute/DQ™ p150 (Biocrates Life Sciences AG, Innsbruck, Austria) applying mass spectrometric analysis as described previously [21]. The metabolomics data set contains hexose, 14 amino acids, free carnitine (C0), 40 acylcarnitines, hydroxylacylcarnitines and dicarboxylacylcarnitines, 15 sphingomyelins, 77 phosphatidylcholines, 15 lysophosphatidylcholines (complete list of the analysed metabolites is shown in Table S1). Potential plate effects were addressed by multiplying metabolite concentrations by a metabolite- and plate-specific correction factor. This factor was calculated as the geometric mean of the six plate-specific geometric means of metabolite concentrations divided by the respective plate-specific geometric mean.

Furthermore, metabolites that did not meet one of the following criteria were excluded: (1) The coefficient of variation (CV) of metabolite concentrations in the five reference samples was ≥ 0.25 for all of the plates; (2) Kendall’s correlation p-value was ≥ 0.1 for correlation between the sample and the reference sample means of the different plates; (3) less than 30% concentration values were exactly zero for this metabolite. 37 metabolites were excluded, leaving 126 for analysis (Table S1). In addition, 45 metabolite sums that were assumed to indicate a certain metabolic state or process were calculated, as proposed in the Biocrates MetaDis/DQ™ kit manual.

**Calculations and statistical analysis**

The area under the curve (AUC) of plasma insulin during the first phase of the ivGTT was calculated according to the trapezoid method as 0.5*[0.5*c1(insulin) +c2(insulin)+c3(insulin)+c4(insulin)] where c1(insulin) denotes the insulin concentration at x minutes after ivGTT. The AUC of the second-phase insulin response (time points 10, 15, 25 and 35 minutes after the glucose challenge) and the glucose response were calculated accordingly. First-phase insulin response (FPiR) was calculated as the sum of the plasma insulin values at time points 1 and 3 minutes. Clamp-derived insulin sensitivity index (ISI) was calculated as glucose infusion rate per kg body weight necessary to maintain euglycemia during the last 45 minutes of the clamp steady state per unit of plasma insulin concentration. Proinsulin conversion was estimated as proinsulin divided by insulin concentration at the indicated time points.

Differences in baseline anthropometric and clinical data between the two genotype groups were assessed using Mann-Whitney U tests. Distributions of the metabolite concentrations and sums, henceforth referred to as metabolite traits, were assessed using quantile-quantile-plots and most were considered to be of log-normal shape. Consequently, log-transformed metabolite traits were used for all models. Genotype and challenge effects on metabolite traits were investigated with linear mixed-effects models using the R package nlme, version 3.1-103 [22]. Specifically, for each of the metabolite traits, we included genotype, coded as 1 (risk allele carriers) and 0 (non-carriers), corresponding to a dominant genetic model, two measurement time indicators (t35 and t240, with fasting measurement as reference category) and the interaction of these with genotype to also explore the modification of challenge effects by genotype. As potential confounders, mean-centered BMI, age, and the interactions of both with measurement time, were included in all models, and random intercepts were included per each measurement time point. Restricted maximum likelihood estimation was used. P-values of genotype main effects and of genotype x measurement time interactions were subjected to correction for multiple testing using the Benjamini-Hochberg procedure [23]. All calculations concerning metabolomics analysis were performed using the statistical environment R, version 2.14.2.

**Results**

**Baseline characteristics**

The baseline characteristics of the probands are given in Table 1. There were no statistically significant differences in anthropometric parameters, age, family history of diabetes, waist circumference, systolic and diastolic blood pressure, plasma triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, creatinine, CRP and HbA1c values between TCF7L2 risk allele carriers and controls. Fasting glucose, insulin, proinsulin, and C-peptide levels were also similar in both study groups.

**Genotype effects in the fasting state**

Fasting results in β-oxidation of fatty acids and gluconeogenesis from amino acids. As expected, we observed high plasma concentrations of non-esterified fatty acids (NEFAs), free short chain acylcarnitines (C2 and C3) and gluconeogenic amino acids (sum of glycine and serine). The sum of branched-chain amino acids (leucine, isoleucine, valine) did not differ between study groups (Table 2 and Figure S1). Higher levels of several sphingomyelins (SMs), lysophosphatidylcholines (lysoPCs) and phosphatidylcholines (PCs) were observed in rs7903146 risk allele carriers (Table 2). After correction for multiple testing, there was no statistically significant genotype effect on any metabolite concentration in the fasting samples.

**Insulin response to intravenous glucose and insulin sensitivity**

The plasma insulin levels at 1, 3 and 5 minutes after the glucose injection and the first-phase insulin response (FPiR, sum of 1 + 3 minutes) were significantly reduced in carriers of the TCF7L2 risk allele compared to the control group (Figure...
Table 1. Baseline characteristics of the study population.

| Metabolite                        | CC genotype (n = 24)     | CT/TT genotype (n = 17) | p-value |
|-----------------------------------|--------------------------|-------------------------|---------|
| **Height (cm)**                   | 179.7 (7.5), [180.0]     | 177.6 (6.3), [177.2]    | 0.58    |
| **Weight (kg)**                   | 87.4 (8.9), [85.5]       | 85.3 (11.8), [84.5]     | 0.55    |
| **BMI (kg/m²)**                   | 27.1 (2.6), [26.9]       | 27.0 (2.7), [25.5]      | 0.74    |
| **Waist circumference (cm)**      | 99.8 (7.5), [100.0]      | 99.9 (9.6), [96.0]      | 0.75    |
| **Age (years)**                   | 51.0 (10.4), [50.5]      | 58.10 (10.0), [80.0]    | 0.07    |
| **Systolic blood pressure (mmHg)**| 140 (18.2), [136]        | 142 (14.2), [140]       | 0.49    |
| **Diastolic blood pressure (mmHg)**| 83 (11.1), [83]         | 85 (10.8), [85]         | 0.57    |
| **Creatinine (mg/dl)**            | 0.9 (0.1), [0.9]         | 0.9 (0.2), [1.0]        | 0.25    |
| **Total cholesterol (mg/dl)**     | 201.1 (28.0), [202.5]    | 206.6 (34.2), [205.0]   | 0.82    |
| **LDL-cholesterol (mg/dl)**       | 119.5 (31.8), [122.5]    | 124 (25.4), [123.0]     | 0.58    |
| **HDL-cholesterol (mg/dl)**       | 53.0 (14.5), [51.5]      | 61.0 (19.3), [60.5]     | 0.31    |
| **Triglycerides (mg/dl)**         | 143.3 (75.8), [126.0]    | 114.0 (50.7), [102.0]   | 0.38    |
| **CRP (mg/dl)**                   | 0.30 (0.6), [0.1]        | 0.22 (0.2), [0.1]       | 0.70    |
| **TSH (µU/ml)**                   | 1.6 (0.8), [1.5]         | 1.9 (1.1), [2.0]        | 0.55    |
| **HbA1c (%)**                     | 5.5 (0.2), [5.6]         | 5.7 (0.3), [5.6]        | 0.29    |
| **Fasting glucose (mg/dl)**       | 96.8 (9.7), [97.5]       | 97.9 (10.9), [100.0]    | 0.73    |
| **2 h glucose (oGTT)**            | 94.4 (29.7), [94.0]      | 93.5 (28.5), [90.0]     | 0.99    |
| **Insulin (pmol/l)**              | 45.9 (18.6), [43.2]      | 37.5 (22.0), [31.7]     | 0.57    |
| **Proinsulin (pmol/l)**           | 3.4 (4.6), [2.3]         | 3.0 (2.6), [1.9]        | 0.30    |
| **C-peptide (ng/ml)**             | 2.8 (1.6), [2.4]         | 2.5 (1.1), [2.0]        | 0.76    |
| **NEFA (mEq/l)**                  | 0.6 (0.4), [0.5]         | 0.55 (0.3), [0.5]       | 0.47    |

Data are given as mean (standard deviation), [median]. Given are the crude p values before correction for multiple testing.

doi: 10.1371/journal.pone.0078430.t001

Table 2. Selected metabolite concentrations (µmol/l) in the fasting state in carriers of TCF7L2 rs7903146 (CT/TT genotype) and non-risk allele carriers (CC genotype).

| Metabolite                        | CC genotype (n = 24)     | CT/TT genotype (n = 17) | p-value |
|-----------------------------------|--------------------------|-------------------------|---------|
| **C0 (µmol/l)**                   | 36.5 (6.5)               | 42.2 (8.8)              | 0.079   |
| **C2 (µmol/l)**                   | 7.9 (4.1)                | 8.12 (2.6)              | 0.914   |
| **C3 (µmol/l)**                   | 0.43 (0.14)              | 0.53 (0.20)             | 0.046   |
| **C4 (µmol/l)**                   | 0.21 (0.12)              | 0.34 (0.43)             | 0.039   |
| **C10 (µmol/l)**                  | 0.38 (0.15)              | 0.59 (0.54)             | 0.046   |
| **Non-essential amino acids (µmol/l)** | 1085.5 (186.5)         | 1226.6 (195.7)          | 0.018   |
| **Sum of glycine and serine (µmol/l)** | 293.2 (60.3)           | 325.6 (55.3)            | 0.086   |
| ** Branched-chain amino acids (µmol/l)** | 515.6 (93.1)           | 550.1 (135.7)           | 0.177   |
| **Proline (µmol/l)**              | 181.8 (39.9)             | 226.8 (57.8)            | 0.005   |
| **IysOPCs (µmol/l)**              | 171.0 (52.2)             | 190.1 (38.2)            | 0.025   |
| **Saturated IysOPCs (µmol/l)**    | 105.9 (24.1)             | 122.0 (21.7)            | 0.006   |
| **IysOPC a C14:0 (µmol/l)**       | 6.2 (0.7)                | 6.6 (0.6)               | 0.032   |
| **IysOPC a C16:0 (µmol/l)**       | 98.2 (23.4)              | 113.9 (21.4)            | 0.007   |
| **IysOPC a C20:4 (µmol/l)**       | 6.5 (3.1)                | 7.44 (2.06)             | 0.021   |
| **PC ae C36:4 (µmol/l)**          | 204.7 (47.6)             | 234.2 (59.2)            | 0.045   |
| **PC ae C38:4 (µmol/l)**          | 13.7 (3.5)               | 15.0 (3.6)              | 0.029   |
| **PC ae C38:5 (µmol/l)**          | 17.9 (4.7)               | 20.81 (5.2)             | 0.025   |
| **PC ae C40:5 (µmol/l)**          | 3.2 (0.7)                | 3.5 (0.6)               | 0.038   |
| **PC ae C44:5 (µmol/l)**          | 1.6 (0.5)                | 1.9 (0.4)               | 0.008   |
| **PC ae C44:6 (µmol/l)**          | 1.0 (0.3)                | 1.2 (0.2)               | 0.030   |
| **SMs (µmol/l)**                  | 328.7 (57.8)             | 377.7 (100.9)           | 0.045   |
| **SC (µmol/l)**                   | 284.5 (49.8)             | 327.5 (87.0)            | 0.045   |
| **Long SM-OH (µmol/l)**           | 1.7 (0.4)                | 2.0 (0.7)               | 0.039   |
| **SM C16:0 (µmol/l)**             | 121.5 (22.5)             | 141.6 (35.9)            | 0.023   |
| **SM-C22:1 (µmol/l)**             | 17.4 (3.8)               | 20.0 (5.8)              | 0.049   |
| **SM-C24:1 (µmol/l)**             | 1.7 (0.4)                | 2.0 (0.7)               | 0.039   |

Data are given as mean (standard deviation). Given are the crude p values before correction for multiple testing. Abbreviation of metabolites are explained in Table S1.

doi: 10.1371/journal.pone.0078430.t002
accompanying a significant increase in carnitine (C0) levels in control subjects but not in TCF7L2 risk allele carriers and a marked decrease of short-chain acylcarnitines (C2, C3) and NEFAs levels in both groups (Figure S1). We observed a significant genotype effect at time-point t240 versus t0 in 17 metabolite traits (Table 4). Fasting insulin as well as FPIR modified genotype effects on metabolites (Table S3). The decrease of 8 SM species and the sum of all SMs were more pronounced in TCF7L2 risk allele carriers and all but one SM-OH (SM-OH C14:1) were significantly altered. The most prominent down-regulation occurred in SM-OH C24:1 (Figure 2B). At t240, three lysoPC species (lysoPC a C16:0, lysoPC a C16:1, lysoPC a C17:0), the sum of all lysoPCs (Figure 2C) and the sum of saturated lysoPCs displayed a significant stronger decline in the TCF7L2 compared to the control group. Change in total PC levels was not different between genotype groups (Figure 2E). However, 7 unsaturated PC species decreased significantly stronger in the TCF7L2 group (PC aa C36:6, PC aa C40:4, PC ae C40:2, PC ae C40:5, PC ae C44:5, PC ae C44:6).

Discussion

Using a targeted metabolomics platform, we identified alterations in sphingolipid, phosphatidylincholine and lysophosphatidylcholine metabolism in subjects at potentially increased risk for T2D defined by the presence of the rs7903146 risk genotype. The most important finding is that these differences were only observed after challenge tests and were present in a state when all conventional markers of glucose metabolism such as fasting and 2-h glucose in oGTT, fasting insulin, proinsulin and insulin sensitivity were still in the normal range.

Measurement of a large number of metabolites by targeted metabolomics is a promising approach to identify and quantify intermediate and endpoint compounds of metabolism related to specific disorders. This method has recently been successfully
used to define metabolomic signatures of cardiovascular diseases, neurological disorders, obesity and diabetes mellitus [15,24–27]. Our hypothesis was that metabolic alterations may precede the development of glucose intolerance and that the disturbed metabolic homeostasis may be detected by differences in plasma metabolite concentrations. We chose to analyse non-diabetic male subjects harbouring the high risk allele TCF7L2 rs7903146 and compared them with age-, sex- and BMI-matched controls. Considering that TCF7L2 is involved in β-cell dysfunction including impaired insulin secretion and disturbed incretin effects as well as an enhanced rate of basal hepatic glucose production [3–7], changes of subsets of metabolites may provide novel insights into biochemical pathways involved in the development of T2D.

In our study we demonstrate the presence of impaired FPIR and normal insulin sensitivity in carriers of the TCF7L2 rs7903146 allele. Our data confirm the finding that TCF7L2 polymorphism is associated with an intrinsic β-cell dysfunction and/or a reduction of β-cell mass [4]. Incretins are thought to partly mediate the impaired insulin secretion in patients with TCF7L2 risk allele [28]. By challenge with intravenous glucose we can exclude any impact of incretins on metabolite concentrations in the present study. Marked differences in the regulation of plasma phospholipids were observed in ivGTT and EH clamp. A stronger reduction of plasma metabolite levels in TCF7L2 risk allele carriers compared to controls was observed for 8 SMs, 6 PCs and 3 lysoPCs (t0 versus t240). This finding may be explained by slightly increased basal metabolite levels at the starting point under fasting conditions and a stronger clearance of SMs, PCs and lysoPCs in the TCF7L2 group after glucose challenge and additional stimulation with high insulin levels. The fact that significant genotype effects were only observed in challenge tests may explain why other studies may have identified the same metabolite profiles in risk allele carriers [13,19].

The three major phospholipid groups (SMs, PCs, lysoPCs) are important structural components of plasma lipoproteins and cell membranes and are involved in regulation of cell function, membrane protein trafficking and inflammation [29]. In human plasma, PCs comprise about 60-70 %, SMs 10-20 % and lysoPCs about 10-20 % of the circulating phospholipids. Increased plasma SM levels have been reported in subjects with subclinical atherosclerosis [30], coronary heart disease [31], obesity [32] and diabetes mellitus [13]. We did not detect significantly increased fasting SM levels in TCF7L2 risk allele carriers. The present study is the first investigating metabol
signatures after challenge tests in subjects with defined genetic risk for T2D. We observed a significant genotype effect in almost all SMs, suggesting a general alteration in the synthesis or breakdown of sphingolipid metabolism. Several studies indicate a role for SMs in the development of T2D by influencing insulin resistance as well as insulin secretion. In SM synthetase knockout mouse models it has been shown that the reduction of plasma membrane SMs increased insulin sensitivity [33,34]. Another study reported a decreased insulin secretion due to mitochondrial dysfunction as a consequence of altered membrane SM and ceramide contents [35]. Inhibition of ceramide synthesis decreased β-cell apoptosis and defective protein trafficking in β-cells exposed to lipotoxicity [37]. Thus, ceramides derived from SM degradation may contribute to lipotoxicity-mediated β-cell apoptosis and may accelerate the observed reduction of FPIR in carriers of the TCF7L2 risk genotype.

While plasma LDL- and HDL-cholesterol and triglyceride levels were similar in all probands, significant genotype effects were detected in long-chain lysoPCs (C16:0, C16:1, C17:0) and long-chain PC species (PCs containing C28-C44). The finding of a disturbed PC/lysoPC metabolism is in agreement with results from previous studies describing that altered PC and lysoPC plasma profiles are associated with T2D (increased PC aa (16:0/18:2); decreased PC aa (16:0/18:0), decreased PC aa (18:0/20:4), increased lysoPCs (C16:0, C18:0, C18:1, C18:2, C20:4)) [13], increased lysoPCs (C14:0, C16:1, C18:1, C20:5, C22:6) [38], impaired glucose tolerance (decreased lysoPC C18:2) ) [14,39] and increased risk for T2D (decreased PC ae (C34:3, C40:6, C42:5, C44:4, C44:5), increased PC aa (C32:1, C36:1, C38:3, C40:5)) [14]. In addition, increased levels of several lysoPCs were reported in subjects with obesity and insulin resistance [40]. Here, we did not find significantly increased basal lysoPC species in subjects at increased risk for T2D, but detected significant alterations in the regulation of PCs with fatty acid aliphatic tails between 36 and 44 carbons. This has not been described before in prediabetes or T2D and may indicate that in parallel with the modification of the long-chain lysoPCs there is also a disturbed regulation of very-long-chain PCs in risk subjects carrying the TCF7L2 risk allele.

LysoPCs are produced by the action of phospholipase A2 (PLA2) or lecithin cholesterol acyltransferase (LCAT) on PCs and the activity of both enzymes is related with plasma lysoPC concentrations. LysoPCs, e.g. produced by increased PC

Figure 2. Metabolites displaying the highest differences between TCF7L2 risk genotype carriers and controls. Concentration of total plasma SM, lysoPC and PC levels and selected SM-, lysoPC-, and PC-species in the fasting state, 35 minutes after the intravenous glucose bolus (t35) and during the steady state of the EH clamp (t240). (A) A significantly higher decrease of total SM plasma levels was observed in TCF7L2 rs7903146 allele carriers (CT/TT genotype) at t35 (p=3.5E-03) and t240 (p = 4.4E-03) compared to CC genotype carriers. (B) SM-OH C24:1 plasma levels displayed the strongest difference between the genotype groups in delta t35 (p = 8.3E-04) and delta t240 (p = 5.4E-04). (C) Delta t35 and delta t240 of total lysoPC was significantly different between the genotype groups (p = 3.0E-03 and p = 3.6E-03). (D) LysoPC a C16:0 presented higher delta t35 (p = 2.6E-03) and deltat240 (p = 7.9E-04) in the subjects carrying the TCF7L2 rs7903146 risk allele. (E) The sum of all PCs did not present significant differences. (F) However, several PC species decreased significantly stronger in risk allele carriers. Delta t35 (p = 2.7E-03) and t240 (p = 4.2E-03) of PC ae C40:5 are given as a representative example. Data are given as mean and standard error of the mean. * = p ≤ 0.05 after correction for multiple testing.

doi: 10.1371/journal.pone.0078430.g002

Metabolomics in TCF7L2 Risk Allele Carriers
Table 4. Metabolic traits with significant genotype effect in response to EH clamp (t0 versus t240).

| Metabolite | Challenge response (clamp) CC genotype | Challenge response (clamp) CT/TT genotype | Genotype effect |
|------------|----------------------------------------|------------------------------------------|-----------------|
|            | β   | se  | p value   | β   | se  | p value   | β   | se  | p value   | p value (corrected) |
| Acylcarnitines |     |     |            |     |     |            |     |     |            |                  |
| CD         | 0.66 | 0.14 | 1.10E-05   | 0.01 | 0.17 | 9.50E-01 | -0.65 | 0.23 | 5.40E-03 | 3.80E-02          |
| Phosphatidyicholines |     |     |            |     |     |            |     |     |            |                  |
| PC aa C36:6 | -0.18 | 0.09 | 4.80E-02 | -0.65 | 0.11 | 1.60E-07 | -0.47 | 0.15 | 2.40E-03 | 3.80E-02          |
| PC aa C40:4 | -0.01 | 0.10 | 9.20E-01 | -0.48 | 0.12 | 1.90E-04 | -0.47 | 0.16 | 4.80E-03 | 3.80E-02          |
| PC aa C40:2 | -0.11 | 0.13 | 3.80E-01 | -0.69 | 0.16 | 4.00E-05 | -0.57 | 0.21 | 7.40E-03 | 4.70E-02          |
| PC aa C40:5 | 0.02 | 0.16 | 9.10E-01 | -0.84 | 0.22 | 2.80E-04 | -0.86 | 0.29 | 4.20E-03 | 3.80E-02          |
| PC ae C44:5 | 0.02 | 0.13 | 8.90E-01 | -0.72 | 0.17 | 4.00E-05 | -0.74 | 0.22 | 1.10E-03 | 2.70E-02          |
| PC ae C44:6 | -0.02 | 0.15 | 9.10E-01 | -0.74 | 0.18 | 1.30E-04 | -0.72 | 0.24 | 3.90E-03 | 3.80E-02          |
| Sphingomyelins |     |     |            |     |     |            |     |     |            |                  |
| lysoPC a C16:0 | 0.33 | 0.16 | 3.90E-02 | -0.56 | 0.19 | 4.70E-03 | -0.88 | 0.25 | 7.90E-04 | 2.20E-02          |
| lysoPC a C16:1 | 0.09 | 0.11 | 4.30E-01 | -0.47 | 0.14 | 9.20E-04 | -0.55 | 0.18 | 2.80E-03 | 3.80E-02          |
| lysoPC a C17:0 | 0.24 | 0.14 | 7.80E-02 | -0.56 | 0.17 | 1.10E-03 | -0.81 | 0.22 | 4.50E-04 | 2.20E-02          |
| lysoPCs | 0.06 | 0.15 | 6.80E-01 | -0.66 | 0.18 | 4.80E-04 | -0.72 | 0.24 | 3.60E-03 | 3.80E-02          |
| Saturated lysoPCs | 0.33 | 0.16 | 3.90E-02 | -0.57 | 0.19 | 4.30E-03 | -0.90 | 0.26 | 7.40E-04 | 2.20E-02          |

PLOS ONE | www.plosone.org

Our challenge tests revealed that changes in metabolic profiles of TCF7L2 risk allele carriers are reversible. Hyperinsulinemia in the EH-clamp was able to reduce the differences between the study groups. Although we did not observe any differences in basal glucose or insulin levels, adjustment for fasting insulin and FPIR attenuated the association of phospholipids in TCF7L2 risk carriers, suggesting that some alterations in metabolic homeostasis are dependent on disturbed insulin secretion, which can be overridden by high insulin doses. The most important sites for the action of insulin are liver, adipose tissue and muscle. Insulin inhibits gluconeogenesis in the liver, promotes the storage of glycogen and induces the cellular uptake of amino acids and fatty acids to produce proteins and lipids. Under high insulin concentrations there is a strong inhibition of lipolysis (e.g. reduction of NEFAs). One possible explanation for the decline of phospholipid species during the EH-clamp in TCF7L2 risk allele carriers is that the high insulin concentrations induce sphingomyelinase, down-regulate SM synthase or induce PL2/LCAT, leading to decreasing plasma concentration of SMs, lysoPCs and PCs. The exact

hydrolysis during LDL oxidation, are strong pro-inflammatory lipid mediators [38] and are related to various pathophysiological conditions including endothelial dysfunction [41] and acute coronary syndrome [42]. Treatment of diabetic patients with metformin led to decreased lysoPC levels [43] and in vitro inhibition of PLA2 prevented palmitic acid-induced insulin resistance in L6 myotubes by reduced generation of lysoPC [44]. In contrast to these results, other studies demonstrated that lysoPCs stimulate glucose uptake in 3T3-L1 adipocytes by elevating glucose transporter type 4 levels at the plasma membrane yielding to lower blood glucose levels in normal and diabetic mice [45]. Treatment of cultured islet cells with phospholipase increased lysoPC contents and insulin secretion [46]. Thus, there may be positive and negative effects of some lysoPC species on insulin resistance and β-cell function. The significant genotype effects on lysoPCs containing C16:0, C16:1 and C17:0 fatty acid chains, which have been observed in the present study, may suggest an increased proinflammatory and proatherogenic state in TCF7L2 risk allele carriers. The underlying molecular mechanism in the regulation of circulating PC species remains unclear.
mechanisms by which high insulin concentrations influence phospholipid synthesis or degradation are incompletely understood and require further experimental and clinical studies.

In contrast to other studies indicating an association of increased plasma levels of several amino acids with prediabetes and T2D [14,27,47], we did not find significant changes in plasma amino acids. Different amino acid concentrations have been shown to be associated with insulin resistance in obese patients [24,48]. One study reported that elevated plasma levels of three branched-chain amino acids (valine, leucine and isoleucine) and two aromatic amino acids (tyrosine and phenylalanine) are correlated with future risk for T2D [27]. In the present study we did not observe any difference in amino acid levels, which may be explained by the fact that our study population was not obese and had normal insulin sensitivity. In addition, only some probands carrying the risk allele may progress to overt T2D. It may be speculated that changes in amino acids occur at a late stage during the development of T2D [39].

Limitations of the present study are the moderate sample size, the lack of prospective follow-up investigation and the complex interaction between the components of glucose, lipid and protein metabolism, which may limit the interpretation of the results. It is obvious that our findings need to be confirmed in other independent populations. We are not able to make any causal statements on the role of metabolic profiles on future development of T2D. We used a targeted approach focussing mainly on lipids. Thus, we cannot exclude associations between TCF7L2 genotype and other metabolic products of carbohydrate or protein metabolism, e.g. metabolites from the tricarboxylic acid cycle. In addition, the exact biochemical mechanisms leading to changes of these metabolites remain unknown and the clarification of the biological significance of these findings requires further investigation.

In conclusion, this study provides evidence for a complex perturbation of phospholipid metabolism possibly linked to early β-cell dysfunction in TCF7L2 risk allele carriers, in a state when conventional parameters of glucose homeostasis are not yet affected. Our findings suggest subtle modifications in the regulation of intermediate metabolism, which can only be detected by metabolic challenge tests. These data may contribute to a better understanding of the biochemical networks underlying the development of T2D in subject with the TCF7L2 risk genotype.

Supporting Information

Figure S1. Change of NEFAs, selected acylcarnitines and amino acid plasma levels during challenge tests.

References
11. Aminosyed L, Körcher F, Faghih N, Fischer R, Seissler J (2011) Kinetics of insulin secretion and glucose intolerance in adult patients with cystic fibrosis. Horm Metab Res 43: 355-360. doi:10.1055/s-0031-1275270. PubMed: 21448848.

12. Römsch-Margl W, Bogumil R, Röhring C, Strueh K, Adamski J (2012) Procedure for tissue separation and metabolite extraction for high-throughput targeted metabolomics. Metabolomics 8: 133-142. doi:10.1007/s11306-011-0293-4.

13. Illig T, Gieger C, Zhai G, Römsch-Margl W, Wang-Sattler R et al. (2010) A genome-wide perspective of genetic variation in human metabolomics. Nat Genet 42: 137-141. doi:10.1038/ng.507. PubMed: 20037589.

14. Laird NM, Ware JH (1982) Random-effects models for longitudinal data. Biometrics 38: 963-974. doi:10.2307/2529876. PubMed: 7168798.

15. Benjamini YHY (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc 57: 289-300.

16. Newgard CB, An JJ, Barn JR, Muehlbauer MJ, Stevens RD et al. (2009) A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab 9: 311-326. doi:10.1016/j.cmet.2009.02.002. PubMed: 1936713.

17. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL et al. (2007) Plasma metabolic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLOS ONE 5: e15234. doi:10.1371/journal.pone.0015234. PubMed: 21170321.

18. Gall WE, Beebe K, Lietman KA, Adam KP, Mitchell MW et al. (2010) Alpha-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a non-diabetic population. PLOS ONE 5: e10883. doi:10.1371/journal.pone.010883. PubMed: 20526369.

19. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP et al. (2010) Metabolite profiles and the risk of developing diabetes. Nat Med 17: 448-453. doi:10.1038/nm.2307. PubMed: 21423183.

20. Gonzalez-Sanchez JL, Martinez-Larrad MT, Zabena C, Perez-Barba M, Serrano-Rios M (2008) Identification of variants of the TCF7L2 gene associated with the risk of type 2 diabetes by a high-throughput targeted metabolomics approach. Metabolomics 8: 133-142. doi:10.1007/s11306-011-0293-4.

21. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

22. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

23. Metabolomics in TCF7L2 Risk Allele Carriers

24. Feige N, Sauter NS, Schulthess FT, Matveyenko AV, Oberholzer J et al. (2009) Metabolite profiles and the risk of developing diabetes. Nat Med 17: 448-453. doi:10.1038/nm.2307. PubMed: 21423183.

25. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

26. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

27. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

28. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

29. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

30. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

31. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

32. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

33. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

34. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

35. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

36. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

37. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

38. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

39. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

40. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

41. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

42. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

43. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

44. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

45. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

46. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

47. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

48. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

49. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

50. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

51. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

52. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

53. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.

54. Zhang X, Wang Y, Hao F, Zhou X, Han X et al. (2009) Human serum metabolomics in TCF7L2 Risk Allele Carriers

55. Rathmann W, Strassburger K, Heier M, Holle R, Thorand B et al. (2012) Adipose tissue TCF7L2 splicing is regulated by weight loss and diet. Mol Syst Biol 4: 214.
type 2 diabetes. Clin Endocrinol (Oxf) 76: 674-682. doi:10.1111/j.1365-2265.2011.04244.x. PubMed: 21956081.

39. Wang-Sattler R, Yu Z, Herder C, Messias AC, Floegel A et al. (2012) Novel biomarkers for pre-diabetes identified by metabolomics. Mol Syst Biol 8: 615. PubMed: 23010998.

40. Pietiläinen KH, Kannisto K, Korsheninnikova E, Rissanen A, Kaprio J et al. (2006) Acquired obesity increases CD68 and tumor necrosis factor-alpha and decreases adiponectin gene expression in adipose tissue: a study in monozygotic twins. J Clin Endocrinol Metab 91: 2776-2781. doi:10.1210/jc.2005-2848. PubMed: 16608891.

41. Kougias P, Chai H, Lin PH, Lumsden AB, Yao Q et al. (2006) Lysophosphatidylcholine and secretory phospholipase A2 in vascular disease: mediators of endothelial dysfunction and atherosclerosis. Med Sci Monit 12: RA5–16. PubMed: 16369478.

42. Dohi T, Miyachi K, Ohtawa R, Nakamura K, Kishimoto T et al. (2012) Increased circulating plasma lysophosphatidic acid in patients with acute coronary syndrome. Clin Chim Acta 413: 207-212. doi:10.1016/j.cca.2011.09.027. PubMed: 21983165.

43. Huo T, Cai S, Lu X, Sha Y, Yu M et al. (2009) Metabonomic study of biochemical changes in the serum of type 2 diabetes mellitus patients after the treatment of metformin hydrochloride. J Pharm Biomed Anal 49: 976-982. doi:10.1016/j.jpba.2009.01.008. PubMed: 19249171.

44. Han MS, Lim YM, Quan W, Kim JR, Chung KW et al. (2011) Lysophosphatidylcholine as an effector of fatty acid-induced insulin resistance. J Lipid Res 52: 1234-1246. doi:10.1194/jlr.M014787. PubMed: 21447485.

45. Yea K, Kim J, Yoon JH, Kwon T, Kim JH et al. (2009) Lysophosphatidylcholine activates adipocyte glucose uptake and lowers blood glucose levels in murine models of diabetes. J Biol Chem 284: 33833-33840. doi:10.1074/jbc.M109.024869. PubMed: 19815546.

46. Ishida-Oku M, Iwase M, Sonoki K, Sasaki N, Imoto H et al. (2010) Expression of secretory phospholipase A2 in insulinis of human transplanted pancreas and its insulinotropic effect on isolated rat islets. Islets 2: 274-277. doi:10.4161/isl.2.5.12728. PubMed: 21099324.

47. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC et al. (2013) Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab 98: E1060-E1065. doi:10.1210/jc.2012-4132. PubMed: 23633210.

48. Pietiläinen KH, Naukkarinen J, Rissanen A, Saharinen J, Ellonen P et al. (2008) Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. PLOS Med 5: e51. doi:10.1371/journal.pmed.0050051. PubMed: 18336063.