Identification of prognostic and diagnostic biomarkers of glucose intolerance in ApoE3Leiden mice

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Wopereis S, Radonjic M, Rubingh C, van Erk M, Smilde A, van Duyvenvoorde W, Cnubben N, Kooistra T, van Ommen B, Kleemann R. Identification of prognostic and diagnostic biomarkers of glucose intolerance in ApoE3Leiden mice. Physiol Genomics 44: 293–304, 2012. First published January 10, 2012; doi:10.1152/physiolgenomics.00072.2011.—The prevalence of diabetes mellitus Type 2 could be significantly reduced by early identification of subjects at risk, allowing for better prevention and earlier treatment. Glucose intolerance (GI) is a hallmark of the prediabetic stage. This study aims at identifying 1) prognostic biomarkers predicting the risk of developing GI later in life and 2) diagnostic biomarkers reflecting the degree of already manifest GI. To this end, disease development was followed over time in mice, and biomarkers were identified using lipidomics and transcriptomics. Young adult ApoE3Leiden mice were treated a high-fat diet for 12 wk to induce GI. Blood was collected before and during disease development. The individual extent of GI was determined with a glucose tolerance test and the area under the curve (AUC) was calculated for each animal. Subject-specific AUC values were correlated to the plasma lipidome (t = 0) and the white blood cell (WBC) transcriptome (t = 0, 6, and 12 wk) to identify prognostic and diagnostic biomarkers, respectively. The plasma ratio of specific free fatty acids prior to high-fat feeding (C16:1/C16:0, C18:1/C18:0 and C18:2/C22:6) was significantly correlated with the AUC and predictive for future GI. Subsequently, the expression level of specific WBC genes (Acss2, Arfgap1, Tfrc, Cox6b2, Barhl2, Abcb4, Cyp4b1, Sars2, Fgfl6, and Tceal8) reflected the individual degree of GI during disease progression. Specific plasma free fatty acids as well as their ratio can be used to predict future GI. The expression levels of specific WBC genes can serve as easy accessible markers to diagnose and monitor already existing GI.

DIABETES MELLITUS TYPE 2 (DM2) is a metabolic disorder whose incidence has rapidly increased over the past several decades (15, 28). DM2 accounts for 90–95% of all diagnosed cases of diabetes and is associated with a high morbidity and mortality rate (1). Glucose intolerance is an indicator of the prediabetic state and a hallmark of the disease process leading to overt DM2 (28). Glucose intolerance is a condition in which an individual has higher than normal levels of glucose in the blood upon fasting or ingestion of a glucose test solution but not high enough to be classified as DM2. This can either be due to an insufficient production of insulin or due to an inefficient response of cells to insulin (insulin resistance). In both cases, the organ uptake of glucose from the blood stream is impaired resulting in a longer residence time and elevated levels of circulating glucose. Glucose intolerance is typically diagnosed with an invasive glucose tolerance test (GTT) in which glucose is given to a subject and blood samples are taken afterward to determine how quickly it is cleared from the blood. The test is time-consuming and elaborate, and new strategies to diagnose the degree of glucose intolerance are needed. This would allow routine monitoring of disease progression as well as testing the efficacy of interventions.

Early identification of subjects at risk would constitute another possibility to improve DM2 prevention and care. Such prognostic markers would allow an early advice on lifestyle, caloric intake, and physical exercise, thereby preventing the development of DM2. Traditional clinical indicators such as body weight and plasma lipids [e.g., triglycerides (TG), cholesterol, and nonesterified fatty acids (NEFA)] may have only limited value to identify individual subjects at risk and are not useful to discriminate between subjects who will develop glucose intolerance/DM2 and subjects who will stay healthy. Hence, reduction of the incidence of DM2 and optimization of treatment requires specific prognostic and diagnostic biomarkers allowing risk estimation on an individual level.

Recent research shows that the first diagnostic and prognostic biomarkers for DM2 are being identified. Plasma α-hydroxybutyrate was identified as an alternative diagnostic biomarker for both insulin resistance and impaired glucose regulation in human (16). Furthermore, five amino acids (iseleucine, leucine, valine, tyrosine, and phenylalanine) (45) and a triacylglycerol signature (34) were identified in the Framingham Heart Study to have highly significant associations with future DM2. Several studies have shown a clear link between dietary fat profile and DM2 risk, particularly that of saturated fat (26, 41, 44).

In the present study, our goal was to identify prognostic and diagnostic markers of glucose intolerance. We used ApoE3Leiden transgenic mice (ApoE3L) displaying a humanized lipid metabolism and mimicking the disease situation in humans (22, 39). In a recent time-resolved study we showed that high fat diet-treated ApoE3L mice develop glucose intolerance within 12 wk (23). A large variation in the degree of glucose intolerance was observed. For biomarker identification, biobank samples of this study were used. The natural variation in development of glucose intolerance enabled us to make correlations with the initial lipidome profiles at t = 0 (prior to onset of disease) to identify prognostic biomarkers. The rationale for this was that research has shown a clear link between plasma lipids and risk for future DM2. The advantage of using a mouse model is that it allows studying individual differences in lipid
metabolism as risk factor for DM2, since there is no dietary variation. Also in a correlation approach, the gene expression profiles of white blood cells (WBC) were used to identify diagnostic biomarkers of glucose intolerance.

**MATERIALS AND METHODS**

**Study design.** For the identification of prognostic and diagnostic biomarkers, we used biobank samples of a reported study (23) investigating the development of glucose intolerance over time. All animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health. All animal experiments were approved by an independent institutional ethical committee on animal care and experimentation (Diererthische Commissie DEC, Zeist, the Netherlands). In brief, male ApoE3L mice (TNO Innovation for life, Metabolic Health Research, Leiden, the Netherlands) were fed a chow diet until the age of 12 wk. Then, mice were treated with a high-fat diet containing 24% beef tallow (diet 4031.05; Hope Farms, Woerden, the Netherlands) to induce obesity and glucose intolerance. The fatty acid composition of the diet is provided in Table 1.

Blood was collected at regular time points over time by tail bleeding into chilled paraxon (Sigma, St. Louis, MO)-coated capillary tubes to prevent lipolysis. Tubes were placed on ice and centrifuged for 10 min at 3,300 g to prepare plasma (storage at −80°C). Groups (n = 15) of mice were euthanized with CO2 at t = 0 and 1, 6, 9, and 12 wk of diet feeding to collect tissues and to isolate WBC. Three days prior to death, a tail blood sample was taken after 5 h of fasting in all animals (t = 0). Then, mice were subjected to a GITT, i.e., an intraperitoneal injection of glucose (2 g/kg body wt), to determine their individual ability to clear a bolus of glucose and to measure their individual degree of glucose intolerance. Glucose tolerance protocols and glucose measurements are reported in Verschuren et al. (40). In brief, blood glucose levels were monitored at t = 0, 15, 30, 60, and 120 min after glucose injection, by a colorimetric assay of glucose hexokinase (Instruchemie, Delfzijl, the Netherlands). Basal (t = 0) levels of insulin after 12 wk of high-fat feeding have been determined and provided in Supplementary data (see Supplementary Data S2).

For the identification of prognostic markers of glucose intolerance, plasma taken at the start of the study (t = 0) was analyzed by lipidomics.

For the identification of diagnostic markers of glucose intolerance, WBC of time points 0, 6, and 12 (n = 8 per time point) were used to extract RNA (18) for microarray transcriptomics analysis. At death, 450 μl blood was collected in syringes filled with 50 μl heparin (final heparin concentration 10% vol/vol), and the collected blood samples were then incubated with lysis buffer (3 M NH4Cl, 0.24 M KHCO3, 0.02 M EDTA; pH = 7.4) for 30 min in ice-cold water for lysis of red blood cells. After centrifugation (10 min, 6,000 rpm at 4°C) the supernatant was removed and the cellular pellet was gently resuspended with 1 ml of lysis buffer. After a second centrifugation step (10 min, 6,000 rpm at 4°C), the pellets were resuspended and incubated with 800 μl RNA-Bee and transferred into Eppendorf tubes for RNA extraction. Briefly, 160 μl chloroform was added, and tubes were shaken for 30 s and then placed on ice for 5 min. Samples were then centrifuged (15 min, 13,000 rpm at 4°C), and the supernatant was transferred into clean 1.5 ml Eppendorf tubes and incubated with 400 μl isopropanol and vortexed. After incubation at room temperature (30 min) samples were centrifuged for 10 min at 11,000 rpm, and the supernatant was removed. The pellet was incubated with 800 μl of a 75% ethanol solution and vortexed, followed by centrifugation (5 min at 7,500 rpm). The supernatant was removed, and the pellet was dried at room temperature and finally dissolved in 10 μl RNase-free water. RNA was stored at −80°C until use.

**Liquid chromatography/tandem mass spectrometry of lipids and free fatty acids.** Plasma lipids and free fatty acids (FFA) were analyzed with electrospray liquid chromatography/tandem mass spectrometry (LC-MS) with a Thermo LTQ mass spectrometer by extracting 10 μl of plasma with 300 μl of isopropanol containing several internal standards (IS) (C17:0 lysophosphatidylcholine, di-C12:0 phosphatidylcholine, tri-C17:0 glycerol ester, C17:0 cholesterol ester and C17:0 fatty acid). This LC-MS lipidomics method has been described in several other peer reviewed publications (5, 6, 12, 19).

FFA were separated on a Alltech Prophere C4 300 Å column [150 × 3.2 mm, 5 μm, temperature (T) = 40°C] using a mobile phase gradient from 70% mobile phase A (5% methanol in 2 mM NH4Ac (pH 7)) to 100% mobile phase B (100% methanol containing 2 mM NH4Ac) in 15 min with a flow of 0.4 ml/min. Mass detection was carried out using electrospray ionization in the negative mode (heater capillary temperature 250°C, spray voltage 3.5 kV, scan range m/z 150–400). Injection volume was 20 μl.

Lipids were separated on a Alltech Prophere C4 300 Å column (150 × 3.2 mm, 5 μm, T = 50°C) using a mobile phase gradient from 80% mobile phase A (5% methanol in water containing 10 mM NH4Ac and 0.1% formic acid) to 100% mobile phase B (100% methanol containing 10 mM NH4Ac and 0.1% formic acid) in 25 min with a flow of 0.4 ml/min. Mass detection was carried out using electrospray ionization in the positive mode (heater capillary temper-

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Table 1. Fatty acid composition of the chow diet (fed until the start of the study) and the HFD used to induce obesity and glucose intolerance

<table>
<thead>
<tr>
<th>Fat [g/100 g diet]</th>
<th>Chow</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8:0</td>
<td>&lt;0.1</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>C10:0</td>
<td>&lt;0.1</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>C12:0</td>
<td>0.3</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>C14:1 c9</td>
<td>&lt;DL</td>
<td>0.4</td>
</tr>
<tr>
<td>C14:1 c9</td>
<td>&lt;DL</td>
<td>0.2</td>
</tr>
<tr>
<td>C15:0</td>
<td>&lt;0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>C15:0 iso</td>
<td>&lt;DL</td>
<td>0.3</td>
</tr>
<tr>
<td>C16:0</td>
<td>15.4</td>
<td>24.9</td>
</tr>
<tr>
<td>C16:1 c9</td>
<td>0.1</td>
<td>2.7</td>
</tr>
<tr>
<td>C16:1 t9</td>
<td>&lt;DL</td>
<td>0.3</td>
</tr>
<tr>
<td>C17:0</td>
<td>&lt;0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>C17:0 iso</td>
<td>&lt;0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.8</td>
<td>23.2</td>
</tr>
<tr>
<td>C18:1 t9</td>
<td>0.2</td>
<td>2.1</td>
</tr>
<tr>
<td>C18:1 c9</td>
<td>16.9</td>
<td>33.4</td>
</tr>
<tr>
<td>C18:2 c9,12</td>
<td>47.3</td>
<td>1.8</td>
</tr>
<tr>
<td>C18:2 c9,12</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>C18:3 c6,9,12</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C18:3 c9,12,15</td>
<td>5.1</td>
<td>0.3</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>C20:1 c11</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C20:2 c11,14</td>
<td>&lt;0.1</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>C21:0</td>
<td>&lt;DL</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C22:0</td>
<td>&lt;0.1</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>C22:3 c13,16,19</td>
<td>0.2</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>C22:4 c7,10,13,16</td>
<td>0.1</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.2</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>Not identified peaks</td>
<td>9.3</td>
<td>35.8</td>
</tr>
<tr>
<td>Saturated fatty acids</td>
<td>19.3</td>
<td>36.6</td>
</tr>
<tr>
<td>MUFA (cis)</td>
<td>17.1</td>
<td>2.5</td>
</tr>
<tr>
<td>PUFA</td>
<td>53.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Unsaturated FA (trans)</td>
<td>&lt;0.1</td>
<td>2.6</td>
</tr>
</tbody>
</table>

HFD, high-fat diet; <DL, below detection limit.

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1 The online version of this article contains supplemental material.
The difference between animals with highest and lowest level of glucose intolerance. AUC values ranged from 607 up to 1,826, which is a 3-fold curve (AUC) was calculated as a measure for the individual degree of glucose glucose (2 g/kg body wt). Glucose concentrations (in mmol/l) were measured standards) of lipids were determined. The lipidomics measurements FFA (IS used: C17:0 FFA). Relative concentrations (to internal C17:0 cholesterol ester), TG [IS used: tri-C17:0 glycerol ester, this IS used: di-C12:0 phosphatidylcholine), sphingomyelines (SPM; IS used: C17:0 lyso-phosphatidylcholine), phosphatidylcholines (PC; nant lipid classes measured are the lyso-phosphatidylcholines (LPC; IS used: C17:0 lyso-phosphatidylcholine), phosphatidylcholines (PC; IS used: di-C12:0 phosphatidylcholine), cholesterolesters (ChE; IS used: C17:0 cholesterol ester), TG [IS used: tri-C17:0 glycerol ester, this IS was also used for monoglycerides (MG) and diglycerides (DG)) and FFA (IS used: C17:0 FFA). Relative concentrations (to internal standards) of lipids were determined. The lipidomics measurements were performed in randomized order. Additional reference control samples were respectively analyzed at regular time points during the measurements to control for a consistent performance of the platform. The lipid concentrations of the complex lipids were determined using internal standards of the respective compound classes, i.e., for LPC, PC, TG, and ChE. Exceptions were made for MG and DG, which were reported relative to tri-C17:0 glycerol ester, and for SPM, which were reported relative to di-C12:0 phosphatidylcholine. The LC-MS lipid and LC-MS FFA data were processed using the LC-Quan software (Thermo). The datasets have been made available as supplementary data (see Supplementary Data S2).

Analysis of fatty acids present in the high-fat diet. Samples were hydrolyzed with hydrochloric acid, fat was extracted with petroleum ether, and the ether was evaporated (ISO 8262-3). To the obtained fat, internal standard (C17:0) was added, and fat was hydrolyzed by reflux with sodium hydroxide in methanol. Fatty acids were derivatized with boron trifluoride in methanol, and the obtained methyl esters were extracted with hexane. The extract was dried and analyzed by gas chromatography with flame ionization detector fitted with a cold on-column injector and a CP-SIL88 column (NEN 6302:1980, 6304: 1992, and 5410:1997).

WBC transcriptomics. The integrity of RNA extracted from WBC was examined by Agilent Lab-on-a-chip technology (RNA 6000 Nano LabChip kit and a bioanalyzer 2100; Agilent Technologies, Amstelveen, the Netherlands) essentially as reported (24). Briefly, the One-Cycle Target Labelling and Control Reagent kit (Affymetrix #900493) and the protocols optimized by Affymetrix were used to prepare biotinylated cRNA (from 5 µg of total RNA) for microarray hybridization (n = 8 per time point). For microarray analysis and the intermediate QC steps we followed a protocol that has recently been described in detail (24).

Quality of obtained microarray data was checked using BioConductor packages (a.o. simpleaffy and affyplm) and the NuGO pipeline that is available as a Genepattern procedure on http://nbx2.nugo.org (11). One microarray from week 6 did not pass the QC criteria. Therefore, the total WBC microarray dataset consisted of 23 samples. The microarray data are MIAME compliant, and the raw data have been deposited in ArrayExpress with the accession number E-TABM-959.

For each tissue, raw signal intensities (from CEL files) were normalized using the GCRMA algorithm (gc-rma slow). Probe sets were remapped and annotated into Entrez gene-IDs using the custom MBNI CDF-file, version 9.0 11 (10). Genes were filtered on expression value >5 in at least four samples. Expression data were log-transformed (base 2). After normalization, annotation, and filtering of genes with a low expression value, expression data for 9,599 genes were used for correlation analysis.

Liver metabolite analysis. For the Pearson correlations between acyl-CoA synthetase short-chain family member 2 (Acss2) gene expression and functionally related liver metabolites we used hepatic relative concentrations of succinate, malate, citrate, and fumarate as

These genes may serve as diagnostic markers to monitor the development of glucose intolerance via the blood. WBC, white blood cell.

Table 2. Overview of WBC genes, whose expression correlated with the intraindividual level of glucose intolerance

<table>
<thead>
<tr>
<th>EntrezID</th>
<th>Common Name</th>
<th>Description</th>
<th>Correlation Factor</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>60525</td>
<td>Acss2</td>
<td>acetyl-CoA synthetase short-chain family member 2</td>
<td>−0.786</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>228998</td>
<td>Afgap1</td>
<td>ADP-riboosylation factor GTPase activating protein 1</td>
<td>−0.750</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>22042</td>
<td>Tfrc</td>
<td>transferrin receptor</td>
<td>0.805</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>333182</td>
<td>Cx3662</td>
<td>cytochrome c oxidase subunit Vb polypeptide 2</td>
<td>0.801</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>104382</td>
<td>Barhi2</td>
<td></td>
<td>0.782</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>18670</td>
<td>Abcb4</td>
<td>ATP-binding cassette, subfamily B (MDR/TAP), member 4</td>
<td>0.780</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>13120</td>
<td>Cyp4b1</td>
<td>cytochrome P450, family 4, subfamily b, polypeptide 1</td>
<td>0.775</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>71984</td>
<td>Sars2</td>
<td>seryl-aminocyl-tRNA synthetase</td>
<td>0.764</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>80903</td>
<td>Fg16</td>
<td>fibroblast growth factor 1</td>
<td>0.761</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>66684</td>
<td>Tceal8</td>
<td>transcription elongation factor A (SII)-like</td>
<td>0.754</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

The genes in Table 2 were selected based on their expression correlation with the individual glucose intolerance. The table shows the Entrez ID, common name, description, correlation factor, and p-value for each gene. The genes may serve as diagnostic markers to monitor the development of glucose intolerance via the blood. WBC, white blood cell.

![Glucose Tolerance Test](chart)

Fig. 1. Individual results of glucose tolerance test after 12 wk of high-fat feeding. ApoE3L mice (n = 15) received an intraperitoneal injection of glucose (2 g/kg body wt). Glucose concentrations (in mmol/l) were measured at 0, 15, 30, 60, and 120 min after glucose injection and the area under the curve (AUC) was calculated as a measure for the individual degree of glucose intolerance. AUC values ranged from 607 up to 1,826, which is a 3-fold difference between animals with highest and lowest level of glucose intolerance.
determined by two-dimensional gas chromatography-mass spectrometry (GC×GC-MS) (23).

Analysis of TG, cholesterol, glucose, and insulin in plasma. Total plasma cholesterol and TG levels were measured after 5 h of fasting, using kits no. 11489437 and 11488872 (Roche Diagnostics, Almere, The Netherlands), respectively, and NEFA with kit WAKO 999–75406 (FFA test NEFA-HR, Instruchemie, Delfzijl, The Netherlands) (23).

Plasma glucose was measured using the glucose hexokinase method (Instruchemie), and plasma insulin concentrations were determined by ELISA (Mercodia, Uppsala, Sweden).

Statistical analysis. Partial least squares (PLS) was used to correlate the individual plasma FFA as well as the plasma lipids determined at $t = 0$ to the area under the curve (AUC) of glucose in the GTT performed at $t = 12$ wk (4). To prevent overfitting and to obtain a model error independently of the model fit, double cross-validation was used to validate the PLS models (36, 47). Plasma FFA data were mean-centered, and plasma lipids data were autoscaled within the validation procedure.

Pearson correlation was used to correlate the WBC log 2 transformed gene expression values of $t = 0$ ($n = 8$), week 6 ($n = 7$), and week 12 ($n = 8$) to the AUC of glucose measured in the same animals during GTT. The total number of tested variables was 9,599, of which 2,627 (27.4%) had a $P$ value that was statistically significant (alpha = 0.05). After false discovery rate correction (Benjamini and Hochberg), 602 (6.3%) $P$ values were considered to be truly statistically significant. Only significant correlations with $r > 0.75$ were considered. Scatter plots were used to determine the significance of $r$.

Pearson correlation was also used to correlate the plasma $Acss2$ expression to concentrations of the metabolites malate, succinate, fumarate, and citrate. Furthermore, it was used to correlate C16:1/C16:0 FFA ratio, C18:1/C18:0 ratio, and C18:2 (n-6)/C22:6 (n-3) ratio to AUC of glucose. Only correlations with $r > 0.6$ were considered. Scatter plots and $|r|$ values ($>|r| > 0.01$) were used to determine the significance of $r$. A more stringent $P$ value cutoff was chosen for the entire gene expression data set compared with only $Acss2$ expression, since many more correlation coefficients were calculated.

Network analysis. Network (Fig. 5) was built in Ingenuity Pathway Analysis version 9.0 (Ingenuity System, Redwood City, CA). This method was used to visualize the relations among WBC genes significantly correlated with the AUC of glucose. The top 10 WBC genes (Table 2) were used as an input for network generation. Furthermore, relations of the genes in network with glucose intolerance was assessed by overlaying the network with the functions “insulin resistance of mice,” “insulin resistance index of mice,” and “glucose tolerance of mice.”

RESULTS

Identification of prognostic early biomarkers of glucose intolerance. ApoE3L mice were treated with an obesogenic high-fat diet for 12 wk to induce glucose intolerance. In $n = 15$ mice glucose intolerance was determined individually at $t = 0$ and in week 12. Figure 1 shows the AUC for the individual GTT. Comparison of the AUC at $t = 0$ to the one in week 12 showed that the mice became glucose intolerant ($P < 0.001$), yet a large interindividual variation in AUC at 12 wk was observed with values ranging from 607 to 1,826 [which is typical for mice on a BL/6 background (42)]. No correlation was observed between glucose AUC at week 0 and 12, indicating that there is no relationship between initial glucose tolerance values and the degree of glucose intolerance developed within an animal on a high-fat diet (results not shown). This indicates that a part of the animals developed severe glucose intolerance while others only hardly developed disease.

There was no significant correlation between the individual body weight gain (body weight in week 12 minus starting...
value) and the AUC at week 12 (Pearson correlation of 0.317). Also, there was no significant correlation between the individual concentrations in plasma TG (Pearson correlation of \(-0.311\)), NEFA (Pearson correlation of \(-0.477\)), and total cholesterol at \(t = 0\) (Pearson correlation of 0.111), and the AUC at week 12 (not shown). In this study, the correlation analyses show that global measures such as body weight gain and basic plasma lipids do not predict the susceptibility of the individual animal to become glucose intolerant.

Therefore, plasma collected at \(t = 0\) was analyzed with a more refined method allowing the analysis of specific lipid classes (lipidomics), and interindividual correlations were made to the corresponding AUC measured in week 12.

A total of 114 FFA and complex lipids were quantified in \(t = 0\) plasma samples, and multivariate regression analysis was performed subsequently on the two separate datasets. PLS was used to determine the relationship between \(t = 0\) baseline data derived from lipidomics and the AUC of week 12. Only the individual baseline FFA profile of an animal predicted the individual susceptibility to become glucose intolerant in week 12 with an association of 64% after double cross-validation (Fig. 2A). The baseline profile of complex lipids (LPC, PC,
sphingomyelines, ChE, and MG, DG, and TG) was not able to predict the susceptibility of the individual animal to becoming glucose intolerant (an association of 47% after double cross-validation) with PLS. The fatty acids C16:0, C16:1, C18:2, C18:0, and C22:6 (in this order of predictive weight) contributed most to the prediction (Fig. 2B). The FFA C16:0, C18:0, and C22:6 showed a positive correlation with glucose intolerance, whereas the FFA C16:1 and C18:2 showed a negative correlation. The fact that positive and negative regression vectors were found in the plasma FFA profile suggests that the activity of endogenous enzymes (e.g., fatty acid desaturase) that control metabolism of these fatty acids may determine the susceptibility of disease.

**Analysis of specific FFA ratios and their predictive value for future glucose intolerance.** To further characterize the relationship between specific FFA at \( t = 0 \) and future glucose intolerance we next analyzed Pearson correlations between the FFA that were identified as most significant (based on the PLS model) and the glucose AUC at week 12. The C16:1/C16:0 ratio (Fig. 3A) and the C18:1/C18:0 ratio (Fig. 3B) at \( t = 0 \) were significantly correlated with the AUC at week 12 with correlations of \(-0.816, P = 0.0002 \) and \(-0.665, P = 0.0068 \), respectively (\( n = 15 \)). Also, the C18:2/C22:6 ratio was significantly correlated to the AUC at week 12 (Fig. 3C), showing a positive correlation of 0.688 with a \( P \) value of 0.0046 (\( n = 15 \)). Correlations between the ratios of FFA and the AUC were throughout higher than the correlations of individual FFA and the AUC (C16:1, \(-0.681 \); C16:0, 0.043; C18:1, \(-0.513 \); C18:0, 0.454; C18:2, 0.584, and C22:6, \(-0.348 \)). Furthermore, no correlations were found between the ratios of FFA at week 0 with the AUC at \( t = 0 \), indicating that the fatty acid ratios are no reflections of glucose intolerance status (results not shown).

Together, these lipidomics analyses demonstrate that the ratio of specific FFA in plasma of a healthy individual can serve as predictive biomarker to estimate its susceptibility to develop glucose intolerance when exposed to a high-fat diet.

**Identification of diagnostic biomarkers of glucose intolerance in circulating cells.** Figure 4 shows the individual AUC data obtained by GTT performed at \( t = 0 \) and in weeks 6 and 12 of high-fat diet feeding. Of note, we used a separate group of ApoE3L mice (\( n = 23 \)) that did not overlap with the animals used above for identification of prognostic biomarkers. The natural variation in glucose intolerance was used to perform correlation analyses with the transcriptome of WBC. The WBC transcriptome was generated by extracting RNA from WBCs that were isolated at death, followed by microarray analysis.

To assess whether the gene expression pattern of specific WBC genes would correspond with the degree of glucose intolerance over time, Pearson correlations were made to correlate the individual WBC transcriptome of \( t = 0 \), week 6, and week 12 to the corresponding AUC. The expression changes of genes that were significantly (\( r > 0.75 \) and \( P \) value <0.0001) correlated with the AUC are listed in Table 2. To evaluate if these genes are related to each other network analysis was performed (Fig. 5). Eight out of 10 WBC genes were found to be closely related (network score 25); only Tceal8 and Barhl2 could not be connected to the network. The projection of the functions glucose tolerance of mice, insulin resistance of mice, and insulin resistance index of mice shows a strong connection to the WBC gene network (P value 9.68E-8, 2.53E-4, and 5.74E-4, respectively) (Fig. 5).

The greatest negative correlation was found for the gene Acss2, and an association of \(-0.786 \) was found between the expression of Acss2 at \( t = 0 \) and weeks 6 and 12, and the corresponding glucose AUC (\( n = 23 \)) (Fig. 6). The expression of Acss2 at each of the individual time points also correlated negatively with the AUCs, and the correlations were \(-0.764 \) (\( t = 0, n = 8 \)), \(-0.684 \) (week 6, \( n = 7 \)), and \(-0.785 \) (week 12, \( n = 8 \)). This demonstrates that the expression of Acss2 gene transcripts in WBC allow accurate monitoring of the individual degree of glucose intolerance over time during progression of the disease.

**Acss2 gene expression in WBC is linked to hepatic concentrations of functionally related metabolites.** Citrate, malate, succinate, and fumarate are metabolites that are functionally related to Acss2 protein activity. Acss2 converts acetate generated by the liver back into Acetyl-CoA for oxidation through the citric acid cycle (29). Figure 6 illustrates this relationship between Acss2 and these citric acid cycle metabolites. To determine whether Acss2 gene expression in WBC is linked to the function of Acss2 protein in the liver, we calculated Pearson correlations between Acss2 gene expression in WBC at \( t = 0 \) and weeks 6 and 12 and the liver concentrations of citrate, malate, succinate, and fumarate determined by GC*GC-MS at the same time points (WBCs and livers were of the same mice). Significant correlations over time were found between Acss2 gene expression and the concentrations of these metabolites: succinate 0.655, malate 0.677, citrate 0.721, and fumarate 0.754 (\( n = 23 \), Fig. 7).
DISCUSSION

This study identified prognostic and diagnostic biomarkers of glucose intolerance. High-fat diet treatment of ApoE3L mice resulted in obesity and glucose intolerance. The variation in glucose intolerance seen in this study is typical for mouse models of diet-induced obesity (42) and likely due to epigenetic alterations as seen in monozygotic twin studies (17). This natural variation in the extent of glucose intolerance was used for correlation and regression analysis to identify biomarkers.

We found that the plasma FFA profile of ApoE3L mice prior to the high-fat diet intervention was predictive for the degree of glucose intolerance that developed after 12 wk of high-fat diet treatment. No relationship was found between initial glucose tolerance values and baseline FFA ratios, indicating that the identified FFA ratios are predictors for the susceptibility of the individual animal to become glucose intolerant, rather than indicators of the current level of glucose intolerance. FFA present in plasma are mainly derived from adipose tissue via lipolysis (13), and it may be possible that interindividual differences in adipose tissue lipid metabolism exist in ApoE3L mice.

The initial \( t = 0 \) ratio between monounsaturated and saturated palmitic and stearic FFA was found to be predictive for the level of glucose intolerance at a later time point (week

![Network analysis of white blood cell (WBC) genes associated with AUC of glucose over time.](image)

Fig. 5. Network analysis of white blood cell (WBC) genes associated with AUC of glucose over time. Network showing connections between WBC genes associated with glucose intolerance. The functions “insulin resistance of mice,” “insulin resistance index of mice,” and “glucose tolerance of mice” were projected on the network (blue lines). Red indicates WBC genes with a positive association with glucose intolerance; green indicates WBC genes with a negative association with glucose intolerance.

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Moreover, FFA ratios had higher correlations with glucose intolerance than the individual FFA identified using a multivariate PLS model. Plasma fatty acid ratios C16:1/C16:0 and C18:1/C18:0 are commonly used as estimated measure (“desaturase index”) for the activity of stearoyl-CoA desaturase (SCD) (9, 51). Assuming that metabolism is at equilibrium after an overnight fast, this suggests that the fatty acid desaturase activity of SCD at baseline \((t = 0)\) in ApoE3L mice is predictive for the degree of glucose intolerance developed later in life (2, 25). The negative association between the desaturase index and glucose AUC indicates that ApoE3L mice with a lower activity of SCD prior to high-fat diet feeding become more glucose intolerant when treated with high-fat diet. One of the products of SCD1, C16:1n7-palmitoleate, has recently been identified as a lipokine since this adipose tissue-derived lipid hormone strongly stimulates muscle insulin action (8). This links SCD action to glucose metabolism and provides an explanation for the glucose intolerance prediction in ApoE3L mice.

In mice, four different SCD isoforms have been identified (SCD1, 2, 3, and 4) (21, 30, 31, 50), whereas only two SCD enzymes have been identified in humans (SCD1 and 2) (27, 43, 49). In adipose tissue, the SCD1 isoform is most abundant in both human and mice (33). Since circulating FFA mainly stem from adipose tissue, it is possible that the observed differences in the desaturation index are related to differences in activity of SCD1 in adipose tissue. Interestingly, some mouse studies...

Fig. 6. Scatter plot of acyl-CoA synthetase short-chain family member 2 (Acss2) expression in WBC against GTT values. The WBC Acss2 expression at \(t = 0\) and in week 6 and 12 was plotted against the AUC of the respective GTT. A negative Pearson correlation of 0.786 was found between these Acss2 \(\log_2\) expression values and glucose AUCs. \(P\) value \(\leq 0.0001\). Blue dots represent ApoE3L mice at \(t = 0\), red and green dots animals of week 6 and 12, respectively.

Fig. 7. Acetyl-CoA synthetase 2-related pathway and connection to citric acid cycle. In the mitochondria from tissue cells Acss2 converts acetate generated by the liver back into Acetyl-CoA for oxidation through the citric acid cycle to produce ATP. Significant Pearson correlations were found between WBC Acss2 \(\log_2\) expression value and concentrations of citric acid cycle intermediates from liver tissue over time. The correlations were, respectively, 0.655 for succinate, 0.677 for malate, 0.721 for citrate, and 0.754 for fumarate. Scatter plots of WBC Acss2 \(\log_2\) expression values of \(t = 0\) and week 6 and 12 against respective liver metabolite concentrations are shown.
suggested a positive correlation between SCD1 and the development of obesity and glucose intolerance (33) and therefore SCD1 has been proposed to be a therapeutic target for treatment of obesity and its comorbidities (20). For example, Scd1-deficient mice or mice treated with Scd1-targeted antisense oligonucleotides show several metabolic changes that protect them from obesity, cellular lipid accumulation, and glucose intolerance (20, 32). In humans, data concerning SCD in relation to glucose intolerance are scarce. Evidence for an adverse role of SCD1 activity has not been found, and SCD1 might even protect from development of glucose intolerance in humans (33), which is in line with our findings in ApoE3L mice but contrary to the findings in above mouse studies, all of which employed standard wild-type mice. These contradictory observations might be explained by the different lipid and fat metabolism of standard mice, which differs from humans and ApoE3L mice (33). The human transgenes present in ApoE3L mice result in a lipid metabolism and a lipoprotein profile (48) that are very similar to those of humans and may possibly provide an explanation for the similarities observed in ApoE3L and humans regarding the role of SCD1.

Subsequently, it was found that the FFA ratio C18:2/C22:6, further referred to as n-6/n-3 FFA ratio, prior to the intervention predicted glucose intolerance at week 12 and that this ratio showed higher correlation to glucose intolerance than the individual FFA as identified with a multivariate PLS model. Docosahexaenoic acid (DHA, C22:6 FFA) is the end-product of the elongation and desaturation chain of the essential omega-3

![Fig. 8. The shared series of desaturation and elongation reactions in n-6 and n-3 fatty acid pathways.](image-url)
fatty acids, whereas linoleic acid (LA, C18:2 FFA) is the start-product of the elongation and desaturation chain of the essential omega-6 fatty acids (Fig. 8). LA cannot be synthesized by mice (and humans) and therefore must be obtained from the diet. Since all ApoE3L mice consumed the same diet, differences in the ratio of LA to DHA may reflect a difference in polyunsaturated fatty acid (PUFA) metabolism. The positive association between the n-6/n-3 FFA ratio and glucose AUC suggests that ApoE3L mice with relative high plasma amounts of LA (n-6) and low plasma amounts of DHA (n-3) at the start (t = 0) will become more glucose intolerant on high-fat diet. Figure 8 shows that omega-3 and omega-6 fatty acids use the same set of desaturase and elongase enzymes (3). Our findings suggest that ApoE3L mice with a relatively lower activity of elongase and/or desaturase enzymes at baseline more prone to develop glucose intolerance.

Comprehensive analysis of the changes in lipid composition over time in white adipose tissue, liver, and plasma in the biobank samples used for this study showed that saturated fatty acids increased in all tissues, whereas the content of (poly)unsaturated fatty acids decreased significantly, suggesting that the ratio of saturated to unsaturated fatty acids may indeed be an important indicator for the observed development of glucose intolerance (7, 23). This is also in line with the present finding that the capacity to desaturate and/or to elongate fatty acids is associated with the susceptibility to become insulin resistant.

The data also support results made in human studies indicating that desaturase and elongase activities play important roles in the pathogenesis of glucose intolerance (9, 37, 51, 52).

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Ten different genes expressed in WBC were identified as potential diagnostic biomarkers of glucose intolerance. Network analysis of these genes showed a strong connection with glucose tolerance indexes, suggesting that the WBC genes are related to glucose intolerance itself and not merely to a symptom of the disease such as inflammation. Interestingly, the genes *Acss2* and *SCD1* are known to be co-regulated by insulin and the inflammatory cytokite tumor necrosis factor-α (TNF-α) during and after adipocyte differentiation (46). *Acss2* expression had strongest negative correlation with the glucose AUC over time. This means that a low expression level of *Acss2* in WBC is indicative of a high degree of glucose intolerance. *Acss2* encodes for the protein acyl-CoA synthetase short-chain family member 2. Acetyl-CoA synthetase catalyzes the ligation of acetate (derived from β-oxidation of fatty acids) with CoA to produce acetyl-CoA for oxidation through the citric acid cycle to produce ATP and CO₂ (14, 35). This mitochondrial enzyme is essential for energy expenditure under ketogenic conditions, such as fasting, low-carbohydrate diet feeding, and diabetes (see also Fig. 7). Consistent with this, we found significant correlations between *Acss2* expression in WBC and concentrations of liver intermediates from the citric acid cycle over time (0, 6, and 12 wk), suggesting that *Acss2* expression in WBC is related to the hepatic citric acid cycle.

The present study reports on novel prognostic markers and WBC-derived diagnostic markers of glucose intolerance that may serve as a tool to monitor glucose intolerance during disease progression and to assess the efficacy of interventions. Both sets of markers require further validation in future studies. When validated, these markers may be used for diagnostic purposes to assess the risk and stage of glucose intolerance, but also for the development and efficacy testing of pharmaceuticals/nutraceuticals.

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DISCLOSURES

Authors have a potential conflict of interest as their organization may benefit from a product or patent generated on the basis of the published data. In these cases, the authors will however not receive additional salary, additional personal income, or any form of financial support.

AUTHOR CONTRIBUTIONS


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