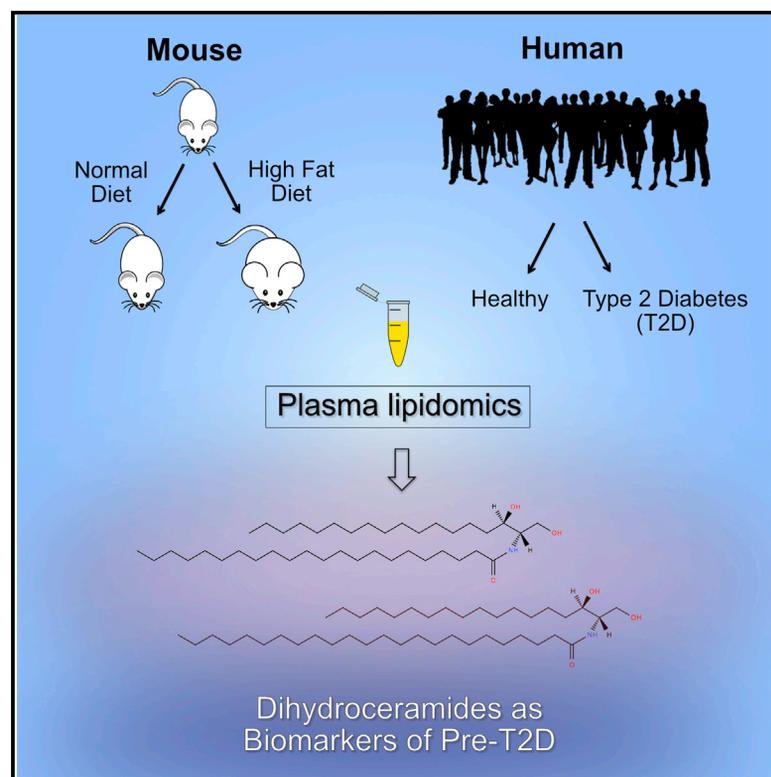# Plasma Dihydroceramides Are Diabetes Susceptibility Biomarker Candidates in Mice and Humans

Graphical Abstract

Authors

Leonore Wigger,
Céline Cruciani-Guglielmacci,
Anthony Nicolas, ..., Christophe Magnan,
Mark Ibberson, Bernard Thorens

Correspondence

mark.ibberson@sib.swiss (M.I.),
bernard.thorens@unil.ch (B.T.)

In Brief

Wigger et al. find that several sphingolipids in mouse plasma correlate with glucose tolerance and insulin secretion. Quantitative analysis of these and closely related lipids in human plasma from two cohorts reveal that dihydroceramides are significantly elevated in individuals progressing to diabetes, up to 9 years before disease onset.

Highlights

- Shotgun lipidomics was performed on plasma samples from mice and humans

- In mice, several sphingolipids correlate with diabetes-like traits

- In human cohorts progressing to diabetes, dihydroceramide levels are elevated

- A significant increase was found in two cohorts, up to 9 years before disease onset

CrossMark

CellPress

Plasma Dihydroceramides Are Diabetes Susceptibility Biomarker Candidates in Mice and Humans

Leonore Wigger,[1,2] Céline Cruciani-Guglielmacci,[3] Anthony Nicolas,[4,5,6,7] Jessica Denom,[3] Neïké Fernandez,[3] Frédéric Fumeron,[4,5,6,7] Pedro Marques-Vidal,[8] Alain Ktorza,[9] Werner Kramer,[10] Anke Schulte,[11] Hervé Le Stunff,[3,12] Robin Liechti,[1] Ioannis Xenarios,[1] Peter Vollenweider,[8] Gérard Waeber,[8] Ingo Uphues,[13] Ronan Roussel,[4,5,6,7] Christophe Magnan,[3] Mark Ibberson,[1,*] and Bernard Thorens[2,14,*]

[1]Vital-IT Group, SIB Swiss Institute of Bioinformatics, 1015 Lausanne, Switzerland
[2]Center for Integrative Genomics, University of Lausanne, 1015 Lausanne, Switzerland
[3]Unité de Biologie Fonctionnelle et Adaptative (BFA), CNRS UMR 8251, Université Paris Diderot, Sorbonne Paris Cité, 75205 Paris, France
[4]INSERM, Sorbonne Paris Cité, Centre de Recherce des Cordeliers (CRC), UMR_S 1138, 75006 Paris, France
[5]UPMC, Sorbonne Universités, Centre de Recherce des Cordeliers (CRC), UMR_S 1138, 75006 Paris, France
[6]Université Paris Descartes, Sorbonne Paris Cité, Centre de Recherche des Cordeliers (CRC), UMR_S 1138, 75006 Paris, France
[7]Université Paris Diderot, Sorbonne Paris Cité, Centre de Recherches des Cordeliers (CRC), UMR_S 1138, 75006 Paris, France
[8]Department of Medicine, Internal Medicine, Lausanne University Hospital (CHUV), 1011 Lausanne, Switzerland
[9]Recherche de Découverte, PIT Métabolisme, Institut de Recherche Servier (IdRS), 92150 Suresnes, France
[10]Biomedical and Scientific Consulting, 55130 Mainz, Germany
[11]Diabetes Research, Islet Biology Cluster, Sanofi-Aventis Deutschland GmbH, Industriepark Höchst, 65926 Frankfurt am Main, Germany
[12]Institut de biologie intégrative de la cellule (I2BC), CNRS UMR 9198, Université Paris-Sud, Université Paris-Saclay, 91190 Gif-sur-Yvette, France
[13]Cardiometabolic Diseases Research, Boehringer Ingelheim Pharma GmbH & Co. KG, 88400 Biberach (Riss), Germany
[14]Lead Contact
*Correspondence: mark.ibberson@sib.swiss (M.I.), bernard.thorens@unil.ch (B.T.)
http://dx.doi.org/10.1016/j.celrep.2017.02.019

SUMMARY

Plasma metabolite concentrations reflect the activity of tissue metabolic pathways and their quantitative determination may be informative about pathogenic conditions. We searched for plasma lipid species whose concentrations correlate with various parameters of glucose homeostasis and susceptibility to type 2 diabetes (T2D). Shotgun lipidomic analysis of the plasma of mice from different genetic backgrounds, which develop a pre-diabetic state at different rates when metabolically stressed, led to the identification of a group of sphingolipids correlated with glucose tolerance and insulin secretion. Quantitative analysis of these and closely related lipids in the plasma of individuals from two population-based prospective cohorts revealed that specific long-chain fatty-acid-containing dihydroceramides were significantly elevated in the plasma of individuals who will progress to diabetes up to 9 years before disease onset. These lipids may serve as early biomarkers of, and help identify, metabolic deregulation in the pathogenesis of T2D.

INTRODUCTION

Type 2 diabetes mellitus (T2D) is diagnosed based on elevated fasting glycemia and abnormal glucose tolerance tests. These deregulations are caused by genetic and lifestyle factors, which lead to the failure of beta cells to secrete enough insulin to compensate for the insulin resistance of liver, fat, and muscles. The specific pathogenic mechanisms that lead to the imbalance between insulin secretion and insulin action are, however, poorly understood. Nevertheless, it is known that initial defects in specific metabolic, signaling, or differentiation pathways in pancreatic beta cells or insulin target tissues may all induce whole body functional deregulations that ultimately cause T2D. This is exemplified by the various forms of maturity onset diabetes of the young (MODY) (Bonnefond and Froguel, 2015), which are monogenic forms of diabetes affecting mostly pancreatic beta-cell function, or by numerous studies of mice with cell-specific inactivation of genes involved in insulin signaling or insulin secretion, which all develop T2D (Baudry et al., 2002). Thus, T2D may have multiple initial causes and identifying them before the onset of hyperglycemia may help develop preventative strategies or therapeutic approaches based on specific pathogenic mechanisms.

Identification of circulating biomarkers that could predict the susceptibility to T2D and be used to monitor the progression of the disease is important in developing better treatments and in

designing innovative clinical trials. Plasma metabolomic studies may identify such biomarkers. Indeed, it has been hypothesized many years ago (Pauling et al., 1971) that quantitative determination of plasma metabolite concentrations could give precise information about the activity of specific metabolic pathways, the functional state of the organs in which they operate, and their deregulations in pathogenic conditions. Many studies have searched for plasma metabolite biomarkers for T2D susceptibility (Ferrannini et al., 2013; Stancáková et al., 2012; Würtz et al., 2012; Zhao et al., 2010), but predictive biomarkers for T2D are not yet available, in particular, because the combination of several biomarkers may be needed to obtain sufficient prediction power (Cobb et al., 2015). Finding such biomarkers is critical to identify the original metabolic deregulations that cause T2D. These biomarkers can then be used to classify diabetes according to the causal pathogenic mechanisms and develop personalized treatment of the disease.

It would be advantageous to find biomarkers that are similarly predictive of disease susceptibility in mouse and humans. Indeed, animal studies are critical to (1) experimentally determine whether the circulating biomarker correlates with or induces beta-cell dysfunction or insulin resistance in liver, fat, or muscle and (2) identify the tissue that produces the biomarker as this may become a therapeutic target. On the other hand, population-based prospective cohorts are required to validate the prognostic value of the biomarker, which may be coupled, for instance, to already gathered genetic data to potentially provide composite biomarkers of higher predictive power.

It is well established that mice fed a high-fat (HF) diet develop a form of pre-diabetes characterized by obesity, mild fasting hyperglycemia, hyperinsulinemia, insulin resistance, and glucose intolerance (Burcelin et al., 2002; Surwit et al., 1988). However, the metabolic deregulations induced by high-fat diet feeding are largely influenced by the genetic background (Andrikopoulos et al., 2005; Rossmeisl et al., 2003; Surwit et al., 1995). Thus, studying adaptation of mice from diverse genetic backgrounds to metabolic stress offers a powerful way to identify the changes in plasma metabolites that are most relevant to assess the risk of developing T2D. The availability of population-based prospective cohorts such as data from an epidemiologic study on the insulin resistance syndrome (DESIR) cohort (Balkau et al., 2008) and Cohorte Lausannois (CoLaus) (Firmann et al., 2008), which comprise hundreds of individuals who have progressed from a healthy state to T2D, provide a unique resource for the validation of the biomarkers identified in pre-clinical models.

In the present study, we report the identification of a group of sphingolipids associated with impaired glucose tolerance and altered insulin secretion in mice exposed to metabolic stress. Targeted analysis of these sphingolipids in over 250 individuals from two different prospective cohorts who developed T2D revealed that the plasma concentrations of dihydroceramide and ceramide species are significantly elevated as compared to control individuals up to 9 years before development of T2D. These sphingolipids are therefore biomarkers for T2D susceptibility and may help identify primary metabolic deregulation in the pathogenesis of T2D.

RESULTS

Plasma Levels of Certain Ceramides and Ceramide Derivatives Correlate to Glucose Tolerance and Insulin Secretion in Metabolically Challenged Mice

Mice from six genetically different mouse strains (C57BL/6J, DBA/2J, 129S2/SvPas, AKR/J, A/J, and BALB/cJ) were fed a high-fat, high-sucrose (HFHS) or regular chow (RC) diet for 2, 10, 30, or 90 days, after which time several phenotypic measurements were performed, including glycemia, insulinemia, glucose tolerance, and in vitro glucose-stimulated insulin secretion (detailed study to be reported separately). At each time point and for each mouse strain and diet, plasma samples were taken from six different mice and analyzed using a combined shotgun and targeted lipidomics strategy. In this strategy, 135 molecular lipids of 19 different lipid classes were quantified including sphingolipids [ceramides Cer(d18:1), dihydroceramides Cer(d18:0), ceramide derivatives, and sphingosine] and triacylglycerides (TAGs) (see Experimental Procedures). The plasma lipids showed distinct profiles across samples, where both genetic background and diet strongly influenced plasma lipid concentrations. This can be seen by distinct patterns of high and low lipid concentrations across the mouse strains on different diets and different time points in the study (Figure S1). We next correlated the lipids to each of the phenotypic traits in order to identify those lipids that were potentially associated with mouse phenotypes related to pancreatic dysfunction and diabetes. Figure 1 shows a network representation of the strongest lipid trait correlations (absolute Spearman correlation $|r_s| \geq 0.4$) with lipids and phenotypic traits as nodes and correlations as edges. Lipids were selected for further investigation (marked with red borders in Figure 1) if they were (1) strongly correlated to glucose tolerance or insulin secretion or both, which we regarded as the primary traits of interest in mice, and (2) in addition to these to one or several of HOMA-B, HOMA-IR, and fasting insulin. Correlation data that were used to generate the network in Figure 1 are included in Table S1. Three ceramides, one dihydroceramide, and two lactosylceramides satisfied these criteria due to their correlations with several traits including glucose intolerance and insulin secretion and were selected for further investigation. Correlation data for these selected lipids with the different phenotypic traits are included as a separate sheet in Table S1. Scatterplots of these lipids showing correlations with six mouse traits are shown in Figure S2. Most of them are positively correlated to glucose intolerance (area under the curve [AUC] of glycemia) and negatively correlated to insulin sensitivity (AUC of insulinemia) as well as positively correlated to fasting insulin, insulin secretion, and HOMA-B, suggesting that they might be early markers of the pre-diabetic phenotype induced by high-fat diet in mice. The plasma levels of many of these sphingolipids were also significantly increased by HFHS diet but to differing degrees in the six mouse strains (Figure S3; Table S2). There are also marked differences between certain strains. For example, Cer(d18:1/22:0) (SwissLipids ID: SLM:000392149) plasma concentration increased over time in both RC and HFHS-fed mice in DBA/2J, but started high and decreased over time in BALB/cJ mice. BALB/cJ mice are interesting since they showed the strongest early diabetic phenotype compared to the other
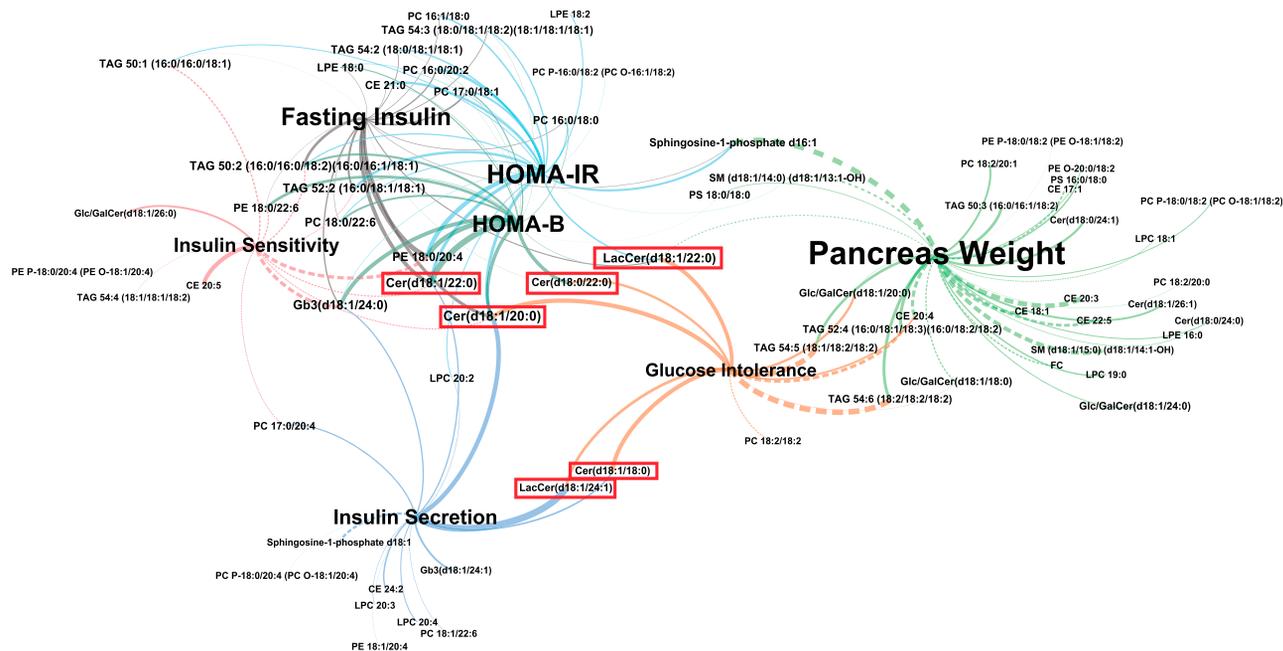
Figure 1. Ceramides Are Correlated to Glucose Intolerance and Insulin Sensitivity in Metabolically Challenged Mouse Strains
Lipid-trait network showing plasma lipid correlations with five measured phenotypic traits. Correlations are represented as edges between lipid nodes and trait nodes. Only correlations with absolute value ≥ 0.4 are shown. The graph was produced using the *ForceAtlas2* layout in *Gephi 0.9.1* with scaling = 10, gravity = 1, and edge weight influence =1, and overlapping labels were adjusted using Label Adjust. Each trait node is depicted as a different color, and edges are colored according to the correlated trait. Edge width is proportional to correlation strength from minimum 0.4 to maximum 0.65. Solid edge lines indicate positive correlations; dashed lines indicate negative correlations. Node label size is proportional to degree (total number of connections). Ceramide lipids that were chosen for further investigation based on their correlations to several mouse traits are boxed in red. (See Table S1 for the correlation data from which the network was constructed.)

strains (C.C.-G., J.D., N.F., R.L., L.W., I.U., I.X., H.L.S., B.T., C.M., M.I., L. Bellini, M. Oshima, P. Normandie-Levi, X.P. Berney, N. Kassis, C. Rouch, J. Dairou, T. Gorman, D. Smith, A. Marley, D. Kuznetsov, F. Burdet, A.-L. Lefèvre, I. Wehrle, T. Hildebrandt, W. Rust, C. Bernard, A. Ktorza, G.A. Rutter, and R. Scharfmann, unpublished data). It is therefore intriguing that plasma levels of all six of the sphingolipid species on RC diet were higher in BALB/cJ compared to the other strains (Figure S3), suggesting that plasma levels of these lipids might indicate predisposition to high-fat-induced diabetes in mice.

A Class of Ceramides Is Elevated in Human Plasma 9 Years before Disease Diagnosis
The results in mouse prompted us to investigate whether the same or similar sphingolipid species were associated with pre-diabetes in humans. We therefore performed targeted ceramide lipidomics on plasma samples from a longitudinal human cohort (DESIR). Samples from this cohort were divided into a control group and three groups of cases, corresponding to different time durations from inclusion in the study to disease diagnosis: group 1 contained individuals who were diagnosed with T2D at 3 years; group 2 were diagnosed at 6 years, and group 3 at 9 years. In each group, plasma samples were obtained every 3 years: for group 1, there were two sets of samples (baseline and follow-up at 3 years, n = 81 and n = 82), for group 2 three sets (baseline, 3 and 6 years (T2D diagnosis), n = 48, n = 49,

n = 48) and for group 3 four sets (baseline, 3, 6, and 9 years (T2D diagnosis), n = 62, n = 62, n = 61, n = 61). For the control group, there were also four sets (baseline, 3, 6, and 9 years, n = 105, n = 102, n = 104, n = 97). Within each group, the number of samples n available at each time point differed slightly due to missing samples and samples excluded because of quality issues. The definition of T2D diagnosis was that an individual either had a fasting glucose level above 7 mmol/L or was under antidiabetic drug treatment at the time of sample collection. We performed statistical comparisons correcting for age and sex, comparing cases and controls at baseline, 3, 6, and 9 years for the three groups (see Experimental Procedures). We found that several of the ceramide species that were correlated to glucose intolerance in mouse, namely, Cer(d18:1/18:0) (SwissLipids ID: SLM:000392135), Cer(d18:1/20:0) (SLM:000392142), and Cer(d18:1/22:0) (SLM:000392149), were significantly elevated in the plasma of patients 3 years before T2D diagnosis (Figure 2), but this was only observed in a single group of patients (group 1). Remarkably, we found that the class of dihydroceramides, Cer(d18:0), which are precursors of the ceramides and differ from them by a single double bond (Aimo et al., 2015), were significantly and reproducibly elevated in the plasma of all groups of patients at all time points of the study compared to controls (Figure 2). Dihydroceramide species Cer(d18:0) were elevated in plasma samples of patients up to 9 years prior to T2D diagnosis. Figure 3B shows details of the fold change and
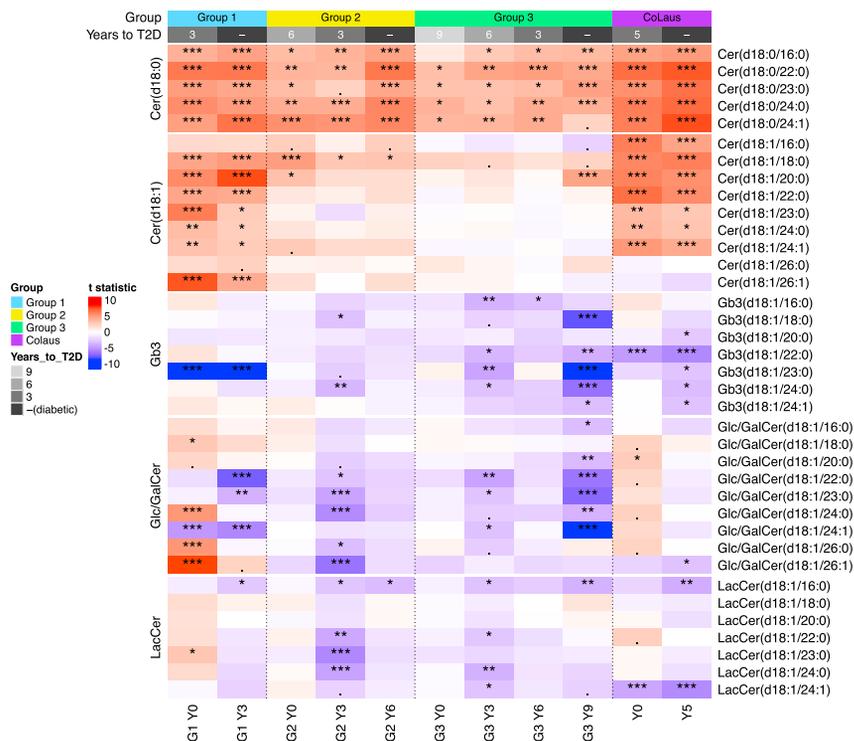
Figure 2. Dihydroceramides Are Elevated in Plasma up to 9 Years before Incident Disease

Heatmap of t statistics calculated between diabetic subjects and the control samples corrected for age and sex. The mean lipid concentration from each subject group at each time point was compared to the mean lipid concentration from the control group taken during the same sample collection period. White, no difference between diabetic and control group; red, higher value in diabetic group; blue, lower value in diabetic group. Asterisks indicate significance of differences between cases and controls (*adjusted $p < 0.05$, **adjusted $p < 0.01$, ***adjusted $p < 0.001$, p values adjusted for multiple correction across 37 lipids by the Benjamini-Hochberg method).

significance for ceramide changes 9 to 3 years before diagnosis compared to controls for group 3, highlighting the Cer(d18:0) class lipids. The fold change difference in mean dihydroceramide Cer(d18:0) concentrations observed between cases and controls remains stable over time (Figure 3A). Additional correction for BMI or low-density lipoprotein (LDL) diminished the statistical significance of case-control differences, but the overall pattern remained the same (Figure S4). Interestingly, one of the dihydroceramides showing the most significant differences between cases and controls, Cer(d18:0/22:0) (SwissLipids ID: SLM:000392085), was also significantly positively correlated to glucose intolerance in mouse (Figures 1 and S2). These results suggest that dihydroceramide plasma levels could be stably associated with T2D predisposition.

Dihydroceramides Are Elevated in Plasma 5 Years before Disease Onset in an Independent Cohort

We sought to validate our findings from the DESIR cohort in an independent cohort. We performed targeted lipidomics analysis on plasma samples from the CoLaus cohort at baseline and 5 years (T2D diagnosis). The number of samples n in each group was 150, less a small number of samples (up to three per group and time point) that were either entirely missing or excluded after the lipidomics analysis (for sample numbers, see Table S6). The results (Figure 2) showed elevated plasma concentrations of the same dihydroceramide Cer(d18:0) species that were significantly elevated in the DESIR cohort at baseline and diagnosis, thus confirming our previous results. Similar to the results obtained for the DESIR cohort, Cer(d18:0) levels are elevated to similar levels at both baseline and diagnosis, indicating that this lipid class could be associated with T2D predisposition

(Figure 4A). Interestingly, many of the Cer(d18:1) lipids (ceramides) that were significantly different between cases and controls in group 1 of the DESIR cohort (baseline 3 years before T2D diagnosis) and in the mouse study (Figures 1 and S2) were also significantly elevated in CoLaus 5 years prior to diagnosis (Figure 2). Additional correctio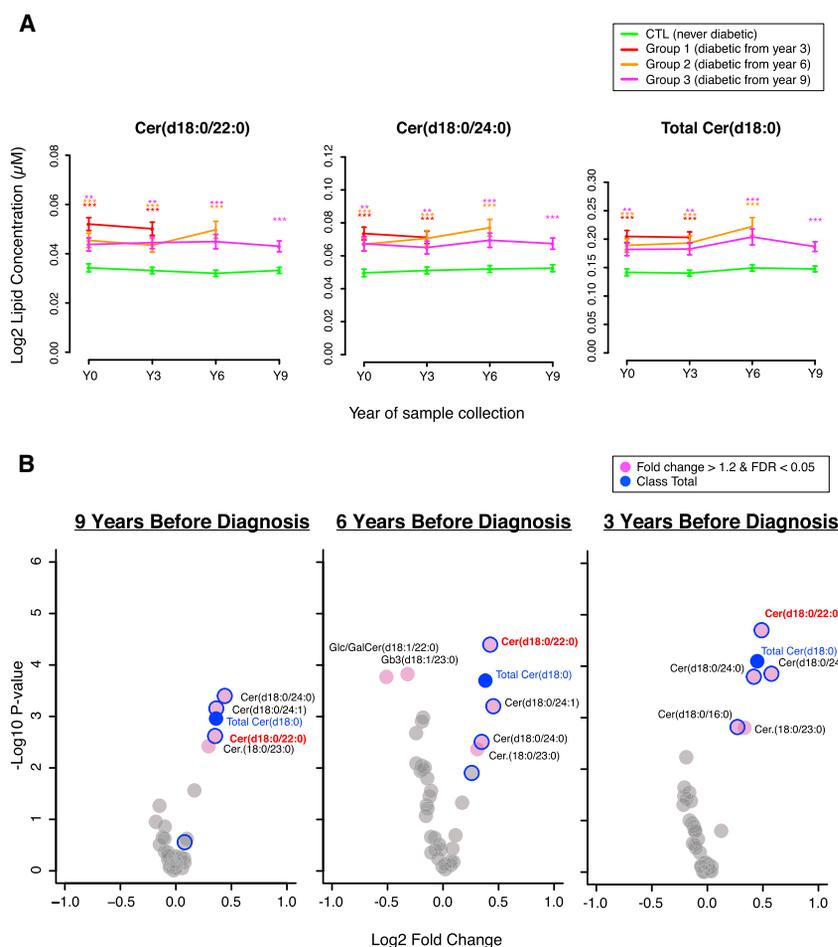n for BMI, LDL, insulin or glycemia had little effect on the significance of observed increases of ceramide Cer(d18:1) or dihydroceramide Cer(d18:0) levels at baseline in CoLaus, although insulin and glycemia had a larger effect on groups 2 and 3 of the DESIR cohort (Figures S4 and S5).

High Plasma Levels of Dihydroceramides May Be Associated with an Increased Risk of Future Diabetes

Logistic regression models were performed on both the DESIR and the CoLaus data in order to compute adjusted odds ratios for Cer(d18:0) and Cer(d18:1) species. Results from the DESIR cohort, from all three groups at all time points before diabetes incidence, are shown in Table S7. The basic model included age and sex as covariates besides the lipid concentration. Additional models included one further covariate (BMI, LDL, fasting glucose, insulin, HOMA2-%B, HOMA2-%S, and waist circumference). The odds ratios provided are "inter-quartile range odds ratios"; that is, they are calculated per increase of the lipid concentration by an amount equal to the difference between the top and bottom quartile. There are marked differences in the results from the three groups of study participants. Results from group 1 (3 years before diabetes) show odds ratios greater than 2 for the total dihydroceramides concentration and for the three Cer(d18:1) ceramides, regardless of the other covariates added to the model. In groups 2 and 3 (6 and 9 years before diabetes, respectively), dihydroceramides have lower but still significant odds ratios than for group 1 from the basic regression model with age and sex, but, if glucose or insulin is added to the model, the odds of belonging to the diabetic group are not significantly increased in patients with higher lipid concentration (confidence intervals of odds ratios tend to span 1 or come close to 1). Similarly, except for Cer(d18:1/18:0) in group 2 at year 0,

A

Cer(d18:0/22:0)   Cer(d18:0/24:0)   Total Cer(d18:0)

CTL (never diabetic)
Group 1 (diabetic from year 3)
Group 2 (diabetic from year 6)
Group 3 (diabetic from year 9)

Log2 Lipid Concentration (μM)

Y0 Y3 Y6 Y9

Year of sample collection

B

Fold change > 1.2 & FDR < 0.05
Class Total

9 Years Before Diagnosis   6 Years Before Diagnosis   3 Years Before Diagnosis

-Log10 P-value

Glc/GalCer(d18:1/22:0)
Gb3(d18:1/23:0)

Cer(d18:0/24:0)
Cer(d18:0/24:1)
Total Cer(d18:0)
Cer(d18:0/22:0)
Cer.(18:0/23:0)

Cer(d18:0/22:0)
Total Cer(d18:0)
Cer(d18:0/24:1)
Cer(d18:0/24:0)
Cer.(18:0/23:0)

Cer(d18:0/22:0)
Total Cer(d18:0)
Cer(d18:0/24:0)   Cer(d18:0/24:1)
Cer(d18:0/16:0)
Cer.(18:0/23:0)

Log2 Fold Change

Figure 3. Mean Lipid Concentration of Dihydroceramides Are Significantly Elevated at All Time Points in the DESIR Study

(A) Mean plasma concentrations of dihydroceramides plotted over time. The left two plots show individual lipid species that represent well the behavior of the class Cer(d18:0/22:0) and Cer(d18:0/24:0). The rightmost plot represents the class total for Cer(d18:0). The group means are consistently higher in diabetes cases as compared to control samples. x axis: time point of collection. y axis: mean lipid concentration in each of the groups. Error bars: SEM of the lipid concentration. Asterisks at each time point represent significance of the statistical test comparing cases to controls (age- and sex-corrected linear model): *adjusted $p < 0.05$, **adjusted $p < 0.01$, ***adjusted $p < 0.001$ (p values adjusted for multiple correction across 37 lipids by the Benjamini-Hochberg method).

(B) Volcano plots of statistical tests comparing 37 lipid species in each group of diabetic subjects versus the control samples from the same sample collection period (linear model, containing factors for sex and age). A single lipid class is robustly increased in all diabetic groups and at all time points, before and after onset of diabetes: Cer(d18:0), dihydroceramides. Cer(d18:0/22:0) (highlighted in red), which was identified in the mouse study as strongly correlated to the AUC of glycemia, was elevated at all time points shown. x axis: log2 fold change between cases and controls. y axis: log10 of the p value. Circles with blue outlines: the five lipid species from class Cer(d18:0). Solid blue circle: the class total from class Cer(d18:0). The plots shown are from DESIR group 3, at 9 (left), 6 (center), and 3 (right) years before subjects were identified as diabetic.

none of the three ceramides from Cer(d18:1) show odds ratios significantly different from 1 in groups 2 and 3.

Results from CoLaus are shown in Table 1. The same logistic regression models were applied as for the DESIR cohort, except that bioimpedance was also included (this was not available for the DESIR cohort). For an individual whose plasma lipid concentration was at the top quartile at baseline, the sex- and age-adjusted odds of belonging to the group that became diabetic were around two to 2.5 times higher than they were for an individual at the bottom quartile, depending on lipid species [example: Cer(d18:0/24:0), adjusted odds ratio 2.37, 95% CI, 1.69–3.33]. In all tests, the lower limit of the 95% confidence interval remains above 1. This suggests that a high plasma level of any of these lipids is associated with an increased risk of future diabetes compared to low plasma level. Inclusion of BMI, LDL, or fasting glucose as covariates in the model results in smaller odds ratios than the basic model, but the lower bound of the confidence intervals remains above 1, indicating that the association remains significant.

Decision curve analysis (Rousson and Zumbrunn, 2011; Vickers and Elkin, 2006) was performed as described in Experimental Procedures. Briefly, this analysis quantifies the predictive value of a potential biomarker using a net benefit measure that can

be plotted as a curve for different probability cutoffs. Decision curve results for CoLaus suggests that the Cer(d18:0) lipid class is a better predictor (higher net benefit) for T2D outcome than LDL, HOMA2-%B, and HOMA2-%S (Figure 5). Fasting glucose by itself is the strongest predictor among the variables that were tested. The combined model of the lipid class and fasting glucose has about the same net benefit as the model with fasting glucose by itself. Adding the lipid does not appear to improve the prediction above glucose alone (Figure 5). Similar results were obtained from analysis of the DESIR cohort (data not shown). On the other hand, the results also suggest that a combined model of the lipid class and BMI is a better predictor than BMI by itself. The same is true for waist circumference, bioimpedance, and insulin (Figure 5), suggesting that Cer(d18:0) lipids may constitute a plasma biomarker that could be used in combination with other measures to help predict T2D.

DISCUSSION

In the present study, we first investigated the plasma lipidome of mice from different genetic backgrounds exposed to a metabolic stress for different periods of time. The rationale for these experiments is that because of their diverse genetic architectures, the
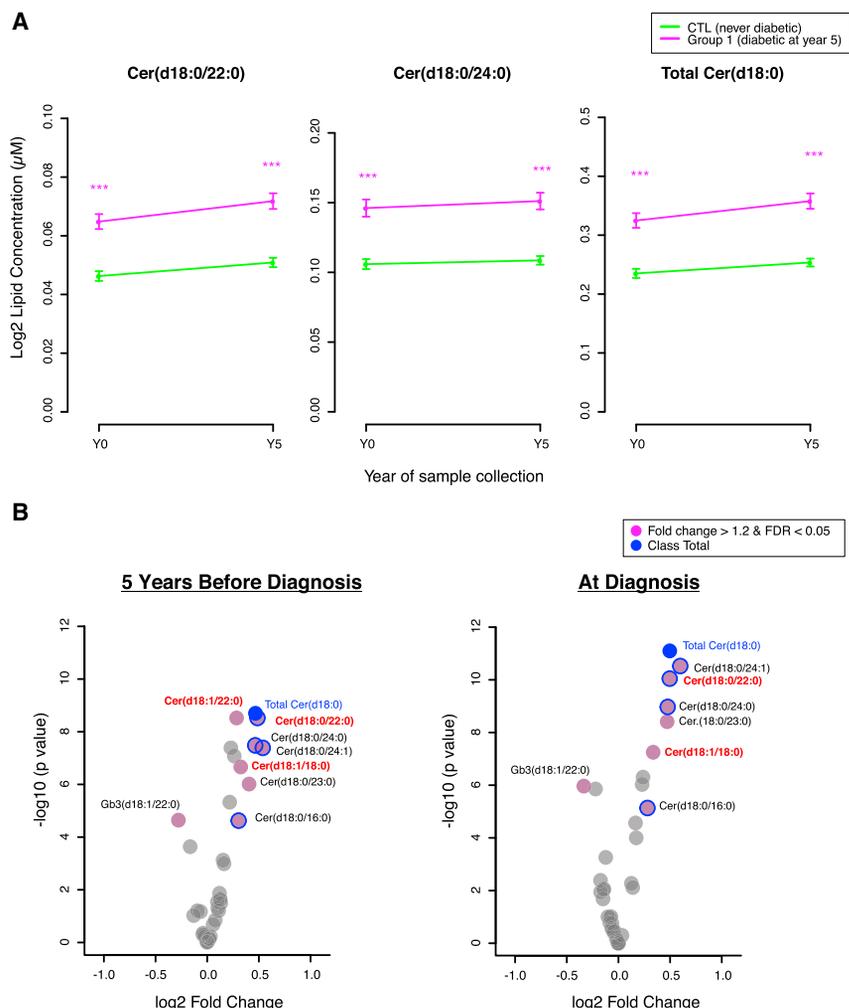
A

The fact that the same group of sphingolipids was associated with susceptibility to T2D in two separate cohorts as well as in mice strongly suggests that they may represent reliable biomarkers. This is further supported by the fact that a significant association of these lipids, but, in particular, the dihydroceramides in DESIR group 1 and in CoLaus samples, with T2D risk is conserved even after correction for LDL, BMI, or glycemic levels as covariates. These observations also suggest that in humans dihydroceramides may play a particular role in T2D progression.

Dihydroceramides are produced in the third step of the de novo ceramide biosynthetic pathway initiated by serine palmitoyl transferase. They are converted into ceramides by ceramide desaturase, of which two isoforms exist, the ubiquitously expressed Des1, and Des2 which is expressed mostly in the gut and skin (Bikman and Summers, 2011). Despite being once considered inert sphingolipid precursors, dihydroceramides have recently been proposed as biomarkers of metabolic dysfunction (Siddique et al., 2015). Circulating levels of dihydroceramides were shown to be elevated in T2D in young adults (Lopez et al., 2013) and have been associated with waist circumference (Mamtani et al., 2014) and severity of insulin resistance (Brozinick et al., 2013). At cellular levels, increased concentrations of dihydroceramides can regulate autophagy, reactive oxygen species production, cell proliferation, and apoptosis (Rodriguez-Cuenca et al., 2015; Siddique et al., 2015). This has been studied in detail in 3T3-L1 adipocytes where silencing of

selected mouse strains display different phenotypic adaptation to HFHS feeding. Thus, if a group of lipids correlates strongly with a given phenotype across strains, it is likely to represent a general biomarker for susceptibility to develop T2D. The overall goal of the mouse experiment was to select the most likely biomarker candidates to follow up in human cohorts. Following this approach, we identified a group of sphingolipids as being correlated with insulin secretion and glucose intolerance. Several of those were also found to be strongly associated with the risk to develop T2D in both the DESIR and CoLaus cohorts.

In mice, three ceramides [Cer(d18:1/18:0, 20:0 and 22:0)], two lactosylceramides (LacCer18:1/22:0 and 24:1), and one dihydroceramide [Cer(d18:0/22:0)] were found at a central interaction point in a lipid-phenotype correlation network between glucose intolerance, insulin secretion, fasting insulin, and HOMA-B. In both human cohorts, the susceptibility to develop T2D was associated with increased plasma levels of several ceramide and dihydroceramide species, of which the ceramides Cer(d18:1/18:0), Cer(d18:1/20:0), Cer(d18:1/22:0), and the dihydroceramide Cer(d18:0/22:0) were also found in pre-diabetes in mice.

Table 1. Adjusted Interquartile-Range Odds Ratios from the CoLaus Cohort at Baseline

Odds Ratios (per increase by the interval between first and third quartile), with 95% CI	Cer(d18:0/16:0)	Cer(d18:0/22:0)	Cer(d18:0/23:0)	Cer(d18:0/24:0)	Cer(d18:0/24:1)	Total Cer(d18:0)	Cer(d18:1/18:0)	Cer(d18:1/20:0)	Cer(d18:1/22:0)
Basic model (lipid, age, and sex)	1.92 (1.39–2.64)	2.59 (1.83–3.66)	2.11 (1.53–2.9)	2.37 (1.69–3.33)	2.47 (1.74–3.51)	2.52 (1.8–3.53)	2.25 (1.62–3.13)	2.53 (1.76–3.65)	2.25 (1.68–3.02)
Basic model plus BMI	1.71 (1.21–2.42)	2.24 (1.56–3.23)	1.85 (1.33–2.58)	2.16 (1.52–3.07)	2 (1.38–2.9)	2.18 (1.53–3.1)	1.78 (1.27–2.51)	2.31 (1.57–3.4)	2.26 (1.65–3.1)
Basic model plus LDL	1.78 (1.26–2.52)	2.42 (1.67–3.5)	2.03 (1.43–2.88)	2.27 (1.57–3.27)	2.37 (1.64–3.43)	2.44 (1.7–3.51)	2.08 (1.46–2.98)	2.28 (1.55–3.34)	2.2 (1.58–3.07)
Basic model plus fasting glucose	1.57 (1.08–2.28)	2.1 (1.4–3.15)	1.63 (1.13–2.37)	1.86 (1.26–2.76)	1.64 (1.12–2.41)	1.89 (1.29–2.79)	2.03 (1.38–2.99)	2.3 (1.52–3.46)	2.21 (1.57–3.11)
Basic model plus insulin	2.01 (1.39–2.9)	2.67 (1.79–3.96)	2.07 (1.44–2.97)	2.39 (1.62–3.53)	2.46 (1.64–3.69)	2.56 (1.74–3.77)	2.21 (1.52–3.19)	2.8 (1.86–4.23)	2.5 (1.8–3.48)
Basic model plus waist circumference	2.12 (1.48–3.04)	2.9 (1.96–4.28)	2.32 (1.63–3.31)	2.56 (1.76–3.74)	2.72 (1.85–4.01)	2.82 (1.93–4.12)	2.55 (1.76–3.69)	2.89 (1.93–4.32)	2.48 (1.79–3.43)

Results are shown from all Cer(d18:0) species, the Cer(d18:0) class total, and three Cer(d18:1) species. The adjusted odds ratios were computed per increase by the interval between the top and bottom quartile of lipid plasma concentration ("interquartile range"), and 95% confidence intervals are given in parentheses. They result from a series of logistic regression models with different sets of covariates. See Table S7 for the DESIR cohort.

Des1 expression or its pharmacological inhibition decreases adipocytes proliferation, differentiation, and function (Barbarroja et al., 2015). Addition of fenretinide (N-(4-hydroxyphe- nyl)retinamide, FEN), an inhibitor of Des1 (Bikman et al., 2012), to human cells leads to accumulation of dihydroceramides (Zheng et al., 2006). Increased dihydroceramide levels in HFHS-fed mice treated with FEN are associated with improvements in glucose homeostasis (Mody and Mcilroy, 2014). However, FEN is also an antagonist of retinoic acid receptor (RAR), and the effect of this drug on metabolic diseases is complex and not restricted to the increase in dihydroceramides (Mcilroy et al., 2013, 2016).

Thus, an increase in plasma levels of dihydroceramides may have multiple effects on specific cellular functions, which may, combined with the already well described role of ceramides in induction of insulin resistance (Bikman and Summers, 2011; Chaurasia and Summers, 2015), contribute to induce T2D. The potential impact of elevated dihydroceramides on insulin secretion and insulin action will need to be tested specifically; availability of relevant preclinical models will be important for these purposes. It will also be important to determine which tissue produces these sphingolipids. This could be investigated by assessing the tissue expression of the six different ceramide synthase (Cers) isoforms with a focus on Cers2, Cers3, and Cers4, which preferentially catalyze the formation of very long-chain fatty-acid-containing ceramides (Chaurasia and Summers, 2015). On the other hand, reduced expression of Des1 and Des2 may also explain the increased accumulation of dihydroceramides. Of note, the Des1 gene was found to be associated with increased fat mass accumulation in a genetic screen in HFHS-fed recombinant inbred mice (Parks et al., 2013).

The fact that the identified ceramides and dihydroceramides contain long-chain fatty acids of diverse desaturation levels also suggests that beyond changes in ceramide biosynthetic pathways, changes in fatty-acid-modifying pathways may contribute to the chemical diversity of the ceramides. We know from previous studies (Ding et al., 2013) that the NC diet does not contain fatty acids with chain length ≥20 carbons. Thus, the presence of sphingolipids with ≥20 carbon fatty acids of diverse desaturation levels most likely reflects contribution of elongases and desaturases acting on ingested or newly synthesized fatty acids (Guillou et al., 2010).

Collectively, our data show that combining lipidomic analysis in preclinical models and in human prospective cohorts allows identifying robust biomarkers for susceptibility to develop T2D, up to 9 years before the disease is diagnosed. Future studies should aim at evaluating the potential role of these sphingolipids on insulin secretion and insulin action and whether they can be combined with other biochemical or genetic biomarkers to improve their prediction power.

EXPERIMENTAL PROCEDURES

Animal Housing and Diet

Male mice from six different strains (C57BL/6J, DBA/2J, AJ, Balb/cJ, AKR/J, and 129S2/SvPas) were housed on a 12-hr light/dark cycle and were fed a standard rodent chow (SAFE A04) or HFHS diet (SAFE 235F, with 46% fat and 38.5% carbohydrates expressed in Kcal/kg), ad libitum for 2, 10, 30, and 90 days. At each time point, mice were phenotyped for body weight, pancreas weight, fasting glycemia and insulinemia, glucose and insulin
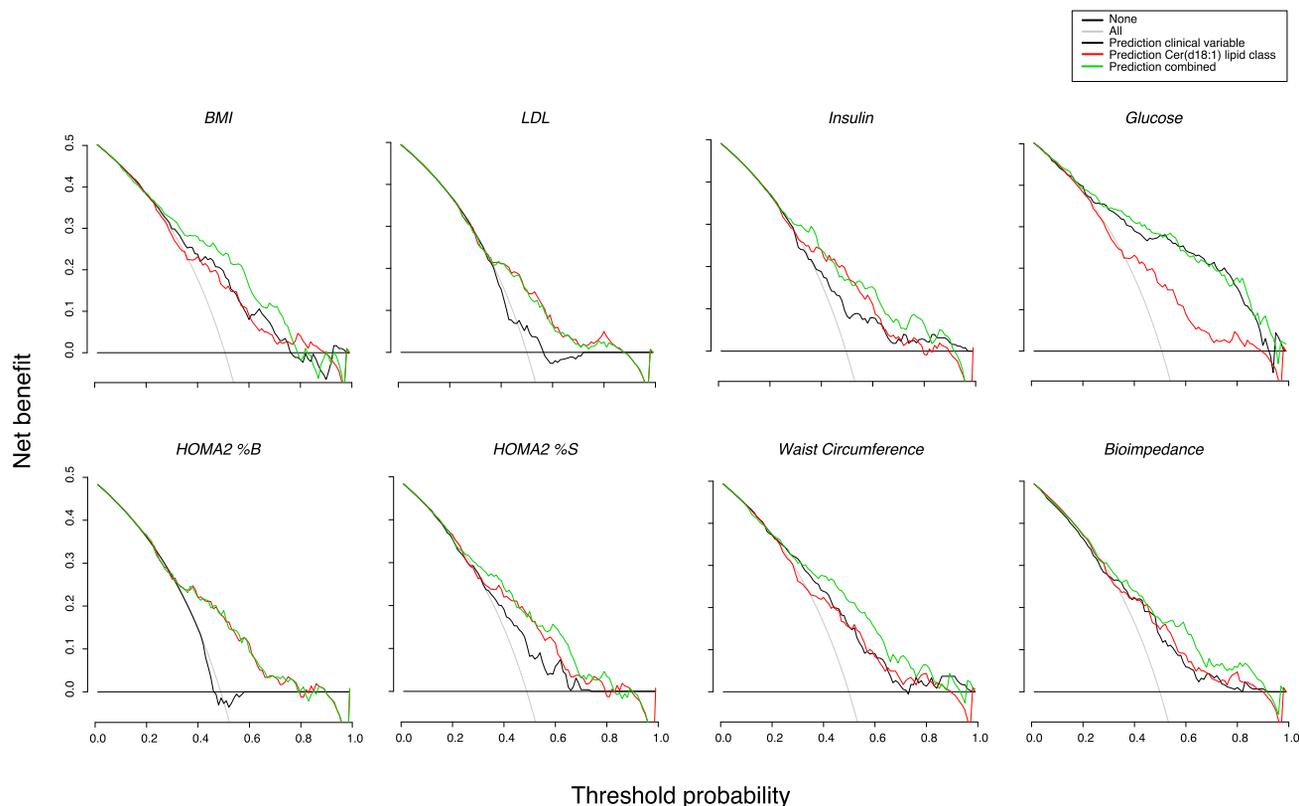
Figure 5. High Plasma Levels of Dihydroceramides May Be Associated with an Increased Risk of Future Diabetes
Decision curves, based on predicted probabilities from logistic regression tests. Standard net benefit is plotted against probability thresholds. Each plot compares three models: model with Cer(d18:0), model with a clinical variable, model with both. All models are adjusted for age and sex. Higher net benefit over a range of probability thresholds means that the model performs better as a predictor of T2D (as long as a threshold in that range is used for classifying individuals as susceptible).

tolerance, as well as glucose-stimulated insulin secretion assessed using purified islets. Mice were fasted for 5 hr before the different tests were performed. The details of this study will be reported separately. The experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40, reference CERFE 2009-033).

Study Populations
Samples from the DESIR Cohort
The DESIR study (Balkau et al., 2008) was a population-based longitudinal study on male and female volunteers between 30 and 65 years of age, conducted in ten health examination centers in western France between 1994 and 2004. Study participants visited a center four times between 1994 and 2004, with an interval of approximately 3 years between visits. The time points of the four visits are labeled year 0 (Y0), year 3 (Y3), year 6 (Y6), and year 9 (Y9). The visits took place in the following time intervals: Y0: 07/1994–02/1996; Y3: 09/1997–11/1998; Y6: 10/2000–11/2001; Y9: 10/2003–10/2004. The study was approved by the ethics committee (CCPPRB) of the Bicêtre Hospital first in 1994 and then for addenda in 1997 and 2000. It was also approved by the National Commission for Data Treatment (CNIL) in 1994, 1997, and 2008. All subjects provided informed written consent.

For this work, three groups of diabetes cases were selected from the study participants: all subjects who were determined to be diabetic for the first time at Y3 (group 1), or at Y6 (group 2), or at Y9 (group 3), respectively. Blood plasma samples for lipidomics analysis were picked from all available time points before subjects were diabetic, and from the time point when they were classified as diabetic for the first time, i.e., for group 1: Y0 and Y3; for group 2: Y0, Y3, and Y6; for group 3: Y0, Y3, Y6, and Y9. Sub-

jects who were already diabetic at Y0 (baseline) were not included. In addition, a group of control subjects was selected at random from the entire pool of DESIR participants, and samples from all four time points (Y0, Y3, Y6, and Y9) were picked. Due to the random selection without prior filtering of subjects, the control group initially contained a small number of participants with diabetes. Subjects with diabetes at any time point were removed from the control group. Baseline clinical characteristics and details of the numbers of samples analyzed in each of the groups are shown in Tables S3 and S4.

Samples from the CoLaus Cohort
From the CoLaus (Cohorte Lausannoise) study (Firmann et al., 2008), a population-based study of men and women living in Lausanne, Switzerland, plasma samples for lipidomics analysis were obtained from two time points, year 0 (Y0) and year 5 (Y5). A group of incident diabetes cases was picked, consisting of participants who were diabetic at year 5 but not yet at year 0 and in the same age range as the DESIR subjects. A group of sex- and age-matched control subjects was also selected. Baseline clinical characteristics and details of the numbers of samples analyzed in each of the groups are shown in Tables S5 and S6. The institutional Ethics Committee of the University of Lausanne, which afterward became the Ethics Commission of Canton Vaud, approved the CoLaus baseline study in 2003 (reference 16/03) and renewed the approval for two follow-ups in 2009 and 2014 (references 33/09 and 26/14). All participants gave their signed informed consent.

Definition of Diabetes Cases
In both cohorts, subjects were identified as incident diabetic cases either when their fasting glucose level exceeded 7 mmol/L or when they were under treatment for diabetes at the time of the health center visit.

Lipidomics Analysis

Lipid extraction, mass spectrometry (MS)-based lipid detection and data processing for mouse and human samples was performed by Zora Biosciences Oy (Jung et al., 2011). See Supplemental Experimental Procedures, Lipid sample preparation and extraction and Mass spectrometric analyses and data processing.

Data Preprocessing

Lipid concentrations that could not be measured and were missing from the original lipidomics data files were imputed (see Supplemental Experimental Procedures, Imputation of missing lipidomics values). In the DESIR cohort, a batch effect in the lipidomics data, which resulted from processing the lipid samples in two batches several months apart, was corrected using the ComBat method (see Supplemental Experimental Procedures, Removing unwanted variation due to batch effect, DESIR cohort). Clinical data were log2-transformed (except age and categorical or binary variables). Prior to the log2 transformation, waist circumference in females was adjusted to account for the difference in waist size between males and females (see Supplemental Experimental Procedures, Data preprocessing in clinical variable").

Statistical Tests, DESIR Cohort

The main focus of this work was to compare the concentrations of ceramides and related lipids in diabetes cases, before and after diabetes onset, to those in the control group. Each group of cases from a certain time point (sample collection period) was compared to the control samples from the same time point. Time points were not mixed and not compared directly to one another in order to avoid confounding with effects linked to sample storage duration or collection date. This gave the following comparisons:

Year 0: Collection Dates 1994–1996, None of the Included Cases Were Diabetic
- Group 1 (3 years before T2D) versus controls (not diabetic)
- Group 2 (6 years before T2D) versus controls (not diabetic)
- Group 3 (9 years before T2D) versus controls (not diabetic)

Year 3: Collection Dates 1997–1998, Group 1 Was Diabetic at This Time Point
- Group 1 (diabetic) versus controls (not diabetic)
- Group 2 (3 years before T2D) versus controls (not diabetic)
- Group 3 (6 years before T2D) versus controls (not diabetic)

Year 6: Collection Dates 2000–2001, Group 2 Was Diabetic at This Time Point
- Group 2 (diabetic) versus controls (not diabetic)
- Group 3 (3 years before T2D) versus controls (not diabetic)

Year 9: Collection Dates 2003–2004, Group 3 Was Diabetic at This Time Point
- Group 3 (diabetic) versus controls (not diabetic)

The R language, version 3.2.1 was used. A linear model (R function lm) was used for the comparisons, with variables for age and sex to address confounding factors. The statistical test was performed for each of the 37 lipid species included in the lipidomics study, as well as the total concentrations for each lipid class. Additional tests were run that included one more variable in addition to age and sex, such as BMI, glycemia, or LDL. Within each group/time point, the p values from all 37 lipid species were adjusted using the Benjamini-Hochberg method to correct for multiple testing. In addition, interquartile-range odds ratios were computed using logistic regression models with different covariates. These are the odds ratios per increase in lipid concentration by an amount equal to the interquartile range, i.e., the magnitude of the interval between the top and the bottom quartile. The lrm() function in the R package rms (Harrel, 2016), version 4.4-2, was used with default parameters.

Statistical Tests, COLAUS Cohort

For the CoLaus cohort, the statistical tests with linear models were performed in the same way as for the DESIR cohort. Cases were compared to controls at year 0 and, in a separate test, at year 5. Interquartile odds ratios for year 0 were also computed with the same method as described above for the DESIR cohort.

Decision Curve Analysis

Logistic regression models were used to obtain predicted probabilities (of being a future diabetic) for all individuals. This was done separately for each participant group at each time point with control samples from the same time point. A 10-fold cross-validation scheme was applied: data within each group (consisting of cases and controls) was divided into ten equal-sized parts. Nine parts were used to calculate regression coefficients, and then probabilities were computed for the tenth part. This was repeated until each data part had been left out once, and all individuals had a predicted probability assigned to them. All logistic regression models included age and sex as potential confounders, and a clinical variable or the Cer(d18:0) concentration or both clinical variable and Cer(d18:0) as the predictors of interest.

Decision curve analysis based on net benefits (Rousson and Zumbrunn, 2011; Vickers and Elkin, 2006) was performed to quantify the predictive value of the plasma concentration of lipid class Cer(d18:0). Decision curve analysis is commonly used on predicted probabilities resulting from logistic regression tests. Similar to an ROC analysis, it assumes that participants will be classified into "positives" and "negatives" by applying a threshold to the predicted probabilities. It allows to compute a "net benefit" of using a particular statistical model for prediction, across all possible probability thresholds.

The same predictor variables (measures known or thought to be associated with diabetes) were used in this analysis as for the computation of odds ratios: BMI, LDL, glucose, insulin, HOMA2-%B, HOMA2-%s, waist circumference, and bioimpedance. For each of these variables, a logistic regression model was defined that also included age and sex as covariates, and the same was done for the lipid class Cer(d18:0). A decision curve plot was prepared for each clinical variable to visually compare the performances of (1) the model with the lipid, (2) the model with the clinical variable, and (3) an additional model with both clinical variable and lipid. Our principal interest was to see if combining the clinical variable and the lipid in a model improved prediction over the model with only the clinical variable. We looked at the range of risk thresholds between 0.6 and 0.8 to compare the performance of predictive models. The net benefit was not adjusted for population prevalence of diabetes, so the risk thresholds on the x axis of the plots reflect the risk within the case-control study setting, where about half of all participants are cases (i.e., not the risk of an individual picked at random from the population). This does not matter for comparing decision curves with one another.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.02.019.

REFERENCES

Aimo, L., Liechti, R., Hyka-Nouspikel, N., Niknejad, A., Gleizes, A., Götz, L., Kuznetsov, D., David, F.P., van der Goot, F.G., Riezman, H., et al. (2015). The SwissLipids knowledgebase for lipid biology. Bioinformatics 31, 2860–2866.

Andrikopoulos, S., Massa, C.M., Aston-Mourney, K., Funkat, A., Fam, B.C., Hull, R.L., Kahn, S.E., and Proietto, J. (2005). Differential effect of inbred mouse strain (C57BL/6, DBA/2, 129T2) on insulin secretory function in response to a high fat diet. J. Endocrinol. 187, 45–53.

Balkau, B., Lange, C., Fezeu, L., Tichet, J., de Lauzon-Guillain, B., Czernichow, S., Fumeron, F., Froguel, P., Vaxillaire, M., Cauchi, S., et al. (2008). Predicting diabetes: Clinical, biological, and genetic approaches: Data from the Epidemiological Study on the Insulin Resistance Syndrome (DESIR). Diabetes Care 31, 2056–2061.

Barbarroja, N., Rodriguez-Cuenca, S., Nygren, H., Camargo, A., Pirraco, A., Relat, J., Cuadrado, I., Pellegrinelli, V., Medina-Gomez, G., Lopez-Pedrera, C., et al. (2015). Increased dihydroceramide/ceramide ratio mediated by defective expression of degs1 impairs adipocyte differentiation and function. Diabetes 64, 1180–1192.

Baudry, A., Leroux, L., Jackerott, M., and Joshi, R.L. (2002). Genetic manipulation of insulin signaling, action and secretion in mice. Insights into glucose homeostasis and pathogenesis of type 2 diabetes. EMBO Rep. 3, 323–328.

Bikman, B.T., and Summers, S.A. (2011). Ceramides as modulators of cellular and whole-body metabolism. J. Clin. Invest. 121, 4222–4230.

Bikman, B.T., Guan, Y., Shui, G., Siddique, M.M., Holland, W.L., Kim, J.Y., Fabriàs, G., Wenk, M.R., and Summers, S.A. (2012). Fenretinide prevents lipid-induced insulin resistance by blocking ceramide biosynthesis. J. Biol. Chem. 287, 17426–17437.

Bonnefond, A., and Froguel, P. (2015). Rare and common genetic events in type 2 diabetes: What should biologists know? Cell Metab. 21, 357–368.

Brozinick, J.T., Hawkins, E., Hoang Bui, H., Kuo, M.S., Tan, B., Kievit, P., and Grove, K. (2013). Plasma sphingolipids are biomarkers of metabolic syndrome in non-human primates maintained on a Western-style diet. Int. J. Obes. 37, 1064–1070.

Burcelin, R., Crivelli, V., Dacosta, A., Roy-Tirelli, A., and Thorens, B. (2002). Heterogeneous metabolic adaptation of C57BL/6J mice to high-fat diet. Am. J. Physiol. Endocrinol. Metab. 282, E834–E842.

Chaurasia, B., and Summers, S.A. (2015). Ceramides - Lipotoxic inducers of metabolic disorders. Trends Endocrinol. Metab. 26, 538–550.

Cobb, J., Eckhart, A., Perichon, R., Wulff, J., Mitchell, M., Adam, K.P., Wolfert, R., Button, E., Lawton, K., Elverson, R., et al. (2015). A novel test for IGT utilizing metabolite markers of glucose tolerance. J. Diabetes Sci. Technol. 9, 69–76.

Ding, J., Loizides-Mangold, U., Rando, G., Zoete, V., Michielin, O., Reddy, J.K., Wahli, W., Riezman, H., and Thorens, B. (2013). The peroxisomal enzyme L-PBE is required to prevent the dietary toxicity of medium-chain fatty acids. Cell Rep. 5, 248–258.

Ferrannini, E., Natali, A., Camastra, S., Nannipieri, M., Mari, A., Adam, K.P., Milburn, M.V., Kastenmüller, G., Adamski, J., Tuomi, T., et al. (2013). Early metabolic markers of the development of dysglycemia and type 2 diabetes and their physiological significance. Diabetes 62, 1730–1737.

Firmann, M., Mayor, V., Vidal, P.M., Bochud, M., Pécoud, A., Hayoz, D., Paccaud, F., Preisig, M., Song, K.S., Yuan, X., et al. (2008). The CoLaus study: A population-based study to investigate the epidemiology and genetic determinants of cardiovascular risk factors and metabolic syndrome. BMC Cardiovasc. Disord. 8, 6.

Guillou, H., Zadravec, D., Martin, P.G., and Jacobsson, A. (2010). The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. Prog. Lipid Res. 49, 186–199.

Harrel, F.E.J. (2016). rms: Regression Modeling Strategies. R package version, 4.4-2.

Jung, H.R., Sylvänne, T., Koistinen, K.M., Tarasov, K., Kauhanen, D., and Ekroos, K. (2011). High throughput quantitative molecular lipidomics. Biochim. Biophys. Acta 1811, 925–934.

Lopez, X., Goldfine, A.B., Holland, W.L., Gordillo, R., and Scherer, P.E. (2013). Plasma ceramides are elevated in female children and adolescents with type 2 diabetes. J. Pediatr. Endocrinol. Metab. 26, 995–998.

Mamtani, M., Meikle, P.J., Kulkarni, H., Weir, J.M., Barlow, C.K., Jowett, J.B., Bellis, C., Dyer, T.D., Almasy, L., Mahaney, M.C., et al. (2014). Plasma dihydroceramide species associate with waist circumference in Mexican American families. Obesity (Silver Spring) 22, 950–956.

Mcilroy, G.D., Delibegovic, M., Owen, C., Stoney, P.N., Shearer, K.D., McCaffery, P.J., and Mody, N. (2013). Fenretinide treatment prevents diet-induced obesity in association with major alterations in retinoid homeostatic gene expression in adipose, liver, and hypothalamus. Diabetes 62, 825–836.

Mcilroy, G.D., Tammireddy, S.R., Maskrey, B.H., Grant, L., Doherty, M.K., Watson, D.G., Delibegović, M., Whitfield, P.D., and Mody, N. (2016). Fenretinide mediated retinoic acid receptor signalling and inhibition of ceramide biosynthesis regulates adipogenesis, lipid accumulation, mitochondrial function and nutrient stress signalling in adipocytes and adipose tissue. Biochem. Pharmacol. 100, 86–97.

Mody, N., and Mcilroy, G.D. (2014). The mechanisms of Fenretinide-mediated anti-cancer activity and prevention of obesity and type-2 diabetes. Biochem. Pharmacol. 91, 277–286.

Parks, B.W., Nam, E., Org, E., Kostem, E., Norheim, F., Hui, S.T., Pan, C., Civelek, M., Rau, C.D., Bennett, B.J., et al. (2013). Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. Cell Metab. 17, 141–152.

Pauling, L., Robinson, A.B., Teranishi, R., and Cary, P. (1971). Quantitative analysis of urine vapor and breath by gas-liquid partition chromatography. Proc. Natl. Acad. Sci. USA 68, 2374–2376.

Rodriguez-Cuenca, S., Barbarroja, N., and Vidal-Puig, A. (2015). Dihydroceramide desaturase 1, the gatekeeper of ceramide induced lipotoxicity. Biochim. Biophys. Acta 1851, 40–50.

Rossmeisl, M., Rim, J.S., Koza, R.A., and Kozak, L.P. (2003). Variation in type 2 diabetes–related traits in mouse strains susceptible to diet-induced obesity. Diabetes 52, 1958–1966.

Rousson, V., and Zumbrunn, T. (2011). Decision curve analysis revisited: Overall net benefit, relationships to ROC curve analysis, and application to case-control studies. BMC Med. Inform. Decis. Mak. 11, 45.

Siddique, M.M., Li, Y., Chaurasia, B., Kaddai, V.A., and Summers, S.A. (2015). Dihydroceramides: From Bit Players to Lead Actors. J. Biol. Chem. 290, 15371–15379.

Stancáková, A., Civelek, M., Saleem, N.K., Soininen, P., Kangas, A.J., Cederberg, H., Paananen, J., Pihlajamäki, J., Bonnycastle, L.L., Morken, M.A., et al. (2012). Hyperglycemia and a common variant of GCKR are associated with the levels of eight amino acids in 9,369 Finnish men. Diabetes *61*, 1895–1902.

Surwit, R.S., Kuhn, C.M., Cochrane, C., McCubbin, J.A., and Feinglos, M.N. (1988). Diet-induced type II diabetes in C57BL/6J mice. Diabetes *37*, 1163–1167.

Surwit, R.S., Feinglos, M.N., Rodin, J., Sutherland, A., Petro, A.E., Opara, E.C., Kuhn, C.M., and Rebuffé-Scrive, M. (1995). Differential effects of fat and sucrose on the development of obesity and diabetes in C57BL/6J and A/J mice. Metabolism *44*, 645–651.

Vickers, A.J., and Elkin, E.B. (2006). Decision curve analysis: A novel method for evaluating prediction models. Med. Decis. Making *26*, 565–574.

Würtz, P., Tiainen, M., Mäkinen, V.P., Kangas, A.J., Soininen, P., Saltevo, J., Keinänen-Kiukaanniemi, S., Mäntyselkä, P., Lehtimäki, T., Laakso, M., et al. (2012). Circulating metabolite predictors of glycemia in middle-aged men and women. Diabetes Care *35*, 1749–1756.

Zhao, X., Fritsche, J., Wang, J., Chen, J., Rittig, K., Schmitt-Kopplin, P., Fritsche, A., Haring, H.U., Schleicher, E.D., Xu, G., et al. (2010). Metabonomic fingerprints of fasting plasma and spot urine reveal human pre-diabetic metabolic traits. Metabolomics *6*, 362–374.

Zheng, W., Kollmeyer, J., Symolon, H., Momin, A., Munter, E., Wang, E., Kelly, S., Allegood, J.C., Liu, Y., Peng, Q., et al. (2006). Ceramides and other bioactive sphingolipid backbones in health and disease: lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy. Biochim. Biophys. Acta *1758*, 1864–1884.