Abstract

Identification of pre-diabetic individuals with decreased functional β-cell mass is essential for the prevention of diabetes. However, in vivo detection of early asymptomatic β-cell defect remains unsuccessful. Metabolomics emerged as a powerful tool in providing read-outs of early disease states before clinical manifestation. We aimed at identifying novel plasma biomarkers for loss of functional β-cell mass in the asymptomatic pre-diabetic stage. Non-targeted and targeted metabolomics were applied on both lean β-Phb2-/- mice (β-cell-specific prohibitin-2 knockout) and obese db/db mice (leptin receptor mutant), two distinct mouse models requiring neither chemical nor diet treatments to induce spontaneous decline of functional β-cell mass promoting progressive diabetes development. Non-targeted metabolomics on β-Phb2-/- mice identified 48 and 82 significantly affected metabolites in liver and plasma, respectively. Machine learning analysis pointed to deoxyhexose sugars consistently reduced at the asymptomatic pre-diabetic stage, including in db/db mice, showing strong correlation with the gradual loss of β-cells. [...]
Metabolomics identifies a biomarker revealing *in vivo* loss of functional β-cell mass before diabetes onset

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ABSTRACT

Identification of pre-diabetic individuals with decreased functional β-cell mass is essential for the prevention of diabetes. However, in vivo detection of early asymptomatic β-cell defect remains unsuccessful. Metabolomics emerged as a powerful tool in providing read-outs of early disease states before clinical manifestation. We aimed at identifying novel plasma biomarkers for loss of functional β-cell mass in the asymptomatic pre-diabetic stage. Non-targeted and targeted metabolomics were applied on both lean β-Phb2-/- mice (β-cell-specific prohibitin-2 knockout) and obese db/db mice (leptin receptor mutant), two distinct mouse models requiring neither chemical nor diet treatments to induce spontaneous decline of functional β-cell mass promoting progressive diabetes development. Non-targeted metabolomics on β-Phb2-/- mice identified 48 and 82 significantly affected metabolites in liver and plasma, respectively. Machine learning analysis pointed to deoxyhexose sugars consistently reduced at the asymptomatic pre-diabetic stage, including in db/db mice, showing strong correlation with the gradual loss of β-cells. Further targeted metabolomics by GC-MS uncovered the identity of the deoxyhexose with 1,5-anhydroglucitol displaying the most significant changes. In conclusion, this study identified 1,5-anhydroglucitol associated with the loss of functional β-cell mass and uncovered metabolic similarities between liver and plasma, providing insights into the systemic effects caused by early decline in β-cells.
INTRODUCTION

Both type 1 and type 2 diabetes (T2D) are chronic diseases primarily characterized by β-cell defect. While the former is an autoimmune disease resulting in the inexorable destruction of the β-cells (1), the latter is usually associated with obesity-induced insulin resistance eventually leading to β-cell failure that prompts diabetes (2; 3). Indeed, insufficient insulin secretion has emerged as the determining factor for the onset of T2D (4; 5). Although loss of β-cell function and mass both contribute to impaired insulin secretion (6; 7), these two parameters are not well correlated and their relative contribution to T2D pathogenesis remains a subject of debate (8; 9). Identification of robust and sensitive in vivo biomarkers that could reflect the integrated assessment of β-cell function and mass, i.e. the functional β-cell mass, would be essential for the identification of high-risk, but yet asymptomatic, individuals for the prevention of diabetes.

Metabolomics is a powerful method to measure subtle biochemical changes caused by underlying pathologies (10; 11). The main metabolites consistently appearing in human T2D studies include branched-chain amino acids (BCAAs) (12; 13), aromatic amino acids (13), fructose (14; 15), mannose (14; 16), α-hydroxybutyrate (17; 18) and phospholipids (18-20). Most of these identified T2D-related metabolites are associated with insulin resistance and obesity (21; 22) rather than the direct alteration of the β-cells. This is partially due to the fact that the clinical practice for diabetes detection, such as fasting glucose and glucose tolerance test, cannot efficiently discriminate the respective contributions of insulin resistance versus insulin secretion (23; 24). Therefore, we still lack biomarkers reflecting the loss of the functional β-cell mass as the triggering event.

The main obstacle for the identification of biomarkers that would be specific for early β-cell defects is the scarce availability of pancreas samples from pre-diabetic subjects. By essence, one cannot study isolated human pancreas from an individual who would later eventually develop T2D; likewise pancreatic tissues are only available upon autopsy or invasive surgery (25). Animal models are useful surrogates but typically require special diets or drug injections to induce diabetes, which may generate confounding factors. To address these issues, we used two mouse models, i.e. the β-cell-specific Prohibitin-2 knockout (β-Phb2−/-) mice (26) and the leptin receptor-deficient mice (db/db) mice (27).
Both mouse models exhibit progressive β-cell failure and diabetes development, while the genetic cause of the disease differs. Deletion of Phb2 in the β-cells causes mitochondrial alterations and β-cell dysfunction, then apoptosis and a progressive decline in the β-cell mass starting at 4 weeks of age resulting in the onset of diabetes in β-Phb2−/− mice at 6 weeks of age (26). The db/db mice carry a single-gene spontaneous mutation in the leptin receptor that leads to hyperphagia and obesity (28), diabetes appearing approximately at the age of 8 weeks (29) when the decline in functional β-cells results in the failure to compensate for insulin resistance (30). In both mouse models, the critical event triggering diabetes, subsequently uncovered by hyperglycemia, is the loss of a critical mass of functional β-cells, similarly to human subjects (5; 31). Using non-targeted and targeted metabolomics, we profiled the liver and plasma metabolome of β-Phb2−/− and db/db mice from the earliest post-weaning age (4 weeks) to diabetes onset and later. Along with the assessment of the β-cell mass, the simultaneous measurements of hundreds of metabolites (32) led to the identification of common changing metabolites. Ultimately, we have found a shared metabolite among the mouse models, which levels linearly correlate with the decline of the functional β-cell mass already in the asymptomatic pre-diabetic state.

**RESEARCH DESIGN AND METHODS**

**Animals**

All animal experiments were conducted at the University of Geneva Medical Centre with the approval by the animal care and experimentation authorities of the Canton of Geneva (#GE/128/15). Mice were maintained on a 12-h dark/light cycle with water and food ad libitum and genotyped using primers listed in Supplementary Table S1. Since the phenotype of β-Phb2−/− mice is similar between males and females (26), only males were used for experiments and the age of the mice is specified for each experiment. All β-Phb2−/− and Phb2+/+ mice were generated as previously described (26) and maintained on a mixed genetic background (C57BL/6J × 129/Sv) to avoid inbred strain-specific phenotypes. BKS.Cg-Dock7m+/+Leprdb/J mice (db/db and heterozygous db/+ mice as controls) were purchased from Charles River, Italy.
**Plasma and tissue collection**

On the day of sacrifice, food was removed from cages at 8:30 and sacrifice was done from 11:30 to noon. In mice, this 3-hour fasting period corresponds to a physiological overnight fast in human subjects, *i.e.* before total exhaustion of hepatic glycogen stores (33), while starving mechanisms are not yet operating (34). Body weights and glucose levels were monitored just before sacrifice. Blood glucose values were measured using Accu-Check Aviva glucometer (Roche Diagnostics). Blood was collected into EDTA-coated tubes (Sarstedt #20.1341) via retro-orbital bleeding and centrifuged at 2,000 rpm at 4°C to separate plasma. C-peptide was measured with a commercial kit (mouse C-peptide ELISA kit #90050, Crystal Chem. Inc.) and HOMA-IR index (35) was calculated as follows: insulin (µU/mL) x fasting glycemia (mmol/L) / 22.5. Tissues of interest were collected, weighed, snap-frozen in liquid nitrogen and stored at -80°C. Pancreas was collected for fixation and further immunohistochemistry.

**Immunostaining and β-cell mass quantification**

Upon sacrifice, pancreas was fixed for 2 h in 4% paraformaldehyde and embedded in paraffin. Sections of 5 µm were incubated overnight with primary antibody and then with secondary antibody for 1 h (Supplementary Table S2). Images were captured by confocal microscopy (LSM 800, Zeiss). For assessment of the β-cell mass, 5 µm sections at an interval of 150 µm throughout the pancreas were stained for insulin with the primary antibody and horseradish peroxidase-conjugated secondary antibody, and then visualized by DAB Substrate-Chromogen system (Dako K3468). Sections were scanned by widefield slide scanner (Axio Scan.Z1, Zeiss) and brown areas were quantified using Definiens software, adjusted to total pancreas weight.

**Insulin secretion on isolated islets**

Pancreatic islets were isolated by collagenase digestion and cultured overnight in complete RPMI 1640 medium as described (36). For secretion assays, islets were pre-incubated with Krebs-Ringer bicarbonate HEPES (KRBH) buffer supplemented with 2.8 mmol/L glucose and 0.1% BSA for 1 h. Subsequently, batches of 10 islets were hand-picked and incubated with basal 2.8 mmol/L and stimulatory 22.2 mmol/L glucose at 37°C for 1 h. At the end of the incubation period, supernatants
were collected, and islets were re-suspended in acid ethanol for determination of insulin contents.
Insulin in supernatants and extracted islets was measured using radioimmunoassay (Millipore).

**Metabolome extraction**

The metabolome extractions were performed from a 50-100 mg of frozen liver. The samples were initially homogenized by TissueLyser II (Qiagen) in 1 mL cold 70% ethanol. Extraction was continued with addition of 7 mL hot 70% ethanol (75°C) for 2 min with thorough mixing every 30 sec, followed by removal of cell debris by centrifugation. Extracts were stored at -20°C until mass spectrometry analysis. Plasma was extracted by adding 90 µL of 80% methanol to 10 µL volume of plasma, followed by thorough vortex mixing. After 1 h incubation at 4°C, centrifugation was done at maximum speed for 10 min and supernatant was used for mass spectrometry measurement.

**Non-targeted metabolomics**

Untargeted metabolomics was performed by flow injection analysis on a 6550 Agilent QTOF instrument as described (32). Briefly, profile spectra were recorded in negative ionization from m/z 50 to 1000 mode at 4 GHz high-resolution mode. Ion annotation was based on accurate masses using a tolerance of 0.001 amu and KEGG mmu database, accounting for –H+ and F- ions, sodium and potassium adducts, and heavy isotopes. The further data analysis involved calculating log2 fold changes between different groups (e.g. knockout vs control at week 4), with statistical significance calculated by two-sample Student’s t-test, additionally corrected for multiple testing and expressed as q-values (37). The data analysis was performed in MATLAB (The MathWorks).

For targeted metabolomics by LC-MS, polar metabolome extracts were dried and re-suspended in deionized water. To adjust for the differences in tissue sample weight, dried samples were re-suspended in 10 µL of deionized water per 1 µg tissue. Centrifuged cold supernatant aliquots of 10 µL were injected into a Waters Acquity UPLC (Waters Corporation, Milford, MA) with a Waters Acquity T3 column coupled to a Thermo TSQ Quantum Ultra triple quadrupole instrument (Thermo Fisher Scientific) with negative-mode electrospray ionization. Gradient of two mobile phases (A) 10 mmol/L tributylamine, 15 mmol/L acetic acid and 5% (v/v) methanol, and (B) 2-propanol was used.
for separation of compounds (38). Peak integration of mass spectra was performed by in-house software (Begemann and Zamboni, unpublished).

**Metabolomics targeted to deoxysugar separation**

Targeted metabolite profiling to separate deoxysugars was performed as detailed previously (39; 40) by gas chromatography coupled to electron impact ionization/time-of-flight mass spectrometry, GC/EI-TOF-MS, using an Agilent 6890N24 gas chromatograph (Agilent Technologies, Böblingen, Germany; http://www.agilent.com) with split and splitless injection onto a VF-5ms capillary column, 30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness (Agilent Technologies, Waldbronn, Germany), which was connected to a Pegasus III time-of-flight mass spectrometer (LECO Instrumente GmbH, Mönchengladbach, Germany) and by enhanced targeted metabolite profiling by gas chromatography-atmospheric pressure ionization-quadrupole time of flight-mass spectrometry, GC/APCI-qTOF-MS, (Bruker Daltonik GmbH, Bremen, Germany) as was previously described (41). Aliquots of 50μL from the polar metabolite fractions (metabolome extracts, see above) were dried by vacuum concentration and stored dry under inert gas at -20°C until further processing. Metabolites were methoxyaminated and trimethylsilylated manually prior to GC/EI-TOF-MS analysis (39; 40). Retention indices were calibrated by addition of a C_{10}, C_{12}, C_{15}, C_{18}, C_{19}, C_{22}, C_{28}, C_{32}, and C_{36} n-alkane mixture to each sample (42).

GC/EI-TOF-MS chromatograms were acquired, visually controlled, baseline corrected and exported in NetCDF file format using ChromaTOF software (Version 4.22; LECO, St. Joseph, USA). GC-MS data processing into a standardized numerical data matrix and compound identification were performed using the TagFinder software (43). Compounds were identified by mass spectral and retention time index matching to the reference collection of the Golm metabolome database (GMD, http://gmd.mpimp-golm.mpg.de/; (44)) and to the mass spectra of the NIST17 database (https://www.nist.gov/srd/shop). Guidelines for manually supervised metabolite identification were the presence of at least 3 specific mass fragments per compound and a retention index deviation < 1.0% (Strehmel et al., 2008). Authenticated reference compounds for identification were obtained
from, Sigma Aldrich GmbH (Steinheim, Germany), namely 1,5-anhydro-D-glucitol (A7165, CAS 154-58-5), \textit{D-(+)}-fucose (F8150, CAS 3615-37-0) and \textit{L-(−)}-fucose (F2252, CAS 2438-80-4). \textit{D-}/ \textit{L-} stereo isomers were not distinguished by this method. Identification of 1,5-anhydro-D-glucitol was supported by earlier reports (45) and by exact masses (± 5 mDa) of the molecular ion (M\textsuperscript{+}), proton adducts (M+H\textsuperscript{+}) and M-CH\textsubscript{3}\textsuperscript{+} fragments that were observed by \textit{GC/APCI-qTOF-MS}.

All mass features of an experiment were normalized by sample fresh weight or volume (plasma) and internal standard and subsequently maximum scaled (%) for visualization of relative concentration changes.

\textbf{Machine learning algorithm}

The machine learning method to classify the disease state consists of a composition of a pre-processing step and a classification step. For the pre-processing step, we tested the following algorithms: Principal Component Analysis (PCA), L1-regularized Logistic Regression and correlation thresholding, which consists of ranking the metabolites according to their Pearson's sample correlation coefficient and selecting those with highest correlation coefficient (the number of chosen features is a parameter of the algorithm, coded in python). For the classification step, we tested linear discriminant analysis, L1-regularized Logistic Regression, Gradient Boosting, and Random Forests (46). After testing all the possible combinations of pre-processing and classification algorithms listed above, we selected the one yielding the best score on a leave-20-out cross validation (CV) procedure repeated 100 times. Overall, the percentage of sampling data into validation sets is 18\% and 28\% of the total dataset for \textit{β-Phb2\textsuperscript{−/−}} and \textit{db/db} mice, respectively. Each time, the validation set was resampled exclusively from the set of metabolomics profiles corresponding to the pre-diabetic stage.

The whole machine learning pipeline was coded in python, making extensive use of the “sklearn” package (47). The latest version of the program used for this analysis is available for download (https://github.com/agazzian/ml_project).

The best CV scores were consistently obtained by combining a correlation thresholding method for the pre-processing step with Random Forests algorithm for the classification step. The metabolites were finally ranked according to their importance in the classification process (attribute
feature_importances_ of the sklearn.RandomForestClassifier estimator). It should be noted that new random forest models were generated for each classification run since the goal was not to find a universal classifier but rather to perform feature extraction, i.e. to investigate the classifying power of the selected metabolites.

Statistical Analysis

Data presented as dot plots are shown as mean ± SEM. The boxplots are presented as median with 25/75% percentiles (boxes) and 10/90% percentiles (bars). Statistical parameters (numbers and p values) are included in each figure legend, except for number of mice used for metabolomics analysis, which is listed in Supplementary Table S3. For statistical analysis, Mann-Whitney U test was used to compare two groups, unless differently described. A p value < 0.05 was considered significant.

RESULTS

Phenotypic characterization of β-Phb2−/− and db/db mice

The β-Phb2−/− mice exhibit a spontaneous progression from euglycemia to diabetes over a 2-week period, independently of any chemical or diet treatments (26). The primary cause of diabetes, efficiently treated by insulin therapy (26), is a β-cell failure rooted in mitochondrial dysfunction and accompanied by the loss of the β-cell mass. We further assessed the β-Phb2−/− mouse model through phenotyping at the pre-diabetic stage (4 and 5 weeks of age) and at the diabetic stage (6 and 10 weeks of age), see Figure 1. At the age of 4 and 5 weeks, β-Phb2−/− mice had normal levels of blood glucose, whereas at 6 weeks they became hyperglycaemic (glucose > 11.1 mmol/L) and severely diabetic by the age of 10 weeks. The body weights of β-Phb2−/− mice were similar to the controls at the pre-diabetic stage. Once β-Phb2−/− mice developed diabetes, their body weights became lower than controls (Figure 1B). The adipose tissue was comparable between β-Phb2−/− mice and the controls until diabetes onset, and then remarkably reduced when β-Phb2−/− mice developed diabetes (Figure 1C).

Consistent with previous characterization of the widely studied mouse model for T2D (28-30), we observed that db/db mice developed diabetes at the age of 8 weeks (Figure 1D). In contrast to β-Phb2−/− mice, db/db mice had higher body weights than their controls at the pre-diabetic stage (4 and 6
weeks of age) and at diabetes onset (8 weeks of age), Figure 1E. The adipose tissue was much higher in db/db mice versus their controls at all the examined time points (Figure 1F).

**Islet morphology and β-cell mass in β-Phb2−/− mice**

Before the appearance of symptoms, the disease development from pre-diabetes to diabetes was accompanied by marked changes in the islet morphology of β-Phb2−/− mice. There was a gradual decrease of insulin-positive cells and intrusion of glucagon-positive cells within the core of the islets (Figure 2A). Notwithstanding β-Phb2−/− mice of 4 and 5 weeks of age exhibited euglycemia (Figure 1A), quantitative analysis revealed a clear reduction (58-64%) of their β-cell mass compared to controls (Figures 2B, C). This correlated with severely impaired glucose-stimulated insulin secretion tested on islets isolated from 4-week old β-Phb2−/− mice (26). At the age of 6 weeks, the observed 68% reduction of the β-cell mass was then associated with hyperglycemia (Figure 1A). Neither circulating C-peptide (Figure 2D) nor HOMA-IR (Figure 2E) could reflect the reduction of β-cells before the appearance of hyperglycemia. Of note, at 10 weeks of age the C-peptide was undetectable in β-Phb2−/− mice (data not shown). Therefore, β-Phb2−/− mice present asymptomatic early-onset reduction of the β-cell mass before the appearance of hyperglycemia at the age of 6 weeks. This indicates a threshold around one third of the β-cell mass for maintenance of euglycemia in such non-obese animals.

**Islet morphology and β-cell mass in db/db mice**

As opposed to β-Phb2−/− mice, db/db mice preserved their insulin-positive cells during diabetes development (Figure 3A). The β-cell mass of db/db mice doubled from the age of 4 to 8 weeks and was significantly higher versus their controls at all examined time points (Figure 3B, C). Consistent with the β-cell mass, insulin contents per islet increased in db/db mice compared to db/+ controls at 4 and 8 weeks of age (35.0±3.7 versus 18.5±2.4 and 48.4±2.5 versus 14.6±3.8 ng/islet, respectively), along with higher circulating C-peptide (Figure 3D). In parallel, the insulin secretory responses on isolated islets from db/db mice dramatically deteriorated as diabetes developed (Supplementary Table 4). At the age of 4 weeks, db/db mice exhibited similar glucose-stimulated insulin secretion compared with the controls. However, upon appearance of hyperglycaemia at 8 weeks, db/db mice presented a sharp decline of β-cell function as reflected by a ~80% reduction of glucose-stimulated insulin
secretion, compared with their controls. This implies that, despite the observed increase in insulin-positive cells, the functional β-cell mass of db/db mice actually decreased during diabetes progression, consistent with previous reports (30). The HOMA-IR index (Figure 3E) did not correlate with the β-cell mass. Taken together, data show that both β-Phb2−/− and db/db mice share a progressive decline of the functional β-cell mass at a pre-diabetic asymptomatic stage.

**Metabolite changes in liver and plasma**

For metabolomics analyses of the two mouse models, we have extracted polar metabolomes of the mouse liver and their plasma samples before and after diabetes onset. Metabolome extracts were analysed in a non-targeted fashion by the flow-injection analysis time-of-flight mass spectrometry (FIA-TOF-MS) in negative ionization mode. Upon the sample spectra acquisition, metabolites were identified by matching ions of the unique mass-to-charge (m/z) ratios to the KEGG database (48). Since this method cannot distinguish isomers, e.g. isoleucine and leucine, multiple annotations are possible and fully disclosed in the Supplementary Table S5. Upon processing and annotation, 756 and 755 metabolites were detected and putatively annotated in liver and plasma of β-Phb2−/− mice, respectively. The principal component analysis (PCA) of the liver metabolic profile of β-Phb2−/− mouse model revealed partial separation between β-Phb2−/− mice and their controls at the age of 4 weeks. This separation became larger with the disease progression (Figure 4A). Regarding the db/db mouse model, 653 and 595 metabolites ions were detected and putatively annotated in the liver and plasma, respectively. PCA revealed that the liver metabolic profile of db/db mice separated completely from the controls at the age of 4 weeks, converged at 6 weeks and later separated again upon diabetes onset at 8 weeks (Figure 4B).

Next, we analysed metabolome changes between β-Phb2−/− and control mice. For each week of development, we performed univariate analysis to identify significant markers (|log2(FC)| > 1 and q-value < 0.01) (Supplementary Table S6). We found 48 and 82 metabolites to be affected in liver and plasma, respectively. A similar differential analysis was done for db/db mice (Supplementary Tables S6); 35 and 10 metabolites were filtered in liver and plasma, respectively.
We analysed the metabolome with a special focus on amino acids and carbohydrate metabolism (Figure 4C). The most consistent diabetes-related metabolites from human T2D studies are the BCAAs, i.e. valine, leucine and isoleucine (12-14), although their cause-effect relationship with diabetes per se remains unclear. BCAAs have been reported to increase in plasma years before diabetes diagnosis (13; 14), probably contributed by obesity-related insulin resistance, therefore not reflecting β-cell failure. In the plasma of β-Phb2−/− mice, we found modest increase of BCAAs at the early pre-diabetic stage (4 weeks) and a more pronounced increase upon severe diabetes (10 weeks), which might be due to sarcopenia associated with cachectic state (Figure 1B, C). In the plasma of fatty db/db mice (Figure 1E, F), BCAAs showed marked increases at the early pre-diabetic stage (4 weeks), consistent with their association with insulin resistance (49). Apart from BCAAs, two of the aromatic amino acids, tyrosine and phenylalanine, have been positively associated with diabetes (13; 19). Similar to the pattern we observed with BCAAs, the increment of plasma aromatic amino acids was higher in db/db mice compared with β-Phb2−/− mice during the pre-diabetic stage (4 weeks).

Regarding carbohydrate metabolites analysed by FIA-TOF-MS (Figure 4C), hexose sugars (e.g. glucose, mannose, fructose), denoted as Glucose (m/z ~179.056), increased in the plasma of both mouse models as diabetes progressed. Interestingly, in the liver of db/db mice, the increment of hexose sugars paralleled diabetes development, which could be explained by both higher food intake and increased endogenous glucose production caused by insulin resistance. On the contrary, hexose sugars remained unchanged in the liver of β-Phb2−/− mice, except for a slight increase at diabetes onset (6 weeks). Another striking difference between these two mouse models was the alteration in hepatic tricarboxylic acid (TCA) cycle at the pre-diabetic stage (4 weeks). In the liver of β-Phb2−/− mice, the TCA cycle intermediates fumarate, succinate and malate slightly increased at the age of 4 weeks. This observation was further substantiated by targeted metabolomics using LC-MS (Supplementary Figure S1). In db/db liver, fumarate, succinate, malate and citrate dramatically decreased (Figure 4C), suggesting important hepatic cataplerosis not compensated by the replenishment of the TCA cycle, i.e. anaplerosis. Malate and citrate exit the TCA cycle to fuel gluconeogenesis and lipogenesis, respectively, potentially contributing to the early-onset insulin resistance in db/db mice. In parallel,
the anaplerotic metabolites glutamate, glutamine and aspartate were reduced in the liver of db/db mice at the age of 4 weeks, indicating lower hepatic anaplerosis.

In summary, alterations of BCAAs, aromatic amino acids and TCA cycle-related metabolites in prediabetes were much more pronounced in obese db/db mice than in lean β-Phb2−/− mice, pointing to an association of these specific metabolites with insulin resistance rather than β-cell defect per se.

**Identification of metabolites shared between β-Phb2−/− and db/db metabolome reprogramming**

In order to identify putative metabolites predictive of diabetes development in both db/db and β-Phb2−/− mice, we performed machine learning analysis with the Random Forests classification algorithm. A randomly sampled training set of data was used for the analysis and the left-out set of data was used for internal cross validation. The analysis pipeline was conducted as follows: (i) in the first preprocessing step the features displaying the highest correlation with the diabetic state were selected to reduce the dimensionality of the data; (ii) in the second classification step, the selected metabolites from the β-Phb2−/− cohort were ranked according to their contribution to the classification of the individuals into diabetic and non-diabetic conditions (Table 1). Random Forest analysis clearly ranked m/z ~163.061 as the most important metabolite feature to classify diabetic and non-diabetic animals in both liver and plasma of β-Phb2−/− mice (Supplementary Figure S2). This m/z corresponds to C₆H₁₁O₅−, i.e. deprotonated deoxyhexose, and might relate to one or multiple isomers such as rhamnose, 1,5-anhydroglucitol or fucose. Consistent with the machine learning data, the levels of deoxyhexose sugars were significantly lower in the liver and plasma of β-Phb2−/− mice at the euglycemic pre-diabetic stage (4 weeks) compared with their controls (Figure 5A). Furthermore, the difference of deoxyhexose sugars between β-Phb2−/− and control mice became gradually more pronounced with the development of diabetes. Since the primary tissue alteration upstream of diabetes development in β-Phb2−/− mice is the progressive decline in functional β-cells, deoxyhexose sugars emerged as direct marker of the β-cell mass.

These observations were further substantiated in the db/db mice (Figure 5B). In their liver, the levels of deoxyhexose sugars were significantly reduced at the age of 6 weeks when mice were still euglycemic. In agreement with the β-Phb2−/− model, the difference in hepatic deoxyhexose sugars
between db/db and their controls became larger at the diabetic stage 2 weeks later (8 weeks). In the plasma of db/db mice, marginal differences were observed in the pre-diabetic stage, whereas significant changes appeared with diabetes (8 weeks).

We then performed targeted metabolomics by GC-MS to disambiguate the molecular identity of the m/z ~163.061 deoxyhexose sugar that contributes most to the differentiation at the pre-diabetic stage. Metabolite profiling by GC-MS identifies isomers with the identical molecular mass but different structures by differential chemical derivatization and gas chromatographic separation. GC-MS analyses performed on plasma and liver samples by conventional GC/EI - TOF - MS and enhanced GC/APCI - qTOF - MS (39; 41) led to the identification of 2 analytes, i.e. 1,5-anhydroglucitol (Figure 6A) and fucose (Figure 7A). This corresponds to the m/z ~163.061 ion that was detected by FIA-TOF-MS. Consistent with untargeted metabolomics results by TOF-MS, the level of 1,5-anhydroglucitol was significantly lower in the liver and plasma of β-Phb2−/− mice at the pre-diabetic stage (5 weeks) compared with their controls (Figure 6B), the difference becoming more pronounced as diabetes progressed. In the liver of db/db mice (Figure 6C), 1,5-anhydroglucitol levels were also significantly reduced at the early diabetic stage (8 weeks). However, at the pre-diabetic stage the difference between db/db mice and their controls did not reach significance (p=0.093). In light of these results, we took advantage of previously published studies reporting 1,5-anhydroglucitol plasma levels in alternative rodent models of diabetes secondary to β-cell loss. Non-obese NOD mice spontaneously develop diabetes by the age of 15-25 weeks (50). A decrease in plasma 1,5-anhydroglucitol was consistently observed 10-30 days ahead of the hyperglycemia (51), see Supplementary Figure S3A. Regarding toxin-induced diabetes, injection of streptozotocin (STZ) exerts a rapid cytotoxic action on β-cells inducing diabetes within hours, which was preceded by a decline in 1,5-anhydroglucitol plasma levels (52), see Supplementary Figure S3B.

For both db/db and β-Phb2−/− mouse models, plasma levels of the m/z ~163.061 deoxyhexose fucose were unchanged, while there were some differences in their livers (Figure 7B-C). In β-Phb2−/− mice at pre-diabetic stage (4 weeks), fucose levels were slightly higher in the liver compared to controls, a difference vanishing with time (Figure 7B). For db/db mice, fucose levels were reduced in the liver at
the pre-diabetic stage (6 weeks) and remained lower compared to controls when mice became hyperglycaemic (Figure 7C). Thus, as opposed to its isomer 1,5-anhydroglucitol, fucose levels did not correlate with β-cell failure.

In conclusion, both β-Phb2−/− and db/db mouse models exhibited early changes specifically in the deoxyhexose 1,5-anhydroglucitol, in association with the development of diabetes triggered by a decline of the functional β-cell mass.

**DISCUSSION**

A sensitive and robust biomarker of the functional β-cell mass is an unmet medical need. To date, non-invasive imaging of total β-cell mass is not yet available at the clinical level (53). Using metabolomics in two mouse models with complementary and well characterized spectrum of β-cell defects, we identified the deoxyhexose 1,5-anhydroglucitol as a biomarker that closely associated with functional β-cell mass preceding the appearance of hyperglycaemia. Specifically, the liver and plasma levels of 1,5-anhydroglucitol lowered along with functional β-cell mass, through an unidentified mechanism, preceding diabetes onset and progressing further as diabetes manifested to a severe stage. Consistently, inverse association of deoxyhexose sugars with T2D has been reported about 6 years before T2D diagnosis in an untargeted study on a German cohort (54). In this cohort, the deoxyhexose levels were poorly correlated with other risk factors, such as age and body mass index (BMI) (54). In line with these results, a Korean study has shown that low serum levels of 1,5-anhydroglucitol in euglycemic pre-diabetic subjects were associated with reduced insulinogenic index (a metric of insulin secretion capacity) and not with higher HOMA-IR (55). Regarding type 1 diabetes, a recent GC-MS analysis identified circulating metabolites in children who later progress to autoimmune diabetes, showing lower plasma levels of the sugar class 1,5-anhydrohexitol (e.g. 1,5-anhydroglucitol) at 6 months of age compared to control subjects (56).

The deoxyhexose 1,5-anhydroglucitol has been previously reported as an index of short-term glycaemic control in T2D patients (57). Similar changes in 1,5-anhydroglucitol have been measured in ob/ob and db/db mice (58). In human cohorts, 1,5-anhydroglucitol has been reported to be 38%
lower in diabetic patients compared to control subjects (59) and the deoxyhexose correlated with impaired fasting glycemia and T2D (14; 15). 1,5-Anhydroglucitol is a naturally occurring polyol found in nearly all foods and hardly metabolized in human (45). It can also be synthesized from glycogen in the liver (60), although its de novo synthesis accounts for a small percentage of its whole body storage (61). The primary route for 1,5-anhydroglucitol disposal is urinary excretion, while hyperglycaemia has been shown to promote such a mechanism resulting in the lowering of plasma levels of 1,5-anhydroglucitol (45; 62). Indeed, 1,5-anhydroglucitol can be reabsorbed by the sodium/glucose co-transporter 4 (SGLT4) in the renal tubule (63) but its reabsorption is inhibited when blood glucose levels reach 10 mmol/L. Accordingly, it has been suggested that 1,5-anhydroglucitol is sensitive in reflecting postprandial glycaemic excursions (64). The impaired glucose clearance in ß-Phb2−/− secondary to ß-cell failure (26) may contribute to reduced renal reabsorption of 1,5-anhydroglucitol and lowering of its plasma levels. In parallel, the decrease in hepatic 1,5-anhydroglucitol concentrations observed in ß-Phb2−/− mice could be the consequence of lower glycogen-derived biosynthesis, reducing its efflux normally occurring across the cell membrane (60; 65). These mechanisms may contribute to the lowering in plasma 1,5-anhydroglucitol levels, reflecting the progressive decline of the functional ß-cell mass in ß-Phb2−/− mice. Of note, we did not analyse the metabolome of the ß-cells since pancreata were fixed for the assessment of the ß-cell mass by immunohistochemistry in parallel to liver and plasma metabolomics. Therefore, the changes in liver and plasma 1,5-anhydroglucitol might be induced indirectly by the ß-cell decline, possibly by early modifications of hepatic metabolism in response to a pre-diabetic stage. Another constraint in our study was the limited amount of samples, in particular plasma volumes. We therefore focused on a single analytical metabolomics approach and negative mode ionization. In the future, the breadth of the analysis could be expanded by e.g. including positive mode ionization or complementary methods for the analysis of lipid extracts.

The vast heterogeneity of human genetics cannot be recapitulated in one mouse model, although investigating a highly heterogeneous background may introduce multiple confounding factors precluding the identification of a metabolite of significance. In order to reduce the putative impact of
the genetic background and to widen the mechanisms promoting diabetes, we extended our analyses to db/db mice. They are characterized by β-cell mass expansion (28; 29) as a sign of compensation for insulin resistance. However, such expansion of the insulin-positive cells does not prevent the development of hyperglycaemia, observed at the age of 8 weeks, as a consequence of β-cell dysfunction. Glucose-stimulated calcium responses and insulin exocytosis are reduced in pre-diabetic db/db mice (30). The present study reveals similar changes in deoxyhexose levels in pre-diabetic db/db and β-Phb2−/− mice, while being less pronounced in the former. BCAAs and aromatic amino acids are recognized predictors of insulin resistance (12; 66). Accordingly, our data revealed higher increments of these amino acids in the plasma of overweight db/db mice compared to lean β-Phb2−/− mice. These changes indicate that insulin resistance in db/db mice has a great impact on metabolome in pre-diabetes, not directly related to the β-cell mass.

In conclusion, the common denominator for pre-diabetic db/db and β-Phb2−/− mice was restricted to 1,5-anhydroglucitol, a sugar that can be easily measured by enzymatic-based colorimetric assay. This deoxyhexose reflects progressive decline of functional β-cell mass at asymptomatic pre-diabetic stage. Although this points to a valuable biomarker of early asymptomatic β-cell decline, validation of our findings in human cohorts would be the ultimate goal. If combined with already known clinical risk factors, such as BMI and fasting glucose, such a biomarker could add value in predicting progression to T2D and to enable tailored treatment strategies.

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Data and Resource Sharing and Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Funding. This study was financed by Sinergia (#CRSII3_147637 to PM and NZ) grant from the Swiss National Science Foundation.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.
Author Contributions. Conceived and designed the experiments: LL, NZ and PM; performed experiments on mice and related parameters: LL and JM-L; performed the metabolomics: PK; performed and analysed the targeted GC-MS based metabolomics: AE and JK; analysed the data: LL, PK, AA, NZ, and PM; wrote the paper: LL, PK and PM with contributions by NZ, AE, AA and JK. PM is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES


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Table 1. Ranking of prediction power on β-Phb2<sup>−/−</sup> metabolites by Random Forest/correlation thresholding analysis.

<table>
<thead>
<tr>
<th>Score</th>
<th>m/z</th>
<th>Top annotated metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1246</td>
<td>163.061</td>
<td>Deoxyhexose</td>
</tr>
<tr>
<td>0.0442</td>
<td>450.263</td>
<td>Terpendole G</td>
</tr>
<tr>
<td>0.0333</td>
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<td>Fructoselysine 6-phosphate</td>
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<td>365.233</td>
<td>Urocortisol</td>
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<td>0.0221</td>
<td>565.047</td>
<td>UDP-Glucose</td>
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<td>0.0203</td>
<td>361.202</td>
<td>Cortisol</td>
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<td>0.0182</td>
<td>320.092</td>
<td>gamma-L-Glutamyl-L-cysteinyl-beta-alanine</td>
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<td>0.0141</td>
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<td>UDP-N-acetyl-alpha-D-glucosamine</td>
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<td>0.0119</td>
<td>195.139</td>
<td>Ethyl(E,Z)-decadienoate</td>
</tr>
<tr>
<td>Plasma</td>
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<tr>
<td>0.0987</td>
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<td>Deoxyhexose</td>
</tr>
<tr>
<td>0.0343</td>
<td>517.097</td>
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<td>0.0313</td>
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</tr>
<tr>
<td>0.0127</td>
<td>279.057</td>
<td>Sulfamonomethoxine</td>
</tr>
</tbody>
</table>

FIGURE LEGENDS

Figure 1. β-Phb2<sup>−/−</sup> and db/db mice develop hyperglycaemia with different changes in body weight and adipose tissue

(A) Blood glucose levels measured from the age of 4 to 10 weeks in β-cell specific prohibitin knockout β-Phb2<sup>−/−</sup> and control Phb2<sup>fl/fl</sup> floxed mice (n=8-16). The dashed line shows the 11.1 mol/L threshold indicating the diabetic state.

(B) Body weights of β-Phb2<sup>−/−</sup> and control Phb2<sup>fl/fl</sup> mice from the age of 4 to 10 weeks (n=8-16).

(C) Epididymal adipose tissue (eWAT/body weight ratio) of β-Phb2<sup>−/−</sup> and control Phb2<sup>fl/fl</sup> mice from the age of 4 to 10 weeks (N=7-11).

(D) Blood glucose levels measured from the age of 4 to 8 weeks in db/db and control db/+ mice (n=8-17). The dashed line shows the 11.1 mol/L threshold indicating the diabetic state.

(E) Body weights of db/db and control db/+ mice from the age of 4 to 8 weeks (n=8-22).

(F) Epididymal adipose tissue (eWAT/body weight ratio) of db/db and control db/+ mice from the age of 4 to 8 weeks (n=6 per group).

Values are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 between two groups.
Figure 2. Islet morphology and β-cell mass in β-Phb2−/− mice during diabetes development

(A) Representative pancreatic sections of immunofluorescence staining for insulin (green) and glucagon (red). Scale bar, 20 µm (applies to all panels).

(B) Representative pancreatic sections of immunohistochemistry for insulin using DAB (brown) in β-Phb2−/− and control floxed mice. Scale bar, 20 µm (applies to all panels).

(C) Quantification of the β-cell mass as defined by insulin-positive area (DAB staining from B) normalized to total pancreas weight (n=5-8).

(D) Plasma C-peptide concentrations (n=6).

(E) HOMA-IR index (n=6-11).

Values are expressed as mean ± SEM; *p<0.05, **p<0.01 between two groups.

Figure 3. Islet morphology and β-cell mass in db/db mice during diabetes development

(A) Representative pancreatic sections of immunofluorescence staining for insulin (green) and glucagon (red). Scale bar, 20 µm (applies to all panels).

(B) Representative pancreatic sections of immunohistochemistry for insulin using DAB (brown) in db/db and control db/+ mice. Scale bar, 20 µm (applies to all panels).

(C) Quantification of the β-cell mass as defined by insulin-positive area (DAB staining from B) normalized to total pancreas weight (n=4-6).

(D) Plasma C-peptide concentrations (n=6).

(E) HOMA-IR index (n=6).

Values are expressed as mean ± SEM; *p<0.05, **p<0.005 between two groups.

Figure 4. Changes over time in liver and plasma metabolome in β-Phb2−/− and db/db mice

(A and B) Principal component analysis of liver metabolome from β-Phb2−/− (A) and db/db (B) mice compared with their respective Phb2fl/fl and db/+ controls at different stages of diabetes development.

(C) Differential analysis of amino acid and carbohydrate metabolism between β-Phb2−/− and db/db mice with their respective controls at the each week of development. Color corresponds to log2 fold change ratios between them, while the size of the bubble corresponds to –log10 q-value (Student’s t-test followed by multiple testing correction); n=6-9, see Supplementary Table S3 for detailed n numbers per group. Top metabolite name is included for each annotated ion, with m/z indicated.

Figure 5. Boxplot of deoxyhexose sugars in the liver and plasma of β-Phb2−/− and db/db mice

Untargeted metabolomics by TOF-MS have identified a group of deoxyhexose sugars (m/z~163.061, in deprotonated form, C₆H₁₁O₅).
(A) Levels of deoxyhexose sugars in the liver and plasma of control Phb2^{fl/fl} (blue) and β-Phb2^{-/-} mice (red) at different ages.

(B) Levels of deoxyhexose sugars in the liver and plasma of control db/+ mice (blue) and db/db mice (red) at different ages. *p<0.05, **p<0.01, ***p<0.001 between two groups.

Values in the boxplot are given as median, 25/75% percentiles (boxes), 10/90% percentiles (bars); *p<0.05, **p<0.01, ***p<0.001 between two groups.

Figure 6. Boxplot of 1,5-anhydroglucitol in the liver and plasma of β-Phb2^{-/-} and db/db mice
Targeted metabolomics by GC-MS annotated 1,5-anhydroglucitol as a potential biomarker.

(A) Chemical structure of 1,5-anhydroglucitol.

(B) Levels of 1,5-anhydroglucitol in the liver and plasma of control Phb2^{fl/fl} mice (blue) and β-Phb2^{-/-} mice (red) at different ages.

(C) Levels of 1,5-anhydroglucitol in the liver and plasma of control db/+ mice (blue) and db/db mice (red) at different ages.

GC-MS data in liver was normalized to n-docosane and fresh liver weight (maximum scaled [%]); GC-MS data in plasma was calculated based on area (maximum scaled [%]); Values in the boxplot are given as median, 25/75% percentiles (boxes), 10/90% percentiles (bars); *p<0.05, **p<0.01, ***p<0.001 between two groups.

Figure 7. Boxplot of fucose in the liver and plasma of β-Phb2^{-/-} and db/db mice
Targeted metabolomics by GC-MS annotated fucose as a potential biomarker.

(A) Chemical structure of fucose.

(B) Levels of fucose in the liver and plasma of control Phb2^{fl/fl} mice (blue) and β-Phb2^{-/-} mice (red) at different ages.

(C) Levels of fucose in the liver and plasma of control db/+ mice (blue) and db/db mice (red) at different ages.

GC-MS data in liver was normalized to n-docosane and fresh liver weight (maximum scaled [%]); GC-MS data in plasma was calculated based on area (maximum scaled [%]); Values in the boxplot are given as median, 25/75% percentiles (boxes), 10/90% percentiles (bars); *p<0.05, **p<0.01, ***p<0.001 between two groups.
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217x220mm (300 x 300 DPI)
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SUPPORTING INFORMATION

Metabolomics identifies a biomarker revealing in vivo loss of functional β-cell mass before diabetes onset

Li et al.

Table S1. Primers for β-Phb2⁻/⁻ mice genotyping.

Table S2. Antibodies.

Table S3. Number of mice for mass spectrometry analysis.

Table S4. Glucose-stimulated insulin secretion from islets isolated from db/db mice.

Table S5. Full annotation list

Table S6. Univariate analysis of metabolome data

Figure S1. Targeted metabolomics by LC-MS on the liver of β-Phb2⁻/⁻ mouse model represented on metabolic pathways.

Figure S2. ROC curves.

Figure S3. Progression of glycemia and 1,5-anhydroglucitol plasma levels in non-obese diabetic (NOD) mice and streptozotocin (STZ) rats.
### Table S1. Primers for β-Phb2<sup>−/−</sup> mice genotyping.

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<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>Phb2, mouse</td>
<td>Microsynth</td>
<td>5’ATC GTA TTG GTG GCG TGC AGC A-3’</td>
<td>5’CGA GGT CTG GCC CGA ATG TCA T-3’</td>
</tr>
<tr>
<td>Cre, mouse</td>
<td>Microsynth</td>
<td>5’TGC CAC GAC CAA GTG ACA GC-3’</td>
<td>5’CCA GGT TAC GGA TAT AGT TCA TG-3’</td>
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### Table S2. Antibodies used

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<tr>
<th>Antibodies</th>
<th>Source</th>
<th>Cat. number</th>
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<td>Dako</td>
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<tr>
<td>Goat anti-guinea pig IgG (H+L) secondary antibody, Alexa Fluor 488 conjugate 1:200</td>
<td>Invitrogen</td>
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</tr>
<tr>
<td>Goat anti-guinea pig antibody, peroxidase-conjugated 1:100</td>
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<td>106-035-008</td>
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<td>Mouse anti-glucagon 1:500</td>
<td>Sigma-Aldrich</td>
<td>G2654</td>
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<tr>
<td>Goat anti-rabbit IgG (H+L) secondary antibody, Alexa Fluor 546 conjugate 1:200</td>
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<td>A11035</td>
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### Table S3. Number of mice for mass spectrometry analysis.

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<tr>
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<td>n=7 for Phb2&lt;sup&gt;fl/fl&lt;/sup&gt; mice</td>
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<td>n=9 for β-Phb2&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>n=7 for Phb2&lt;sup&gt;fl/fl&lt;/sup&gt; mice</td>
<td>n=6 for β-Phb2&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
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<table>
<thead>
<tr>
<th>db/db model</th>
<th>4 weeks</th>
<th>6 weeks</th>
<th>6 weeks</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n=6 for both db/+ and db/db</td>
<td>n=6 for both db/+ and db/db</td>
<td>n=6 for both db/+ and db/db</td>
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Table S4. Glucose-stimulated insulin secretion from islets isolated from db/db mice. Secretory responses were tested as static incubation at basal 2.8 mmol/L and stimulatory 22.2 mmol/L glucose concentrations on isolated islets. Insulin secretion rate is expressed as % insulin release normalized to total islet insulin content (n=6-8 animals per genotype at indicated age). Values are expressed as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001 between two groups.

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<tr>
<th>Age</th>
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<th>Stimulatory glucose (22.2 mmol/L)</th>
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<tr>
<td>4 weeks</td>
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<td>0.32±0.16</td>
<td>1.72±0.48</td>
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<td>Statistics: db/db vs. db/+</td>
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<td>***</td>
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Table S5. Full annotation list, see pdf file:

- **Supplementary Dataset 1** contains the ion annotation data for liver Phb mice, with reported ion m/z, average intensity, annotation scores, names, IDs and formulas.
- **Supplementary Dataset 2** contains the ion annotation data for plasma Phb mice, with reported ion m/z, average intensity, annotation scores, names, IDs and formulas.
- **Supplementary Dataset 3** contains the ion annotation data for liver db/db mice, with reported ion m/z, average intensity, annotation scores, names, IDs and formulas.
- **Supplementary Dataset 4** contains the ion annotation data for plasma db/db mice, with reported ion m/z, average intensity, annotation scores, names, IDs and formulas.

Table S6. Univariate analysis of metabolome data:

Complete xls files upon request to Pierre.Maechler@unige.ch

- **Supplementary Dataset 1** contains the full list of differential scores between liver Phb and corresponding controls at each week of development. Fold changes, p-values and q-values have been reported for each ion studied, with multiple ion annotation shown under the same ionIdx and m/z.
- **Supplementary Dataset 2** contains the full list of differential scores between plasma Phb and corresponding controls at each week of development. Fold changes, p-values and q-values have been reported for each ion studied, with multiple ion annotation shown under the same ionIdx and m/z.
- **Supplementary Dataset 3** contains the full list of differential scores between liver db/db and corresponding controls at each week of development. Fold changes, p-values and q-values have been reported for each ion studied, with multiple ion annotation shown under the same ionIdx and m/z.
- **Supplementary Dataset 4** contains the full list of differential scores between plasma db/db and corresponding controls at each week of development. Fold changes, p-values and q-values have been reported for each ion studied, with multiple ion annotation shown under the same ionIdx and m/z.
Figure S1. Targeted metabolomics by LC-MS on the liver of β-Phb2−/− mouse model represented on metabolic pathways. Red represents Phb2+/+ samples and blue represents β-Phb2−/− samples. Different age groups (4-week, 5-week, 6-week and 10-week) were from left to right.
**Figure S2. ROC curves.** Receiver Operating Characteristics (ROC) curves for the best classifiers (correlation thresholding combined with random forests) applied to the different untargeted metabolomics databases at hand: (A) β-PhB2−/− liver, (B) β-PhB2−/− plasma (C) db/db liver, (D) db/db plasma. Each plot indicates the corresponding value of the Area Under the Curve (AUC). The red curve is an average ROC curve obtained from 100 independently sampled leave-20-out test sets sampled exclusively from data points corresponding to the pre-diabetic stage.
Figure S3. Progression of glycemia and 1,5-anhydroglucitol plasma levels in non-obese diabetic (NOD) mice and streptozotocin (STZ) rats. Changes in plasma 1,5-anhydroglucitol levels (circles) and glycemia (squares) in (A) four NOD mice and (B) three STZ rats. Individual values were extracted from Kametani et al. 1987 for NOD mice (1) and Yamanouchi et al. 1986 for STZ rats (2).

A

NOD mice:

- 1,5-AG
- Glycemia

B

STZ rats:

- 1,5-AG
- Glycemia

1,5-AG: drop in 1,5-AG
D: diabetes onset
Content

Supplementary Dataset 1 contains the ion annotation data for liver Phb mice, with reported ion m/z, average intensity, annotation scores, names, IDs and formulas.

Supplementary Dataset 2 contains the ion annotation data for plasma Phb mice, with reported ion m/z, average intensity, annotation scores, names, IDs and formulas.

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Supplementary Dataset 4 contains the ion annotation data for plasma db/db mice, with reported ion m/z, average intensity, annotation scores, names, IDs and formulas.
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