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Obesity Development in a Miniature Yucatan Pig Model: A Multi-Compartmental Metabolomics Study on Cloned and Normal Pigs Fed Restricted or *Ad Libitum* High-Energy Diets.

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ABSTRACT. Miniature pig models for human metabolic disorders such as obesity and Metabolic Syndrome are gaining popularity. However, in-depth knowledge on the phenotypic and metabolic effects of metabolic dysregulation is lacking and ad libitum feeding is not well characterized in these pig breeds. Therefore, an investigation was performed into the metabolome of Yucatan minipigs fed ad libitum or restrictive. Furthermore, we used cloned and conventional minipigs to assess if cloning reflects a presumably lowered variation between subjects. For five months, 17 female Yucatan minipigs were fed either ad libitum or restrictive a western style diet. Serum, urine and liver tissues were collected and analyzed by non-targeted liquid chromatography–mass spectrometry metabolomics and by biochemical analyses. Several metabolic pathways were deregulated as a result of obesity and increased energy dense feed intake, particularly the hepatic glutathione pathway and the pantothenic acid and tryptophan metabolic pathways in serum and urine. Although cloned minipigs were phenotypically similar to wild-type, metabolomics analysis of serum and liver tissues showed several altered pathways, such as amino acid and purine metabolism. These changes, as an effect of cloning, could limit the use of cloned models in dietary intervention studies and provides no evidence of decreased variability between subjects.

KEYWORDS. Yucatan, minipigs, LC-MS, metabolomics, high-energy dense diet, cloned swine, inter-individual variation.

INTRODUCTION

Obesity is a condition characterized by abnormal or excessive fat accumulation which leads to an altered state of metabolism and physiology. Obesity in humans has been described as a major contributing factor to the development of metabolic syndrome¹, as well as being associated with a long list of debilitating diseases: insulin resistance, hypertension, non-alcoholic fatty liver disease, dyslipidemia, irregular appetite and hormonal imbalance, adipose tissue inflammation and abnormal energy balance²⁻³. Furthermore, persons with severe forms of obesity can suffer from reduced mobility, osteoarthritis, and even obstructive sleep apnoea⁴⁻⁵. Due to its status as a global epidemic, the state of metabolic dysfunction in obesity is being explored intensively as to understand its development mechanisms and possible mitigation strategies.

In order to achieve consistent and accurate results in studies which cannot involve human subjects, the pig model for obesity has gained in popularity over the last decade⁶. They present many similarities to humans⁷ and allow for repeated sampling of tissues and fluids without a high level of distress to the animal⁸⁻⁹. Both commercial livestock breeds and miniature pigs have been used as models to investigate obesity, diabetes or atherosclerosis. However, the Landrace or Yorkshire are large in size and difficult to handle during medical procedures, and as a result the miniature breeds are preferred due to their reduced size and ease of handling when fed ad libitum, comparable anatomy and reduce costs of feeding and maintenance. The Yucatan minipig breed, together with the Ossabaw and Göttingen, are currently the preferred models for obesity and metabolic syndrome (MetS) research⁸⁻¹⁰. The Yucatan and Ossabaw breeds have often shown a higher predisposition to the development of atherosclerotic plaque and stenosis after a prolonged high-fat/high-carbohydrate diet¹¹. The long-term effects of obesogenic diets are markedly increased by the use of an ad libitum feeding strategy¹². A major component of obesity development within the human population is the free access and frequent consumption of energy-dense foods. As such, the ad libitum fed minipig

model becomes a closer model to daily human habitual practices and might better reflect physiological changes during the development of obesity and MetS. Furthermore, we explored a possible advantage from using genetically identical clones in order to lessen variation within experimental groups. Cloned offspring obtained via somatic cell nuclear transfer assume a decreased variation because the genetic material originates from a single donor cell culture and as such, we expect metabolically more identical responses when compared to wild-type minipigs. However, recent studies involving cloned conventional breeds, such as Danish Landrace x Yorkshire¹³⁻¹⁴ and Göttingen minipigs¹⁵, revealed differences in the metabolome of cloned animals compared to normal wild-type pigs but presented no compelling evidence towards a reduction in variation between animals, such as a lower coefficient of variation or significant F values.

To get a better understanding of the biochemical mechanisms involved in the development of obesity, as well as to the effects of cloning, metabolomics has proven to be a good investigative approach¹⁶. Non-targeted high-resolution mass spectrometry (HRMS) is an instrumental technique which allows the study of specific small molecular-weight metabolites within a biological system¹⁷. When coupled with liquid chromatography, LC-MS metabolomics offers a highly sensitive and selective approach in characterizing a wide span of metabolites from different biological matrixes. Recently, MS-based techniques have been successfully used in large-scale studies to identify biomarkers of disease and to further explore the basic mechanisms involved in the development of diabetes, metabolic syndrome, type 2 diabetes and cardiovascular diseases¹⁶. In addition to the biochemical parameters and phenotypical characteristics, we used non-targeted LC-MS metabolomics to explore the metabolome of three different compartments (i.e. serum, urine and liver tissue) under the effect of ad libitum feeding of a Western Style Diet (WSD), as a primary objective. We aimed at identifying metabolic mechanisms through LCMS metabolomics, during an increased intake of energy-dense feed as they relate to metabolic dysregulation and development of obesity.

The second objective of this study was to determine whether cloning would reduce the variability between cloned animals with regards to measured phenotypical and metabolic responses. In addition, we hypothesised that cloned animals would be more metabolically homogeneous to the wild type and thus suitable models for studies of nutrition or metabolic diseases.

MATERIALS AND METHODS

Animals, experimental design, and diets

The animal experiment was conducted in compliance with procedures approved by the Danish Animal Experiments Inspectorate in accordance with the Danish Ministry of Justice Law no. 253/03-08-2013, concerning experiments with animals and care of experimental animals.

Cultured ear fibroblasts isolated from Yucatan minipigs were used as donor cells for cloning in a procedure previously described by Schmidt et al.¹⁸ Briefly, cumulus-oocyte complexes (COCs) were obtained from slaughterhouse-derived Large White (Landrace x Yorkshire x Duroc) sows. The donor cells together with the matured oocytes were used for cloning, resulting in viable embryos which were surgically transferred to surrogate sows 5 to 6 days after cloning. The piglets were born by either spontaneous farrowing up to 114 days or by cesarean section if this period was exceeded. Control animals were normal Yucatan minipig litters obtained by standard artificial insemination. Ten cloned female pigs from three surrogate sows (sow 1 – four littermates; sow 2 – two littermates; sow 3 – four littermates) were used together with seven normal female pigs from one surrogate sow (control Yucatan). Embryo transfer, farrowing and pig rearing were done in the experimental stables at Aarhus University (AU Foulum, Tjele, Denmark).

After weaning, the pigs were fed a standard diet until 5 months of age, when control animals weighed 29.4 kg ($\pm$ 1.6 SEM) and clones 28.1 kg ($\pm$ 1.3 SEM). Following the next 5 months, animals were switched to a high-energy wheat-based diet (Table 1) high in sugars (10% sucrose) and high in

fat (10% soybean oil). Samples of the experimental diets were collected after production and stored at -20 °C prior to analysis. Five clones and 3 controls were fed ad libitum and the average values from these animals were used as reference for the 5 clones and 4 control animals that were fed restrictive to 60% of total feed consumption of the ad libitum group. The diets were provided in two daily meal allowances. The cloned littermates were assigned identically in both restrictive and ad libitum groups as follows: two littermates from sow 1, one piglet from sow 2, two litter mates from sow 3. Pigs were individually penned but physical and visual contact with neighboring pigs through railings was still possible. The animals had access to ad libitum water intake and were weighed every two weeks.

Slaughtering, sample collection and CT scanning

At 10 months of age, animals were euthanized using a bolt pistol after an overnight fasting period (16 hours). Blood serum was obtained from blood collected at exsanguination in 9 mL tubes with Z serum clot activator. Blood tubes were left at room temperature for 1 hour and centrifuged for 10 minutes at 1620 x g. Urine was collected by removing the urinary bladder and direct puncture. Liver tissue was obtained from the midsection of the right hepatic lobe and 12 sections of 0.5 x 0.5 x 0.5 cm were immediately snap frozen in liquid nitrogen. Serum, urine and liver tissue stored at -80°C until analysis. The animal carcasses were sectioned in two sides using a circular saw and the left side was scanned using a portable computed tomography machine (HiSpeed CT/i, GE Medical Systems, General Electric, US). A tube voltage and current of 120kV and 110mA, respectively, were used with a slice thickness of 10.0 mm. The standard convolution kernel was used for scanning (a scanner-specific parameter contributing to spatial resolution and noise texture appearance of the image). CT scans were saved in the DICOM (Digital Imaging and Communications in Medicine) format at 512x512 pixels in 16-bit grayscale depth.

Analytical methods

Chemical analyses of the diets were performed in triplicate on freeze-dried samples. Ash was analyzed as described in the Association of Analytical Communities (AOAC) method no. 942.05 and dry matter was determined by drying to constant weight at 103°C. N was analyzed by Kjeltec (Kjeltec™ 2400; FOSS Analytical) and protein was calculated as $N \times 6.25$. Fat content was measured using the Stoldt procedure with diethyl ether extraction after HCl hydrolysis¹⁹. The Larsson and Bengtsson method was used to analyze the content of fructans and sugars (glucose, fructose, sucrose)²⁰. Starch and NSP (cellulose, soluble non-cellulosic polysaccharides, and insoluble non-cellulosic polysaccharides) measurements were performed as described by Bach Knudsen²¹. Klason lignin was determined as the sulphuric acid-insoluble residue using the Theander and Aman method²². Gross energy of the diet was analyzed using a 6300 Automatic Isoperobol Calorimeter system (Parr Instruments, Moline, IL, USA).

Serum glucose, triglycerides, total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), nonesterified fatty acids (NEFA) and lactate were measured using the ADVIA 1650 Chemistry System (Siemens Healthcare Diagnostics, Deerfield, IL, U.S.A). Glucose was determined using the glucose hexokinase II and enzymatic colorimetric method²³. Serum triglycerides were analysed by measurement of glycerol after hydrolysis of mono-, di- and triglycerides²⁴ and total cholesterol by enzymatic hydrolysis of cholesterol esters, oxidation of free cholesterol and release of hydrogen peroxide²⁵. HDL and LDL were determined after selective isolation and liberation as described by Okada et al²⁶ and Izawa et al²⁷. NEFAs were determined using the Wako NEFA C ACS-ACOD assay (Wako Chemicals GmbH, Neuss, Germany), and lactate was measured using the method described by Noll²⁸. Insulin-like growth factor 1 (IGF-1) was measured using an EIA kit from R&D Systems Inc. (Minneapolis, MN, U.S.A) and insulin was measured by time-resolved fluoroimmunoassay as described by Løvendahl and Purup²⁹.

Chemical solvents and standards for metabolomics analysis

High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from VWR (West Chester, PA, USA), formic acid from Fluka (Merck KGaA, Darmstadt, Germany) and MilliQ grade water (Millipore, MA) were used for solvents and eluents in the untargeted metabolomics analysis. Glycocholic acid (Glycine-1-¹³C) and 4-chloro-DL-phenylalanine purchased from Sigma (Merck KGaA, Darmstadt, Germany) were used as internal standards. All other standards used for compound identification were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and Cayman Chemical (MI, USA).

Sample Preparation of Blood, Urine and Liver Tissue for Metabolomics Analysis

Blood serum for metabolomics was prepared by deproteinization of 200 μ L sample with 600 μ L ice-cold acetonitrile (ACN) containing an internal standard mix of glycocholic acid (glycine-1-¹³C) and p-chlorophenylalanine to a final concentration of 0.01 mg/mL. The samples were immediately vortexed and incubated at 4°C for 20 minutes. After centrifugation at $13200 \times g$ for 10 minutes, the supernatant was evaporated to dryness in a vacuum centrifuge set at 2000 rpm and 30°C. The resulting dry residue was re-dissolved in 100 μ L water/acetonitrile/formic acid (95:5:0.1, v/v/v) and centrifuged for 10 minutes at $1000 \times g$. The supernatant was transferred to LC-MS vials with inserts for the analysis.

Urine samples (450 μ L) were mixed with 50 μ L ice-cold ACN containing an internal standard mix of glycocholic acid (glycine-1-¹³C) and p-chlorophenylalanine to a final concentration of 0.01 mg/mL. After an immediate vortex, the samples were incubated 20 minutes at 4°C for protein precipitation. After a centrifugation at $13200 \times g$ for 10 minutes, the supernatant was collected and transferred to LC-MS vials with inserts.

Liver tissue samples were prepared using the aqueous extraction protocol described by Want et al³⁰. Briefly, approximately 50 mg of tissue (weight was noted) was weighted in a 2 mL Eppendorf tube with 100 mg glass beads (SiLibeads Glass beads Type S, 0.15-0.25mm, VWR International)

followed by the addition of 500 μ L ice-cold methanol/water (50:50, vol/vol) containing a mix of glycocholic acid (glycine-1- ^{13} C) and p-chlorophenylalanine to a final concentration of 0.01 mg/mL. Samples were homogenized on a tissue lyser (StarBeater, VWR International) using the following sequence: two cycles of 2.5 min at 25 Hz, 5 min rest on ice, one cycle of 2.5 min at 30 Hz. Another 500 μ L of ice-cold methanol/water (50:50, vol/vol) was added to the samples and immediately vortexed followed by incubation at 4°C for 20 minutes. Samples were centrifuged for 10 min at 13200 $\times$ g and 250 μ l supernatant was transferred to a new Eppendorf tube which was evaporated to dryness in a vacuum centrifuge (30°C, 2000 rpm). The dry residues were re-solubilized in 120 μ L water/acetonitrile/formic acid (95:5:0.1 v/v/v) and centrifuged for 5 min at 1350 $\times$ g. The collected supernatant was transferred to LC-MS vials with inserts.

Quality control of metabolomics analysis

Quality control samples (QCs) were prepared for all sample types as a way of monitoring the quality of the chromatographic runs, the stability of the system, and the accuracy of intra-lab sample preparation. For serum, urine and liver tissue, 5 to 6 quality controls were obtained by pooling aliquot amounts and subjected to the same protocol as the samples. For the liver, the pooled sample was obtained by pipetting aliquots of each sample after the tissue homogenization and centrifugation. In order to monitor the stability of the LC system, for each metabolomics analysis, one of these QCs was injected multiple times throughout the run. The remainder QCs were injected in series at the beginning and end of the analysis, which allowed us to observe any changes caused by liquid handling inconsistency. Three types of blank samples were run during the chromatographic analysis to monitor any possible external contamination, degradation of chemical reagents, carryover effects: simple blank containing only ACN or methanol, blank containing the internal standard mix, blanks containing water which were subjected to the same protocols of sample preparation. Samples were

randomized for the chromatographic running order in order to eliminate biases and reduce noise or variance in the results and at the same time ensuring that each sample group is affected equally.

Ultra-High Pressure Liquid Chromatography-Mass Spectrometry

Samples were analysed using a Dionex UltiMate 3000 (Dionex, Sunnyvale, CA, USA) ultra-high pressure liquid chromatography system (UHPLC) and metabolite profiles were obtained with a Impact HD Quadrupole Time-of-Flight (QTOF) mass spectrometer (Bruker daltonics GmbH, Bremen, Germany) operating in positive electrospray ionization mode (ESI⁺) and negative electrospray ionization mode (ESI⁻). For the acquisition of mass spectrometry (MS) data, a scan range from m/z 50 to 1000 was used. Other ESI⁺ and ESI⁻ parameters were as follows: capillary voltage set to 4500 V (ESI⁺) and 3600 (ESI⁻); dry carrier gas temperature, 200°C; dry gas flow, 10.0 L min⁻¹; nebulizer pressure, 2.2 bars; collision energy during the MS scan was set to 6 eV. When MS/MS fragmentation was used for identification of compounds the collision energy was set from 15 to 30 eV depending on the characteristics of the compound analyzed. A solution of lithium formate clusters in water/isopropanol/formic acid (50:50:0.2, v/v/v) at a concentration 5 mmol/L was injected at the beginning of each chromatographic run and used for automatic internal calibration.

The chromatographic separation of compounds was performed on an Acquity HSS T3 1.7 µm 100 × 2.1 mm (Waters Corporation, Milford, MA) column equipped with a VanGuard Pre-column, 100Å, 1.8 µm, 2.1 mm × 5 mm (Waters Corporation, Milford, MA). The column temperature was set to 30°C, samples were kept in the autosampler at 10°C for the whole duration of the analysis and the injection volume was set to 5 µL for all three sample types. The chromatographic analysis was performed under a gradient of water/formic acid (100:0.1, v/v, mobile phase A) and acetonitrile/formic water (100:0.1, v/v, mobile phase B). For the analysis of serum and urine, the column was equilibrated for two minutes at 5% B followed by a linear increase to 100% B for 12 minutes. For the liver analysis, the gradient was increased to 13 minutes. Mobile phase was kept

isocratic (100% B) for 3 minutes and then returned to 5% B for the next injection. The flow rate was set to 400 $\mu\text{L}\times\text{min}^{-1}$ and total analysis time was 17 minutes for serum and urine and 18 minutes for liver.

Data analysis

Statistical analysis. Statistical analysis of the biochemical parameters and weight development of the animals was performed using SAS Enterprise Guide 5.1 (SAS Inst. Inc., Cary, NC) using a linear mixed model with the PROC MIXED procedure:

$$Y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the dependent variable, μ the overall mean, α_i is the type effect (i = clone or control), β_j is the diet effect (j = ad libitum or restricted), $\alpha\beta_{ij}$ is the interaction term, and ε_{ij} represents the residual error component. In the statistical model date of sampling and surrogate mother were included as random effects. Comparing the groups was done using ANOVA and multiple comparisons were adjusted using Tukey-Kramer. Values were reported as LSMEANS $\pm$ SEM and statistical significance was set as $P < 0.05$ and $0.05 \leq P < 0.10$ are considered trends.

Differences in variability between cloned animals and controls were tested using an F-test under the alternative hypothesis assumption that variances are different between the two groups ($H_a: \sigma_A^2 \neq \sigma_B^2$). The test was performed in RStudio 1.1.442 using the “var.test” function, normality was checked using the Shapiro-Wilk’s test, and for data not normally distributed a logarithm transformation was applied. No significant difference between the variances of tested groups is considered when $P > 0.05$.

LCMS data pre-treatment. Mass spectra acquired using Impact HD QTOF MS were calibrated and converted into the mzXML file format. R based XCMS package was used to for the extraction of the mass features; peak peaking was performed using ‘centWave’ method and retention time aligned using ‘Obiwarped’; missing values were substituted using the ‘fillPeaks’ method; adducts, fragments,

and isotopes were annotated using CAMERA. Exported data tables were filtered to eliminate features present in blanks, retention times were truncated to contain only portions with chromatographic peaks and masses higher than 700 m/z were discarded. Normalization of the serum and urine data was done using the peak area of the internal standard to compensate for variation in sample processing and instrumental drift. For the liver metabolomics data, a within batch normalization strategy was employed, using the QC samples and the Van der Kloet procedure³¹.

Multivariate data analysis. Data tables were imported to LatentIX 2.12 (Latent5 Aps.) and Pareto scaled to reduce the relative importance of large values and keep the data structure partially intact³². An initial principal component analysis (PCA) was performed to check the quality of the data sets and eliminate potential outliers. Partial least squares discriminant analysis models (PLS-DA) were built in order to determine the metabolites responsible for the greatest differences between groups (Table S1). Validation of the models was done using full cross-validation (leave-one-out) and assessed using the explained variation in Y, actual versus predicted plots and the coefficient of variation (R^2). For improving the models a variable selection strategy was used by excluding low importance variable based on the VIP (Variable Importance in Projection) scores. To select metabolites for identification a combination of scaled regression coefficients and VIP scores for those features were used. Differences between metabolite ion intensities were tested using a two-sample T-test in the online software MetaboAnalyst 4.0³³. In order to correct for multiple comparisons, false discovery rate (FDR) q values were calculated with significance threshold set at $q < 0.10$.

Metabolite identification and pathway analysis. Accurate mass and mass fragmentation patterns of discriminating features were used for compound identification based on queries in the following databases: Human Metabolome Data Base (<http://www.hmdb.ca/>), METLIN (<http://metlin.scripps.edu/>), MetFrag database (<https://msbi.ipb-halle.de/MetFragBeta/>). Annotated features were classified on different levels of identification according to Sumner et al.³⁴ as follows:

MSI level 1 – compounds identified using commercially available standards, MS/MS fragmentation patterns and retention time; MSI level 2 – compounds identified using MS/MS fragmentation patterns from databases or previously published articles; MSI level 3 – unidentified compounds assigned to a chemical compound class based on fragmentation patterns and retention time; MSI level 4 – unidentified compounds which cannot be matched in databases, based on accurate mass or fragmentation pattern. Compounds that were identified and assigned a pathway classifier from Kyoto Encyclopaedia of Genes and Genomes or Human Metabolome Database (KEGG/HMDB) were used to build pathway analysis maps using MetaboAnalyst 4.0³³. Compounds were matched against the *Homo sapiens* pathway library, pathway enrichment analysis was performed using a “Global Test” algorithm based on the intensities of the compounds and difference between groups and a “Relative-betweenness Centrality” algorithm for the pathway topology analysis.

CT image analysis. Measurement of the total adipose tissue area (TAT) was based on single abdominal cross-sectional CT imaging. Differentiation between subcutaneous adipose tissue and visceral adipose tissue was restricted because organs and gut content were removed prior to CT scanning. Slides representing measurements between the lumbar vertebrae 1-2 (L1-L2) were chosen for all animals in order to maintain the same location using the distal part of ribs 14-15 as a reference points (Figure S1). Image visualisation and interpretation was done using RadiAnt DICOM Viewer 4.6.5, image processing and data extraction was performed in the freeware FIJI (ImageJ 1.5d, <http://imagej.nih.gov/ij>). Briefly, TAT was outlined using the Weka Trainable Segmentation plugin 3.2.27³⁵, region of interest (ROI) were selected using the colour threshold function and measured in squared cm based on pixel count on each of the digital images (Figure 2).

RESULTS

Diet and animal characteristics

The experimental diet (Table 1) provided the animals a gross energy of 19.24 MJ/kg DM. In terms of relative energy contribution, the animals received a substantial part from carbohydrates (47.3%) in the form of sucrose (119 g/kg DM) and starch (375 g/kg DM). Fat provided 28.6% energy, and as intended for a WSD, protein and dietary fiber played a minor role in terms of energy, only 19.7% and 4.4%, respectively. Following a 20-week dietary intervention, ad libitum fed Yucatan minipigs gained significantly more weight, reaching a daily weight gain of 0.32 ± 0.01 kg compared to restrictive fed animals (0.18 ± 0.01 kg; $P < 0.001$). Two weeks after the start of the dietary intervention the ad libitum fed group began to significantly gain more weight approaching 50 kg by week 10 (Figure 1). Following completion of the feeding trial, ad libitum fed animals weighed on average 80.6 ± 3.23 kg compared to 57.8 ± 2.97 kg in restrictive feeding (Table 2). Total adipose tissue (TAT) measured on abdominal cross-sectional CT images revealed increased adipose mass deposition subcutaneously as well as in the pararenal areas in the ad libitum fed swine (Figure 2, Table 2). No significant change in weight was observed as a result of cloning. No feed residues were collected during the trial and as a result, a daily feed or energy intake could not be estimated.

Biochemical parameters in fasting serum and urine samples of the Yucatan minipigs were analyzed (Table 2). Significant changes were seen with regards to the feeding strategy, with higher cholesterol ($P = 0.003$) and LDL ($P = 0.002$) levels in ad libitum fed minipigs and trending higher levels of serum HDL ($P = 0.058$) and urinary glucose ($P = 0.069$). Other parameters such as glucose, triglycerides, NEFA, lactate, IGF or insulin measured in serum remained unchanged between ad libitum and restricted fed animals. None of the biochemical markers investigated showed significant changes between cloned minipigs and control wild-type minipigs and no diet and cloning interaction was observed either.

Non-targeted multi-compartmental metabolomics analysis

After processing of the extracted data tables from each compartment and each ionization mode 12 PLS-DA models were constructed to discover discriminating features in serum, urine or liver samples in either positive (ESI⁺) or negative (ESI⁻) mode. Models were evaluated based on several criteria (Table S1) that resulted in valid models for the discovery of metabolic features. One exception was observed with the urine metabolome of cloned and control animals, for which the predictive powers of the model were low indicating no significant features that lead to separation between the two groups (Figure 4E,F). The strongest models with a high separation were observed for the urinary metabolome of ad libitum versus restrictive fed animals (Figure 3E, F) in both positive and negative mode, PLS component 1 describing 40.1% and 28.7% of feature variation in positive and negative mode, respectively. Similarly, serum metabolome (ESI⁺, Figure 3A) showed good separation between samples and model stability ($R^2 = 0.498$). Score plots of liver PLS-DA analysis on feeding strategy show a good percentage of explained variance by PLS component 1 in positive (26.4%) and negative (19.8%) mode, however, in positive mode, a higher explained variation was associated with differences between ad libitum fed animals, as seen in PLSC2. Differences between cloned and control animals were seen in the PLS-DA models of serum and liver. For serum analysis in ESI⁺ and ESI⁻, PLS component 1 accounts for 17.2% and 9.6%, respectively (Figure 4A,B). The largest differences based on the explained variance of PLSC1 were seen in the liver tissues with 27.6% in positive and 17.8% in negative mode (Figure 4C,D).

Metabolome changes, effect of feeding strategy

Significant and annotated discriminating features found between ad libitum and restrictive fed Yucatan minipigs are presented in Table 3. Generally, an up-regulation of metabolites in ad libitum fed animals was observed in all three compartments; 10 out of 13 metabolites in serum, 13 out of 15 hepatic metabolites and 43 urinary metabolites out of 56.

Tryptophan together with tryptophan dimers and trimers were significantly upregulated in the serum of ad libitum fed animals. Indolelactate ($P = 0.026$), a downstream degradation product of tryptophan, as well as other indolic compounds (3-methylindole, indole-3-carboxaldehyde), were also found increased in the serum of ad libitum minipigs. Lipids and lipid-like molecules such as 2-aminoheptanoic acid, LysoPC (17:0/0; 1-heptadecanoyl-sn-glycero-3-phosphocholine) or PC (16:0/5:0(CHO)) were the only significant molecules to decrease in the serum of ad libitum fed animals.

The liver metabolome showed a significant increase in the tripeptide glutathione ($P = 0.001$, ESI^+) together with its component amino acid L-glutamate ($P = 0.0037$, ESI^+). Other organic acids were found up-regulated, such as L-alpha-aminobutyric acid, D-2-hydroxyglutaric acid, glycerophosphoglycerol, N-acetylneuraminic acid. A glycine-conjugated bile acid, glyoursodeoxycholic acid, was significantly higher in intensity in the hepatic tissue of ad libitum fed animals ($\text{FC}=1.39$; $P=0.0037$), whereas the nucleotide sugar uridine diphosphate glucose (UDP-glucose) and ribosylated imidazoleacetic acid were down-regulated -1.37 and -1.30 fold, respectively.

As expected, a high number of sulphated or glucuronated metabolites was seen in urine (Table 3). Among these, a particular group of metabolites that belong to the flavonoids compound subclass were significantly ($P < 0.001$) enriched in the urine of ad libitum fed animals: baicalein (m/z 269.0462), baicalein-7-glucuronide (m/z 446.0819), diadzein 4-O-glucuronide (m/z 429.0833), diadzein 7-O-glucuronide (m/z 429.0833). Untargeted metabolomics allowed for the identification of a high number of fatty acyl molecules together with their glucuronated counterparts. With ad libitum feeding, we observed an increase in urinary excreted 7-oxo-11-dodecenoic and 7-oxo-11-dodecenoic acid glucuronide, tetradecaenoic acid and tetradecaenoic acid glucuronide, dodecanedioic acid glucuronide, hexadecanedioic acid glucuronide. Two putatively classified molecules of oxylipins, m/z 389.1814 HpODE (1) and m/z 424.2184 HpODE (2), and monoacylglycerol m/z 559.3032

PG(22:4/0:0) showed high fold changes of 2.38, 2.57 and 4.66, respectively. Docosahexaenoic acid was the only significant fatty acyl compound ($P = 0.025$) which was higher in restrictive fed minipigs. Several carboxylic acids and derivate compounds like 4-hydroxy-3-methoxy-cinnamoylglycine, DL-2-aminoadipic acid, hydroxyphenylacetylglycine, N-acetyl-DL-tryptophan, N-phenylacetylaspatic acid, phenylacetylglycine, picolinoylglycine were upregulated in ad libitum fed pigs. On the other hand, a large number of compounds classified as indolines or containing an indole moiety were down-regulated together with their glucuronated or sulphated form: adrenochrome glucuronide ($P = 0.015$), adrenochrome sulphate ($P = 0.046$), 4-formyl indole ($P = 0.028$), dihydroxy-1H-indole glucuronide ($P=0.02$), 3-indole carboxylic acid glucuronide ($P = 0.014$), 5-hydroxy-6-methoxyindole glucuronide ($P = 0.015$), 6-hydroxy-5-methoxyindole glucuronide ($P = 0.024$). Two indolic compounds presented opposite activity and were found in much higher concentrations in the urine of ad libitum minipigs: 5,6-dihydroxyindoline (leukoaminochrome; $P = 0.0006$) and leukoaminochrome glucuronide ($P = 0.04$). Two significantly different quinolone compounds were detected in higher amounts in urine, kynurenic acid ($P = 0.0002$, $FC = 2.16$) and xanthurenic acid ($P < 0.0001$, $FC = 2.33$). The remaining unidentified features and non-significant compounds together with the previously described metabolites can be examined in Table S2.

Metabolome changes, effect of cloning

The metabolome of cloned animals (Table 4) expressed an increased intensity of purine nucleosides such as adenosine ($[M+H]$, $[M+Cl]$), adenosine monophosphate ($[M+H]$, $[M-H]$, $[M+Na]$ and $[M+CH_3COOH]$) and inosine ($[M-H]$, $[M+Na]$, $[2M+Na+H]$). Lower molecular weight carboxylic acids were down-regulated in cloned animals as reflected by amino acids L-phenylalanine, L-histidine, L-methionine, and putatively assigned prolyl-cysteine. Pantothenic acid ($P = 0.026$), creatine ($P = 0.014$), or acetylcarnitine ($P = 0.026$) were also lower in cloned animals compared to the control phenotype. Lipid and lipid-like molecules were upregulated in serum: tricosahexaenoic

acid (TrHA, $P = 0.025$), hydroxymyristic acid ($P = 0.014$), sphingosine-1-phosphate ($P = 0.03$). Methylsuccinic acid, a normal metabolite usually found in human fluids was the highest most upregulated compound among all detected metabolites with a fold change of 3.96 ($P = 0.0002$). Indole metabolite serotonin ($[M+H]$, $[M-NH_4]$, $[M+H-NH_4]$), was significantly increased ($P=0.003$) in the serum of cloned animals, whereas levels of serotonin precursor L-tryptophan were not changed ($P=0.08$, Table S2).

A major shift in amino acid levels was observed in the liver compared to the metabolome of serum, where L-phenylalanine ($P=0.0004$), L-lysine ($P=0.0003$), L-histidine ($P=0.026$), L-methionine ($P < 0.0001$), L-threonine ($P=0.03$), L-tryptophan ($P=0.009$), taurine ($P=0.01$), L-tyrosine ($P=0.08$) and putatively classified compound m/z 237.0881 glycyl-tyrosine ($P=0.02$) were found up-regulated, however, all metabolites had a FC < 1.5 . Taurine was the only down-regulated ($P=0.011$) amino acid out of the ones detected. Glycerophosphocholine ($[M+H]$, $[M+K]$) together with 3-dehydroxycarnitine were two lipids that were found in higher intensities in the liver of cloned animals, whereas sn-glycero-3-phosphoethanolamine and 2-hydroxyadipic acid were lower. Within the liver metabolome, organo-oxygen compounds were also detected in higher amounts: D-glucose ($P=0.006$), D-glucose-6-phosphate ($P=0.025$). In contrast D-glucuronic acid ($P=0.026$), gluconic acid ($P=0.08$), glycolic acid ($P=0.005$) were decreased in the liver tissue of cloned animals. Calculated PLS-DA models (Table S1) showed poor characteristics and as a result, no metabolites were identified from the urinary metabolome of cloned Yucatan minipigs compared to the control wild-type. Other non-significant compounds and unidentified metabolic features that were detected in the serum and liver of cloned and control Yucatan minipigs and can be inspected in Table S3.

Metabolic pathway analysis.

Identified compounds irrespective of their significance level between groups were assigned KEGG compound identifiers and were used for pathway topology analysis. Using the intensities of the

metabolites, a quantitative enrichment analysis was also performed. Figure 5A presents several pathways that were linked to metabolites from the serum of ad libitum and restrictive fed animals. The first five pathways in order of impact (Table S4) and the metabolites involved were: pantothenate and CoA biosynthesis (map00770; pantothenic acid, L-valine), arginine and proline metabolism (map00330; L-glutamyl-5-phosphate, L-proline, creatine), phenylalanine metabolism (map00360; L-phenylalanine, L-tyrosine, phenyllactic acid, 2-hydroxycinnamic acid, phenylacetyl glycine), tryptophan metabolism (map00380; L-tryptophan, indolelactic acid). Only the tryptophan pathway was affected significantly ($P=0.003$) due to a high upregulation of both metabolites found in that pathway. Six metabolites were found belonging to the aminoacyl-tRNA biosynthesis pathway (map00970), however, no impact on the pathway enrichment and topology was observed. Liver metabolism (Figure 5B) was primarily altered by the presence of L-glutamate and glutathione, both found in the glutathione metabolism pathway (map00680). The amino acid L-glutamate was located also in the alanine, aspartate and glutamate metabolism (map00250), as well as in the D-glutamine and D-glutamate metabolism (map00471). The hepatic starch and sucrose metabolism (map00500) was influenced by the presence of uridine-diphosphate-glucose and D-glucose-6-phosphate, the latter also found in the amino sugar and nucleotide sugar metabolism pathway (map00520). Similar to serum, the presence of pantothenic acid in urine (Figure 5C) had a high impact on the urinary metabolome reflecting changes in the pantothenate and CoA biosynthesis (map00770). Several urinary metabolites were assigned to the tryptophan metabolism (map00380) and are direct products of tryptophan catabolism: kynurenic acid, xanthurenic acid, 3-methyldioxyindole, and 3-hydroxyanthranilic acid sulphate. Other metabolites identified in urine such as DL-2-aminoadipic acid belongs to the lysine biosynthesis pathway (map00300), creatine and creatinine are found in the arginine and proline metabolism pathway (map00330) and hydroxyphenyllactic acid can be identified in the ubiquinone and other terpenoid-quinone biosynthesis metabolism (map00130). Although

having less impact on the urinary metabolome, three other metabolites were found significant in the tyrosine metabolism pathway (map00350): hydroxyphenyllactic acid; hydroxyphenylacetyl glycine and dihydroxy-1H-indole glucuronide.

There are 24 pathways affected in the serum of cloned versus normal phenotype minipigs that were described based on identified metabolites (Figure 6A). Out of these, phenylalanine metabolism (map00360) had the highest impact on the serum metabolome where 5 out of 45 metabolites in the pathway were identified using our LC/MS platform: L-phenylalanine, D-phenyllactic acid, 2-phenylacetamide, hippuric acid, phenylacetyl glycine. Among other high impact pathways, we identified histidine metabolism (map00340; L-histidine, histamine), arginine and proline metabolism (map00330; L-proline, creatinine), purine metabolism (map00230; adenosine monophosphate, adenosine, inosine, hypoxanthine, guanine), vitamin B6 metabolism (map00750; pyridoxine). Two metabolic pathways, although having a high impact in serum, were not significant according to the pathway enrichment analysis: pantothenate and CoA biosynthesis (map00770; pantothenic acid, L-valine) and tryptophan metabolism (map00380; L-tryptophan, serotonin, indolelactic acid). Aminoacyl-tRNA biosynthesis (map00970) although not significant ($P=0.22$) contained the highest number of hits among all other pathways in serum: L-histidine, L-phenylalanine, L-valine, L-methionine, L-leucine; L-tryptophan, L-proline. This amino acid homeostasis was disrupted in the liver of cloned animals where the upregulation of numerous amino acids led to changes in pathways associated with these compounds (Figure 6B, Table S5). Out of 32 metabolic pathways identified based on the liver metabolome, 20 of them are related to amino acid metabolism. D-glucose, D-glucuronic acid and D-glucose-6-phosphate were together or individually found in several metabolic pathways such as: pentose and glucuronate inter-conversions pathway (map00040), pentose phosphate pathway (map00030), ascorbate and aldarate metabolism (map00053), starch and sucrose metabolism (map00500), galactose metabolism (map00052), amino sugar and nucleotide sugar

metabolism (map00520), glycolysis or gluconeogenesis pathway (map00010), inositol phosphate metabolism (map00562).

DISCUSSION

The main objective of this study was to examine changes in the metabolism of Yucatan minipigs and to explore the molecular mechanisms involved in the development of diet-induced obesity by using explorative non-targeted metabolomics in a multi-compartmental approach. Ad libitum feeding of the minipigs considerably increased their body weight, animals showing visible signs of fat accretion. Animals gained 28% more weight compared to the restrictive feeding of the same WSD. A similar study in which male Yucatan minipigs were fed restrictively a high-fat/high-cholesterol atherogenic diet for 43 weeks resulted in animals weighing 58 kg on average¹¹ and very high levels of total cholesterol (TC), HDL and LDL levels. Interestingly, the minipigs in our study reached a similar weight after only 20 weeks of dietary intervention even though energy levels deriving from fat were only 28.6% compared to 47%. Diets formulated in the present study included higher levels of carbohydrates, by addition of sucrose, from which we can speculate that refined carbohydrates had a higher obesogenic effect than fat alone. However, gender effect should not be overlooked as female minipigs have a higher predisposition to rapid and increased fat accumulation³⁶⁻³⁷. The supplementation of cholesterol and sodium cholate¹¹, increased levels of blood cholesterol to 10.86 mmol/L whereas in our study 20 weeks of high-fat/high-sucrose diet only resulted in 2.17 mmol/L circulating TC. Ad libitum feeding markedly increased levels of TC, HDL, and LDL, however, these values are still below what has been previously reported in hypercholesterolaemic miniature swine. An increased level of plasma lactate in the fasting state together with higher glucose levels has been associated with increased tissue adiposity³⁸, which could lead to obesity and type 2 diabetes (T2D). No changes in lactate or glucose levels were observed indicating that although ad libitum fed minipigs developed an increased state of obesity, there were no indications of a pre-diabetic state. Blood

glucose remained unchanged and in agreement to similar fasting levels seen in other minipig studies^{11, 39}. A glucose or insulin challenge might have provided, however, more information about the effects of ad libitum feeding.

The multi-compartmental non-targeted metabolomics approach used in this study revealed intriguing differences between ad libitum and restricted fed minipigs. Increased hepatic levels of glutathione and glutamine point towards the activation of the glutathione metabolism pathway. Glutathione (γ -glutamylcysteinylglycine, GSH) is a known key hepatic tripeptide involved in detoxification reactions and protective functions⁴⁰. GSH responds to reactive oxygen species and neutralizes the free electrons that induce the high reactivity and threatening status of such molecules⁴¹. Deficiency in GSH and other hepatic antioxidants has been described as a key mechanism in the pathophysiology of non-alcoholic fatty liver diseases (NAFLD)⁴²⁻⁴³ and GSH administered orally in a clinical trial resulted in decreased levels of triglycerides, NEFA, ferritin and ALT levels in humans with NAFLD⁴⁴. The increased levels we observed in ad libitum fed minipigs can reflect a direct response to the influx of nutrients and potential free radical species with deleterious effects on hepatocyte health. This is the first time increased hepatic GSH has been observed as a direct effect of feeding a WSD in minipigs. Other studies reporting on GSH were focusing on the effect of ethanol as an inducing agent of fatty liver disease⁴⁵.

Shared pathways identified in serum and urine of ad libitum fed minipigs were the pantothenate and CoA biosynthesis (map00770), tryptophan metabolism (map00380), phenylalanine metabolism (map00360), arginine and proline metabolism (map00330) and tyrosine metabolism (map00350). Pantothenic acid is a major component of coenzyme A (CoA) together with cysteine and adenosine triphosphate (ATP)⁴⁶. Fluctuations in levels of pantothenic acid and implicitly CoA have a direct consequence on the TCA cycle. The increase of pantothenic acid and other TCA cycle intermediates⁴⁷ could be described as an internal protection mechanism to an increased effect of lipogenesis through

an energy-dense diet. We speculate that an enhanced urinary excretion of pantothenate reflects a direct loss of energy from carbon-containing molecules as a mean to reduce lipid synthesis intermediates and deregulate lipid accretion⁴⁸. Tryptophan (Trp) metabolism has previously been reported as an activated pathway in obesity and metabolic diseases⁴⁹⁻⁵⁰. Increased levels of Trp and its degradation products were found in serum and urine. Trp is an important constituent in protein synthesis and is used as a precursor in the kynurenine and serotonin synthesis⁵¹. Approximately 95% of dietary Trp catabolism is defined by the synthesis of kynurenine⁵¹⁻⁵² and significant higher amounts of kynurenic acid (KA) were measured in the urine of ad libitum fed animals compared to restrictive feeding. Similarly, xanthurenic acid (XA), a two-step derivate of kynurenine was detected two-fold higher in urine. Trp is one of eight essential amino acids (AA) and increased levels in serum and urine together with KA, XA, and indolelactate is indicative primarily of feed intake. Since up to 90% of Trp degradation is believed to occur in the liver⁵², we speculate that elevated levels of Trp and N-acetyl-tryptophan in urine reflect an excess of non-metabolised dietary Trp. However, recent findings associate high urinary and plasma Trp, KA and the Kynurenine:Tryptophan Ratio (KTR) with higher risk incidence of T2D and cardiovascular disease⁵³. The involvement of KA in various signaling pathways has been described in several studies as an immunomodulatory effect, both in homeostatic and inflammatory conditions⁵⁴. However, it is uncertain whether changes in KA levels reflect a disease derived abnormality or a compensatory response to cellular stress and low-grade inflammation.

Further evidence of an increased feed intake was reflected in the detection of a large number of diet originating flavonoids detected in the urine, mostly as glucuronated compounds. Daidzein is a naturally occurring and frequent isoflavone found in soybeans⁵⁵ and ferulic acid is a common phytochemical identified in plant cell walls bound to arabinoxylans⁵⁶. Baicalein and its glucuronated

counterpart (baicalin) have also been putatively identified; these plant flavonoids increased in intensity with ad libitum feeding.

Very often obesity studies have reported alterations in the lipid profiles of humans and minipigs^{14, 16}. Although our LC/MS analysis platform is not specific for lipid detection, we putatively identified the presence of three lipids: 2-amino-heptanoic acid, 1-heptadecanoyl-sn-glycero-3-phosphocholine and PC(16:0/5:0(CHO)). These three compounds were down regulated with ad libitum feeding in the plasma of the minipigs. This potential decrease in phosphocholine release from adipocytes has been previously reported in a study on *in-vitro* cultured adipocytes from obese adults⁵⁷. This study finds a high number of PCs that were down regulated in the extracellular space and in plasma compared to an up-regulation observed intracellular. Imbalances in omega 3-fatty acids such as docosahexaenoic acid (DHA) were also observed, this metabolite decreasing almost three fold in the urine of ad libitum feed minipigs. DHA is a lipid synthesized from alpha-linolenic and dependent on the dietary intake of polyunsaturated fatty acids (PUFA)⁵⁸. Supplementation of DHA has shown in several studies to have a beneficial effect on obesity and metabolism imbalances and even to decrease liver fat in NAFLD cases⁵⁹⁻⁶⁰. Lower pro-inflammatory (IL-2, IL-6, TNF- α) and higher anti-inflammatory (IL-10, TGF- β) cytokines have been associated with DHA and other PUFAs intake, reducing overall levels of chronic inflammation induced by obesity^{59, 61-62}. A decrease in DHA could potentially enhance the effects of inflammation in obese individuals.

Among different amino acid pathways identified in the urinary metabolome of the minipigs, the lysine biosynthesis pathway stands out. Within this pathway, we observed a 2.36 fold increase of 2-aminoadipic acid (AAA) with ad libitum feeding. AAA is an intermediate in the breakdown of lysine and has been strongly associated with the risk of developing type 2 diabetes (T2D)⁶³ and found to be even higher in cases of renal disease or sepsis⁶⁴.

In the present study, we made use of cloned and normal phenotype Yucatan minipigs in order to elucidate whether a cloned model for nutritional studies would exhibit a lower inter-subject variability. This could potentially limit the number of animals used in further studies by placing more focus on animal welfare and on the “reduction” aspect of the 3Rs used in animal research. Irrespective of the feeding strategy, cloned and control Yucatan minipigs were hardly distinguishable based on phenotypic characteristics or biochemical parameters analyzed in serum and urine. Based on tests evaluating homogeneity in the cloned versus control group, all tested parameters showed no decrease in variation within the cloned Yucatan minipigs. PCA (data not shown) or PLS-DA models were also not conclusive concerning the reduction of inter-individual variation based on the metabolic profile. This is in agreement with several other studies on cloned conventional or minipig breeds, which were unable to show a reduction in variation between cloned animals^{13-15, 65}. However, some interesting differences were found in the metabolome of serum and liver. Significant changes were found with several AA, e.g. L-phenylalanine, methionine, and histidine, which were up-regulated in the liver of cloned minipigs but down-regulated in the serum of cloned minipigs compared to wild-type animals, suggesting a potentially increased liver AA catabolism in cloned animals. Furthermore, methylsuccinic acid was detected almost four-fold higher in the serum of cloned minipigs compared to control. This compound normally found in human biofluids has been reported to be increased in disorders of the isoleucine metabolism caused by short or medium chain acyl-CoA dehydrogenase deficiency⁶⁶⁻⁶⁷. In contrast to other metabolomics studies investigating the effect of cloning, we detected significant changes in levels of adenosine, adenosine monophosphate and inosine within the serum metabolome. Adenosine and inosine are two nucleosides constituted from ribose linked to adenine and hypoxanthine, respectively. Both these compounds are involved in the purine metabolism and are linked by the activity of adenosine deaminase (ADA) which irreversibly deaminates adenosine to inosine⁶⁸. Given that all three compounds share the same metabolic pathway, i.e. purine

metabolism, and present similar activities, we hypothesise that cloning might have unexpectedly affected this pathway. Modest differences were observed in the fold change between adenosine and inosine in clones compared to controls from which we could speculate that not all adenosine is converted to inosine. Furthermore, hypoxanthine, the primary degradation product of inosine, showed no increase in cloned animals compared to control. This points towards a disturbance in the ADA levels or activity. ADA has been associated with immune system maintenance and development, and a deficiency in this enzyme can lead to severe lymphopenia and immunodeficiency⁶⁸. Regarding the urinary metabolome, we observed no effect of cloning, whereas diet had a significant impact. Similar findings were seen in cloned conventional pigs where no differences were seen in urine using NMR metabolomics¹³. This suggests that even though changes exist in the liver and serum metabolome between cloned and control animals, kidney physiology, and detoxification functions are not impaired. On the other hand, changes in several metabolites and pathways of a cloned pig model indicate different responses to a dietary intervention. This could lead to a fallible interpretation of metabolism physiology, especially in relation to human nutrition and human metabolic diseases. How these changes occur by process of cloning remain, however, uncertain and although metabolomics provides a good wealth of information about differences between cloned and wild phenotypes, more studies are needed to elucidate the exact mechanisms.

Interpretation of the changes in several metabolic pathways described in this study should be done with care, as the LC-MS techniques employed only presents a fraction of the entire metabolome and the changes occurring upon a dietary intervention or cloning strategy. When combined in a multi-compartmental fashion, analysis of metabolic pathways with data generated from LC-MS metabolomics is on the other hand, a fast, explorative and hypothesis generating way of identifying changes across different tissues and biofluids.

CONCLUSIONS

The current study presents the impact of ad libitum feeding on the phenome and metabolome of minipigs during the development of obesity, as well as exploring the effect of cloning on the metabolism of this animal model for disease. The higher intake of calorically dense feed via ad libitum feeding resulted in a markedly elevated state of obesity with indications of metabolic imbalance and signs of metabolic syndrome. Biochemical parameters were changed and the regulation of several metabolic pathways was disturbed. The use of non-targeted metabolomics in a multicompartment approach allowed us to identify several pathways that were affected by feeding strategy and conserved across compartments. We observed similar metabolite responses indicating common biological feedback and revealing pathways that are deregulated during the development of obesity. Hepatic metabolome exploration revealed novel changes in the Yucatan minipig model with increased levels of antioxidant components as a response to ad libitum feeding. This could explain why minipigs are generally more resilient towards developing a fatty liver induced by diet alone. For the first time, this paper presents changes occurring in a cloned Yucatan minipig phenome and metabolome. Where cloned conventional pigs show greater differences, the cloned Yucatan minipig was phenotypically similar to the wild type. However, metabolomics managed to reveal minute changes within different compartments indicating that several metabolic pathways are affected. This implies that responses to a similar dietary intervention are different in cloned animals compared to wild-type. This may impair any translatability to humans when discussing cloned pigs as models for diseases of metabolism, and at the same time provide no gain in terms of reducing animal numbers in an experimental setting.

Figure 1. Body weight of Yucatan minipigs fed ad libitum (●) or restrictive (●) during a 20 weeks feeding trial.

Figure 2: Quantification of total adipose tissue (TAT) area on abdominal cross-sectional CT images.

(a) Example of ad libitum fed Yucatan minipig; anatomical structures: 1 - superficial subcutaneous

adipose tissue; 2 – deep subcutaneous adipose tissue; 3 – vertebra; 4 – kidney; 5 – perirenal adipose tissue; 6 – pararenal adipose tissue; 7 – muscular tissue; (b) TAT measured in ad libitum fed Yucatan minipig; (c) example of restricted fed Yucatan minipig; (d) TAT measured in restricted fed Yucatan minipig.

Table 1. Ingredients, Analyzed Chemical Composition of the Diet and Energy Distribution

Ingredients	g/kg
<i>White wheat flour</i>	480
<i>Wheat bran</i>	185
<i>Sugar</i>	100
<i>Soybean oil</i>	100
<i>Casein</i>	110
<i>Limestone</i>	7
<i>Salt</i>	4
<i>Dicalcium phosphate</i>	12
<i>Vitamin and mineral premix¹</i>	2
Chemical composition (g/kg DM)	g/kg
<i>Dry matter %</i>	91.38
<i>Ash</i>	38
<i>Protein (N x 6.25)</i>	207
<i>Fat</i>	138
<i>Digestible carbohydrates</i>	
<i>Available sugars</i>	122
<i>Glucose</i>	2
<i>Fructose</i>	1
<i>Sucrose</i>	119
<i>Starch</i>	375
<i>Non-digestible carbohydrates</i>	

<i>Fructan</i>	1
<i>Total NSP</i>	74
<i>Cellulose</i>	13
<i>Total NCP</i>	61
<i>Klason lignin</i>	24
<i>Total Dietary fiber</i>	98
<i>Energy (cal/g)</i>	5032.4
<i>Gross energy (MJ/kg DM)²</i>	19.24
<i>Relative energy contribution (%)</i>	<i>(%) (kJ/kg DM)</i>
<i>Protein</i>	19.7 3517
<i>Fat</i>	28.6 5113
<i>Carbohydrates</i>	47.3 8442
<i>Dietary Fibre</i>	4.4 781

¹quantities per kilogram complete diet: 4400 IU vitamin A, 1000 IU vitamin D3, 60 mg alpha-tocopherol, 2.2 mg menadione, 2.2 mg thiamine, 4 mg riboflavin, 3.3 mg pyridoxine, 11 mg D-pantothenic acid, 22 mg niacin, 0.055 mg biotin, 0.022 mg vitamin B12, 50 mg Fe as FeSO₄*7H₂O, 80 mg Zn as ZnO, 27.7 mg Mn as MnO, 20 mg Cu as CuSO₄*5H₂O, 0.2 mg I as KI, 0.3 mg Se as Na₂SeO₃; ²calculated gross energy.

Table 2. Phenotypic measurements and biochemical characteristics of ad libitum and restricted fed Yucatan minipigs and cloned versus control

Yucatan minipigs; serum and urine fasting values collected at exsanguination (mean $\pm$ SEM)

	<i>Ad libitum</i>	<i>Restricted</i>	<i>Cloned</i>	<i>CV¹</i>	<i>Control</i>	<i>CV¹</i>	<i>P values</i>			<i>F test ratio³</i>	<i>F-test P values³</i>
	n = 8	n = 9	n= 10	(%)	n = 7	(%)	Diet	Type	Diet x Type		
<i>Start Body weight (kg)</i>	28.32 $\pm$ 1.41	29.15 $\pm$ 1.29	28.05 $\pm$ 1.22	13.8	29.42 $\pm$ 1.47	13.3	0.67	0.48	0.73	3.03	0.19
<i>End Body weight (kg)</i>	80.62 $\pm$ 3.23	57.82 $\pm$ 2.97	68.74 $\pm$ 2.80	12.9	69.70 $\pm$ 3.38	12.8	<0.001	0.83	0.63	1.36	0.73
<i>TAT area (cm²)²</i>	182.1 $\pm$ 13.2	99.8 $\pm$ 12.2	124.6 $\pm$ 11.5	29.1	157.3 $\pm$ 13.9	23.3	<0.001	0.09	0.42	0.84	0.79
<i>Daily weight gain (kg)</i>	0.32 $\pm$ 0.01	0.18 $\pm$ 0.01	0.26 $\pm$ 0.01	15.5	0.24 $\pm$ 0.02	17.1	<0.001	0.31	0.27	1.33	0.76
<i>Glucose (mM)</i>	4.65 $\pm$ 0.28	4.64 $\pm$ 0.22	4.63 $\pm$ 0.26	17.5	4.67 $\pm$ 0.26	14.7	0.97	0.90	0.25	0.34	0.22
<i>Triglycerides (mM)</i>	0.41 $\pm$ 0.13	0.41 $\pm$ 0.12	0.42 $\pm$ 0.13	94.6	0.40 $\pm$ 0.13	86.9	0.97	0.89	0.45	0.62	0.58
<i>Cholesterol (mM)</i>	2.68 $\pm$ 0.19	2.17 $\pm$ 0.17	2.47 $\pm$ 0.18	23.1	2.38 $\pm$ 0.19	21.2	0.003	0.53	0.40	0.62	0.57
<i>HDL (mM)</i>	1.21 $\pm$ 0.09	1.04 $\pm$ 0.08	1.17 $\pm$ 0.09	23.9	1.08 $\pm$ 0.09	23.1	0.06	0.36	0.95	1.66	0.55
<i>LDL (mM)</i>	1.06 $\pm$ 0.06	0.72 $\pm$ 0.04	0.86 $\pm$ 0.05	19.8	0.91 $\pm$ 0.05	14.3	0.002	0.51	0.19	0.21	0.08
<i>TC/HDL ratio</i>	2.22 $\pm$ 0.06	2.10 $\pm$ 0.05	2.12 $\pm$ 0.06	8.6	2.21 $\pm$ 0.05	6.3	0.18	0.27	0.28	0.36	0.24
<i>NEFA (μeq/L)</i>	290.12 $\pm$ 46.55	261.94 $\pm$ 34.21	246.61 $\pm$ 42.66	54.7	305.45 $\pm$ 38.94	33.7	0.64	0.34	0.99	0.17	0.05
<i>Lactate (mM)</i>	2.90 $\pm$ 0.78	4.41 $\pm$ 0.58	3.77 $\pm$ 0.72	60.4	3.55 $\pm$ 0.66	49.0	0.16	0.83	0.18	0.54	0.47
<i>IGF (ng/mL)</i>	173.08 $\pm$ 18.35	180.54 $\pm$ 14.68	158.18 $\pm$ 16.82	33.6	195.44 $\pm$ 16.42	22.2	0.76	0.16	0.77	0.39	0.28
<i>Insulin (ng/mL)</i>	0.13 $\pm$ 0.05	0.13 $\pm$ 0.04	0.13 $\pm$ 0.04	111.4	0.14 $\pm$ 0.04	81.6	0.99	0.82	0.54	0.21	0.09
<i>Urine glucose (mM)</i>	0.35 $\pm$ 0.23	0.23 $\pm$ 0.04	0.32 $\pm$ 0.04	44.7	0.26 $\pm$ 0.05	56.8	0.069	0.33	0.84	0.82	0.76
<i>Urine creatinine (μM)</i>	15845 $\pm$ 2284	11452 $\pm$ 2098	13990 $\pm$ 1978	160.8	13307 $\pm$ 2389	207.7	0.18	0.83	0.93	0.58	0.45
<i>Urine total protein (mg/L)</i>	978.3 $\pm$ 444.2	524.8 $\pm$ 435.1	729.2 $\pm$ 370.8	37.8	773.9 $\pm$ 508.2	56.6	0.17	0.90	0.59	1.38	0.71

¹Coefficient of variation; ²Total adipose tissue area; ³Calculated for comparison between cloned and control Yucatan minipigs.

Figure 3 – Partial least squares – discriminant analysis (PLS-DA) score plots comparing ad libitum fed (●) with restrictive fed (◆) Yucatan minipigs in ESI⁺, ESI⁻ metabolome of serum (A, B), liver (C, D) and urine (E, F).

Figure 4 – Partial least squares – discriminant analysis (PLS-DA) score plots comparing cloned (●) with control phenotype (◆) Yucatan minipigs in ESI⁺, ESI⁻ metabolome of serum (A, B), liver (C, D) and urine (E, F).

Table 3. List of metabolites identified from metabolomics analysis discriminating ($P < 0.05$) between pigs that have been fed ad libitum or restricted high-fat/high-sucrose diets.

Metabolite	ionization mode	M/Z ratio	RT ¹ (min)	Level of identification ²	P value ³	FC ⁴	Regulation (vs restricted)	FDR ⁵ q value	KEGG/HMDB ⁶
Serum ⁷									
3-Methylindole	ESI+	132.0809	2.61	Level 1	0.021	1.2	Up	0.089	C08313
2-amino-heptanoic acid	ESI+	146.1177	2.33	Level 2	*0.0464	-1.68	Down	0.168	0034252
N-Methyl-4-pyridone-3-carboxamide	ESI+	153.0661	1.39	Level 1	0.015	1.44	Up	0.089	C05843
L-Tryptophan	ESI+	205.0973	2.61	Level 1	0.021	1.19	Up	0.089	C00078
[2M+H] L-Tryptophan	ESI+	409.1873	2.6	Level 1	0.015	1.13	Up	0.089	C00078
[M+K] 1-heptadecanoyl-sn-glycero-3-phosphocholine	ESI+	548.3719	9.95	Level 2	0.010	-1.36	Down	0.089	C04230
Indole-3-carboxaldehyde	ESI-	144.0456	4.66	Level 1	0.002	1.37	Up	0.025	C08493
p-Cresol sulphate	ESI-	187.0072	4.04	Level 1	0.051	1.62	Up	0.119	C01468
L-Tryptophan	ESI-	203.0825	2.60	Level 1	*0.0016	1.15	Up	0.025	C00078
Indolelactic acid	ESI-	204.0668	4.33	Level 1	0.026	1.39	Up	0.080	C02043
Pantothenic Acid	ESI-	218.1033	2.08	Level 1	0.009	1.32	Up	0.045	C00864
PC(16:0/5:0(CHO))	ESI-	592.3619	9.94	Level 3	0.022	-1.33	Down	0.080	N/A
[3M-H] L-Tryptophan	ESI-	611.2618	2.60	Level 1	*0.001	1.28	Up	0.025	C00078
Liver ⁸									
L-Glutamate	ESI+	148.0607	0.71	Level 1	0.037	1.19	Up	0.056	C00025
Glutathione	ESI+	308.0914	0.92	Level 1	0.001	1.12	Up	0.003	C00051
Glycochenodeoxycholate	ESI+	450.3221	7.70	Level 1	0.009	1.43	Up	0.018	C05466
[2M+H] Glutathione	ESI+	615.1756	0.92	Level 1	0.001	1.18	Up	0.003	C00051
L-Alpha-aminobutyric acid	ESI-	102.0561	0.72	Level 3	0.050	1.17	Up	0.063	C02356
L-Glutamic acid	ESI-	146.0459	0.72	Level 1	0.035	1.18	Up	0.055	C00025
D-2-Hydroxyglutaric acid	ESI-	147.0299	1.03	Level 1	0.009	1.3	Up	0.016	C01087

<i>Arabinonic acid/L-Xylonate</i>	ESI-	165.0404	0.74	Level 2	*0.0016	1.39	Up	0.007	C00878
<i>Glycerophosphoglycerol</i>	ESI-	245.0432	0.74	Level 2	*0.0025	1.19	Up	0.007	C03274
<i>Glutathione</i>	ESI-	306.0764	0.94	Level 1	0.002	1.07	Up	0.007	C00051
<i>N-Acetylneuraminic Acid</i>	ESI-	308.0986	0.75	Level 1	0.001	1.22	Up	0.007	C19910
<i>Imidazoleacetic acid ribotide</i>	ESI-	337.0441	1.13	Level 2	0.006	-1.3	Down	0.012	C04437
<i>Glycochenodeoxycholate</i>	ESI-	448.3066	7.73	Level 1	*0.0037	1.39	Up	0.009	C05466
<i>Uridine diphosphate glucose</i>	ESI-	565.0471	1.44	Level 2	0.045	-1.37	Down	0.063	C00029
<i>[2M-H] Glutathione</i>	ESI-	613.1600	0.94	Level 2	0.003	1.15	Up	0.007	C00051
Urine⁹									
<i>4-formyl Indole</i>	ESI+	146.0598	3.46	Level 2	0.028	-1.99	Down	0.039	N/A
<i>N-Methyl-4-pyridone-3-carboxamide</i>	ESI+	153.0660	1.40	Level 1	0.001	2.11	Up	0.004	C05843
<i>Picolinoylglycine</i>	ESI+	181.0611	2.77	Level 2	*0.0002	1.77	Up	0.001	N/A
<i>Kynurenic acid</i>	ESI+	190.0503	2.77	Level 1	0.0002	2.16	Up	0.001	C01717
<i>[M+Na] Hippuric acid</i>	ESI+	202.1079	3.34	Level 1	*< 0.0001	2.9	Up	0.001	C01586
<i>Hydroxyphenylacetyl glycine</i>	ESI+	210.0765	2.38	Level 1	0.009	1.83	Up	0.019	C05596
<i>7-Oxo-11-dodecenoic acid</i>	ESI+	213.1493	5.23	Level 2	*0.0002	2.28	Up	0.001	N/A
<i>Pantothenic Acid</i>	ESI+	220.1184	2.08	Level 1	0.001	2.09	Up	0.004	C00864
<i>N-Acetyl-DL-tryptophan</i>	ESI+	247.1079	4.30	Level 2	0.002	1.72	Up	0.004	0013713
<i>4-Hydroxy-3-methoxy-cinnamoylglycine</i>	ESI+	252.0873	3.46	Level 2	0.010	2.54	Up	0.020	C02564
<i>Tetradecanedioic acid</i>	ESI+	259.1911	5.05	Level 2	0.006	1.53	Up	0.015	C11002
<i>3-Methylglutaryl carnitine</i>	ESI+	290.1603	2.00	Level 2	0.020	1.67	Up	0.032	N/A
<i>Dihydroxy-1H-indole glucuronide</i>	ESI+	326.0874	2.49	Level 2	0.018	2.1	Up	0.031	C04185
<i>p-Acetamidophenyl glucuronide</i>	ESI+	328.1033	3.37	Level 2	0.025	-1.97	Down	0.036	N/A
<i>Docosaheptaenoic acid</i>	ESI+	329.2471	6.55	Level 2	0.025	-2.88	Down	0.036	C06429
<i>3-Indole carboxylic acid glucuronide</i>	ESI+	338.0876	1.65	Level 2	0.014	-2.11	Down	0.028	C19837
<i>6-Hydroxy-5-methoxyindole glucuronide</i>	ESI+	340.1034	1.93	Level 2	0.024	-1.66	Down	0.036	C03033
<i>HpODE (1)</i>	ESI+	389.1814	5.23	Level 3	*0.0006	2.38	Up	0.004	C04717
<i>HpODE (2)</i>	ESI+	424.2184	4.42	Level 3	0.001	2.57	Up	0.004	C04717
<i>3-Hydroxy-5, 8-tetradecadienecarnitine-glucuronate</i>	ESI+	560.3075	4.97	Level 2	0.054	1.9	Up	0.073	N/A

<i>Leukoaminochrome</i>	ESI-	150.0563	4.02	Level 2	*0.0006	2.3	Up	0.002	C17756
<i>DL-2-Aminoadipic acid</i>	ESI-	160.0616	1.46	Level 2	0.001	2.36	Up	0.002	C00956
<i>Hydroxyphenyllactic acid</i>	ESI-	181.0508	2.66	Level 1	*0.0152	1.4	U	0.029	C03672
<i>p-Cresol Sulphate</i>	ESI-	187.0073	3.93	Level 1	0.014	2.56	Up	0.029	C01468
<i>Kynurenic acid</i>	ESI-	188.0355	2.78	Level 1	0.001	2.17	Up	0.002	C01717
<i>Pyrocatechol sulphate</i>	ESI-	188.9865	2.72	Level 2	0.001	2.27	Up	0.002	N/A
<i>Hydroxyhippuric acid</i>	ESI-	194.0462	4.02	Level 2	*0.0006	2.37	Up	0.002	N/A
<i>Xanthurenic acid</i>	ESI-	204.0305	2.54	Level 1	< 0.0001	2.33	Up	0.001	C02470
<i>Hydroxyphenylacetyl glycine</i>	ESI-	208.0617	2.38	Level 1	0.030	1.47	Up	0.048	C05596
<i>Pantothenic Acid</i>	ESI-	218.1036	2.07	Level 1	0.0001	1.85	Up	0.001	C00864
<i>N-Acetyl-L-tyrosine</i>	ESI-	222.0775	2.73	Level 1	*0.001	1.53	Up	0.003	C01657
<i>N-Phenylacetyl aspartic acid</i>	ESI-	250.0724	3.45	Level 2	*0.0206	1.95	Up	0.036	N/A
<i>Adrenochrome sulphate</i>	ESI-	258.0079	2.12	Level 2	0.047	-1.67	Down	0.063	N/A
<i>[M-176] Baicalein</i>	ESI-	269.0462	4.17	Level 2	*< 0.0001	1.93	Up	0.001	C10025
<i>8,12-Epoxy-4(15),7,11-eudesmatrien-1-one sulphate</i>	ESI-	309.1353	5.08	Level 2	0.039	-1.86	Down	0.058	N/A
<i>3-Methyloxindole glucuronide (3)</i>	ESI-	322.0938	4.21	Level 2	0.010	-2.94	Down	0.023	C02366
<i>3-Methyloxindole glucuronide (2)</i>	ESI-	322.0939	3.1	Level 2	0.004	-2.37	Down	0.010	C02366
<i>3-Methyloxindole glucuronide</i>	ESI-	322.0939	4.72	Level 2	0.040	-2.03	Down	0.058	C02366
<i>Dihydroxy-1H-indole glucuronide I</i>	ESI-	324.0727	2.45	Level 2	0.044	1.76	Up	0.061	C04185
<i>Leukoaminochrome glucuronide</i>	ESI-	326.0883	2.78	Level 2	0.037	1.55	Up	0.056	C17756
<i>Leukoaminochrome glucuronide (2)</i>	ESI-	326.0886	3.36	Level 2	0.030	-1.97	Down	0.048	C17756
<i>5-Hydroxy-6-methoxyindole glucuronide</i>	ESI-	338.0885	2.88	Level 2	0.015	-1.82	Down	0.029	C03033
<i>Adrenochrome glucuronide</i>	ESI-	354.0830	1.47	Level 2	0.015	-2	Down	0.029	N/A
<i>Ferulic acid 4-O-glucuronide</i>	ESI-	369.0832	2.69	Level 3	0.001	2.85	Up	0.002	N/A
<i>3,7-Dimethyl-3-octene-1,2,6,7-tetrol glucuronide</i>	ESI-	379.1615	3.91	Level 2	0.004	1.63	Up	0.010	N/A
<i>7-Oxo-11-dodecenoic acid glucuronide</i>	ESI-	387.1669	5.22	Level 2	*0.0003	1.86	Up	0.002	N/A
<i>Dodecanedioic acid glucuronide</i>	ESI-	405.1772	4.41	Level 2	0.005	1.83	Up	0.011	N/A
<i>Daidzein 4'-O-glucuronide</i>	ESI-	429.0833	3.91	Level 3	*0.0002	2.08	Up	0.001	N/A
<i>Daidzein 7-O-glucuronide</i>	ESI-	429.0833	3.55	Level 2	*0.0003	1.9	Up	0.002	N/A

<i>Tetradecanedioic acid glucuronide</i>	ESI-	433.2087	5.39	Level 2	0.043	1.41	Up	0.061	C11002
<i>Baicalin, Baicalein 7-glucuronide</i>	ESI-	445.0785	4.17	Level 2	* < 0.0001	1.95	Up	0.001	C10025
<i>[M+H] Baicalin, Baicalein 7-glucuronide</i>	ESI-	446.0819	4.17	Level 2	* < 0.0001	1.96	Up	0.001	C10025
<i>3-Hydroxytetradecanedioic acid glucuronide</i>	ESI-	449.2034	4.03	Level 2	0.031	1.59	Up	0.049	N/A
<i>Hexadecanedioic acid glucuronide (2)</i>	ESI-	461.2400	6.26	Level 2	*0.0152	1.7	Up	0.029	C19615
<i>Cortolone-3-glucuronide</i>	ESI-	541.2656	4.77	Level 2	0.027	1.67	Up	0.045	N/A
<i>PG(22:4(7Z,10Z,13Z,16Z)/0:0)</i>	ESI-	559.3032	6.38	Level 3	*0.0006	4.66	Up	0.002	N/A

¹RT – retention time; ²identified metabolites (level 1), putatively annotated compounds (level 2), putatively characterised compound classes (level 3), unknown compounds (level 4); ³ values calculated using Student T-test; values with * are calculated by the Wilcoxon Mann Whitney test; ⁴FC – fold change (ad libitum vs. restricted), positive values reflect an upregulation in the ad libitum feeding strategy; ⁵ FDR – false discovery rate; ⁶ N/A – not available; ⁷N_{ad libitum} (ESI⁺) = 7, N_{ad libitum} (ESI⁻) = 8, N_{restricted} = 9; ⁸N_{ad libitum} = 8, N_{restricted} = 8; ⁹N_{ad libitum} = 7, N_{restricted} = 9;

Table 4. List of metabolites identified from metabolomics analysis discriminating ($P < 0.05$) between cloned and wild type Yucatan minipigs

Metabolite	ionization mode	M/Z ratio	RT ¹ (min)	Level of identification ²	P value ³	FC ⁴	Regulation (vs wild-type)	FDR ⁵ q value	KEGG/HMDB ⁶
Serum ⁷									
<i>Histamine</i>	ESI+	112.0872	0.56	Level 1	0.037	1.56	Up	0.071	C00388
<i>Creatine</i>	ESI+	132.0770	0.68	Level 1	*0.014	-1.50	Down	0.051	C00300
<i>L-Methionine</i>	ESI+	150.0587	0.91	Level 1	*0.033	-1.21	Down	0.069	C00073
<i>[M+Na] Creatine</i>	ESI+	154.0587	0.69	Level 1	0.016	-1.72	Down	0.053	C00300
<i>L-Histidine</i>	ESI+	156.0771	0.56	Level 3	0.011	-1.22	Down	0.043	C00135
<i>[M-NH4] Serotonin</i>	ESI+	160.0758	1.73	Level 1	0.002	1.82	Up	0.031	C00780
<i>[M-NH4+H] Serotonin</i>	ESI+	161.0791	1.73	Level 1	0.004	1.74	Up	0.031	C00780
<i>L-Phenylalanine</i>	ESI+	166.0864	1.94	Level 1	0.005	-1.14	Down	0.031	C00079
<i>Serotonin</i>	ESI+	177.1026	1.73	Level 1	0.003	1.63	Up	0.031	C00780
<i>Pyridoxine</i>	ESI+	192.0610	0.79	Level 3	*0.025	-1.15	Down	0.068	C00314
<i>Acetylcarnitine</i>	ESI+	204.1235	0.86	Level 1	0.026	-1.25	Down	0.068	C02571
<i>Prolyl-Cysteine; Cysteinyl-Proline</i>	ESI+	219.0828	0.61	Level 3	0.004	-1.53	Down	0.031	N/A
<i>Adenosine</i>	ESI+	268.1045	1.11	Level 1	0.006	2.07	Up	0.034	C00212
<i>[M+Na] Inosine</i>	ESI+	291.0705	1.29	Level 1	0.022	1.33	Up	0.068	C00294
<i>TrHA / Docosahexaenoic Acid methyl ester</i>	ESI+	343.2640	7.73	Level 2	0.025	1.31	Up	0.068	N/A
<i>Adenosine monophosphate</i>	ESI+	348.0709	0.73	Level 1	0.041	1.97	Up	0.074	C00020
<i>[M+Na] Adenosine monophosphate</i>	ESI+	370.0529	0.74	Level 1	0.038	2.02	Up	0.071	C00020
<i>Sphingosine-1-phosphate</i>	ESI+	380.2569	8.04	Level 2	0.034	1.34	Up	0.069	C06124
<i>[M+2Na] Adenosine 5'-phosphate</i>	ESI+	392.0346	0.74	Level 1	0.029	2.05	Up	0.069	C00020
<i>[2M+Na+H] Inosine</i>	ESI+	560.1546	1.29	Level 1	0.007	2.02	Up	0.034	C00294
<i>Methylsuccinic acid</i>	ESI-	131.0351	2.29	Level 2	0.000	3.96	Up	0.009	C08645
<i>3-Cresotinic acid</i>	ESI-	151.0402	5.55	Level 2	0.022	-1.68	Down	0.071	C14088
<i>[M+Cl] Glucose</i>	ESI-	215.0327	0.75	Level 1	*0.007	2.31	Up	0.053	C00031

<i>Pantothenic Acid</i>	ESI-	218.1033	2.08	Level 1	0.026	-1.27	Down	0.071	C00864
<i>2-Hydroxymyristic Acid</i>	ESI-	243.1967	8.04	Level 2	*0.014	1.36	Up	0.071	0002261
<i>Inosine</i>	ESI-	267.0732	1.29	Level 1	0.045	1.41	Up	0.100	C00294
<i>[M+Cl] Adenosine</i>	ESI-	302.0657	1.12	Level 1	0.009	1.95	Up	0.059	C00212
<i>Adenosine monophosphate</i>	ESI-	346.0556	0.73	Level 1	0.025	2.12	Up	0.071	C00020
<i>Norchenodeoxycholic acid</i>	ESI-	377.2698	7.73	Level 3	0.024	1.31	Up	0.071	N/A
<i>Sphingosine-1-phosphate</i>	ESI-	378.2415	8.03	Level 2	0.042	1.33	Up	0.098	C06124
<i>[M+CH3COOH] Adenosine monophosphate</i>	ESI-	404.0141	0.73	Level 1	0.018	2.16	Up	0.071	C00020
<i>[M+FA] Norchenodeoxycholic acid</i>	ESI-	423.2751	7.73	Level 3	0.022	1.33	Up	0.071	N/A
Liver⁷									
<i>2-Aminoethylphosphonic acid</i>	ESI+	126.0221	0.74	Level 2	0.015	-1.39	Down	0.030	C03557
<i>3-Dehydroxycarnitine</i>	ESI+	146.1179	2.68	Level 2	0.037	1.19	Up	0.043	C05543
<i>L-Lysine</i>	ESI+	147.1131	0.60	Level 1	0.000	1.13	Up	0.011	C00047
<i>L-Methionine</i>	ESI+	150.0586	0.94	Level 1	< 0.0001	1.2	Up	0.000	C00073
<i>L-Phenylalanine</i>	ESI+	166.0867	2.11	Level 1	0.000	1.22	Up	0.002	C00079
<i>Guanidinosuccinic acid</i>	ESI+	176.067	0.71	Level 2	*0.019	1.23	Up	0.032	C03139
<i>3-amino-2-naphthoic acid</i>	ESI+	188.071	2.87	Level 2	0.003	1.08	Up	0.012	N/A
<i>L-Tryptophan</i>	ESI+	205.0975	2.87	Level 1	0.009	1.08	Up	0.030	C00078
<i>Glycerophosphocholine</i>	ESI+	258.1105	0.69	Level 1	0.046	1.2	Up	0.050	C00670
<i>[M+K] Glycerophosphocholine</i>	ESI+	296.0662	0.65	Level 1	0.031	1.22	Up	0.042	C00670
<i>Glycolic acid</i>	ESI-	75.0089	0.74	Level 3	0.005	1.16	Up	0.021	C03547
<i>L-Threonine</i>	ESI-	118.051	0.69	Level 1	0.032	1.08	Up	0.039	C00188
<i>Taurine</i>	ESI-	124.0074	0.72	Level 1	0.011	-1.38	Down	0.030	C00245
<i>Leucine</i>	ESI-	130.0873	1.27	Level 1	< 0.0001	1.17	Up	0.000	C00123
<i>L-Methionine</i>	ESI-	148.0437	0.98	Level 1	0.036	1.27	Up	0.039	C00073
<i>L-Histidine</i>	ESI-	154.0622	0.63	Level 1	0.026	1.06	Up	0.036	C00135
<i>2-Hydroxyadipic acid</i>	ESI-	161.0461	0.7	Level 3	0.001	-1.17	Down	0.009	C02360
<i>DL-Phenylalanine</i>	ESI-	164.0717	2.15	Level 1	0.001	1.24	Up	0.006	C00079
<i>Guanidinosuccinic acid</i>	ESI-	174.052	0.74	Level 2	0.015	1.44	Up	0.031	C03139

<i>D-Glucose</i>	ESI-	179.0561	0.7	Level 1	0.006	-1.15	Down	0.023	C00031
<i>D-Glucuronic acid</i>	ESI-	193.0353	0.74	Level 1	0.026	1.18	Up	0.036	C00191
<i>Gluconic acid</i>	ESI-	195.051	0.74	Level 1	0.018	1.12	Up	0.035	C00257
<i>L-Tryptophan</i>	ESI-	203.0827	2.91	Level 1	0.007	1.08	Up	0.023	C00078
<i>sn-glycero-3-Phosphoethanolamine</i>	ESI-	215.0328	0.72	Level 2	0.012	-1.13	Down	0.030	59660
<i>Glycyl-Tyrosine</i>	ESI-	237.0881	1.84	Level 3	0.020	1.23	Up	0.035	n/a
<i>D-Glucose 6-phosphate</i>	ESI-	259.0224	0.75	Level 1	0.025	-1.16	Down	0.036	C00092
<i>Dephospho-CoA</i>	ESI-	686.141	2.53	Level 2	0.013	1.23	Up	0.030	C00882

¹RT – retention time; ²identified metabolites (level 1), putatively annotated compounds (level 2), putatively characterised compound classes (level 3); ³ values calculated using Student T-test; values with * are calculated by the Wilcoxon Mann Whitney test; ⁴FC – fold change (clone vs control), positive values reflect an upregulation in the cloned pig group; ⁵ FDR – false discovery rate; ⁶ N/A – not available; ⁷N_{cloned} = 10, N_{control} = 7.

Figure 5. Metabolome view of pathway analysis generated with MetaboAnalyst 4.0 based on metabolites from ad libitum versus restrictive fed Yucatan minipigs in different compartments. Node radius size and importance (X-axis) reflects the pathway impact values calculated using betweenness centrality which takes into consideration the global network structure and measures the number of shortest paths going through metabolites within the node. Node color and direction (Y-axis), on the other hand, is based on the calculated P-value of the enrichment analysis (low p-values describing significant changes of the metabolites involved in that metabolic pathway). (A) Metabolic pathways affected in serum. (B) Metabolic pathways affected in the liver. (C) Metabolic pathways affected in urine.

Figure 6. Metabolome view of pathway analysis generated with MetaboAnalyst 4.0 based on metabolites from cloned versus control Yucatan minipigs in different compartments. Node radius size and importance (X-axis) reflects the pathway impact values calculated using betweenness centrality which takes into consideration the global network structure and measures the number of shortest paths going through metabolites within the node. Node color and direction (Y-axis), on the other hand, is based on the calculated P-value of the enrichment analysis (low p-values describing significant changes of the metabolites involved in that metabolic pathway). (A) Metabolic pathways affected in serum. (B) Metabolic pathways affected in the liver.

ASSOCIATED CONTENT

Supporting Information.

Figure S1. Position of CT abdominal cross-section used in quantifying of total abdominal fat area; marked by red dotted line

Table S1. Partial Least Squares Discriminant Analysis models information

Table S2. List of metabolic features detected from metabolomics analysis of serum, liver, and urine in pigs fed ad libitum (n = 8) or restricted (n = 9) a Western Style Diet.

Table S3. List of metabolic features detected from metabolomics analysis of serum and liver in cloned (n = 10) and control (n = 7) Yucatan minipigs.

Table S4. Metabolic pathway analysis using MetaboAnalyst 4.0: ad libitum versus restrictive fed Yucatan minipigs in serum, liver and urine metabolomics.

Table S5. Metabolic pathway analysis using MetaboAnalyst 4.0: cloned versus wild type Yucatan minipigs in serum and liver metabolomics.

AUTHOR INFORMATION

Author Contributions

Data analysis and drafting of the manuscript was done by M.V.C; animal cloning was performed by H.C.; experimental setup and animal sampling was done by M.S.H., J.S.; the manuscript was written through contributions of all authors: K.E.B.K, H.C., S.P, J.S., M.S.H.; All authors have given approval to the final version of the manuscript.

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MASS SPECTROMETRY DATA

The mass spectrometry data utilized in this manuscript have been deposited to the Chorus public repository (<https://chorusproject.org/>). Data can be retrieved via: <https://chorusproject.org/anonymous/download/experiment/-1301551505608884422> (Project name: Yucatan Obesity Metabolomics 2018)

SYNOPSIS

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