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Liquid Chromatography–Mass Spectrometry Based Metabolomics Study of Cloned versus Normal Pigs Fed Either Restricted or Ad Libitum High-Energy Diets

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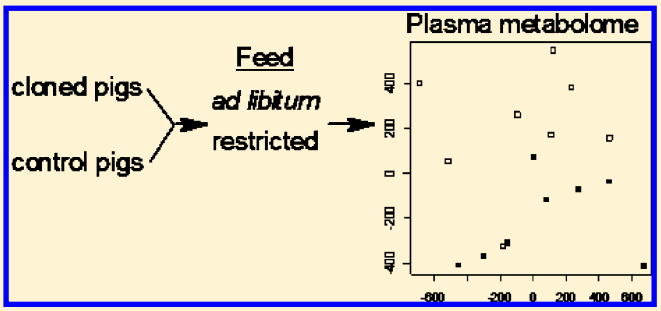
ABSTRACT: Genetically identical cloned pigs should in principle eliminate biological variation and provide more pronounced effects when subjected to, e.g., dietary interventions, but little is known about how phenotype and phenotypic variation is affected by cloning. Therefore, an investigation of the metabolome of cloned pigs compared to normal control pigs was performed to elucidate the variation and possible differences in the metabolic phenotypes during a dietary intervention. A total of 19 control pigs and 17 cloned pigs were given the same high-energy dense diet either ad libitum or in a restricted manner (60% of ad libitum) for ∼6 months, and plasma was subjected to liquid chromatography–mass spectrometry nontargeted metabolomics and biochemical analyses. Low systemic levels of IGF-1 could indicate altered growth conditions and energy metabolism in cloned pigs. In response to ad libitum feeding, clones had a decreased energy intake and lower weight gain compared to controls, and plasma lipid profiles were changed accordingly. Elevated lactate and decreased creatine levels implied an increased anaerobic metabolism in ad libitum fed clones. Less interindividual variation between cloned pigs was however not established, suggesting a strong role for epigenetics and/or the gut microbiota to develop variation.



KEYWORDS: *cloned pigs, high-energy dense diet, interindividual variation, metabolomics, LC–MS*

INTRODUCTION

Obesity is a condition in which surplus body fat accumulates to an extent that might affect one's health.[1] It is associated with several comorbidities including the metabolic syndrome that increases the risk of developing type 2 diabetes mellitus and cardiovascular diseases.[2,3] The metabolism of obesity and its related dysfunctions together with possible treatment or prevention strategies are being investigated intensively due to the epidemic proportions of the condition. To better understand the underlying biochemical mechanisms, genomics, proteomics, and metabolomics have gained considerable interest. Metabolomics is the study of the metabolome, i.e., the quantification and identification of all metabolites in a biological system at a given time point.[4] Liquid chromatography–mass spectrometry (LC–MS) is increasingly applied to metabolomics-driven research, as it is a very useful technique to detect and measure a variety of small biological molecules.[5,6] LC–MS is furthermore highly sensitive and provide spectral information that contributes to the identification of metabolites.[6] Nontargeted LC–MS metabolomics has been proven in several recent human and animal studies to elucidate biomarkers of dietary and cultural influences related to obesity and the metabolic syndrome. These are, e.g., high-fat diet induced obesity,[7] impaired glucose tolerance and reduced insulin sensitivity,[8,9] type 2 diabetes,[10] atherosclerosis,[11] dietary metabolic effects of an oral glucose tolerance test,[12] and dietary carbohydrate interventions.[13,14]

Rodent models have been widely used in obesity and metabolic syndrome research including metabolomics, but metabolic and physiological differences between humans and rodents complicate the translation into effective prevention or intervention therapies.[15,16] The pig is an attractive model for nutritional intervention studies since it displays nutritional and digestive aspects comparable with those of humans.[15,17] Furthermore, use of genetically identical, cloned pigs could be advantageous, provided they exhibit less interindividual variation compared with normal litter mates. Theoretically, this would allow a more sensitive detection of the effects of dietary interventions and require fewer experimental animals. Clones are expected to be metabolically identical to the controls as no genetic changes have been introduced during the cloning process. However, a recent NMR-based study of biofluids from cloned pigs compared to normal controls revealed differences in the metabolome.[18]

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The aim of this study was to investigate differences in the metabolome of cloned pigs compared to normal control pigs when fed ad libitum or diet restricted with a high-energy dense diet by conventional biochemical parameters as well as LC−MS metabolomics. Our focus was to examine if cloned pigs express less interindividual variation compared with normal litter mates and if they were suitable for nutritional intervention studies in obesity and metabolic syndrome research. Plasma from clones and controls was used for obtaining metabolic fingerprints.

EXPERIMENTAL SECTION

Animals and Sampling

Cloning was performed as previously described by Kragh et al.[19] with donor cells from cultured ear fibroblasts of a 65% Danish Landrace × 35% Yorkshire sow. Controls were normal litters (75% Landrace × 25% Yorkshire) obtained by standard artificial insemination. A total of 17 female clones from 5 surrogate sows and 19 female controls from 6 litters were acquired. All pigs were reared in the experimental stables at Aarhus University (AU Foulum, Tjele, Denmark).

The pigs were weaned at 28 days of age and fed a standard diet until 3 months of age, where the clones weighed 61.2 kg (±0.3 SEM) and controls 61.2 kg (±0.9 SEM). The next 6 months they all received the same wheat-based high-energy density diet (Table 1). Nine clones and 10 controls were fed ad libitum, whereas the remaining 8 clones and 9 controls were restricted to get 60% of total feed consumed by the ad libitum fed pigs. The diet still provided sufficient amounts of all micronutrients. The weight of the pigs was monitored weekly. Blood plasma samples were collected from the jugular vein in heparinised Vacutainers after an overnight fast at 8 and 1/2 months of age; the blood samples were stored on ice and centrifuged within 1.5 h at 1800*g* for 10 min at 4 °C.

The animal experiment was conducted according to protocols approved by the Danish Animal Experiments Inspectorate and complied with the guidelines of the Danish Ministry and Justice concerning animal experimentation and care of animals under study.

Table 1. Ingredients and Chemical Composition of the Experimental Diet

ingredient, g/kg, as-is			
white wheat flour	480		
wheat bran	185		
sugar	100		
soy oil	100		
casein	110		
limestone	7		
salt	4		
dicalcium phosphate	12		
vitamin and mineral premix[a]	2		
chemical composition, % of dry matter		**energy distribution[b], %**	
dry matter, %	91.3		
ash	3.9		
crude protein	20.7	19	
crude fat	13.4	27	
carbohydrates	57.0	53	
sugars	12.1	11	
starch	37.4	35	
nonstarch polysaccharides (NSP)	7.5	7	
dietary fiber (NSP + lignin)	10.1		
gross energy, MJ/kg DM	20.9		
metabolizable energy,[b] MJ/kg DM	18.5		

[a]Provided are the quantities of vitamins and minerals per kilogram of complete diet: 4400 IU of vitamin A, 1000 IU of vitamin D_3, 60 mg of alpha-tocopherol, 2.2 mg of menadione, 2.2 mg of thiamin, 4 mg of riboflavin, 3.3 mg of pyridoxine, 11 mg of D-pantothenic acid, 22 mg of niacin, 0.055 mg of biotin, 0.022 mg of vitamin B_{12}, 50 mg of Fe as $FeSO_4 \cdot 7H_2O$, 80 mg of Zn as ZnO, 27.7 mg of Mn as MnO, 20 mg of Cu as $CuSO_4 \cdot 5H_2O$, 0.2 mg of I as KI, and 0.3 mg of Se as Na_2SeO_3. [b]Calculated.

Analytical Methods

Chemical analyses of the diet were performed with classical methods as described in the paper by Bach Knudsen et al.[20]

Plasma glucose, nonesterified fatty acids (NEFA), lactate, triglycerides, total cholesterol, low-density lipoproteins (LDL), and high-density lipoproteins (HDL) cholesterol were analyzed using an ADVIA 1650 Chemistry System (Siemens Healthcare Diagnostics, Deerfield, IL, U.S.A.). Plasma glucose was determined using glucose hexokinase II and enzymatic colorimetric determination.[21] NEFA were determined using the Wako NEFA C ACS-ACOD assay method (Wako Chemicals GmbH, Neuss, Germany), and lactate was measured according to the method described by Noll.[22] Triglycerides were analyzed by determination of glycerol after hydrolysis of mono-, di-, and triglycerides according to Fossati and Prencipe.[23] Total cholesterol was determined by the enzymatic hydrolysis of cholesterol esters, oxidation of free cholesterol, and concomitant liberation of hydrogen peroxide.[24] LDL and HDL cholesterol were determined after selective isolation and liberation according to the principles of Okada et al.[25] and Izawa et al.,[26] respectively. Plasma insulin was analyzed by time-resolved fluoroimmunoassay as described by Løvendahl and Purup,[27] and insulin-like growth factor 1 (IGF-1) was measured by an EIA kit from R&D Systems, Inc. (Minneapolis, MN, U.S.A.), according to manufacturer's instructions.

Standards and Chemicals for Metabolomics Analysis

HPLC-grade acetonitrile and methanol were purchased from VWR (West Chester, Pennsylvania, U.S.A.). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Internal and external standards were obtained from Sigma-Aldrich, Avanti polar lipids (Alabaster, Alabama, U.S.A.), and Merck (Merck, Darmstadt, Germany).

Sample Preparation of Blood Plasma for Metabolomics Analysis

Plasma samples of 200 μL were mixed with 20 μL of 100 $\mu g \cdot mL^{-1}$ internal standard mix of glycocholic acid (Glycine-1 ^{13}C) and lysophosphatidylcholine (17:0). The samples were added to 600 μL ice-cold methanol, immediately vortexed, and incubated at 4 °C for 20 min for deproteinization. The supernatants were collected after centrifugation at 13 200*g* for 10 min and evaporated to dryness under a stream of N_2. The resulting dry residues were dissolved in 200 μL of water/acetonitrile/formic acid (95:5:0.1 v/v/v) and filtered through 0.22 μm Corning Spin-X centrifuge tube filters at 10 621*g* for 10 min. Samples were stored at −20 °C.

Reversed-Phase High Performance Liquid Chromatography Mass Spectrometry

For the LC−MS analysis, an Ultimate 3000 (Dionex, Sunnyvale, CA, U.S.A.) HPLC system was coupled to a MicrOTOF-Q II mass spectrometer (Bruker Daltonik GmbH, Germany) operating in ESI^+ and ESI^- ion modes. The scan

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Table 2. Biochemical Characteristics of Clone and Control Pigs at 8 and 1/2 Months of Age; Fasting Values (Mean ± SEM)[a]

	ad libitum		diet restricted		P values[b]		
	clone (n = 9)	control (n = 10)	clone (n = 8)	control (n = 9)	type[c]	diet[d]	type × diet
feed intake[e] (kg DM/d)	2.48 ± 0.13b	2.97 ± 0.16a	1.37c	1.37c	<0.001	<0.001	0.18
energy intake[f] (MJ/d)	45.8 ± 2.2b	54.8 ± 2.7a	25.3c	25.3c	<0.001	<0.001	0.18
daily weight gain[g] (kg)	0.89 ± 0.04b	1.11 ± 0.03a	0.45 ± 0.03c	0.37 ± 0.01d	<0.001	<0.001	<0.001
glucose (mM)	5.08 ± 0.28a	4.39 ± 0.16b	4.61 ± 0.10b	4.50 ± 0.09b	<0.001	0.76	0.59
cholesterol (mM)	2.29 ± 0.05b	3.18 ± 0.14a	2.01 ± 0.05c	2.06 ± 0.07bc	<0.001	0.09	0.01
HDL (mM)	0.78 ± 0.06b	1.07 ± 0.05a	0.81 ± 0.02b	0.90 ± 0.05b	<0.001	0.28	0.06
LDL (mM)	1.24 ± 0.04b	1.74 ± 0.09a	0.83 ± 0.04c	0.82 ± 0.04c	<0.001	0.01	0.05
LDL/HDL	1.70 ± 0.62a	1.66 ± 0.36a	1.03 ± 0.12b	0.94 ± 0.23b	0.66	<0.001	0.94
triglyceride (mM)	0.43 ± 0.03	0.40 ± 0.05	0.26 ± 0.02	0.27 ± 0.03	0.54	0.13	0.85
NEFA[h] (μ ekv./L)	169.7 ± 47.1b	162.8 ± 32.7b	304.2 ± 33.2a	300.1 ± 22.0a	0.84	0.02	0.91
lactate (mM)	6.23 ± 0.79a	2.52 ± 0.44b	2.08 ± 0.48b	1.42 ± 0.31b	<0.001	0.10	0.05
insulin (ng/mL)	0.14 ± 0.07	0.17 ± 0.03	0.03 ± 0.01	0.07 ± 0.02	0.71	0.15	0.87
IGF-1[I] (ng/mL)	113.4 ± 13.2b	200.6 ± 19.9a	55.2 ± 9.2c	123.8 ± 14.3b	<0.001	0.11	0.82

[a]Means with the same letter are not significantly different. [b]Linear mixed model. [c]Type refers to clones and controls. [d]Diet refers to ad libitum feeding or diet restriction. [e]The pigs were given 1.5 kg of feed when diet restricted of which they ate all. [f]Calculated from metabolizable energy in the diet (Table 1) and feed intake. [g]Estimated by linear regression. [h]NEFA: nonesterified fatty acids. [I]IGF-1: insulin-like growth factor 1.

range was from 50 to 1000 m/z. The capillary voltage was 4500 V both in the positive and negative mode, the nebulizing gas pressure was 1.8 bar, and the drying gas flow and temperature were 8.0 L·min^{-1} and 200 °C, respectively. Lithium formate at a concentration of 5 mM in water/isopropanol/formic acid (50:50:0.2 v/v/v) was employed as an external calibrant. It was infused directly into the mass spectrometer before each sample injection. For the following metabolite identification, MS/MS analysis was performed using argon as collision gas, and collisions were carried out at energies from 6 to 60 eV.

The chromatographic separation was performed on a Discovery HS C_{18} column (15 cm × 2.1 mm, 3 μm) used together with a C_{18} precolumn. The column was held at 30 °C. The injection volume was 5 μL. Mobile phase A consisted of water/formic acid (100:0.1 v/v), and mobile phase B consisted of acetonitrile/formic acid (100:0.1 v/v). The column was equilibrated for 5 min before the gradient started at 10% B for 1 min and then linearly increased to 100% B within 20 min. The mobile phase was kept at isocratic conditions (100% B) for 5 min and then turned back to 10% B. Total analysis time was 31 min, and the flow rate was 200 μL·min^{-1}.

A sample of pooled plasma from pigs was reinjected after each 6 samples for quality control of the LC−MS run. All samples were first analyzed in ESI$^+$ ion mode, and the same samples were reanalyzed in ESI$^-$ ion mode. A homemade metabolomics standard of 47 different physiological compounds was analyzed together with the plasma samples for quick identification of common metabolites.

Data Analysis

LC−MS Data Pretreatment. Acquired mass spectra were calibrated and converted into mzXML file format to carry out LC−MS data analysis. MZmine 2.2[28] was employed for preprocessing of data using a centroid peak detector algorithm and the RANSAC aligner.

Multivariate Analysis. Multivariate analysis was performed on the output table from MZmine using the open-source program R (version 2.10.1) (R Development Core Team 2009).[29] After pareto scaling, principal components analysis (PCA) was carried out using the ade4[30,31] and MASS[32] packages. Outliers were removed based on 95% confidence intervals and plots of residual variance versus Hotelling's T^2. Linear dicriminant analysis (LDA) was applied on PCA scores to test the class prediction probabilities of the PCA models, and the prediction accuracy was measured using leave-one-out cross-validation. PC1 and PC2 scores were used for the LDA models as these principal components seemed to reflect the main variation and separation in data.

Statistical Analysis. Statistical analysis was performed using the open source program R.[29] For metabolomics data, differences in the intensities of metabolite ions between clone and control pigs were tested by Welch's two-sample t test. In order to correct for multiple comparisons, false discovery rate (FDR) q values were calculated,[33] with significance threshold set at $q < 0.25$.[34] Biochemical data were analyzed as a linear mixed model using the lme4[35] package:

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

where Y_{ij} is the dependent variable; μ is the overall mean; α_i is the pig effect (i = clone or control); β_j is the diet effects (j = ad libitum or diet restricted); $\alpha\beta_{ij}$ is the interaction term, and ε_{ij} is the residual error component. The statistical model included date of sampling as a random effect. ANOVA and Fisher's least significant difference was used for comparing groups using the agricolae package.[36] Daily weight gain was estimated by linear regression. Statistical significance was set as $P < 0.05$ and $0.05 \leq P < 0.10$ as trends.

Metabolite Identification. Accurate mass and mass spectrometric fragmentation patterns were used to search databases for metabolite identification. The following databases were used: KEGG (http://kegg.com), PubChem compound database (http://pubchem.ncbi.nlm.nih.gov), METLIN (http://metlin.scrips.edu), and the human metabolome database (http://www.hmdb.ca). Available standards were used for the confirmation of the identity of compounds.

RESULTS

Animal Characteristics

Daily weight gain (Table 2) showed the clones to gain more weight when fed restrictively, even though they had the same amount of feed intake as the controls. However, when subjected to ad libitum feeding, the clones had a lower energy

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intake (45.8 ± 2.2 MJ/d) than the controls (54.8 ± 2.7 MJ/d) and therefore gained less weight.

All fasting plasma samples from both clones and control pigs were subjected to analysis of biochemical parameters (Table 2). Cholesterol showed a significant pig type × diet interaction ($P = 0.01$) and trends in HDL, LDL, and lactate ($P < 0.06$), which revealed that the pigs responded differently to ad libitum and restrictive feeding. Clones only showed minor differences in cholesterol, HDL, and LDL between ad libitum and restrictive feeding, whereas controls showed more marked concentration increases when being fed ad libitum. The LDL/HDL ratios were, however, not different between clones and controls. NEFA showed significant changes due to diet, but no differences between clones and controls. Lactate was numerically higher in diet restricted clones compared to diet restricted controls, whereas ad libitum fed clones exhibited highly elevated lactate compared to controls. Glucose and insulin-like growth factor 1 (IGF-1) additionally showed significant concentration differences between clones and controls ($P < 0.001$). Fasting plasma glucose was higher in clones compared to controls, while IGF-1 was approximately 2-fold lower. No significant differences were observed for triglyceride and insulin.

Metabolomics Analysis

HPLC-ESI-qTOF-MS was applied for the plasma metabolic pattern analysis of clones and controls. Plasma samples from the 17 clones and 19 controls were analyzed in both positive and negative ionization mode. Representative base peak chromatograms (BPC) are shown in Figure 1. After preprocessing, the data sets contained 850 metabolite ions in positive and 469 in negative ESI mode. The preprocessed data were further investigated using multivariate analysis that included PCA and LDA subdivided by diet. PCA reflects the main variation in data, and less interindividual variation between ad libitum fed cloned animals compared to normal litter mates did not seem to be evident from PCA scores plot (Figure 2A,C). A slight discrimination between ad libitum fed clones and controls can, however, be observed (Figure 2A,C), and the goodness of prediction from LDA models based on PC1 and PC2 scores were found to be 0.94 and 0.89 in positive and negative ionization mode, respectively. Diet restricted clones seemed to show a slightly smaller variation than controls in positive ionization mode and with no clear distinction between groups, which is also reflected in the goodness of prediction of 0.65 (Figure 2B). In negative ionization mode, however, less variation cannot be observed for diet restricted clones, but a separation appears with a goodness of prediction of 0.81 (Figure 2D).

Metabolite ions causing differences between clones and controls were located and identified (Tables 3 and 4). Diet restricted clones had a high content of isoleucine and tryptophan in plasma, whereas creatine, valine, glycochenodeoxycholic acid, and lysophosphatidylcholine (Lyso PC) 18:2 were decreased compared to controls. Glucose was the only compound significantly increased in plasma of ad libitum fed clones, while creatine, 4-sulfobenzyl alcohol, and a number of Lyso PC species were decreased. The identified metabolites were primarily nonpolar. The metabolomics platform involved reversed-phase chromatography, which is why highly polar potential biomarkers were not detected.

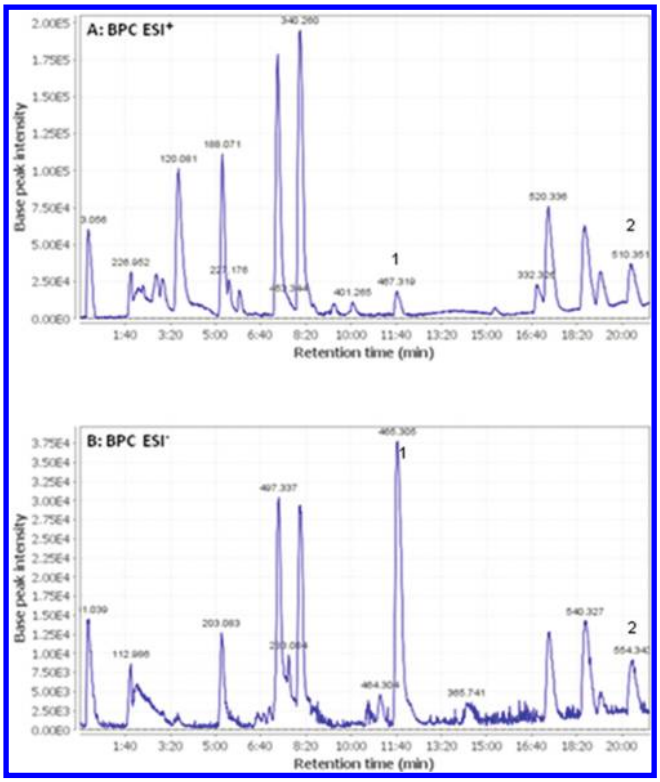

Figure 1. Representative reversed-phase high performance liquid chromatography coupled to electrospray ionization quadropole time-of-flight mass spectrometry base peak chromatograms (BPC) of a pig plasma sample (fasted state) analyzed in positive (A) and negative (B) electrospray ionization (ESI) modes. Internal standards are numbered (1) glycocholic acid (Glycine-1 ^{13}C) and (2) lysophosphatidylcholine (17:0). Details on the separation conditions are given in the experimental section.

DISCUSSION

Characterization of a cloned pig model compared to normal control pigs exposed to a high-energy diet either restricted or ad libitum was performed by biochemical analyses and nontargeted LC−MS metabolomics. The objective was to identify possible differences between cloned and normal pigs and elucidate if the cloned pig model in terms of variability is a better model for nutritional intervention studies than normal pigs. Both clones and controls showed signs related to the metabolic syndrome when fed the energy dense diet ad libitum compared to diet restriction. However, several parameters indicate that the response of cloned pigs and control pigs to high-energy intake is not the same. The PCA models additionally showed some differences between clones and controls in both feeding regimes, and metabolites responsible for this discrimination were identified. The breeding cross difference between clones and controls (65% × 35% versus 75% × 25% Landrace and Yorkshire, respectively) could have a slight impact on the results but is probably only of minor importance as the difference was very small. Less interindividual variation of cloned pigs was furthermore not clearly observed from the PCA models. Single somatic cell nuclei were in the cloning process introduced into enucleated oocytes containing a small subset of maternal mitochondrial DNA, which may have contributed to the variation. Otherwise, epigenetics, gut microbiota, or other environmental factors encountered in utero and/or in the postnatal period may have had a stronger

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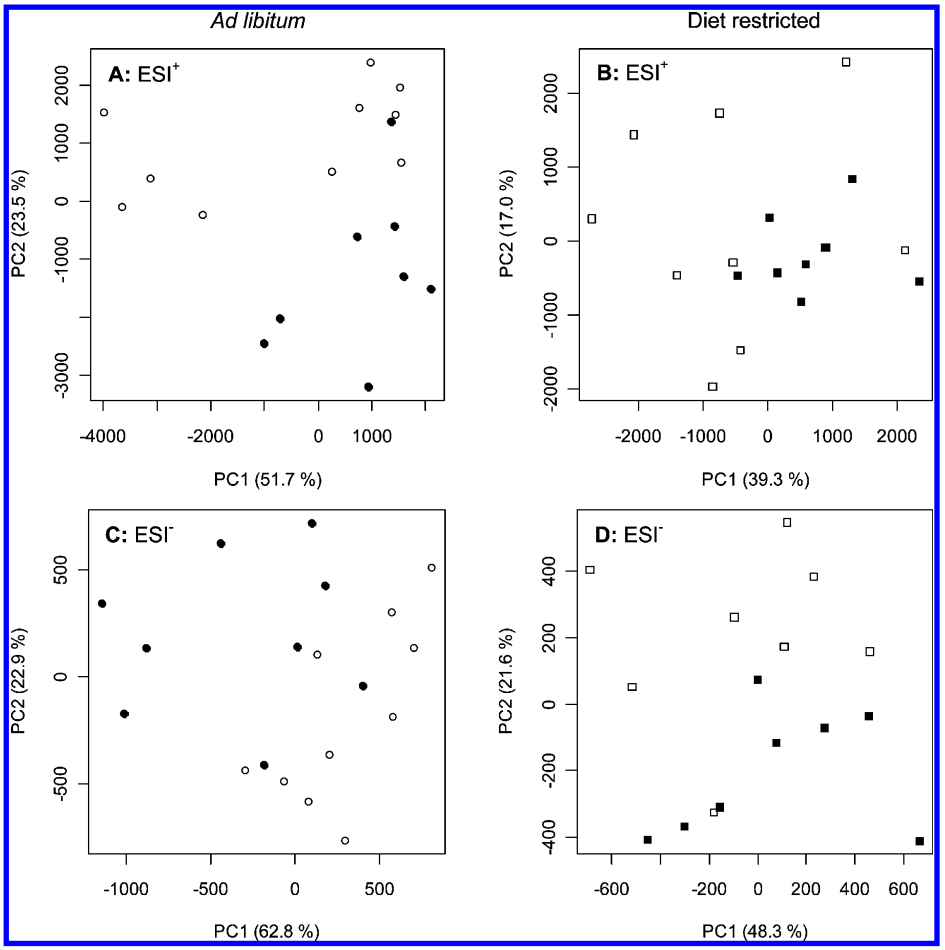

Figure 2. Comparison of clone and normal control pigs fed either ad libitum or in a restricted manner. PCA scores plot in ESI^+ mode (A,B) and ESI^- mode (C,D). ●, clone ad libitum (n = 8 in ESI^+; n = 9 in ESI^-); ○, control ad libitum (n = 10); ■, clone diet restricted (n = 8); □, control diet restricted (n = 9 in ESI^+; n = 8 in ESI^-).

Table 3. List of Identified Metabolites from the Metabolomics Analysis Discriminating between Clone (n = 8) and Control (n = 9) Pigs That Have Been Diet Restricted

metabolite	ionization mode[a]	retention time (min)	m/z	mass accuracy (ppm)	fold change[b] (vs control)	P value[c]	FDR q value[d]
fragment of isoleucine[e]	ESI^+	2.83	86.0945	4.9	1.20	0.008	0.199
isoleucine[e]	ESI^+	2.83	132.0997	20.6	1.23	0.014	0.149
isoleucine[e] $[M + Na]^+$	ESI^+	2.83	154.0813	19.9	1.38	0.040	0.209
fragment of tryptophan[e]	ESI^-	5.42	116.0506	5.2	1.90	<0.001	0.002
fragment of creatine[e]	ESI^+	2.18	90.0533	24.6	0.36	0.043	0.216
valine[e]	ESI^+	2.20	118.0863	0.3	0.60	0.007	0.110
glycochenodeoxycholic acid[e]	ESI^-	13.71	448.3061	0.4	0.28	0.012	0.135
Lyso PC 18:2[f]	ESI^+	17.26	520.3388	2.9	0.65	0.056	0.249

[a]ESI^+, positive electrospray ionization mode; ESI^-, negative electrospray ionization mode. [b]Fold change was calculated by dividing the mean of normalized intensities of each metabolite from cloned pigs by the mean intensity of the same metabolite from control pigs. [c]P value was analyzed by a two-sample t test. [d]FDR q value, false discovery rate and multiple testing correction (q value). [e]Identification confirmed by standard comparison. [f]Lyso PC, lysophosphatidylcholine. Lyso PCs were identified from characteristic MS fragments of 184, 104, and 86 in positive ESI mode.

role in developing variation between animals than genetics. Other studies with cloned swine[37] and doe[38] have shown similar results in variability and phenotypic differences between cloned and control animals. The sample size in the present study was, however, small with only 8−10 animals in each group, and a larger sample size would be more indicative of variation between animals in the multivariate analysis.

Ad libitum fed clones showed highly elevated concentrations of plasma lactate compared to controls. Lactate is produced from pyruvate by lactate dehydrogenase during normal metabolism and exercise from anaerobic glycolysis. Excess lactate is usually removed by oxidation back to pyruvate by well-oxygenated muscle cells or by conversion into glucose via gluconeogenesis in the liver. Elevated plasma levels of lactate in the fasting state have been associated with obesity and type 2 diabetes due to an increase in adipose tissue mass.[39,40] This is with concomitant accelerated gluconeogenesis and elevated fasting glucose,[41] and the clones did show slightly elevated

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Table 4. List of Identified Metabolites from the Metabolomics Analysis Discriminating between Clone (n = 9) and Control (n = 10) Pigs Fed Ad Libitum

metabolite	ionization mode[a]	retention time (min)	m/z	mass accuracy (ppm)	fold change[b] (vs control)	P value[c]	FDR q value[d]
glucose[e]	ESI^+	2.15	203.0525	3.3	1.15	0.013	0.151
isoleucine[e]	ESI^+	2.84	132.0997	20.6	1.23	0.072	0.333
fragment of tryptophan[e]	ESI^-	5.42	116.0506	5.2	1.31	0.066	0.322
indoleacrylic acid $[M + Na - 2H]^-$	ESI^-	5.40	208.0389	4.3	2.06	0.053	0.309
fragment of creatine[e]	ESI^+	2.18	90.0529	24.6	0.22	0.014	0.153
4-sulfobenzyl alcohol	ESI^-	8.09	187.0079	7.8	0.48	0.059	0.318
Lyso PC 18:3[f]	ESI^+	15.92	518.3214	6.2	0.39	0.014	0.154
Lyso PC 20:4[f]	ESI^+	17.21	544.3374	5.3	0.34	<0.001	0.014
Lyso PC 18:2[f]	ESI^+	17.26	520.3388	2.9	0.37	<0.001	0.011
Lyso PC 18:2[f] $[M - CH_3]^-$	ESI^-	17.26	504.3085	0.9	0.42	0.002	0.046
Lyso PC 20:5[f]	ESI^+	17.27	542.3199	8.9	0.39	<0.001	0.014
Lyso PC 16:0[e,f]	ESI^+	18.63	496.3384	2.8	0.43	0.001	0.036
Lyso PC 16:0[e,f] $[M + CH_2O_2 - H]^-$	ESI^-	18.63	540.3286	3.8	0.54	0.005	0.085
Lyso PC 16:0 dimer[e,f]	ESI^+	18.67	991.6647	7.1	0.22	0.029	0.234
Lyso PC 18:1[e,f]	ESI^+	19.21	522.3537	4.4	0.34	<0.001	0.006
Lyso PC 18:1[e,f] $[M + Na]^+$	ESI^+	19.21	544.3358	2.6	0.21	0.004	0.078

[a]ESI^+, positive electrospray ionization mode; ESI^-, negative electrospray ionization mode. [b]Fold change was calculated by dividing the mean of normalized intensities of each metabolite from cloned pigs by the mean intensity of the same metabolite from control pigs. [c]P value was analyzed by a two-sample t test. [d]FDR q value, false discovery rate and multiple testing correction (q value). [e]Identification confirmed by standard comparison. [f]Lyso PC, lysophosphatidylcholine. Lyso PCs were identified from characteristic MS fragments of 184, 104, and 86 in positive ESI mode.

fasting glucose concentrations when fed ad libitum compared to controls observed with both analysis methods. Another explanation could be a possible perturbation of mitochondrial metabolism that may cause excess production or insufficient clearing of lactate from the blood. An NMR-based metabolomics study of clone and control pigs performed by Clausen et al.[18] also revealed higher lactate levels in clones. A suggestion was that an increased oxidative stress during in vitro culture of embryos could have an impact on stress sensitivity postnatally, and a high lactate level could be a reflection of this increased sensitivity. Oocytes and embryos are inevitably exposed to oxidative stress generated by reactive oxygen species during in vitro culture.[42] Irrespective of the cause, the high plasma lactate concentrations implies an increased anaerobic metabolism, which is associated with depletion of phosphocreatine/creatine,[43] and lower creatine levels were in fact detected in ad libitum and diet restricted clones. Creatine increases muscle stores of phosphocreatine that is used to resynthesize ATP from ADP in response to an increased energy demand.

IGF-1 was approximately half the level in plasma from cloned pigs. IGF-1 regulates growth and whole body energy metabolism and glucose homeostasis at the cellular level.[44] A low concentration of IGF-1 could therefore explain the lower weight gain of clones when fed ad libitum. A known polymorphism in the gene for IGF-1 gives rise to low systemic levels of IGF-1, which is associated with lower body height and increased risks of developing type 2 diabetes and myocardial infarction.[45] When fed restrictively, however, the clones did not seem to be affected by low systemic levels of IGF-1 as they gained more weight than controls despite of equal energy intake. Decreased serum concentrations of IGF-1 have also been observed in cloned Holstein calves.[46]

Ad libitum fed control pigs showed signs of hypercholesterolemia with increased fasting concentrations of plasma cholesterol, LDL, and HDL when compared to diet restriction. This was not as prominent for the ad libitum fed clones, which could be due to the significant lower feed intake and less weight gain. Diet restricted clones also had a decreased content of a glycine conjugated (primary) bile acid in plasma, which could indicate a decreased uptake of lipids and cholesterol. NEFA concentrations were, however, unaltered between clones and controls despite of the different feed intakes on the ad libitum diet. The higher NEFA content when diet restricted compared to ad libitum indicated an increased mobilization of fat for energy supply due to the lower energy intake.

Several Lyso PC species were also identified and found to be decreased in cloned pigs fed ad libitum and one in diet restricted clones. Plasma Lyso PC originates from hydrolysis of phosphatidylcholine by phosholipase A2 in LDL and cell membranes, hepatic secretion, and transesterification by lecithin-cholesterol acyltransferase. Decreased Lyso PC levels could as a result be connected to the lower LDL concentrations and lower feed intake. However, other metabolomics studies have found decreased levels of Lyso PC species in high-fat diet induced obese mice[7] and humans with impaired glucose tolerance,[8] which suggest that decreased Lyso PC concentrations are connected to increased feed/food intake. Therefore, another mechanism or dietary differences between these studies may cause the observed result.

A fragment of tryptophan were identified and showed increased concentrations in plasma of clones compared to controls, however only significant when diet restricted. Tryptophan is an essential amino acid necessary for protein synthesis and is furthermore a precursor for the biosynthesis of serotonin that can depress hunger. Brain serotonin inhibits food intake, and depletion promotes weight gain.[47] An increased satiety could explain the low feed intake of the ad libitum fed clones compared to controls. It has been shown that obese people with metabolic syndrome have decreased plasma tryptophan concentrations.[48] Indoleacrylic acid, a breakdown product of tryptophan, also showed a tendency to increase in plasma of ad libitum fed clones. Indoleacrylic acid is most probably formed by intestinal microorganisms that catabolise tryptophan to indole derivatives, which are then absorbed and

converted to its final form.[49] 4-Sulfobenzyl alcohol, which also arises from microbial fermentation, was furthermore identified and showed a tendency to be decreased in ad libitum fed clones. These two metabolites could indicate metabolic differences or changes in the gut microbiota of the clones compared to controls. As the ad libitum fed clones had a decreased energy intake, this might reduce the metabolism of the microflora as well. Additionally, it is known that the gut microbiota is influenced and rapidly shift in response to changes in nutrient uptake and adiposity.[50] Differences in the gut microbiota between clones and controls are in the process of being analyzed separately.

Two branched chain amino acids (BCAA) were identified, of which isoleucine was found to be elevated and valine decreased in diet restricted clones. BCAA catabolism involves conversion into branched chain alpha-keto acids that are further degraded in the liver to fuel the TCA cycle.[51] If the mitochondria were perturbed in the ad libitum state, as could be indicated by the high plasma lactate and low creatine concentrations, a decreased BCAA catabolism might occur with increased plasma BCAA concentrations as a consequence. However, why isoleucine was increased and valine decreased in the diet restricted state is unknown; possibly the dietary protein requirements were different for the cloned pigs as they additionally grew at a different rate.

■ CONCLUSIONS

In summary, a number of differences were revealed between cloned and normal control pigs when fed a restricted or ad libitum high-energy diet. Alterations in feed intake and weight gain were observed, which were also reflected in the plasma metabolome. Differences in metabolites related to the gut microbiota, growth, and energy metabolism were furthermore found. Cloned pigs, however, did not seem to exhibit less variation for the detected metabolites compared with normal pigs but could provide a desirable phenotype for certain metabolic disorders. Further characterization of the cloned pig model would be needed if applying them in obesity and metabolic syndrome research.

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Notes

The authors declare no competing financial interest.

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