Controlling the Uncontrolled Variation in the Diet Induced Obese Mouse by Microbiomic Characterization

Valeriia Bondarenko

University of Copenhagen

Cecillie Løkke

University of Copenhagen

Peter Dobrowolski

GVG Genetics

Caroline M. Junker Mentzel

University of Copenhagen

Josué L. Castro-Meija

University of Copenhagen

Camilla Hartmann Friis Hansen

University of Copenhagen

Dorte Bratbo Sørensen

University of Copenhagen

Dennis Sandris Nielsen

University of Copenhagen

Lukasz Krych

University of Copenhagen

Axel Komerup Hansen (✉ akh@sund.ku.dk)

University of Copenhagen

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Abstract

C57BL/6NTac (B6NTac) and C57BL/6NRj (B6NRj) mice were fed a high calorie diet and treated with liraglutide. 42 mice would have been needed in an ordinary one-way ANOVA to show a reduction in glycosylated hemoglobin (HbA1c) in B6NTac mice, but incorporating the sequenced fecal microbiota in a two-way ANOVA reduced the group size needed to obtain a statistical significance to 12 mice. In B6NRj mice there was no impact of liraglutide on HbA1c neither with or without microbiota incorporation. In both sub-strains, the liraglutide effect on glucose tolerance and body weight was powered by incorporation of microbiota clusters. Although B6NTac mice were genetically far more conform than the B6NRj mice, incorporation of a genetic characterization by short tandem repeats had only little impact on the outcome of data evaluation. In conclusion, incorporation of microbiota characterisation powers data evaluation in diet induced obesity mouse strains, which are influenced by the microbiota.

Introduction

The diet-induced obese (DIO) mouse, i.e. C57BL/6 (B6) mice fed a high-calorie diet for up to four months is a commonly used animal model for diabetes and obesity ¹. It has an acceptable predictive validity, i.e. ability to predict an outcome in humans, compared many other animal models in biomedical research. For instance, the effect of glucagon-like peptide 1 receptor agonists (GLP-1RA), such as liraglutide, in DIO mice is highly comparable to humans ², i.e. it lowers body weight ³, insulin resistance ⁴ and long term blood glucose (HbA1c) ⁵. B6 sub-strains can be divided into a main cluster of sub-strains originating from either the Jackson Laboratories (C57BL/6J or B6J) or from the US National Institutes of Health (C57BL/6N or B6N).⁶ Even within these clusters sub-strains differ genetically ⁶ and phenotypically ⁷. For example, in some intervention studies in or lab we can get a significant difference in HbA1c, when using B6NTac from Taconic Biosciences (LI. Skensved, Denmark), while not when using B6NRj from Janvier Labs (Le Genest-Saint-Isle, France).

The high inter-individual variation of DIO mice ⁸ reduces power and leads to the need for increased group sizes to show significant differences, as group sizes in animal studies are calculated from estimates on (a) standard deviation (s.d.) and (b) effect size combined with (c) the preferred power (μ) and (d) significance level (p) ⁹. In some studies the gut microbiota variation has been shown to account for 30-40% of variation in glucose related parameters, such as HbA1c in the DIO model ¹⁰, and the obese phenotype can be transferred between mice by fecal matter transplantation ¹¹. Therefore, microbiota impact may be a simple explanation for problems of obtaining enough power to show a specific difference. Hypothetically, some colonies of B6 mice may have a microbiota composition, which renders them less sensitive to eliciting certain glucose related responses Characterization by short tandem repeats (STRs), i.e. short runs of repetitive nucleotides in the non-coding DNA, reveals that even within a defined sub-strain mice are not genetically homogenic ¹².

Up till today variation in animal models has to a high extent been attempted to be controlled by standardization of factors such as diet and housing conditions ¹³. It is, theoretically, possible to standardize an experiment by using the sub-strain most applicable for the purpose, as e.g. proposed within rheumatoid arthritis ¹⁴. Recently it has been realized that much inter-individual variation is due to microbiota variation, ¹⁵ and that microbiota composition differs substantially between colonies of the same strain ^{7,16}. Relatively little is still known to take robust decisions on what would be the most applicable and translational microbiota for diabetes and obesity research. Even mice of the same sub-strain may be delivered by the same vendor but from different breeding rooms with different microbiota ¹⁷. In fact, the standardization approach has been questioned, as standardization in one environment may make experiments irreproducible in another environment ¹⁸. As an alternative it has been proposed that the variation is systematically dispersed to each study group in a so-called multifactorial heterogenized design and accounted for in a multifactorial data evaluation, which can be shown to be more reproducible ^{19,20}.

Genomic and microbiomic characterizations have become cheaper and more available. Compared to single nucleotide polymorphisms (SNPs), STRs have a higher mutation rate which benefits the arising of new alleles and makes them highly polymorphic and make them an ideal tool for analysing genetic variability within the same colony of inbred mice ^{12,21}. If All mice of a study are microbiota characterized and clustered, as far as each cluster is represented in each group, it can be accounted for in multi-factorial data evaluation as for any other parameter. The STRs with the highest variability of alleles or a very even distribution of the different alleles may be identified, and be used as a factor in a multifactorial data evaluation. We, therefore, hypothesized that if all DIO mice in an intervention study were microbiota and genome characterized, this could be incorporated in data evaluation, and thereby lead to lower group sizes or increased power of a DIO mouse study. We test this we used HbA1c as primary read-out.

Methods

Animals

Experiments were in accordance with Danish Animal Experiments Act on protection of animals used for scientific purpose (Order 12 of 07/01/2016) and Directive 2010/63/EU of the European Parliament and the Council of 22/09/2010, and it was licensed accordingly by the Animal Experimentation Committee under the Danish Ministry of Food, Fisheries, and Agriculture (License No 2017-15-0201-01262. 32 C57BL/6NTac (B6NTac; Taconic Biosciences, Lille Skensved, Denmark) and 46 C57BL/6NRj (B6NRj; Janvier Labs, Le Genest-Saint-Isle, France) four week old male mice were feces sampled and ear-marked at the vendor three days before delivery (B6NTac) or at receipt (B6NRj) at our AAALAC-accredited barrier protected rodent facility, where there was 22°C±2°C, 55% ± 10% humidity, and light cycle 6 am to 6 pm. Mice were housed by four and changed bi-weekly in 1290D Eurostandard Type III cages (Scanbur A/S, Karlslunde, Denmark) with a wire lid and aspen bedding (Tapvei, Paekna, Estonia) with ad libitum access to bottled tap water, Enviro-Dri paper nesting material, aspen biting blocks (L × W × H: 50 × 10 × 10 mm) in aspen wood (all Tapvei), a cardboard tube and a shelter (both from LBS serving Biotechnology, Horley, United Kingdom). Health monitoring according to FELASA guidelines ²² revealed no pathogens. All mice were tunnel handled (from six weeks of age) or cupped without restraint (from 15 weeks of age) ²³.

Experimental design

Samples sizes were determined by power analysis⁹ on estimated intervention effect and variation in the primary read-out, i.e. HbA1c, based upon in-house experience primarily from B6NTac mice setting $p < 0.05$ and power = 90%. Group sizes for BL6NRj mice were increased with approximately 50%. All mice were fed a synthetic high fat diet (HFD) (D12492 Research Diets, Brunswick, USA. Protein: 20% kcal, Fat: 60% kcal, Carbohydrate: 20% kcal) ad libitum from the age of four weeks to termination (Figure 1) monitoring food intake per cage and individual body weights Monday, Wednesday, and Friday. At 16 weeks of age mice were divided into liraglutide or vehicle treated groups (Taconic mice $n = 16/\text{group}$; Janvier mice $n = 23/\text{group}$) (Figure 1) and hereafter for four weeks treated subcutaneously with approximately 0.4 mg/kg liraglutide (as 1:49 dilution of Victoza $\rightarrow$, 6 mg/ml, Novo Nordisk, Denmark) once per day at 15-17 p.m. in volumes of 0.1, 0.125 or 0.15 ml depending on their body weight: 20-30 g, 31-40 g or 41-50 g respectively. Vehicle treated mice ($n=16$) received the corresponding amount of sterile filtered dilution buffer (50 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 70 mM NaCl and 0.05% polysorbate 80, pH adjusted to 7.5). For adaptation, the first two days of intervention experimental animals received a half dose of liraglutide (1:99 dilution) to avoid anorexia.

Oral glucose tolerance test (OGTT), glycated hemoglobin (HbA1c), insulin and leptin measurements

HbA1c (mmol/mol) was measured at 4 (baseline), 16 (pre-intervention) and 20 (post-intervention) weeks of age (DCA Vantage Analyzer, Siemens Healthcare Diagnostics, Ballerup, Denmark) on 1 μL tail vein blood. To test oral glucose tolerance (OGTT) before and after liraglutide intervention fasting glucose ($t=0$) was measured (Freestyle Mini Glucometer, Hermedico, Copenhagen, Denmark) on mice fasted for six hours (from 7 a.m.) after tail vein puncture followed by gavage with 2 mg/g BW glucose (500 mg/ml glucose solution, Amgro I/S, Copenhagen, Denmark) and subsequent blood glucose monitoring at 15, 30, 60, 90 and 120 min. At termination, animals fasted for six hours were anesthetized with 0.1 ml/10 g BW with 25% Midazolam $\rightarrow$ (5mg/ml midazolam, B.Braun, Melsungen, Germany) and 25% Hypnorm $\rightarrow$ (0.315 mg/ml of fentanyl citrate and 10 mg/ml of fluanisone, Skanderborg Pharmacy, Denmark) diluted in saline. Retro-orbital blood was collected into an autoclaved 1.5 ml Safe-Lock Eppendorf $\rightarrow$ microcentrifuge tubes (Buch & Holm A/S, Herlev, Denmark) and stored on ice for several hours, and later at 4°C for 1 day for blood coagulation. After centrifugation for 10 min at 10000 rpm at 4°C supernatants were stored at -80°C. Animals were killed by cervical dislocation. Caecum contents were collected immediately after into sterile Eppendorf $\rightarrow$ tubes on dry ice during collection, with subsequent storage at -80°C. Before post-mortem sampling mice were blinded to the reader. Fasting serum insulin and leptin levels were measured (Mouse Metabolic Kit, Meso Scale Diagnostics, Rockville, United States) following the manufacturer's instructions. Samples from both strains were randomized and measured on one plate. Results were read using MESO QuickPlex SQ 120 (Mesoscale). Leptin measurements with values > 100000 pg/ml were reported as 100000 pg/ml due to limits in detection sensitivity of the test.

Gut microbiota sequencing

Faecal samples were stored at -80°C. DNA was purified using Bead-Beat Micro AX Gravity kit (A&A Biotechnology, Gdynia, Poland, Cat# 106-100) according to manufacturer's protocol. Purified DNA was stored at -80°C until analysis. Concentration and purity were verified on Nano-Drop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Based on Nano-Drop measurements the samples were normalised to 10 ng/ μL with nuclease-free water using Biomek 4000 Workstation (Beckman Coulter Life Sciences, United States). The V3 variable region of the bacterial 16S rRNA gene was amplified using the primers Nxt_{388_F} (5'-TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGACWCCTA CGGGWGGCAGCAG-3') and Nxt_{518_R} (5'-GTCTCGTGGG CTCG-GAGATG TGTATAAGAG ACAGATTACC GCGGCTGCTGG-3') compatible with the Nextera Index Kit (Illumina, San Diego, United States). PCR was performed in a final reaction volume of 25 μL composed of 5 μL PCR BIO buffer, 0.5 μL primer mix (10 uM), 0.25 μL PCR BIO HiFi polymerase (PCR Biosystems Ltd, London, UK), 5 μL of genomic DNA, and nuclease-free water up to 25 μL . It was run on SureCycler 8800 (Agilent Technologies, Santa Clara, CA, USA), and the applied thermal cycling conditions were as follows: Enzyme activation for 2 min at 95 °C, 33 cycles of denaturation for 15 sec at 95 °C, annealing for 15 sec at 55 °C, and extension for 20 sec at 72 °C. Lastly, a final extension step for 20 sec at 72 °C. Quality of PCR amplicons was assessed by gel electrophoresis using 1.5% agarose gel and 0.5% TBE buffer at 120 V for 40 min. A second PCR was run on SureCycler 8800 to label adapter-ligated DNA fragments with indices. PCR reactions contained 5 μL PCR BIO buffer, 0.25 μL PCR BIO HiFi polymerase, 2 μL of each primer (P5 and P7), 2 μL PCR product, and nuclease-free water to a total volume of 25 μL . Applied thermal cycling conditions were as follows: enzyme activation for 1 min at 95 °C, 13 cycles of denaturation for 15 sec at 95 °C, annealing for 15 sec at 55 °C, and extension for 15 sec at 72 °C. Lastly, a final extension step for 5 min at 72 °C. Labelled adapter-ligated DNA fragments were purified by use of Biomek 4000 Workstation (Beckman Coulter Life Sciences, USA) using AMPure CP binding Beads pooled in equimolar concentrations and sequenced with the Illumina NextSeq550 platform. The raw dataset with pair-ended reads and corresponding quality scores was merged and trimmed using `fastq_mergepairs` and `fastq_filter` scripts implemented in the USEARCH pipeline as described previously²⁴. Purging the dataset from chimeric reads and construction of zero radius Operational Taxonomic Units (zOTU) was conducted using the UNOISE. The Greengenes (13.8) 16S rRNA gene collection served as a reference database.

Genetics

The analysis of tail tips for genotyping was performed by GVG Genetic Monitoring GmbH as previously described¹². A chromosomal panel of 244 highly polymorphic STR markers evenly distributed in the non-coding regions of the genome was used (Data S1). Snooplex FastPrep PCR reagents (GVG Genetic Monitoring, Leipzig, Germany) in combination with the GVG Genetic Monitoring standard marker panel (GVG Genetic Monitoring) were used for amplification of fluorescence-labelled PCR products (Bio-Rad C1000 Touch Thermal Cycler, Bio-Rad Laboratories, Hercules, USA). The standard cycling parameters were 94°C for 2 min followed by 38 cycles of 94°C for 30 s, 58°C for 1 min, 68°C for 2 min and a final elongation step of 68°C for 10 min. Amplification products were electrophoresed on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA). Briefly, 1.8 μL of amplicons and 12 μL of a 250:1 mixture of deionized formamide and internal lane size standard (MapMarker Custom, BioVentures Inc., Murfreesboro, USA; sizes: 60, 80, 90, 100, 120, 140, 160, 180, 200, 220, 240, 250, 260, 280, 300, 320, 340, 360, 380, 400, 425, 450, 475, 500, 525 and 550 bp labeled with Dy-632) were combined. PCR products were injected (15 s) and electrophoresed at 15 kV in Performance Optimized Polymer 4 (POP4, Applied Biosystems). Data analysis was performed with GeneMapper v5 (Applied Biosystems).

Statistics

OGTT was calculated as area under the curve (AUC). For OGTT, body weight and HbA1c the the effectss were expressed as Δ -values, i.e. the percentage difference between pre- and post-intervention values out of the pre-intervention value. Data were tested for Gaussian distribution by Anderson-Darling test and equal variances by Levene's test (Minitab 19.0, Minitab Inc., Coventry, UK). For further analysis as factors in two-way ANOVAs all mice were clustered after their microbiome at time 1 and 2 in a group-independent cluster analysis using Bray-Curtis dissimilarities on zOTU tables rarefied to 10,000 reads. Hierarchical clustering was based on the Ward's method and the optimal number of clusters were chosen based on ensuring the maximum possible number of clusters, when each cluster was represented in both treatment and control groups. One-hundred permutations on a generalized-linear model (GML – "lmer4" package R, R Statistics, New York, USA) were performed and final cluster denotation was as random effect for each permutation. For further analysis as factors in two-way ANOVAs from each we selected for each sub-strain the STRs with the most even distribution of alleles within the locus, and those which had the highest number of different alleles within the locus. The degree of conformity of the genotype of an individual with the consensus STR-genotype of its strain was calculated by comparing STR alleles for all STR markers. The value of 1 was allocated if both alleles were identical with the consensus allele, a value of 0 if both alleles differed and a value of 0.75 if there is a heterozygous matched. The resulting sum of all values was then divided by the number of markers and a percentage figure describing the match between the individual and the consensus STR-genotype was obtained.

All data were compared by both a one-tailed, one-way ANOVA (Minitab: ANOVA/One-Way) as well as two-way ANOVAs with the factors treatment and either cluster or allele (Minitab, ANOVA/General Linear Model/Fit general linear model). For one-way ANOVA the observed differences and standard deviations (s.d.) were used in a power analysis for minimum sample size and power estimation (Minitab, Power and sample size/One-Way ANOVA), while for the two-way ANOVAs the mean weighted differences and s.d. based upon the differences between treatments and s.d. within each cluster (Table S2) were used for estimation of the minimum sample size and power estimation (Minitab, Power and sample size/General full factorial design) (Figure 1). Data not following a Gaussian distribution and/or having equal variances were ranked before test.

Bacterial abundances from feces sampled at time 1 and 2 and genetic conformities were compared between sub-strains by a t-test with Welsch's correction (GraphPad Prism, San Diego, USA). Bacterial abundances from both feces and cecum after liraglutide treatment (time 2) were checked for normality (Prism) and equal variances (Minitab) and ranked in case of failing the assumptions before comparing by a two-way ANOVA (Minitab, ANOVA/General Linear Model/Fit general linear model) with the factors strain and treatment. Multiple comparison p-values were corrected by the Holm-Sidak correction (Prism).

Results

The two sub-strains differed essentially in microbiota composition.

B6NTac mice harboured *Bifidobacterium* spp at time 1, while these were absent in B6NRj mice at time 1 and in both substrains on time 2 (Table S1). B6NTac mice had more *Bacteroides* spp., while the B6NRj mice had more *Prevotella* spp. (Table S1), and at time 2 *Prevotella* spp. were absent in B6NTac mice (Table S2 and S3). B6NTac mice had more *Lactobacillus* spp. at time 1, while B6NRj had more *Lactococcus* spp. at time 2 (Table S1). At both times and both in feces and cecum the relative abundances of *Akkermansia muciniphila* was highest in the B6NRj mice (Table S1-3). Liraglutide increased the relative abundances of *Lactobacillus* spp, and Clostridiaceae in feces and cecum and Enterococcaceae in feces (Table S2), while it decreased the relative abundances to below detection of segmented filamentous bacteria (*Candidatus arthromitus*) in feces of both sub-strains (Table S2). B6NTac could be separated into three microbiomic clusters at time 1 and 2, while B6NRj could be separated into four clusters at time 1 and 2. One B6NTac mouse at time 2, 8 B6NRj mice at time 1 and 11 B6NRj mice at time 2 did not yield enough reads to be clustered (Data S2).

Some STRs of B6NRj mice were highly polymorphic

The genetic conformity of the B6NTac mice was significantly higher than the B6NRj mice ($p < 0.0001$, Figure 2). STRs of B6NTac mice were not polymorphic, as none of them expressed more than three different alleles, while in the B6NRj mice three STRs expressed five-six different alleles (Table 2). The STRs D1S113, D9S218 and D5S519 in the B6NTac mice and the STRs D11S112 and D15S215 in B6NRj mice showed the most even distribution of the alleles, while (Table 2).

Table 1.

C57BL/6NTac

C57BL6/NRj

	Δ OGTT AUC%	Δ Body weight %	<u>Primary readout</u> Δ HbA1c%	Insulin pg/ml	Leptin pg/ml*	Fasting glucose mmol/l	Δ OGTT AUC%	Δ Body weight %	<u>Primar readou</u>
Group size in study N	16	16	16	16	16	16	23	23	23
Liraglutide (Mean $\pm$ s.d.)	-40.32 $\pm$ 13.71	-11.94 $\pm$ 4.12	-11.60 $\pm$ 16.29	1235 $\pm$ 780	7490 $\pm$ 5304	5.81 $\pm$ 0.93	-1.47 $\pm$ 22.25	-10.52 $\pm$ 4.05	-4.27 $\pm$
Control (Mean $\pm$ s.d.)	-14.41 $\pm$ 18.77	0.09 $\pm$ 3.55	0.09 $\pm$ 16.27	1568 $\pm$ 967	23688 $\pm$ 12103	7.09 $\pm$ 1.25	-16.51 $\pm$ 19.90	3.48 $\pm$ 5.21	-2.93 $\pm$
One-way ANOVA									
Effect	-25.91	-12.03	-11.69	-335	-16198	-1.28	-15.05	-14.00	-1.34
Pooled s.d.	16.44	3.84	16.28	878	9344	1.1	21.11	4.67	15.64
Significance, difference between treatments, p	0.000	0.000	<i>0.051</i>	0.292	0.000#	0.003	0.020	0.000	0.772
Power	0.99	1.00	0.50	0.18	1.00#	0.89	0.66	1.00	0.06
Group size for p< 0.05 and power>0.9	10	4	42	146	8#	17	43	4	2864
Two-way ANOVA									
<i>Microbiome time 1</i>									
Efficient group size	16	16	16	16	16	16	15	15	15
Weighted effect	-26.66	-12.28	-13.00	-457	-16472	-1.23	-17.85	-13.38	-1.99
Pooled s.d.	16.69	3.92	16.49	803	9422	1.10	20.11	4.80	16.11
Significance, difference between treatments, p	0.000	0.000	0.033	0.133	0.000#	0.001	0.034	0.000	0.681
Significance, difference between clusters, p	0.747	0.307	0.164	0.040	0.230#	0.270	0.968	0.415	0.435
Power at N _{one-way} animals	1.00	1.00	<i>0.80</i>	0.50	1.00#	0.98	<i>0.87</i>	1.00	0.07
Group size for p< 0.05 and power>0.9	6	3	21	40	5#	11	17	3	830
<i>Microbiome time 2</i>									
Efficient group size	15	15	15	15	15	15	12	12	12
Weighted effect	-28.54	-12.48	-17.20	-407	-16610	-1.35	-27.30	-14.67	-0.97
Pooled s.d.	17.23	3.71	15.95	863	10038	1.12	17.46	4.84	13.68
Significance, difference between treatments, p	0.000	0.000	0.038	0.154	0.000#	0.013	0.004	0.000	0.805#

Significance, difference between clusters, p	0.361	0.905	0.376	0.217	0.926#	0.614	0.970	0.337	0.155#
Power	0.99	1.00	0.96	0.34	1.00#	0.98	0.99	1.00	0.06#
Group size p< 0.05 and power>0.9	6	2	12	58	4#	10	7	3	1482#
# Tested on ranked data due failure in unequal variances									
Group size	31-40	21-30		10-20		<10			

Table 2

	B6NRj STRs N (STR) = 243	B6NTac STRs N (STR) = 243
<i>Expression of the most prevalent allele in the STRs</i>		
100%	72 (29.6%)	138 (56.8%)
≥ 90% < 100%	101 (41.6%)	89 (36.6%)
≥ 80% < 90%	36 (14.8%)	10 (4.1%)
≥ 70% < 80%	14 (5.8%)	3 (1.2%)
≥ 60% < 70%	8 (3.29%)	2 (0.8%)^d
≥ 50% < 60%	10 (4.1%)	1 (0.4%)^e
≥ 40% < 50%	2 (0.8%) ^a	0 (0.0%)
<i>No of alleles expressed in the STRs</i>		
6	2 (0.8%)^b	0 (0.0%)
5	1 (0.4%)^c	0 (0.0%)
4	7 (2.9%)	0 (0.0%)
3	48 (19.8%)	31 (12.8%)
2	113 (46.5%)	74 (30.5%)
1	72 (29.6%)	138 (56.8%)
^a D11S112, D15S215; ^b D12S206, D9S312; ^c D16S147; ^d D1S113, D9S218; ^e DXS312		

HbA1c only had a significant effect, if microbiomic clusters were incorporated in data evaluation, and only in B6NTac.

HbA1c was only borderline significantly lowered by the Liraglutide treatment in B6NTac mice (p = 0.051, Table 1), while the effect was minimal in B6NRj mice (p = 0.190, Table 1). A two-way ANOVA including the microbiomic clusters revealed a significant effect of liraglutide at both time 1 (p = 0.033) and 2 (p = 0.038) (Table 1). The group size estimated for obtaining significance on the HbA1c effect in B6NTac mice was estimated to be 42 in a one-way ANOVA, while in a two-way ANOVA it was 21 at time 1 and 12 at time 2 (Table 1). Inclusion of the microbiomic clusters in two-way ANOVAs did not change anything in the B6NRj mice (Table 1).

Liraglutide had the expected effect and by including the microbiomic clusters in a two-way ANOVA power was increased

Liraglutide had the expected effect on glucose intolerance (OGTT), body weight, leptin and blood glucose (Data S3). Liraglutide lowered glucose intolerance (OGTT) in both B6NTac (p = 0.000, Table 1) and B6NRj (p = 0.020, Table 1) mice. However, 43 B6NRj mice would be needed in each group to obtain a power of 0.90 (Table 1) and the actual power with 23 mice was only 0.66, while only 10 B6NTac mice would be needed and the actual power with the 16 mice was 0.99 (Table 1). In the B6NRj mice microbiomic cluster inclusion in a two-way ANOVA decreased the estimated group size needed to show an OGTT effect to 7 at time 2 and increased the actual power to 0.99 in spite of the fact that the lack of reads at time 2 reduced the efficient group size from 23 to 12 (Table 1). Fasting blood glucose was significantly lowered by liraglutide in both strains (p (B6NRj) = 0.001, p (B6NTac) = 0.003), and there was some increase in power and decrease in group sizes by a microbiomic cluster including two-way ANOVA. This was particularly evident in the B6NTac mice, in which it reduced group sizes and increased power from 17 and 0.89 to 10-11 and 0.98, while in B6NRj mice at time 2 the reduction in efficient group size from 23 to 12 due to the lack of reads seemed to be counterproductive (Table 1). One-way ANOVA for the effect on body weight and leptin was powerful (p = 0.000 – 0.001) making it

difficult to assess whether a microbiomic cluster two-way ANOVA had any powering effect on any of the strains (Table 1). B6NRj mice showed a significantly insulin lowering effect of liraglutide in a microbiomic cluster two-way ANOVA at time 1 but not in a one-way ANOVA ($p = 0.022$, Table 1).

Including the STRs in a two-way ANOVA had little impact on power and group sizes.

In neither of the sub-strains the inclusion of the selected STRs as factors in a two-way ANOVA had a major impact on the power and the estimated group sizes (Table 3 and 4). Most remarkably inclusion of D12S206 resulted in a $p = 0.006$, a power of 0.93 and an estimated group size of 21 needed for evaluation of insulin compared to $p = 0.022$, power = 0.64 and an estimated group size of 44 if based upon a one-way ANOVA (Table 4).

Table 3

	Δ OGTT AUC%	Δ Body weight %	Primary readout Δ HbA1c%*	Insulin pg/ml	Leptin pg/ml*	Fasting glucose mmol/l
Group size _y	16	16	16	16	16	16
Liraglutide (Mean $\pm$ s.d.)	-40.32 $\pm$ 13.71	-11.94 $\pm$ 4.12	-11.60 $\pm$ 16.29	1235 $\pm$ 780	7490 $\pm$ 5304	5.81 $\pm$ 0.93
Control (Mean $\pm$ s.d.)	-14.41 $\pm$ 18.77	0.09 $\pm$ 3.55	0.09 $\pm$ 16.27	1568 $\pm$ 967	23688 $\pm$ 12103	7.09 $\pm$ 1.25
One-way ANOVA						
Effect	-25.91	-12.03	-11.69	-335	-16198	-1.28
Pooled s.d.	16.44	3.84	16.28	878.44	6.84	1.1
Significance, difference treatments, p	0.000	0.000	<i>0.051</i>	0.292	0.000	0.003
Power μ	0.99	1.00	0.50	0.18	1.00	<i>0.89</i>
Group size for p< 0.05 and power>0.9	10	4	42	146	8	17
Two-way ANOVA						
	<i>Genome (STRs with most even gene frequencies)</i>					
	D1S113					
Weighted effect	22.86	12.40	12.39	167	-16401	1.18
Pooled s.d.	15.23	3.25	17.22	870.43	9695.3	1.03
Significance, difference treatments, p	0.000	0.000	<i>0.054</i>	0.555	0.000#	0.002
Significance, difference alleles, p	0.099	0.016	0.865	0.301	0.323#	0.113
Power	0.99	1.00	0.65	0.09	1.00#	0.98
Group size for p< 0.05 and power>0.9	7	2	28	386	5#	12
	D9S218					
Weighted effect	-22.78	-11.72	-5.83	-433	-14507	-1.27
Pooled s.d.	17.24	3.68	14.58	823.69	9599.22	1.08
Significance, difference treatments, p	0.001	0.000	0.254	0.129	0.000#	0.002
Significance, difference alleles, p	0.877	0.232	0.013	0.044	0.482#	0.267
Power	0.99	1.00	0.27	0.44	0.99#	0.99
Group size for p< 0.05 and power>0.9	8	2	80	47	6#	10
	DXS312					
Weighted effect	-26,34	-11,98	-11.38	-342	-15757	-1.28
Pooled s.d.	17.85	4,20	17.55	948,53	9897.92	1.19
Significance, difference treatments, p	0.000	0.000	<i>0.062</i>	0.294	0.533#	0.003
Significance, difference alleles, p	0.569	0.787	0.699	0.861	0.000#	0.981
Power μ	1.00	1.00	0.72	0.29	1.00#	0.99
Group size for p< 0.05 and power>0.9	6	3	26	82	5#	10
# Tested on ranked data due failure in equal variances						
Group size	31-40	21-30	10-20	<10		

Table 4

	Δ OgTT AUC%	Δ Body weight %	Primary readout Δ HbA1c%*	Insulin pg/ml	Leptin pg/ml*	Fasting glucose mmol/l
Group size _y	23	23	23	23	23	23
Liraglutide (Mean $\pm$ s.d.)	-1.47 $\pm$ 22.25	-10.52 $\pm$ 4.05	-4.27 $\pm$ 18.56	1769 $\pm$ 2247	12865 $\pm$ 11078	4.82 $\pm$ 0.70
Control (Mean $\pm$ s.d.)	-16.51 $\pm$ 19.90	3.48 $\pm$ 5.21	-2.93 $\pm$ 12.02	3731 $\pm$ 3265	43131 $\pm$ 24610	6.00 $\pm$ 1.35
One-way ANOVA						
Effect	-15.05	-14.00	-1.34	-1962	-30266	-1.18
Pooled s.d.	21.11	4.67	15.64	2803	19083	1.08
Significance, difference treatments, p	0.020	0.000	0.772	0.022	0.000#	0.001
Power μ	0.66	1.00	0.06	0.64	1.00#	0.95
Group size for p< 0.05 and power>0.9	43	4	2864	44	7*	19
Two-way ANOVA						
	<i>Genome (STRs with most even gene frequencies)</i>					
	D11S112					
Weighted effect	-15.71	-13.76	-1.21	-1940	-27115	-1.26
Pooled s.d.	22.25	4.85	14.71	2871	16936	1.18
Significance, difference treatments, p	0.000	0.018	0.742	0.027	0.001#	0.000
Significance, difference alleles, p	0.969	0.492	0.065	0.741	0.920#	0.768
Power μ	0.7	1.00	0.06	0.66	1.00#	0.98
Group size for p< 0.05 and power>0.9	36	3	2575	39	6#	16
	D15S215					
Weighted effect	-14.72	-13.98	-3.46	-2119	-34290	-1.45
Pooled s.d.	20.62	4.54	16.50	2734	15209	0.80
Significance, difference treatments, p	0.027	0.000	0.506	0.024	0.000#	0.000
Significance, difference alleles, p	0.717	0.250	0.612	0.698	0.158#	0.104
Power μ	0.78	1.00	0.1	0.85	1.00#	1.00
Group size for p< 0.05 and power>0.9	31	3	351	27	4#	6
	<i>Genome (STRs with highest polymorphism)</i>					
	D9S312					
Weighted effect	-13.26	-12.60	-0.14	-1453	-26332.41	-0.95
Pooled s.d.	20.36	4.95	16.44	2377	19204	1.12
Significance, difference treatments, p	0.153	0.758	0.605	0.089	0.708#	0.721
Significance, difference alleles, p	0.025	0.000	0.990	0.058	0.000#	0.004
Power μ	0.62	1.00	0.05	0.55	1.00#	0.87
Group size for p< 0.05 and power>0.9	42	4	240197	48	8#	25
	D12S206					
Weighted effect	-13.05	-14.07	-1.87	-2635	-33355.89	-1.15
pooled SD	21.92	4.72	16.16	2768	17969.84	1.15
Significance, difference treatments, p	0.040	0.000	0.959#	0.006	0.000	0.002

	Δ OGTT AUC%	Δ Body weight %	Primary readout Δ HbA1c%*	Insulin pg/ml	Leptin pg/ml*	Fasting glucose mmol/l
Significance, difference alleles, p	0.379	0.314	0.504#	0.490	0.211	0.950
Power μ	0.5	1.00	0.09#	0.93	1.00	0.96
Group size for p< 0.05 and power>0.9	53	3	476#	21	5	19
D16S147						
Weighted effect	-14.07	-12.98	-1.58	-1932.67	-30082	-1.18
pooled SD	20.35	4.41	16.26	2676.17	20096	1.12
Significance, difference treatments, p	0.025	0.000	0.803	0.028	0.000	0.001
Significance, difference alleles, p	0.299	0.105	0.829	0.439	0.833	0.573
Power μ	0.75	1.00	0.06	0.79	0.99	0.99
Group size for p< 0.05 and power>0.9	33	3	1632	31	8	15
# Tested on ranked data due failure in equal variances						
Group size	31-40	21-30	10-20	<10		

Discussion

This study shows that inclusion of the microbiota clusters from a sequencing based characterisation as a factor in a two-way ANOVA increases power in some key parameters of the DIO model. This increased the chances of showing a liraglutide lowering impact on HbA1c in the B6NTac mice. In contrast, the effect in B6NRj mice was either absent or minimal, and considering the microbiota characterisation in a two-way ANOVA did not influence this, which strengthens the conclusion that there probably is no effect of liraglutide on HbA1c in B6NRj mice. As HbA1c in some mice seems strongly correlated to gut microbiota composition ²⁵, and the effect of liraglutide is partly mediated by induced changes in the gut microbiota ²⁶, a reasonable explanation could be that a deviating microbiota in the B6NRj makes them rather resistant to HbA1c changes. B6NTac were in contrast to B6NRj mice colonized with *Bifidobacterium* spp., while B6NRj harboured higher relative abundances of *A. muciniphila* both of which are known to control and alleviate the development of diet-induced obesity in both mice ^{27,28} and humans ^{29,30}. In B6NTac mice the family Lactobacillaceae was dominated by *Lactobacillus* spp., which were increased in abundance by the liraglutide treatment, while it was dominated by *Lactococcus* spp. in B6NRj mice. B6NTac mice had more *Bacteroides* spp., while the B6NRj mice had more *Prevotella* spp. This is equivalent to humans in which patients with a microbiota dominated by *Bacteroides* spp. have a higher risk of developing type 2 diabetes ³¹, but they are also more responsive to certain interventions compared to patients with a microbiota dominated by *Prevotella* spp. ³².

As liraglutide lowers HbA1c in humans ⁵, B6NTac compared to B6NRj seems to have a higher predictive validity. Still, the liraglutide induced lower body weight and increased glucose intolerance, known from humans ^{3,4}, could be shown in both B6NTac and B6NRj mice even in a one-way ANOVA. Inclusion of the microbiota clusters in a two-way ANOVA also increased the power of showing an impact on OGTT in the B6NTac mice.

It is critical for the organization of the mice into clusters that there is a sufficient number of reads in the sequencing for each mouse. In the calculation of group sizes and power for the multifactorial evaluation, we used the weighted average effect in the equation. Normally, group sizes and power are determined by the maximum effect, and this more conservative estimate may lead to a less optimistic but eventually more reachable estimate of group sizes.

With only 29.6% of the B6NRj STRs being 100% homozygous and a STR conformity between 91 and 97% this sub-strain may not be in full accordance with expectations to an inbred mouse. However, in 86% of the B6NRj STRs the most prevalent allele had a frequency of more than 80%. Therefore, most likely this genetic heterogeneity is more to be regarded as a trace contamination without the same impact on the parameter responses as the microbiota fluctuations. Our two-way ANOVAs did not indicate that this heterogeneity could explain the lowered or lacking effect of liraglutide on OGTT and HbA1c in the B6NRj mice.

It is an obligation whenever using animals for research to reduce the number of animals used in individual studies as one of the three 3 R's, i.e. replacement, reduction and refinement ³³. Strong microbiota impact has also been described for a number of animal models within a broad spectrum of other biomedical disciplines ³⁴, such as brain-related ³⁵, autoimmune ³⁶, allergic ³⁷ diseases, and cancer ³⁸ diseases. Therefore, our method would likely also be applicable for other models, as well.

In conclusion, the inclusion of microbiota clusters in a two-way ANOVA in the evaluation of the glucose related effects of an anti-diabetic and anti-obesity intervention in the DIO mouse model is an efficient tool for increasing power and reducing group sizes in those B6 mouse sub-strains, which have a parameter influencing microbiota.

Declarations

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Data availability

Clinico-chemical data are available from the Open Science Framework on <https://osf.io/fhgw7/>.

Microbiomic data are available at the NCBI Bioproject on <https://www.ncbi.nlm.nih.gov/bioproject/752003>.

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Authors' relationships and activities

AKH declares that he has collaborated with pharmaceutical industry and received funding from this source, as well as he is the owner of a diabetes related patent as described on <https://ivh.ku.dk/english/employees/?pure=en/persons/107126>. CHFH declares that she has received funding and collaborated with private enterprises as described on <https://ivh.ku.dk/english/employees/?pure=en/persons/306048>. DBS declares that she has received funding and collaborated with private enterprises as described on <https://ivh.ku.dk/english/employees/?pure=en/persons/114880>. PD declares he is an employee of GVG Genetic Monitoring GmbH. Other authors declares no conflicts of interests.

Contribution statement

AKH launched the idea and the hypothesis; VB and CRL performed the animal studies and the clinical assessments; PD performed the genomic characterization; LK, JLCM and DSN did the microbiomic characterization; DBS and VB set up the handling system for the mice; AKH, VB, CMJM, CRL, JLCM and LK did the statistics; AKH and DSN supervised the study. CHFH, VB and CRL performed insulin and leptin measurements; AKH, VB, CRL and CMJM wrote the first draft of the manuscript and all authors commented and accepted the manuscript.

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Figures

Figure 1

Protein: 20 % kcal, Fat: 60 % kcal, Carbohydrate: 20 % kcal) ad libitum from the age of four weeks to termination (Figure 1) monitoring food intake per cage and individual body weights Monday, Wednesday, and Friday.

Figure 2

The genetic conformity of the B6NTac mice was significantly higher than the B6NRj mice ($p < 0.0001$, Figure 2).

Supplementary Files

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