Clinical Study Protocol

Calorie Restriction Intervention Induces Enterotype-associated
BMI Loss in Nonobese Individuals

Principal Investigator	Kaiye Cai, Master
	BGI-Shenzhen
	Shenzhen, Guangdong, China
Protocol identification number	BGI-20170204 (dated 2017-3-20)
Author	Hua Zou, Bachelor
	BGI Education center
	University of Chinese Academy of Sciences
	Shenzhen, Guangdong, China
Version	0.4

Confidentiality Statement

Table of Contents

1.1 Protocol title, number, date

Title: Calorie restriction dietary intervention

Protocol number: BGI-20170204

Version: 0.4

Date: 20 March 2017

1.2 Name and address of investigator

Kaiye Cai

BGI-Shenzhen, Shenzhen, Guangdong, China

1.3 Investigator(s) signature page

The Principal Investigator is responsible for ensuring that the study is adhere to BGI-Shenzhen all regulations and guidelines during and after study completion.

I have read and understood the protocol specified above and agree on its content. I agree to conduct this study according to this protocol and guidelines in BGI-Shenzhen, the Declaration of BGI-Shenzhen, and the pertinent individual country laws/regulations and to comply with its obligations, subject to ethical and safety considerations. I shall not disclose the information contained in this protocol or any results obtained from this study without authorization.

Kaiye Cai	kaiye Cai	BGI - Shenzhen	2017.3.20
Principal Investigator	**Signature**	**Site name or ID number**	**Date**

1.4 Protocol synopsis

Protocol Number and Title: BGI-20170204 Calorie Restriction Dietary Intervention
Meals: The Normal Calorie diet at baseline and Calorie Restriction diet during Calorie Restriction period.
Phase or Type of study: Interventional
Study Objectives: The purpose of this intervention study is to evaluate the effect of calorie restriction on BMI loss, amino acid, and gut microbiota in healthy volunteers of two different enterotypes and provide useful insights for potential application of gut microbiome stratification in personalized nutrition intervention.
Study design: The study included a one-week run-in period (baseline) and a three-week CR dietary intervention period.
Sample Size: 41 individuals (24 females and 17 males aged 30 ± 6 years old) completed the whole intervention.
Eligibility Criteria: Recruited volunteers should meet all the following criteria: 1) BMI less than 28 kg/m^2 and more than 18 kg/m^2; 2) Without antibiotics in the recent 2 months; 3) Without prebiotic or probiotic supplements in the recent 2 months; 4) Without hypertension, diabetes mellitus, gastrointestinal disease and other severe auto-immune diseases; 5) Regular eating and lifestyle patterns; 6) No international travel in the recent 3 months; 7) Without prebiotic or probiotic supplements in the recent 2 months.

Volunteers meeting any of the following criteria will not be eligible to participate in this study:

1) With antibiotics in the recent 2 months;
2) Failure to comply with the experimental requirement;

2.1 Background

Calorie restriction (CR), which has the potential effect on weight loss and blood amino acids, has been demonstrated to associate with gut microbiota in human, especially in obese individuals [1, 2]. Observational studies with long-term CR found that CR resulted in weight loss and decreasing chronic disease risk factors in nonobese persons [3-6]. A 4-week CR intervention improved gut barrier integrity, reduced systemic inflammation on gut microbial diversity and BMI loss in obese women, suggesting a potential association among gut microbiota, CR and BMI [7]. Enterotype, a concept for stratifying individuals based on the gut microbiota, was first described in 2011 and was closely linked to long-term dietary patterns [8, 9]. Plenty of studies have reported that individuals have shown microbial-dependent (enterotypes, *Bacteroides* to *Prevotella* ratio) metabolic responses to the same intervention, including changes of BMI and glycemic indices [9-14]. As for the association between CR and blood amino acids, Biolo *et.al* reported changes in blood amino acids of 9 healthy nonobese men after a 2-week CR diet [15]. Despite the plenty of studies on CR as we mentioned above, to our knowledge, there are no studies for simultaneously evaluating impacts of CR on BMI, gut microbiota and blood amino acids in nonobese individuals in a single study.

2.2 Description of population to be studied, enrollment targets

Volunteer-wanted posters were propagated at the China National Gene Bank in Shenzhen from March to April 2017. A non-obese healthy volunteer was considered if his/her BMI less than 28 kg/m^2 [16]. 50 individuals met all the criteria and were recruited in this study,

and 41 individuals (24 females and 17 males aged 30 ± 6 years old) completed the whole intervention (**Table 1**). In addition, recruited volunteers should meet all the following criteria: 1) without antibiotics in the recent 2 months; 2) without prebiotic or probiotic supplements in the recent 2 months; 3) without hypertension, diabetes mellitus, gastrointestinal disease and other severe auto-immune diseases; 4) regular eating and lifestyle patterns; 5) no international travel in the recent 3 month; 6) without prebiotic or probiotic supplements in the recent 2 months. The study was approved by the institutional review board on bioethics and biosafety of BGI-Shenzhen, Shenzhen (NO. BGI-IRB 17020). All participates were fully informed of the design and purpose of this intervention study and signed a written informed consent letter.

Table 1. Cohort Description

	Cohort (Mean ± SD)
Number of subjects	41
Sex (female/male)	24/17
Age	30 ± 6
BMI (kg/m^2)	23.72 ± 2.81
Weight(kg)	64.84± 11.57

2.3 Study duration

During the first week (run-in period), all healthy volunteers consumed their usual diet and were encouraged to avoid yogurt, high-fat foods and alcohol. The CR diet was comprised of ~50% calories of a normal-calorie diet (female, 1000kcal/day; male, 1200kcal/day). It was designed with carbohydrate, fat and protein as approximately 55%, 30% and 15% of the total energy intake respectively, according to the Dietary Guidelines for Chinese

Residents (2016) and nutritionally balanced [17] and a recent large nutritional study in China [18]. Common foods in low-calorie diets such as rice, vegetables, eggs, pork and beef were prepared in this study center to control experimental variables introduced by different foods and calorie estimation errors. Traditional Chinese cooking style - boiled, stir-fried and stewed, were applied for foods. For each meal, digital scales were used to measure the nutritional and caloric values of different foods and total meals for male and female respectively. BMI, blood and fecal samples of each volunteer were collected at this study center at baseline and after the 3-week CR intervention (**Figure 1**). BMI, blood and fecal samples of each volunteer were collected at this study center at baseline and after the 3-week CR intervention.

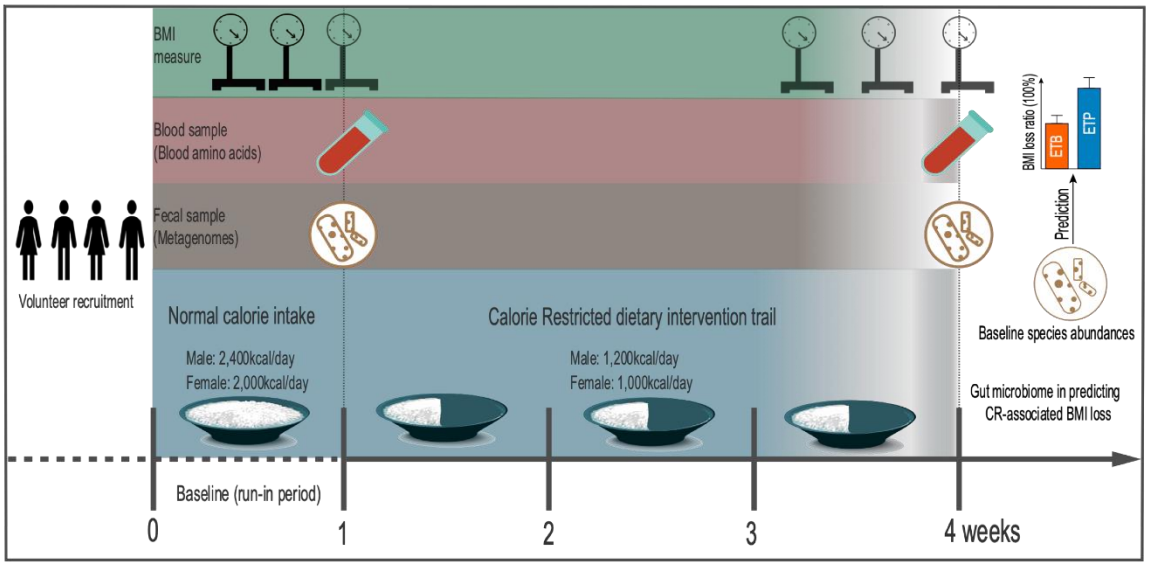

Figure 1. Overview of the experimental design. Illustration of experimental design, including a 1-week run-in period (baseline) and a 3-week calorie restriction (CR) dietary intervention trial with 50% energy deficit diet (male, ~1200Kcal/day; female, ~1000Kcal/day). BMI, fasting blood samples and fecal samples of 41 enrolled healthy subjects were collected before and after the CR intervention to assess its effects on BMI, blood amino acids and gut microbiome in two enterotype groups.

3.1 Description of objectives and purpose

The purpose of this intervention study is to evaluate the effect of calorie restriction on BMI

loss, amino acid, and gut microbiota in healthy volunteers of two different enterotypes and provide useful insights for potential application of gut microbiome stratification in personalized nutrition intervention.

4.1 Primary and secondary outcomes

The primary outcomes for this study are:

- Change in BMI of all volunteers (n=41) following calorie restriction dietary intervention
- Different changes in BMI between enterotypes *Bacteroides* (n=28) and enterotypes *Prevotella* (n=13) following calorie restriction dietary intervention

BMI was measured via the clinic scale. To avoid intra-individual variations, BMI was collected multiple times of each volunteer during the last week of the CR intervention, and the averaged BMI value was used as his/her after-intervention BMI.

The secondary outcomes for this study are:

- Change in the concentration of 31 blood amino acids of 36 volunteers following calorie restriction dietary intervention
- Different in the concentration of 31 blood amino acids between enterotypes *Bacteroides* (n=25) and enterotypes *Prevotella* (n=11) following calorie restriction dietary intervention
- Change in the gut microbiota of all the volunteers (n=41) following calorie restriction dietary intervention
- Different in the gut microbiota of between enterotypes *Bacteroides* (n=28) and enterotypes *Prevotella* (n=13) following calorie restriction dietary intervention

The concentrations of 31 amino acids and derivatives in the fasting serum samples were measured by LC-MS/MS. Fasting blood samples were collected before and after the

intervention for amino acid analysis. These blood samples were then centrifuged, and serum samples were collected and stored at -80 ℃. The concentrations of 31 amino acids and derivatives in the serum samples were then measured via ultra-high pressure liquid chromatography (UHPLC) coupled to an AB Sciex Qtrap 5500 mass spectrometry (AB Sciex, US) as described previously [19].

The gut microbial composition was determined using shotgun metagenomics sequencing of fecal DNA. Fecal samples were self-collected and then transferred to the laboratory on dry ice and kept frozen at -80°C before and after the CR intervention. Fecal DNA was extracted following a manual protocol as described previously [20]. The DNA concentration was estimated by Qubit (Invitrogen). Library construction and shotgun metagenomic sequencing were performed on qualified DNA samples based on the BGISEQ-500 protocol in the single-end 100bp mode [21]. Raw reads of BGISEQ-500 with SE100 mode were trimmed by an overall accuracy (OA) control strategy to control quality [21]. After trimming, averagely, 98.15% of the raw reads remained as high-quality reads. By using SOAP2.22 software, the high-quality reads were aligned to hg19 to remove reads from host DNA (identity $\geqslant$ 0.9). The retained clean reads were aligned to the integrated non-redundant gene catalog (IGC) using SOAP2.22 [22] and the average mapping rate and unique mapping rate were 80.18% and 65.76% respectively (identity $\geqslant$ 0.95). The relative abundance profiles of genes, genera, species and Kyoto Encyclopedia of Genes and Genomes orthologous groups (KEGG, KOs) of each sample were calculated by summing the relative abundances of their assigned IGC genes [22].

5.1 Detailed study procedures

5.1.1 Screening information

Screening information			
Step	**Descriptor**	**Details**	**Appendix**
1	Statement of Informed Consent	Each participate must read, understand and sign the Statement of Informed Consent before enrolled in the study. The Principal Investigator or designer conducting the informed consent discussion must sign the consent form. Note: the participate must be provided with a signed copy of this document.	
2	Demographics	Age, sex, height, weight, body mass index	
3	Healthy history	Record the participates' dietary and medical and international travel history	
4	Eligibility at screening	All responses to Inclusion Criteria questions must be answered "yes", and all responses to Exclusion Criteria questions must be answered "no" for the participate to be considered eligible	

5.1.2 Baseline period for recruiting participates.

Baseline information			
Step	**Descriptor**	**Details**	**Appendix**
1	Baseline clue	Participate will complete the baseline clue measures regarding their own contact dietary habits.	exclude 9 participates
2	BMI clue	Record participates' BMI.	
3	Measuring BMI	BMI was measured via the clinic scale.	
4	Collecting Fasting blood samples	Fasting blood samples were collected at the end of baseline for extracting amino acids	5 participates without fasting blood

5	Collecting fecal samples	Fecal samples were self-collected and then transferred to the laboratory on dry ice and kept frozen at -80°C at baseline.	
6	Eligibility after Baseline Examination	All responses to Inclusion Criteria questions must be answered "yes", and all responses to Exclusion Criteria questions must be answered "no" for the participate to be considered eligible.	

5.1.3 Intervention period for Calorie restriction dietary intervention

Intervention information			
Step	**Descriptor**	**Details**	**Appendix**
1	Prepare low-calorie meals	Common foods in low-calorie diets such as rice, vegetables, eggs, pork and beef were prepared in this study center to control experimental variables introduced by different foods and calorie estimation errors. Traditional Chinese cooking style - boiled, stir-fried and stewed, were applied for foods.	
2	Measuring BMI	BMI was measured via the clinic scale.	
3	Collecting Fasting blood samples	Fasting blood samples were collected for extracting amino acids after the intervention	5 participates without fasting blood
4	Collecting fecal samples	Fecal samples were self-collected and then transferred to the laboratory on dry ice and kept frozen at -80°C after the intervention	
5	Eligibility after Baseline Examination	All responses to Inclusion Criteria questions must be answered "yes", and all responses to Exclusion Criteria questions must be answered "no" for the participate to be considered eligible.	

6.1 Statistical methods to be employed

All outcomes will be presented using descriptive statistics: Wilcoxon rank-sum test is used to detect the significant differences on phenotypes, the concentrations of blood amino acids and the relative abundances of genera and species between enterotypes. Wilcoxon signed-rank test is used to detect the significant differences on phenotypes, the concentrations of blood amino acids and the relative abundances of genera and species in paired samples before and after the intervention. Permutational multivariate analysis of variance (PERMANOVA) is used to detect the association between enterotypes and the overall blood amino acid profile at baseline by using PERMANOVA with 9,999 permutations on enterotypes. Principal coordinate analysis is used to visual overall gut microbial based on the relative abundance profiles between baseline and after the intervention. Principal component analysis is used to visual overall amino acid composition based on the blood amino acid profiles between baseline and after the intervention. To investigate whether we could predict BMI loss ratio using omics features, we will perform a Lasso (Least absolute shrinkage and selection operator) regression analysis between baseline relative abundances of gut common species and the concentrations of blood amino acids (independent variables), and BMI loss ratio (dependent variables). We first normalize values of both independent and dependent variables (R, scale function). We then use the R function cv.glmnet to choose the most appropriate value for λ in the Lasso model (R glmnet package, alpha =1, family="gaussian", nfolds=10, alpha=1, nlambda=100). Here, λ is the tuning parameter ($\lambda > 0$) which controls the strength of the shrinkage of the variables. We then apply the Lasso feature selection process by shrinking the Lasso regression coefficients of non-informative variables to zero and selecting the variables of non-zero coefficients. To reduce overfitting with a limited sample size (n=41), we apply leave-one-out cross validation (LOOCV) to estimate the prediction performance of BMI loss ratio using a generalized linear model (GLM) of the selected features (creatFolds function in R caret package and the glm function in R base package). Likewise, we also use baseline BMI values for LOOCV to estimate its prediction performance for CR-associated BMI loss ratio. Spearman's rho values will be calculated between actual BMI loss ratios and the predicted values. P-value adjustment is applied for multiple hypothesis testing on the concentrations

of blood amino acids, the relative abundances of gut microbial genera and species use Benjamini-Hochberg (BH) method. R 3.5.0 will be used for all statistical analysis.

Reference:

1. Zheng X, Wang S, Jia W: **Calorie restriction and its impact on gut microbial composition and global metabolism**. *Front Med* 2018, **12**(6):634-644.
2. Schmedes MS, Yde CC, Svensson U, Håkansson J, Baby S, Bertram HC: **Impact of a 6-week very low-calorie diet and weight reduction on the serum and fecal metabolome of overweight subjects**. *European Food Research and Technology* 2015, **240**(3):583-594.
3. Redman LM, Huffman KM, Landerman LR, Pieper CF, Bain JR, Muehlbauer MJ, Stevens RD, Wenner BR, Kraus VB, Newgard CB *et al*: **Effect of caloric restriction with and without exercise on metabolic intermediates in nonobese men and women**. *J Clin Endocrinol Metab* 2011, **96**(2):E312-321.
4. Ravussin E, Redman LM, Rochon J, Das SK, Fontana L, Kraus WE, Romashkan S, Williamson DA, Meydani SN, Villareal DT *et al*: **A 2-Year Randomized Controlled Trial of Human Caloric Restriction: Feasibility and Effects on Predictors of Health Span and Longevity**. *J Gerontol A Biol Sci Med Sci* 2015, **70**(9):1097-1104.
5. Martin CK, Bhapkar M, Pittas AG, Pieper CF, Das SK, Williamson DA, Scott T, Redman LM, Stein R, Gilhooly CH *et al*: **Effect of Calorie Restriction on Mood, Quality of Life, Sleep, and Sexual Function in Healthy Nonobese Adults: The CALERIE 2 Randomized Clinical Trial**. *JAMA Intern Med* 2016, **176**(6):743-752.
6. Lefevre M, Redman LM, Heilbronn LK, Smith JV, Martin CK, Rood JC, Greenway FL, Williamson DA, Smith SR, Ravussin E *et al*: **Caloric restriction alone and with exercise improves CVD risk in healthy non-obese individuals**. *Atherosclerosis* 2009, **203**(1):206-213.
7. Ott B, Skurk T, Hastreiter L, Lagkouvardos I, Fischer S, Buttner J, Kellerer T, Clavel T, Rychlik M, Haller D *et al*: **Effect of caloric restriction on gut permeability, inflammation markers, and**

fecal microbiota in obese women. *Sci Rep* 2017, **7**(1):11955.

8. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM *et al*: **Enterotypes of the human gut microbiome**. *Nature* 2011, **473**(7346):174-180.
9. Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R *et al*: **Linking long-term dietary patterns with gut microbial enterotypes**. *Science* 2011, **334**(6052):105-108.
10. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J: **Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates**. *Gut* 2016, **65**(1):57-62.
11. Hjorth MF, Roager HM, Larsen TM, Poulsen SK, Licht TR, Bahl MI, Zohar Y, Astrup A: **Pre-treatment microbial Prevotella-to-Bacteroides ratio, determines body fat loss success during a 6-month randomized controlled diet intervention**. *Int J Obes (Lond)* 2018, **42**(3):580-583.
12. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L *et al*: **Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis**. *Nat Med* 2013, **19**(5):576-585.
13. Zeevi D, Korem T, Zmora N, Israeli D, Rothschild D, Weinberger A, Ben-Yacov O, Lador D, Avnit-Sagi T, Lotan-Pompan M *et al*: **Personalized Nutrition by Prediction of Glycemic Responses**. *Cell* 2015, **163**(5):1079-1094.
14. Korem T, Zeevi D, Zmora N, Weissbrod O, Bar N, Lotan-Pompan M, Avnit-Sagi T, Kosower N, Malka G, Rein M *et al*: **Bread Affects Clinical Parameters and Induces Gut Microbiome-Associated Personal Glycemic Responses**. *Cell Metab* 2017, **25**(6):1243-1253 e1245.
15. Biolo G, Ciocchi B, Stulle M, Bosutti A, Barazzoni R, Zanetti M, Antonione R, Lebenstedt M, Platen P, Heer M: **Calorie restriction accelerates the catabolism of lean body mass during 2 wk of bed rest**. *The American journal of clinical nutrition* 2007, **86**(2):366-372.
16. Potts M: **China's one child policy**. *BMJ* 2006, **333**(7564):361-362.
17. Wang SS, Lay S, Yu HN, Shen SR: **Dietary Guidelines for Chinese Residents (2016): comments and comparisons**. *J Zhejiang Univ Sci B* 2016, **17**(9):649-656.
18. Wan Y, Wang F, Yuan J, Li D: **Optimal dietary macronutrient distribution in China (ODMDC): a randomised controlled-feeding trial protocol**. *Asia Pac J Clin Nutr* 2017, **26**(5):972-980.
19. Jie Z, Liang S, Ding Q, Tang S, Li F, Wang D, Lin Y, Chen P, Cai K, Rao W *et al*: **A multi-omic cohort as a reference point for promoting a healthy human gut microbiome**. *bioRxiv* 2019:585893.
20. Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, Liang S, Zhang W, Guan Y, Shen D *et al*: **A metagenome-wide association study of gut microbiota in type 2 diabetes**. *Nature* 2012, **490**(7418):55-60.
21. Fang C, Zhong H, Lin Y, Chen B, Han M, Ren H, Lu H, Luber JM, Xia M, Li W *et al*: **Assessment of the cPAS-based BGISEQ-500 platform for metagenomic sequencing**. *Gigascience* 2018, **7**(3):1-8.

22. Li J, Jia H, Cai X, Zhong H, Feng Q, Sunagawa S, Arumugam M, Kultima JR, Prifti E, Nielsen T *et al*: **An integrated catalog of reference genes in the human gut microbiome**. *Nat Biotechnol* 2014, **32**(8):834-841.