Characterization of Bile Acid Pathway in Obesity

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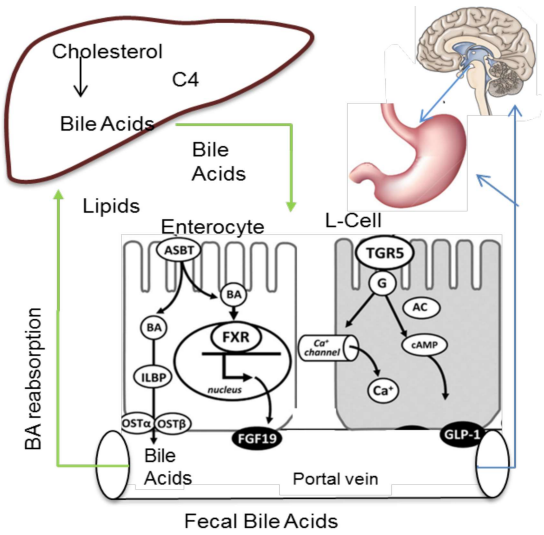
ABSTRACT

Introduction: Bariatric surgery[6] is associated with a 2-fold increase in the concentration of serum bile acids (BA)[7]. In rodents, BA delivered to the ileum induces weight loss and improves glucose homeostasis [8]. These results are mainly mediated by the BA receptor, GPBAR1 (also known as TGR5). We recently identified a *GPBAR1* gene variant associated with lower BMI and food intake[9]. It is unknown whether the bile acid pathway is altered in obesity.

Hypothesis: *Our central hypothesis is that the bile acid pathway* (fasting serum BA, C4, FGF-19, fecal 48h BA excretion) *is altered in obesity resulting in reductions in satiety-related hormones, such as FGF-19, PYY, or GLP-1.* Therefore, there is a critical need to understand the role of BAs in food intake and body weight regulation in obesity compared to health.

Aim: Thus, we propose one specific aim: To characterize the bile acid pathway, key elements of the enterohepatic bile acid regulation and incretin pathway in obesity compared to health (normal weight) at three different metabolic stages: 1) at baseline, 2) stimulated by 100 grams of fat diet (standard, validated diet for testing bile acids) and 3) on a low calorie diet with <3% weight loss.

Fig 1. Bile Acid Pathway



Experimental design: We will screen up to 45 participants to accrue a total of 38 participants [14 individuals with normal weight (BMI 20-25), 24 individuals with obesity (BMI >30)] to compare bile acid pathway, key elements of the enterohepatic bile acid regulation and incretin pathway at three different metabolic stages: 1) at baseline, 2) on a 100 grams of fat diet, and 3) on a low calorie, low fat diet. Participants will be on each diet for 7 days prior to collection of samples; with one week on regular diet between the high fat/calorie diet and the low calorie diet. Fasting and postprandial blood samples will be used to analyze, metabolomics, PYY, GLP-1, FGF-19, C4, BA, and short-chain fatty acids (SCFA) on test days. Saliva sample will be collected for GI peptides and metabolomics. Visual analog scale (VAS) ratings of appetite and satiation will be recorded during the day (on the same days) every 15 minutes for the first 90 minutes, then every 30 minutes. On day 5 and 6 (48 hours), stool from every bowel movement will be collected for fecal BA, SCFA and microbiome. Participants will repeat the stool collection and above studies for a total of three times. On day 5 of 1st, 2nd and 4th week, participants will undergo a flexible sigmoidoscopy with biopsies.

Expected outcome: It is anticipated that the data acquired will lead to future hypothesis-testing studies of the role and interaction of the BA pathway, microbiome and incretins that are critical to circumvent weight regain.

INTRODUCTION

Obesity prevalence continues to increase worldwide[1] and, in the United States, 69% of adults are overweight or obese[2]. Estimated costs to the healthcare system are more than $190 billion annually – 10 percent of the total health budget – according to the Centers for Disease Control and Prevention (CDC 2012). Obesity affects almost every organ system in the body and increases the risk of premature mortality[10]. It is estimated that a man in his twenties with a BMI over 45 will have a 22% reduction (13 years) in life expectancy[11]. Increased severity of obesity correlates with a higher prevalence of the associated co-morbidities. Hence, obesity increases the risk of numerous diseases including type 2 diabetes mellitus (T2DM), hypertension, dyslipidemia, cardiovascular disease, and cancer.

Currently, the most effective **treatment for obesity**, metabolic syndrome and T2DM is bariatric surgery[12]. The efficacy of bariatric surgery in obesity and T2DM treatment, when compared to caloric restriction, demonstrates that the mechanism on weight loss and reversal of insulin resistance and hyperglycemia exceeds the effect of diet restriction seen in duodenal switch or Roux-en Y gastric bypass (RYGB). However, bariatric surgery is invasive with high mortality and morbidity[13] and cannot be considered a public health remedy to the current obesity epidemic. On the contrary, obesity treatment with intense lifestyle interventions (ILI) (defined as low calorie diet, physical activity, and behavioral therapy) result in a mild weight loss (approximately 3-5%) within the first 12 weeks followed by a slow and gradual weight regain and the individual presumption of "failure"[3]. Physiological changes or adaptations to weight loss have a major effect on the failure of weight loss maintenance, thus impeding the health benefits associated with weight loss[4,5]. These adaptations – "pro-orexigenic" – are presumed to be secondary to increased appetite, decreased satiation and decreased thermogenesis.

Recent advances in the **understanding of bariatric surgery** suggest that bile acids play a role in the surgery-related improvement of diabetic profile. RYGB is associated with doubling of the concentration of serum bile acids, and an increase in serum adiponectin and GLP-1. In rodents, BA delivered to the ileum induces weight loss and improves glucose homeostasis[8]. These results are mainly mediated by the BA receptor, GPBAR1 (also known as TGR5)[7,8,14,15]. GPBAR1 stimulation results in a rapid increase in incretins, reduced appetite and improvement in insulin sensitivity[16,17].

Bile acids (BAs) are steroid-derived detergent molecules that are excreted by the liver and are responsible for emulsification of lipids in the small intestine, aiding in lipid digestion and absorption[18]. The key components of the BA pathway (Fig 1) are: a) The hepatocyte synthesizes bile from cholesterol; 7-alpha-hydroxy-4-cholesten-3-one (C4) is a serum marker for bile acid synthesis in humans[19]. C4, a precursor of cholic acid in the classic pathway of bile acid synthesis from cholesterol, correlates with the time-limiting enzymatic step catalyzed by 7-alpha-hydroxylase[19]. b) Bile acids are excreted into the duodenum and form simple micelles or micelles incorporating phospholipids to absorb cholesterol through the brush border membrane of the small intestine. c) In the terminal ileum, bile is reuptaken by the apical NA^+-dependent bile salt transporter [ASBT or also called ileal BA transporter (IBAT)]. In the enterocyte, BA activates farnesoid X receptor (FXR) to synthesize fibroblast growth factor-19 (FGF-19), which initiates a negative feedback to decrease the synthesis of BA. d) Less than 5% of BA is not reabsorbed and is deconjucated and dehydroxylated by colonic bacteria into secondary BA and then excreted in the stool. e) The liver captures and recirculates more than 99% of bile acid, suggesting that "serum" bile acid is a fraction of the BA in the enterohepatic circulation. f) Additionally, BA activates the

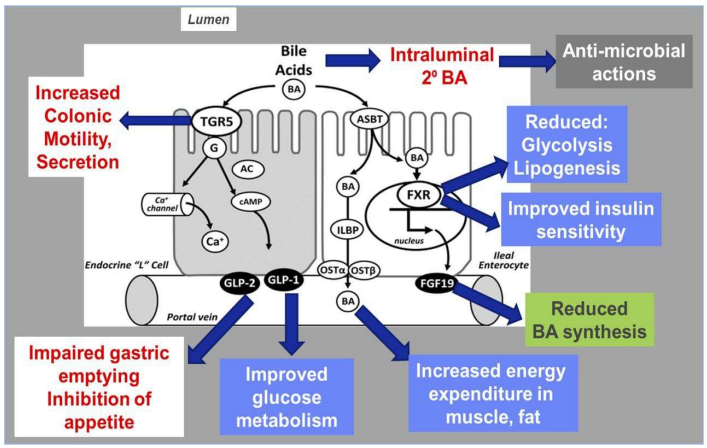


Fig 2. Summary of actions of BAs in gut mucosa [18]

GPBAR1 receptor in the enteroendocrine L-cell which secretes GLP-1 to regulate food intake and glucose homeostasis. Interestingly, each step of the BA pathway can be measured in humans, non-invasively (Fig 1)[18]. Recently, we identified a *GPBAR1* gene variant associated with lower BMI, higher satiation (reduced food intake) and higher GLP-1 concentration; and demonstrated that obesity is associated with decreased fasting fibroblast growth factor 19 (FGF-19)[9], a surrogate of ileal BA reabsorption (Fig 2). Interestingly, in mice, the BA effect on GLP-1 secretion is blunted in absence of GPBAR1 [20]. The synthesis, secretion and regulation of incretin hormones GLP-1, PYY and OXM are controlled by enteroendocrine L-cells in the small intestine and colon.

In summary, it is unknown whether 1) the bile acid pathway is altered in obesity and/or 2) it adapts to weight loss induced by diet only. Thus, there is a ***critical need to understand*** the role of BAs in food intake, absorption of lipids and body weight regulation in obesity compared to health. ***Our central hypothesis is that the bile acid pathway*** **(fasting serum BA, C4, FGF-19, fecal 48h BA excretion)** ***is altered in obesity resulting in reductions in satiety-related hormones, such as FGF-19, PYY, or GLP-1****.* Thus, there is a critical need to understand the role of BAs in food intake and body weight regulation in obesity compared to health.

The understanding of this mechanism will enhance our knowledge about the role of bile acid metabolism and pathway in obesity. Specifically, we hypothesize that the BA pathway –serum BA, FGF-19, GLP-1, PYY, and C4; fecal BA, and fecal microbiome – adapt to a pro-orexigenic mode after diet-induced weight loss to enhance food intake.

INNOVATION

The role of the bile acid pathway in obesity and the metabolic adaptation to weight loss: The outcome of these studies will contribute to the essential understanding of the role of the bile acid pathway in weight loss in humans. Usually, the components of the bile acid pathway are studied independently based on the strengths of each laboratory; for example, study of only "serum" BA". Here, relying on support from the grant mentor, Dr. Michael Camilleri, we have assembled a team to study the whole BA pathway in humans under different interventions. The results will have a robust impact on the BA field and potential applications to obesity and diabetes treatment. Furthermore, the *in-unison* study will contribute to the reveal of a real-time sequence of events regulating or modulating the BA pathways and enterohepatic circulation. This type of analysis in BA physiology and obesity is novel and critically needed, especially with the notion that many BA pathway modulators are under development.

APPROACH

Our central hypothesis is that the bile acid pathway **(fasting serum BA, C4, FGF-19, fecal 48h BA excretion)** ***is altered in obesity resulting in reductions in satiety-related hormones, such as FGF-19, PYY, or GLP-1***. This central hypothesis will be investigated in one specific aim: To characterize the bile acid pathway, key elements of the enterohepatic bile acid regulation and incretin pathway in obesity compare to health (normal weight) at three different metabolic stages: 1) at baseline, and either 2) stimulated by 100 grams of fat diet (standard, validated diet for testing bile acids) or 3) on a low calorie diet with <3% weight loss.

Rationale: Recent advances on the **understanding of bariatric surgery** suggest that bile acids play a role in the surgery-related improvement of diabetes. RYGB is associated with doubling of the concentration of serum bile acids, and an increase in serum adiponectin and GLP-1. In rodents, BA delivered to the ileum induces weight loss and improves glucose homeostasis [8]. These results are mainly mediated by the BA receptor, GPBAR1 (also known as TGR5)[7,8,14,15]. GPBAR1 stimulation results in a rapid increase in incretins, reduced appetite and improvement in insulin sensitivity[16,17]. However, the role of bile acids and the key components of the bile acid pathway and enterohepatic circulation have not been elucidated in individuals with obesity.
Preliminary data: We recently identified a GPBAR1 gene variant associated with lower BMI, higher satiation (reduced food intake) and higher GLP-1 concentration; and demonstrated that obesity is associated with decreased fasting fibroblast growth factor 19 (FGF-19)[9], a surrogate of ileal BA reabsorption. Interestingly, in mice, the BA effect on GLP-1 secretion is blunted in the absence of GPBAR1[20].

Aim: 1a) To characterize the bile acid pathway, key elements of the enterohepatic bile acid regulation and incretin pathway in obesity compared to health (normal weight) at three different metabolic stages: 1) at

baseline, 2) stimulated by 100 grams of fat diet (standard, validated diet for testing bile acids) and 3) on a low calorie diet with <3% weight loss.

Experimental design: We screen up to 45 participants and will study a total of 38 participants[14 individuals with a normal weight (BMI 18.5-25), 24 individuals with obesity (BMI >30)] to compare bile acid pathway, key elements of the enterohepatic bile acid regulation and incretin pathway in obesity compare to health (normal weight) at three different metabolic stages: 1) at baseline, 2) on a 100 grams of fat diet, or 3) on a low calorie, low fat diet. Participants will be on each diet for one week prior to collection of samples; with one week on regular diet between the high fat/high calorie diet and the low calorie diet. Diet will be provided by the metabolic kitchen. Participants will present after an overnight fast to the CRTU on day 5 and day 7 for testing. On day 5 participants will undergo a flexible sigmoidoscopy procedure from which a biopsy will be obtained. A bowel cleansing with water enema will be given by a CRTU nurse before the procedure. On day 7, participants will consume their "scheduled meal" and blood samples will be collected at -15, 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360 minutes. Fasting and postprandial blood samples will be used to analyze, metabolomics, PYY, GLP-1, FGF-19, C4, BA, and short-chain fatty acids (SCFA). Plasma will be collected to analyze EV. Fasting saliva sample will be collected for GI peptide and metabolomics. VAS ratings of appetite and satiation will be recorded during the day every 15 minutes for the first 90 minutes, then every 30 minutes. Prior to discharge from the CRTU, participants will meet with study staff to discuss the following week's diet. On day 5 and 6 (48 hours), stool from every bowel movement will be collected for fecal BA, SCFA and microbiome. Participants will repeat the studies for a total of three times.

Participants (Inclusion and exclusion criteria): Through the use of a natural language processing tool and the electronic medical records, we have an established database of ~1000 patients with obesity and no other comorbidities who reside within ~100 miles, and have been evaluated at Mayo Clinic in Rochester, Minnesota. Many of them have participated in our previous studies[9,22]. The specific inclusion criteria for aim 1a includes: individuals with normal weight (BMI 18.5-25), or obesity (BMI >30); these will be otherwise healthy individuals with no unstable psychiatric disease and not currently on treatment for cardiac, pulmonary, gastrointestinal, hepatic, renal, hematological, neurological, or endocrine disorders.

	Week 1								Week 2							Week 3							Week 4						
Days	0	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Diet	Screen visit	Baseline							High calorie / High fat							Baseline							Low calorie / low fat						
Stool BA						X	X						X	X														X	X	
C4-GI peptides								X							X														X
Flex Sig						X							X														X		

METHODS

Participants: Eligible subjects will be invited to participate by letter. All subjects will be given a verbal explanation of the study, provided time to read and study the written consent form and its information, given opportunities to ask questions and a copy of the consent form. Participants will be informed of their right to withdraw from the study at any time without prejudice to their clinical management now or in the future. Consent will be sought by one of the medical doctor investigators or the study coordinator, and consent will be documented by the participant's signature on the consent form. All recruitment or contact information will be approved by Mayo's Institutional Review Board.

The common inclusion criteria include:

a) Age: 18-65 years.
b) Gender: Men or women. Women of childbearing potential will have negative pregnancy tests within 48 hours of enrolment.
c) BMI: 18.5-25, or ≥ 30

The common exclusion criteria include:

a) History of abdominal surgery including cholecystectomy and other than appendectomy, Caesarian section or tubal ligation.
b) Positive history of chronic gastrointestinal diseases, or systemic disease that could affect gastrointestinal motility, or use of medications that may alter gastrointestinal motility, appetite or absorption, e.g., orlistat, phentermine.
c) Significant untreated psychiatric dysfunction based upon screening with the Hospital Anxiety and Depression Inventory (HAD), a self-administered alcoholism screening test (AUDIT-C) and the Questionnaire on Eating and Weight Patterns (binge eating disorders and bulimia). If such a dysfunction is identified by a HAD score >11 or difficulties with substance or eating disorders, the participant will be excluded and given a referral letter to his/her primary care doctor for further appraisal and follow-up. The AUDIT-C is a 3-item alcohol screening questionnaire that reliably identifies participants who are hazardous alcohol drinkers or have active alcohol use disorders. In men, a score of 4 or more is above the recommended limits will be reviewed by study personnel. In women, a score of 3 or more is above the recommended limits will be reviewed by study personnel. However when the points are above recommended limits, the provider will review the patient's alcohol intake over the past few months to confirm accuracy and determine study eligibility.
d) Intake of medication, whether prescribed or over the counter (except multivitamins), within 7 days of the study. Exceptions are birth control pill, estrogen replacement therapy, and thyroxin replacement therapy.

Participant activities:

a) if recruited for the investigation participants will be asked to refrain from donating blood; refrain from participating in other research studies; avoid taking any additional over the counter or prescription medications or herbal supplements that have not been reviewed and approved by the physician or the study coordinator until the study has been completed

Screening Visit: Height, weight, waist measurement, hip measurement, and vital signs (pulse, blood pressure, respiration rate, and temperature) will be measured. A physical exam and medical history with be completed with the study physician. A urine sample will be collected for a pregnancy test, if applicable. The results from the test must be negative for participant to continue with the study. Subjects will also meet with a dietitian to review food preference and allergies.

Visit 2, 4, 7: These visits occur on 'day 5' of each week. An un-sedated flexible sigmoidoscopy with prior tap water enema will be performed, with a biopsy of the sigmoid colon (n=8) and rectum (n=8).

Visit 3, 5, 8: These visits are 'day 7' of each week. Vital sign, height, weight, waist circumference and hip circumference will be taken. Subjects will be fed a meal that matches the macronutrient make-up of the week. During visit 3, the baseline week, they will be fed a standard CRTU breakfast and lunch. Fasting saliva and blood will be drawn as listed above. VAS ratings of appetite and satiation will be recorded. Before leaving on visits 3 and 5, participants will meet with the study staff.

Visit 6: This visit will occur during week 3. It involves meeting with a study staff regarding week 4 of the investigational study diet.

Diet: During weeks 1 (baseline) and 3 (stabilization), participants will have a daily food diary; The total daily intake of calories and macronutrients will be calculated by our team's registered dietitian. For weeks 2 and 4, we will provide every meal to the participants. The 100 grams of fat diet will consist of 2700 kcal, 100g total fat (33%), 300mg cholesterol, 350g carbohydrates (52%), and 100g protein (15%). The low calorie, low fat diet will consist of 1200 kcal, 26g fat (20%), 78mg cholesterol, 150g carbohydrates (50%), and 100g protein (30%). Meals will be provided by the metabolic kitchen. All uneaten food will be returned to the metabolic kitchen and weighed to determine exact food consumption.

Blood Samples: Fasting and postprandial blood samples will be collected by standard techniques and stored in a -70°C freezer in our laboratory. DNA will be obtained and stored for future studies.

Saliva Samples: fasting saliva samples will be collected by standard techniques and stored in a -70°C freezer in our laboratory. Future studies will include metabolomics and GI peptides.

Collection of Fecal Sample for Fecal Organic Acids: HPLC-tandem MS will be utilized to identify, characterize and quantify total and individual fecal BAs. The 48-hour stool collection will be weighed and divided into aliquots. Samples will then be analyzed for total fat (routine Van de Kamer method at Mayo Medical Laboratory), total BAs, and primary and secondary BAs [by LC/MS as previously described by our group (Shin, 2013b)]. Additional stool samples will be stored for future microbiome studies.

Serum 7α-hydroxy-4-cholesten-3-one (C4) will be measured based on the method adapted from Galman et al.[19] and using HPLC-tandem mass spectrometry[37-39].
Serum FGF-19 levels will be measured by enzyme-linked immunosorbent assay (FGF-19 Quantikine ELISA Kit; R&D Systems, Minneapolis, MN).
Plasma gastrointestinal hormones (GLP-1 and PYY) levels will be measured by radioimmunoassay (Linco Research, Inc., St. Charles, MO).
Metabolomics: We will perform quantitative, targeted metabolomics of salient classes of compounds and untargeted metabolomics in plasma and saliva samples using mass spectrometry. These assays are well-established, validated, and routinely performed in the Mayo Clinic Metabolomics Core Laboratory.
Microbiome: Stool aliquots will be frozen at -70°C freezer in our laboratory for future microbiome studies.
Plasma extracellular vesicales will be analyzed based on the method adapted from Witwer et al. and stored in a -70°C freezer
Flexible sigmoidoscopy will be performed in the CRTU, with a prior tap water enema by the CRTU nurse. Using standard biopsy forceps, 16 mucosal biopsies will be obtained each from the sigmoid colon and the rectum. Eight biopsies will provide adequate tissue for microbial DNA extraction. If any abnormality is seen on sigmoidoscopy, additional biopsies will be obtained for histology. The colonic mucosal microbiota will be compared to that evaluated with a rectal catheter.

STATISTICAL ANALYSIS

Endpoints: *Primary endpoint:* Fecal BA excretion in obesity vs. health and the change from baseline with high fat/high calorie diet vs. low calorie diet. *Secondary endpoints:* (a) fecal individual BA and SCFA excretion; (b) fasting serum C4 and FGF19; (c) serum BA; and (d) serum fasting, peak and AUC postprandial FGF-19, GLP-1 and PYY.
Statistical analysis: The proposed analyses are based on analysis of covariance models, with the responses being each variable compared to baseline. The covariates to be considered include gender and BMI.
Sample size: In healthy volunteers, the normal value for fecal BA is 957 ± 185 micromoles/48hours (SEM)[40]. The table below summarizes data for primary endpoint considering the mean difference between two groups (healthy vs. obesity) and difference between baseline to high fat vs. low fat diet in each group (healthy vs. obesity), n=8 per group; and estimates the effect size detectable with 80% power based on a two sample t-test at a two-sided α level of 0.05. We will accrue 38 participants to achieve the above given n value, assuming a 20% dropout rate.

Response	Mean	SD	Predicted differences [%(absolute #)] n=8/group
Fecal BA, μmoles/48hours	957	185	1) Patient with obesity compared to health: 25% (258 μmoles/48h) 2) Baseline compared to high fat vs. low fat diet: 24% (233 μmoles/48h)

SIGNIFICANCE

The proposed study will provide an understanding of the role of BA and the BA pathway in obesity vs. health influenced by different metabolic states, or weight loss interventions. The expected adaptations seen in the BA pathway and incretin response may suggest novel mechanism to enhance and maintain weight loss in obesity. Additionally, the strength of our results will be based on studying the "whole" BA pathway *in-unison* and the data gathered will support the next aims and future studies in BA and obesity.

Potential Pitfalls, precautions taken and ancillary studies

Feasibility: The study team, including the co-PI, has a significant experience in clinical research and has validated the described techniques. Furthermore, the team frequently recruits and retains participants for similar studies. *Precautions:* Studies will be performed under IRB guidance. *Ancillary studies:* DNA will be stored for future gene variation studies and fecal samples will be stored for future microbiome studies.