COVER PAGE FOR PROTOCOL AND STATISTICAL ANALYSIS PLAN

Official Study Title: Mechanism of microbiome-induced insulin resistance in humans (Aim 1)

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1. RESEARCH PLAN

A) SPECIFIC AIMS

Insulin resistance in peripheral tissues (*i.e.* skeletal muscle) is one of the earliest and most significant abnormalities in the pathogenesis of type 2 diabetes (T2DM). However, the molecular basis for the insulin resistance of T2DM is not fully understood. The human gut hosts an enormous number and variety of microorganisms, including at least 10^{14} bacteria. An increasing number of animal studies suggest that this microbial "organ", also known as the microbiome, is a causative factor in the insulin resistance of obesity and T2DM, and may represent an entirely new therapeutic target against these conditions. These studies suggest that high fat ingestion, obesity, and T2DM, favor a microbiome profile which enhances production and gastrointestinal permeability of lipopolysaccharide *(i.e.* metabolic endotoxemia). Lipopolysaccharide (LPS or endotoxin) is a component of the outer membrane of gram negative bacteria cell walls which induces an inflammatory response by activating the cell surface receptor toll-like receptor-4 (TLR4). Despite accumulating evidence from animal studies suggesting that intestinal microbiota and metabolic endotoxemia could play a key role in the pathogenesis of insulin resistance, obesity, and T2DM, the relevance of the intestinal microbiome and metabolic endotoxemia on human metabolic disease remains unknown. Thus, the goals of this study are to (i) determine the role that intestinal microbiota and metabolic endotoxemia play in the pathogenesis of insulin resistance and T2DM in humans; and (ii) examine the cellular mechanisms by which metabolic endotoxemia causes insulin resistance in human muscle. Specifically, we propose the following Specific Aims:

Aim 1) To determine the effect of high fat consumption on the intestinal microbiome, metabolic endotoxemia, and insulin action, in lean normal glucose tolerant subjects. We will test the hypothesis that a high fat diet given to lean, normal glucose tolerant subjects will modify gut microbiome composition and enhance intestinal permeability, which will increase plasma LPS concentration, induce an inflammatory response in peripheral tissues (skeletal muscle), and impair insulin signaling and sensitivity. Also we will test the hypothesis that the inflammatory response and insulin resistance caused by high fat ingestion can be ameliorated by administering (i) a synbiotic (bifidobacterium and oligofructose) which protects the intestinal epithelial barrier and decreases intestinal translocation of LPS; and (ii) sevelamer, an agent which sequesters LPS in the gastrointestinal tract limiting its translocation into the circulation.

Aim 2) To determine whether microbiome modulation and an experimental reduction in plasma LPS concentration improve inflammation and insulin action in insulin resistant (obese and T2DM) subjects. In this Aim we will test the hypothesis that lowering LPS concentration in the circulation will improve systemic (muscle) inflammation and glucose metabolism in insulin resistant (obese and T2DM) subjects by protecting the intestinal barrier with a synbiotic (bifidobacterium and oligofructose) or by sequestering LPS in the gastrointestinal lumen with sevelamer.

Aim 3) To determine whether the LPS present in the sera from lean high fat fed, obese, and T2DM subjects mediates the insulin resistance, and whether this effect is mediated via TLR4. In this Aim we will test the hypotheses that sera from lean high fat fed, obese, and T2DM subjects (from Aims 1 and 2) will induce an inflammatory response (activation of inflammatory signaling cascades) and impair insulin action in cultured primary myotubes obtained from lean individuals, and that the sera from these groups (lean on high fat diet, obese, and T2DM), when treated with LPS-reducing agents (synbiotic and sevelamer), will blunt the inflammatory response and insulin resistance. We will also specifically neutralize LPS in the sera from these subjects (lean on high fat diet, obese, and T2DM) using polymixin B, and will block TLR4 signaling in the myotubes with short hairpin (sh)RNA and a pharmacologic inhibitor (TAK242), to test the hypothesis that the inflammatory state and insulin resistance caused by the sera occurs via an LPS-TLR4-mediated mechanism.

B. BACKGROUND AND SIGNIFICANCE

The intestinal microbiota and metabolism. A connection between the intestinal microbiota, energy homeostasis, and metabolism, and its role in the pathogenesis of obesity-related metabolic disorders, are increasingly being recognized. The human gut hosts an enormous number and variety of microorganisms (1). The genome size of this microbial "organ", also known as the microbiome, exceeds the size of the human (nuclear) genome by two orders of magnitude, providing additional features and contributing to human

physiological and metabolic diversity (2, 3). In particular, the intestinal microbiota has been considered to be a possible causative factor in metabolic disorders such as insulin resistance and dyslipidemia, as well as a therapeutic target against these conditions (3). Bacteria with beneficial effects such as Bifidobacteria and Bacteroidetes are decreased in obese and type 2 diabetic subjects (4, 5). In addition, germ-free mice transplanted with intestinal microbiota derived from human fecal material have increased body fat relative to mice that do not have the humanized microbiome (6). Moreover, transplantation of microbiota from humans fed a high fat diet (as opposed to low fat diet) into mice leads to obesity (6). These results suggest that intestinal microbiome composition has a significant effect on whole body energy homeostasis and fuel metabolism.

Mechanism of glucose metabolism regulation by the microbiome. The mechanism by which gut microbiota affect glucose metabolism and insulin action is not clear. One hypothesis suggests that microbiome profiles enriched in certain bacterial groups/genus lead to enhanced production and gastrointestinal permeability of lipopolysaccharide *(i.e.* metabolic endotoxemia). Lipopolysaccharide (LPS or endotoxin) is a component of the outer membrane of gram negative bacteria cell walls, which induces an inflammatory response by activating toll-like receptor (TLR)4. Other potential mechanisms by which intestinal microflora can affect glucose metabolism in the host is through the production of substances which alter intestinal wall integrity (7, 8), energy extraction from food (9), energy expenditure, and appetite (10-13). Activation of TLR5 by bacterial flagellin is another mechanism by which intestinal microbioma can affect glucose metabolism (14). Bacterial peptidoglycans, which bind to and activate TLR2, have also been found to impair insulin action in muscle cells (15). In addition, the gut microbiota affects the host's capacity to extract calories from otherwise indigestible polysaccharides (16-18).

TLR4 and downstream signaling. TLRs are a family of pattern recognition receptors that generate immune responses to pathogens by activating a cascade of pro-inflammatory events (6). TLR4, one of the best characterized TLRs, is highly expressed on the surface of immune cells, such as monocytes and macrophages, and insulin target cells, such as myocytes, adipocytes and hepatocytes. LPS is a potent agonist of TLR4 (7). A soluble protein called LPS binding protein (LBP) binds LPS and regulates LPS-dependent responses. Saturated free fatty acids (FFA) are also agonists of TLR4 (9, 10). TLR4 ligand binding leads to the activation of several pro-inflammatory kinases including the mitogen-activated protein kinases (MAPK); c-Jun N-terminal kinase (JNK), p38 and extracellular-signal related kinase (ERK), and the IκB kinase (IKK) complex, which results in the activation of transcription factors such as nuclear factor-κB (NFκB), c-jun, and AP-1 (12) and increased gene transcription of proteins such as tumor necrosis factor-α (TNFα), inducible nitric oxide synthase (iNOS), monocyte chemotactic protein (MCP)-1 and interleukin (IL)-6 (16, 17). Genetic or chemical inhibition of either JNK or IKKβ/NFκB can improve insulin sensitivity (18-20). Moreover, disrupted expression of TLR4 protects mice from developing inflammation and insulin resistance in response to chronic changes in dietary intake of fat (21, 22). These findings provide compelling evidence that insulin action is negatively regulated by TLR4.

Metabolic endotoxemia and insulin resistance. As mentioned above, accumulating evidence suggests that circulating intestinal-generated LPS *(i.e.* metabolic endotoxemia) could play an important role in the pathogenesis of insulin resistance. This emerging hypothesis is of particular interest to our laboratory since we recently observed that obese and T2DM subjects have elevated LPS concentration in plasma (Fig. 2), in association with decreased insulin sensitivity (M) ($r=-0.46$, $P<0.005$). In support of our findings, ingestion of a high fat meal significantly increases circulating LPS level (19, 20). Unlike other situations of endotoxemia, such as sepsis where plasma LPS level may increase ~100-fold (21), in situations of insulin resistance LPS levels are only modestly increased by 2-3 fold, when compared to control subjects (22). Cani et al reported that, in mice, a high fat diet leads to increased plasma LPS concentration in association with alterations in the intestinal microbiota composition (22, 23). Modulation of the intestinal microbiota composition with prebiotics reduced plasma LPS concentration and improved glucose intolerance in obese *ob/ob* and high fat fed mice (7, 23-26). The mechanism by which high fat diets increase plasma LPS concentration is not clear, but postulated mechanisms involve changes in the composition of the microbiome (7, 23-26), lipid- and or micriobial-induced damage of the intestinal epithelial barrier *(i.e.* increased permeability) (24), and post-prandial chylomicron formation which promote transport of LPS (27) (Fig. 1).

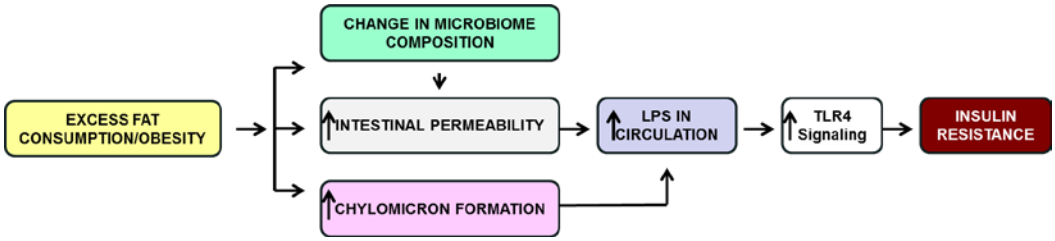


Figure 1. Link between fat consumption/obesity and insulin resistance

In addition to the mouse studies described above, the role of LPS in triggering systemic inflammation also has been evaluated in healthy human subjects. Mehta et al. found that a low dose intravenous bolus of LPS increased TNFα and IL-6 concentrations and decreased insulin sensitivity (28). Ghanim et al reported that a high calorie diet (high in fat and carbohydrates) caused a significant elevation of LPS in plasma, accompanied by increased mononuclear cell expression of TLR4 and NFκB (29). The increases in LPS, TLR4 and NFκB were absent after an American Heart Association-type meal, low in fat (29). The same group performed a study to dissect the effect of high calorie diet (carbohydrate vs. fat), and found that a diet rich in fat, but not in carbohydrates, increased plasma LPS concentration and activated NFκB in circulating mononuclear cells (30). In addition, a dietary survey conducted in 1015 subjects demonstrated that a high intake of fat, but not carbohydrates or protein, was directly associated with plasma LPS concentration (31). Collectively, the results described above suggest that caloric intake and nutrient composition (high fat) affect LPS concentrations, and that metabolic endotoxemia may play a key role in the pathogenesis of insulin resistance and T2DM. However, the relevance of the intestinal microbiome and metabolic endotoxemia on human metabolic disease remains unclear. Thus, the goals of this study are to (i) determine the role that intestinal microbiota and metabolic endotoxemia play in the pathogenesis of insulin resistance and T2DM in humans; and (ii) examine the molecular basis by which metabolic endotoxemia causes insulin resistance. The results from this project could help to develop new interventions against insulin resistance and T2DM.

C) PRELIMINARY RESULTS

Plasma LPS concentration is increased in obese and T2DM individuals. We measured plasma LPS in 11 lean (BMI=24.6 kg/m^2, age=41 y, fasting glucose=93 mg/dl, M value=11.3 mg/kg.min), 9 obese, nondiabetic (BMI=31.5, age=47, glucose=92, M=8.1), and 10 obese, T2DM (BMI=34.2, age=48, glucose=145, M=4.2) subjects. Insulin sensitivity (M) was measured with a hyperinsulinemic (80 mU/m^2.min) clamp. As shown in Fig. 2, the plasma LPS concentration was significantly elevated in the obese and T2DM subjects. It is important to note that a normal value for LPS concentration in human plasma has not been established and there is enormous variability in the concentration reported in normal subjects, from the 0.01 (32, 33) to the 10 EU/ml (34) range, depending on the method employed, reagent manufacturer, and population studied.

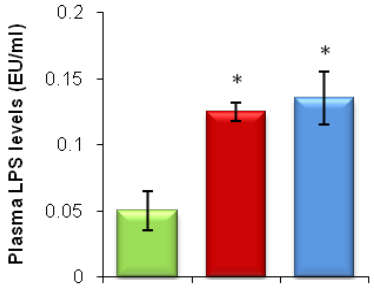
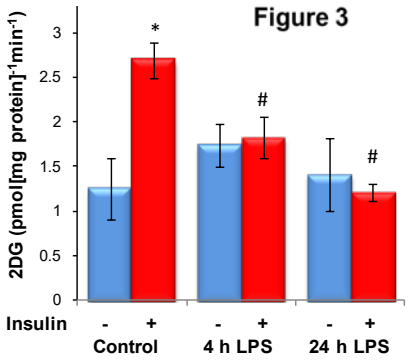
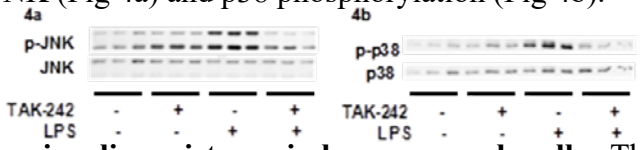


Fig 2. LPS plasma concentration

LPS causes insulin resistance in muscle *in vitro*. We pre-treated cultured L6 (rat) myotubes with 100 ng/ml LPS for 4 and 24 h, followed by stimulation with 100 nM insulin for 20 min. LPS significantly reduced insulin-stimulated glucose (2DG) transport (Fig. 3).

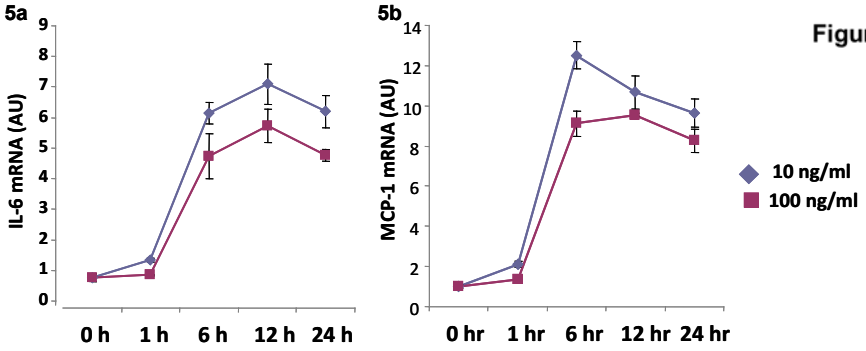


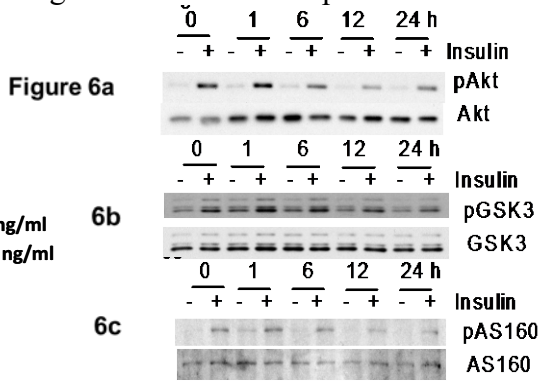
Pharmacologic inhibition of TLR4 protects against LPS-induced inflammation *in vitro*. We examined the effect of TAK242 (Takeda Pharmaceuticals), an inhibitor of TLR4, on LPS-induced inflammation. L6 myotubes were pre-incubated with/without 1μM TAK242 for 1 h, prior to stimulation with 100 ng/ml LPS for 1h. TAK242 completely blocked LPS-induced JNK (Fig 4a) and p38 phosphorylation (Fig 4b).



LPS causes insulin resistance in human muscle cells. The data shown above clearly indicate that LPS impairs insulin action in rodent muscle cells. Also we have tested the effect of

LPS in a primary human muscle cell culture system. Myotubes derived from lean, nondiabetic subjects were treated with 10 and 100 ng/ml LPS for 1, 6, 12 and 24 h. As shown in Fig. 5, LPS caused a robust increase in inflammatory gene expression in a dose- and time-dependent manner. To determine the metabolic consequence of the inflammatory response caused by LPS, human myotubes were treated with 100 ng/ml LPS for 1, 6, 12 and 24 h, followed by insulin stimulation (100 nM for 20 min). LPS reduced Akt (Fig. 6a), GSK3 (6b) and AS160 (6c) phosphorylation. Furthermore, pre-treatment with 100 ng/ml LPS for 12 h impaired insulin-stimulated glucose transport (Fig. 7).





We have conducted experiments in muscle cells using lower (more clinically-relevant) LPS concentrations, and found that doses as low as 0.01 and 0.1 ng/ml can impair insulin-stimulated glucose transport (Fig. 8).

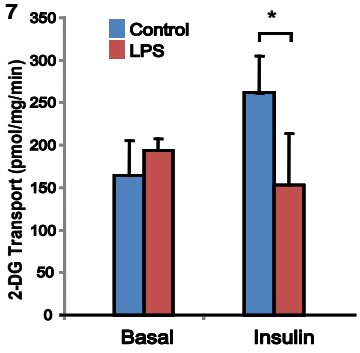
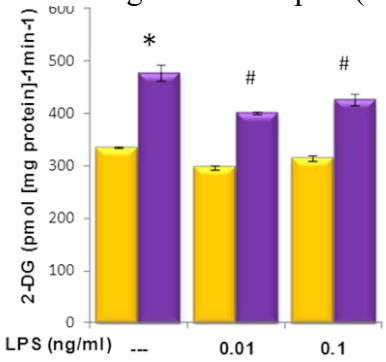
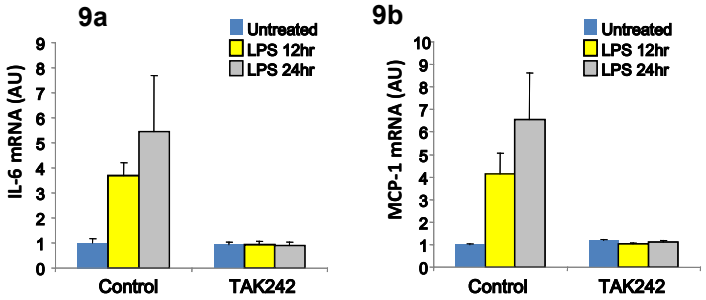
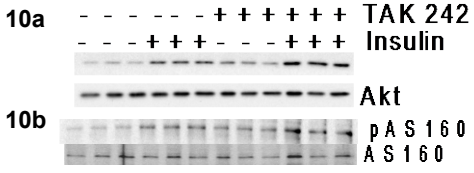


Figure 8. Effect of low dose LPS on glucose transport. Basal in yellow, and 100 nM insulin in purple.



Effect of TLR4 blockade on LPS- induced inflammation in human muscle cells. Human myotubes were treated with 100 ng/ml LPS for 12 and 24 h in the presence/absence of the TLR4 inhibitor TAK242. As shown in Figure 9, LPS caused a robust increase in the gene expression of IL-6 (9a) and MCP-1 (9b) in human muscle cells, an effect which was blocked by the TLR4 inhibitor. We have performed experiments evaluating the effect of TAK242 on insulin signaling. Myotubes derived from a lean, nondiabetic subject were pre-treated for 12 h with 100 ng/ml LPS in the presence/absence of 1μM TAK242, followed by stimulation with insulin. As shown below, the TLR4 inhibitor enhanced insulin-stimulated Akt (Fig. 10a) and AS160 (Fig. 10b) phosphorylation.





Sevelamer reduces LPS concentration and LPS-induced inflammatory responses. Sevelamer is a non-absorbable, cross-linked polymer, which binds to LPS in the GI tract. Several studies have demonstrated sevelamer's ability to reduce LPS plasma concentration in human subjects (35-37). We tested the effect of sevelamer (1.6 g three times a day for 4 weeks; Genzyme, Cambridge, MA) on three T2DM subjects (mean age=65 years), and, consistent with the literature (35-37), sevelamer reduced plasma LPS level in all subjects (Figure 11).

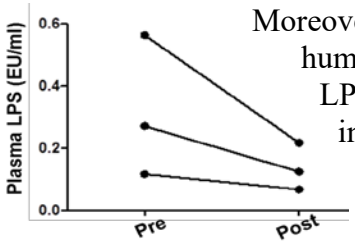
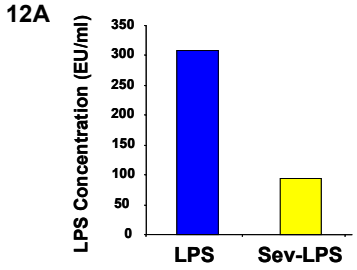
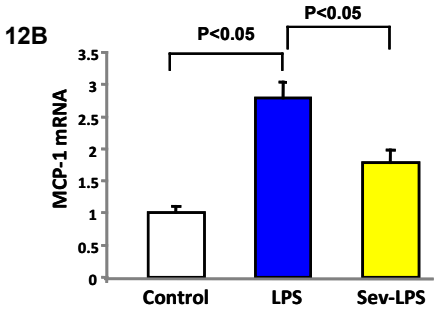
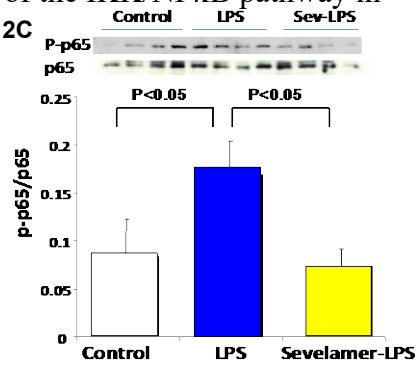


Fig. 11

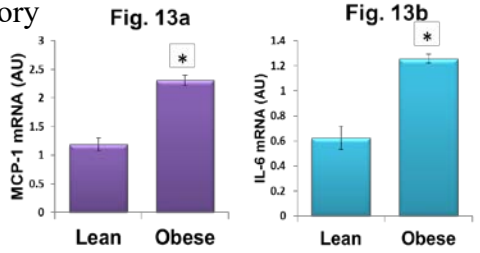
Moreover, we evaluated the effect of sevelamer (ACIC, Coral Springs, FL) using our human myotube culture system, and found that pre-treatment with this agent decreased LPS concentration in the culture media (Fig. 12a), leading to a significant reduction in LPS-stimulated MCP-1 expression (Fig.12b). Further, pretreatment with sevelamer also prevented LPS-induced activation of the IKK/NFκB pathway in isolated rat muscles (Fig. 12c).







Sera from insulin resistant subjects causes an inflammatory response. To examine whether the inflammatory state that occurs in muscle from insulin resistant subjects (38) is caused by a systemic factor, we incubated primary human myotubes derived from a lean person with sera from a lean and an obese person for 24 h. The serum from the obese individual caused a significant inflammatory response as evidenced by increase MCP-1 (Fig. 13a) and IL-6 (Fig. 13b) gene expression. To examine whether LPS is the systemic factor inducing the inflammatory response, during the proposed study we will neutralize serum LPS with polymyxin B and will block TLR4 signaling in the human myotubes using TAK242 and short hairpin (sh)RNA.



D) RESEARCH DESIGN AND METHODS

Specific Aim 1) To determine the effect of high fat consumption on the intestinal microbiome, metabolic endotoxemia, and insulin action, in lean normal glucose tolerant subjects. *Rationale*. A high fat diet increases plasma LPS concentration in rodents (7, 26). Moreover, a high fat ingestion also causes an elevation in LPS levels in human subjects (19, 30) and people who consume more fat have higher plasma LPS concentration (31). In this Aim we will test whether a short-term high fat diet given to lean, normal glucose tolerant subjects increases intestinal permeability, plasma LPS level, and inflammation in the muscle. Also we will test whether the inflammatory response and insulin resistance caused by high fat ingestion is prevented by interventions which reduce/prevent endotoxemia through different mechanisms:

(i) A synbiotic (oligofructose plus Bifidobacterium longum): Probiotics are live bacteria which colonize the intestine after oral administration, prebiotics are products (nutritionals, etc.) that modulate the microbiota, and synbiotics are a combination of a probiotic and a prebiotic. The prebiotic oligofructose is a nondigestible (resists hydrolysis by human digestive enzymes) fructooligosaccharide which is hydrolyzed and used as energy substrate by gut bacteria such as Bifidobacteria. Bididobacteria are a dominant member of the intestinal microbiome and help to maintain integrity of the intestinal barrier (25, 39). Administration of oligofructose and the consequent increase in Bifidobacteria has been shown to protect the intestinal barrier, decrease intestinal permeability, lower plasma LPS concentration, and improve glucose metabolism in numerous studies conducted in animal models of insulin resistance (23-25). In obese insulin resistant subjects with non-alcoholic steatohepatitis the administration of oligofructose and Bifidobacterium longum plus a lifestyle intervention caused a more significant decrease in plasma LPS concentration and improvement in insulin sensitivity (HOMA-IR) than lifestyle intervention alone (40).

(ii) Sevelamer: This agent is a non-absorbable, cross-linked polymer that has strong LPS-binding capacity, and therefore limits intestinal wall translocation of LPS (41). Sevelamer also has affinity for phosphates and it is FDA-approved for use as a phosphate binder in patients with chronic kidney disease. Sevelamer does not cause hypophosphatemia in individuals with normal kidney function (42, 43). We have confirmed sevelamer's ability to reduce LPS concentration *in vitro* and to inhibit LPS-induced inflammatory response using our primary

human muscle cell culture system and isolated rat muscle preparation (Fig.12). A cross-sectional study found that subjects who take sevelamer have lower LPS concentration (35) and prospective studies have shown that sevelamer reduces LPS concentration in humans as much as 80% (36, 37). We have confirmed sevelamer's LPS-lowering effect in our study population (Fig. 11). Based upon its ability to sequester LPS in the intestinal lumen, sevelamer's systemic anti-inflammatory and immune-modulating properties are being tested in a large NIH-sponsored multi-center (20 academic sites) trial, in otherwise healthy HIV-infected subjects with normal renal function (https://actgnetwork.org).

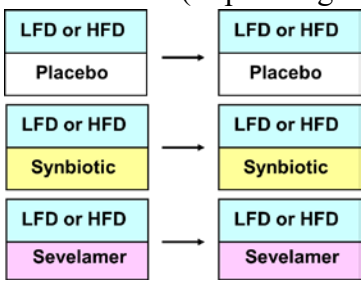


Figure 14. Study design (Aim 1)

Subjects. 36 (completers), healthy, lean (BMI <26 kg/m^2) normal glucose tolerant subjects, aged 18-65 years, without a family history of T2DM, will be enrolled (50% males). Subjects will be randomized to receive a low or a high fat diet for 4 weeks. After a 10-12 week washout period, subjects who first received the low fat diet, will then receive the high fat diet for 4 weeks, and vice versa (each subject will be its own control). During the time the subjects receive these diets, they will simultaneously receive in a randomized double-blind fashion (i) placebo, (ii) synbiotic or (iii) sevelamer (n=12 completers per group). The protocol is outlined in Fig. 14.

Inclusion Criteria: 1) Both genders. All races and ethnic groups.
2) Premenopausal women in the follicular phase, non-lactating, and with a negative pregnancy test. Postmenopausal women on stable dose of or not exposed to hormone replacement for ≥6 months.
3) HCT≥ 34%, serum creatinine ≤ 1.4 mg/dl, electrolytes, urinalysis, and coagulation tests. LFTs up to 2X.
4) Stable body weight (±2%) for ≥ 3 months. Two or less sessions of strenuous exercise/wk for last 6 months.

Exclusion Criteria: 1) Presence of diabetes or impaired glucose tolerance based on ADA criteria.
2) Current treatment with drugs known to affect glucose and lipid homeostasis. If the subject has been on a stable dose for the past 3 months, the following agents will be permitted: calcium channel blockers, β-blockers, ACE inhibitors, angiotensin receptor blockers, and statins. History of allergy to sevelamer.
3) Non-steroidal anti-inflammatory drugs or systemic steroid use for more than a week within 3 months.
4) Current treatment with anticoagulants (warfarin). Aspirin (up to 325 mg) and clopidogrel will be permitted if these can be held for seven days prior to the biopsy in accordance with the primary physician.
5) Use of agents that affect gut flora (e.g. antibiotics, colestyramine, lactulose, PEG) within 3 months.
6) History of heart disease (New York Heart Classification greater than grade II; more than non-specific ST-T wave changes on the ECG), peripheral vascular disease, pulmonary disease, smokers.
7) Poorly controlled blood pressure (systolic BP>170, diastolic BP>95 mmHg).
8) Active inflammatory, autoimmune, hepatic, gastrointestinal, malignant, and psychiatric disease.
9) History of gastrointestinal surgery or gastrointestinal obstruction within two years.

Design. Subjects will be recruited through the General Medicine Clinics of the Texas Diabetes Institute and the Audie L. Murphy VA Medical Center, and through local advertisement. Study visits will be performed in the Bartter Research Unit (BRU) located in the VA Medical Center, which is funded by a Clinical and Translational Science Award (CTSA) to the University of Texas Health Sciences Center at San Antonio (UTHSCSA).

Visit 1: Subjects will come to the BRU at 7 AM for a medical history, physical examination (including anthropometric measurements), screening tests (CBC, chemistry, lipid profile, HbA_{1c}, PT, PTT, UA), and ECG.
Visit 2: Within 3-7 days from Visit 1, eligible subjects will return at 7 AM after an overnight fast for an oral glucose tolerance test (OGTT). A blood (LPS) and stool sample will be collected before the OGTT.

Nutritional Intervention: After this visit subjects will begin a high or a low fat diet to be ingested for 28 days. The subjects will meet with a nutritionist (Celia Darland, RD) who will perform an assessment of macronutrient and energy intake. The meals will be prepared in the Metabolic Kitchen of the BRU. The nutritionist will design the nutritional components and provide dietary education. The high and low fat diets will be designed based on the subjects' usual dietary habits and energy intake to ensure palatability and adherence. The high fat diet will provide 60% of energy from fat (of which 50% from saturated fat), 15% of energy as CHO and 25% from protein. The low fat diet will provide 55% of energy from CHO, 20% from fat, and 25% from protein.

The diets will be isocaloric in relation to the subjects' habitual energy intake. Ready-made meals will be provided to subjects. Oil, butter, heavy cream, eggs, mayonnaise and cheese will be used as the primary fat sources. Subjects will record food intake and will maintain their usual physical activity level.

Pharmacological/nutraceutical intervention: On the same day that the nutritional intervention is started, subjects will be randomized to receive, in a double-blind fashion, placebo (maltodextrin, 6 g three times a day), the synbiotic [5 g of oligofrucose + 1 g Bifidobacterium longum (4x10^10 CFU/g) three times a day], or sevelamer (1.6 g sevelamer + 4.4 g maltodextrin three times a day), for 4 weeks, and discharged home. Sevelamer is obtained from Amgen (Renvela, Cambridge, MA), oligofructose from Orafti (Tienen, Belgium), Bifidobacterium from Danisco (Copenhagen, Denmark) and maltodextrin from American International Foods (Grand Rapids, MI). Randomization and drug preparation/dispensing is performed by the Research Pharmacist of the BRU. For each dose the powder will be included in identical packets. The subjects from the 3 groups (placebo, synbiotic, sevelamer) will ingest the powder contained in one packet with water 3 times /day for 28 days. Before discharge, subjects are given printed instructions and information about potential side effects.

Visit 3: On day 3 of the nutritional intervention subjects will come to the BRU at 7 AM for evaluation of postprandial LPS concentration. Subjects will ingest their breakfast according to their assigned meal plan. Plasma LPS, chylomicron LPS, insulin, and glucose levels are measured at times -30, +60, +120, and +180 min.
Visit 4: On day 24 of the intervention subjects will come to the BRU at 7 AM in the fasting state to undergo stool sample collection and an 8 h intestinal permeability (lactulose/mannitol) testing.
Visit 5: On day 28 subjects will undergo whole body DEXA, a euglycemic hyperinsulinemic clamp and indirect calorimetry as described (44, 45). DEXA is performed to examine the effect of the interventions on body composition. At time -30 min a biopsy of the vastus lateralis muscle will be performed. At time 0 min, insulin will be infused at 60 mU/m^2.min for 180 min. A 2nd biopsy is performed on the contralateral leg at 180 min. After Visit 6, subjects will undergo a washout period of 10-12 weeks. Subjects will not receive any treatment and will maintain their usual (isocaloric) alimentary habits and physical activity level. They will meet every two to four weeks with the nutritionist to monitor their body weight.
Visit 6: After the 10-12 week washout period, subjects will come to the CRC for stool collection and plasma LPS measurement. Subject will then begin the second nutritional intervention. If subjects were placed on a low fat diet first, now they will begin a high fat diet, and vice versa. During the second nutritional intervention subjects will receive the same treatment (synbiotic, sevelamer or placebo) assigned during the first intervention.
Visit 7: On day 3 of the second nutritional intervention subjects will undergo the same procedure as Visit 3 (plasma LPS, insulin and glucose, and LPS content in chylomicrons, in the postprandial state).
Visit 8: On day 24 subjects come to the BRU for stool collection and intestinal permeability testing.
Visit 9: On day 28 of the second nutritional intervention subjects will come to the BRU at 7 AM to undergo DEXA, insulin clamp, indirect calorimetry, and biopsies of the muscle (2) as described above for Visit 5.

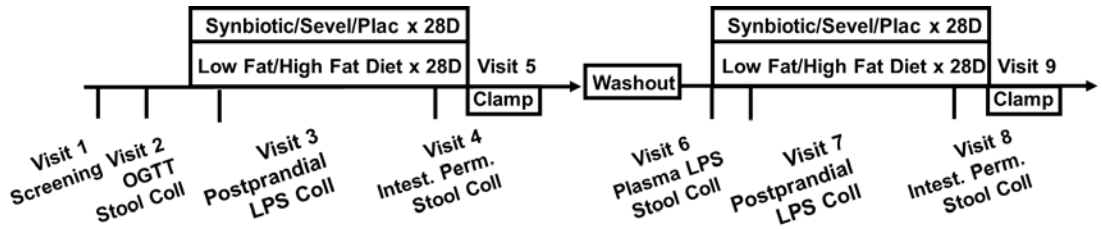


Figure 15. Study Visit Schedule (Aim 2)

Analyses. Plasma LPS (LAL assay, Lonza, Walkersville, MD), LBP (Cell Sciences, Canton, MA), soluble CD14 (R&D Systems, Minneapolis, MN), IL-6 (R&D Systems), and TNFα (R&D Systems) concentration will be measured in all visits to examine the dynamics of LPS concentration and action (LPB, soluble CD14) during the course of the nutritional and pharmacologic/nutraceutical interventions. Because turbidity from triglycerides can falsely increase LPS level (46), 0.25 vol of 50% acetic acid are added to each sample to eliminate turbidity (19). In the basal muscle samples we will perform assays of TLR4 [IKK/NFκB and MAPK (JNK, p38, ERK)] signaling (75 mg tissue), TLR4 content (20 mg), inflammatory gene (TLR4, IL-6, MCP-1, iNOS, TNFα) expression (15 mg) and IRS-1 ser phosphorylation (20 mg) as described by our group (38, 47, 48). These measurements will establish whether elevations in plasma LPS caused by the high fat diet stimulate TLR4 and enhance IRS-1 ser phosphorylation

which impairs insulin action. On all muscle samples we will perform assays of insulin signaling (75 mg). These measurements will establish whether high fat diet-induced increases in LPS level and stimulation of TLR4 lead to impaired insulin-stimulated IRS-tyr phosphorylation, PI 3K activity, and Akt and AS160 phosphorylation (49). Stool samples will be collected for characterization of the intestinal microbiome through 16S DNA sequencing. This will allow us to evaluate the effect of the nutritional and pharmacologic/nutraceutical interventions on intestinal microbiota composition and how this relates to intestinal permeability, changes in LPS, inflammation (TLR4 signaling), and insulin action.

Interpretation and limitations. Based on prior animal studies we anticipate that that the high fat diet will modify the composition of the intestinal microbiota towards an unfavorable microbiome profile. Specifically, we anticipate that there will be a reduction in Bifidobacteria content (22). Since Bifidobacteria protect the integrity of the intestinal barrier (24, 39) we expect that the high fat diet will enhance intestinal permeability, which will lead to increased plasma LPS concentration. If our hypothesis is correct, the elevation in LPS will trigger an inflammatory response in muscle, manifested by increased flux through TLR4 (TLR4-MyD88 association, activation of IKK//NFκB and MAPK). We predict that TLR4 stimulation will result in impaired insulin signaling (enhanced IRS-1 ser phosphorylation and decreased IRS-1 tyr phosphorylation, PI 3-kinase activity, and Akt-AS160 phosphorylation) and reduced peripheral insulin sensitivity (low Rd) during the clamp. Based on studies in rodents (24, 39), we predict that the synbiotic will ameliorate the damage to the intestinal epithelium (*i.e*. permeability) and the rise in plasma LPS caused by the high fat diet, which in turn will blunt the inflammatory response (TLR4 signaling) and improve insulin resistance.

In this Aim the effect of the LPS-binding agent sevelamer also will be assessed to provide an independent way for reducing plasma LPS concentration. Based on previous human studies (35, 36) and our preliminary studies (Fig. 11) demonstrating that sevelamer decreases LPS concentration, and its capacity to bind LPS and inhibit its inflammatory effect (Fig. 12) (41), we anticipate that sevelamer-induced reductions in plasma LPS concentration will lead to reduced inflammatory signaling (IKK/NFκB, MAPK, inflammatory genes) in muscle. Because sevelamer's effect is to reduce LPS, also we anticipate that flux through TLR4 (TLR4-MyD88 association) will be lower. In addition, we expect that the reductions in LPS concentration will predict (correlate with) changes (reductions) in TLR4 signaling and improved insulin signaling and sensitivity.

We anticipate that the microbiome changes and increases in plasma LPS will result in peripheral insulin resistance. However, some (50, 51), but not all studies (52) have found reductions in insulin sensitivity in healthy subjects given short term isocaloric high fat diets. The discrepant results likely are related to differences in nutritional interventions and populations studied. We recognize that there may be variability in individual responses to the high fat diet. In fact, we believe that is an advantage of the proposed experiments, which will allow us the test each subjects' susceptibility to the nutritional intervention, and whether individual variations in microbial changes, correlate (predict) with changes in intestinal permeability, plasma LPS, and insulin sensitivity. These findings would lead to future studies designed to determine the mechanisms underlying such variability.

Insulin resistance is a multifactorial condition. In addition to microbiome changes (tested in this Aim), other potential mechanism by which excess fat supply likely impairs insulin action include changes in free fatty concentration, toxic intracellular lipid metabolites, adipocyte- and macrophage-derived cytokines and mitochondrial function. Because of these different mechanisms, and the interplay between them, we do not anticipate that targeting the microbiome and its products (LPS) solely will result in complete prevention of high fat diet-induced insulin resistance. This proposal is based on the concepts that metabolic endotoxemia is an important contributing mechanism towards insulin resistance, and that targeting the microbiome and endotoxemia will ameliorate the deleterious effect of high fat consumption on inflammation and glucose metabolism. While assessing the role of these different factors (lipid metabolites, mitochondrial function, etc.) is beyond the scope of the current application, the data and specimens collected during the study can be utilized in the future to examine the contribution of all these different mechanisms.

This Aim will examine whether high fat ingestion increases plasma LPS by altering intestinal permeability (Fig. 1). However, another potential mechanism involves postprandial formation of chylomicrons which carry LPS (27). An in-depth analysis of this mechanism is beyond the scope of this grant. However, samples will be

collected in the postprandial state, and this will allow us to evaluate acute postprandial changes in chylomicron LPS content and plasma LPS level, and how these relate to measures of insulin action.

Specific Aim 2) To determine whether microbiome modulation and an experimental reduction in plasma LPS concentration improve inflammation and insulin action in insulin resistant (obese and T2DM) subjects. *Rationale.* Obese and T2DM subjects have alterations in the intestinal microbiome (lower content of beneficial bacteria such as Bifidobacteria) (5), abnormal gut permeability (24, 53), increased plasma LPS (preliminary data) (54, 55), and elevated TLR4 signaling in muscle (38) and monocytes (56). In this Aim we will test whether protecting the intestinal barrier with a synbiotic will restore normal gut permeability, reduce LPS, and ameliorate inflammation (TLR4 signaling) and insulin resistance. To assess the role of LPS independently, also we will reduce plasma LPS concentration with sevelamer.

Subjects. 36 (completers) lean (BMI<26 kg/m^2) normal glucose tolerant, 36 (completers) obese (BMI=30-37) normal glucose tolerant, and 36 (completers) obese (BMI=30-37) T2DM subjects will be studied. 12 subjects per group will receive the symbiotic (oligofructose plus Bididobacterium longum), 12 sevelamer, and 12 placebo. We will study an equal number of males and females. Other inclusion and exclusion criteria are similar as described in Aim 1, except that T2DM subjects ($Hb_{A1c} \leq 8.5\%$) will be enrolled. The only antidiabetic drugs allowed are sulfonylureas, due to their minimal effect on muscle physiology and inflammation. Sulfonylureas will be held for 48 h before any procedure to avoid hypoglycemia during fasting.

Design. *Visit 1*: Subjects will undergo a medical history, physical examination, and screening blood tests.
Visit 2: Within 3-14 days eligible subjects will return for OGTT.
Visit 3: Within 3-14 days subjects undergo blood (LPS) and stool collection and an intestinal permeability test.
Visit 4: Within 7-14 days from Visit 3, subjects will return to undergo a whole body DEXA, indirect calorimetry and an insulin clamp. Muscle biopsies will be performed before (-30 min) and at the end (+180 min) of the clamp. Following completion of these studies, subjects will be randomized to receive in a double-blind fashion, placebo (maltodextrin, 6 g three times a day), the synbiotic [5 g of oligofrucose + 1 g Bifidobacterium longum (4x10^10 CFU/g) three times a day], or sevelamer (1.6 g sevelamer + 4.4 g maltodextrin three times a day), for 4 weeks, and discharged to home. While at home, subjects will maintain their habitual (isocaloric) diet and physical activity level.

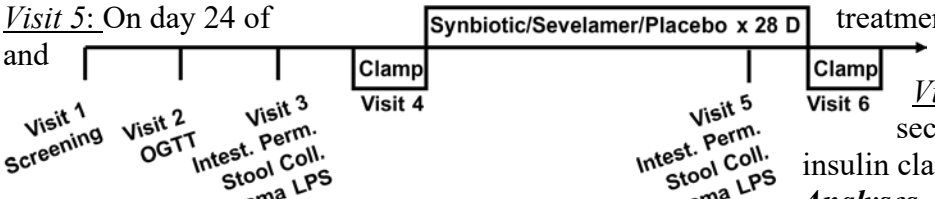


Fig 16. Study Visit Schedule (Aim 1)

Visit 5: On day 24 of treatment blood (LPS) and stool are collected and an intestinal permeability test is done. *Visit 6*: On day 28, subjects undergo a second DEXA, indirect calorimetry, and insulin clamp with muscle biopsies.

Analyses. Plasma LPS, LBP, soluble CD14, IL-6, TNFα and phosphate will be measured before, during, and after treatment (Visits 3, 4, 5 and 6). Assays of TLR4 and insulin signaling in muscle are performed as described for Aim 1. Stool samples will be collected before and during treatments. This will allow us to evaluate differences in microbiome composition between lean, obese, and T2DM subjects at baseline, and establish the relationship between treatment-induced changes in specific bacteria composition/distribution with changes in inflammation and insulin action.

Interpretation and limitations. We expect that the obese and T2DM subject will have an unfavorable intestinal microbiome profile (decreases in Bifidobacteria and Bacteroidetes) (54, 55) which will be associated with enhanced intestinal permeability and increased LPS level. Thus, we expect that these subjects will have an elevated inflammatory tone (TLR4 signaling) in muscle, which will reduce insulin action. Based upon previous animal studies (23-25), we anticipate that the synbiotic will restore gut epithelial integrity, decrease circulating LPS level, and ameliorate insulin resistance in obese and T2DM subjects. We also anticipate that sevelamer will decrease LPS concentration (36, 37) and will lead to reduced inflammatory signaling (IKK/NFκB, MAPK, inflammatory genes) in muscle. Because sevelamer's effect is to reduce LPS, also we anticipate that flux through TLR4 (TLR4-MyD88 association) will be lower. In addition, we expect that the reductions in LPS concentration will predict (correlate with) changes (reductions) in TLR4 signaling and improved insulin action.

Aim 1 will test whether a fat-rich diet alters the gut microbiome and disrupts the intestinal barrier in lean subjects resulting in increased LPS translocation. The goal of Aim 2 is different; in this Aim we are proposing a proof-of-principle experiment to evaluate whether modulating the microbiome and lowering plasma LPS improves insulin action in obese and T2DM subjects. We anticipate that modulating the microbiome and plasma LPS concentration will decrease inflammation and insulin resistance in these individuals. If these changes are the result of protection of gut barrier integrity from the increased fat intake which is common in obese and T2DM subjects, then sevelamer and the synbiotic also should prevent the inflammatory response and insulin resistance caused by the high fat diet in the lean subject from Aim 1. If the lean subjects are not protected from the high fat diet, but the obese and T2DM subjects benefit from sevelamer and the synbiotic, then improvements in inflammation and insulin action are likely caused by a mechanism different from protection against fat-induced damage. Other possible mechanisms by which LPS sequestration and/or microbiome modulation could improve inflammation and insulin action include changes in fructose-mediated endotoxemia (57), food energy extraction (9), appetite (10-13), bacterial flagellin levels (14), and peptidoglycans (15).

One limitation of the proposed experiment is that the synbiotic could alter inflammation and glucose metabolism through other mechanisms, such as by inducing changes in the concentration of FFA and cytokines. Sevelamer also could lead to other effects; this agent can bind to uric acid and reduce its plasma concentration (58), although uric acid *per se* generally is not thought to induce insulin resistance. A primary human cell culture system will be employed in Aim 3 which will allow us to test whether the differences in inflammation and insulin sensitivity between groups and the effect of the interventions are due to changes in circulating LPS.

The proposed studies focus on the impact that the gut microbiome and endotoxemia have on inflammation and insulin action at the level of the skeletal muscle. We are focusing on this tissue because it is the main site for insulin-mediated glucose disposal. Nonetheless, the liver, adipose tissue, brain, and inflammatory cells (monocytes) also are potentially subjected to the influence of the microbiome and LPS. Studying these organs/tissues is beyond the scope of this grant, but they are important areas for future investigation.

Specific Aim 3) To determine whether the LPS present in the sera from lean high fat fed, obese, and T2DM subjects mediates the insulin resistance, and whether this effect is mediated via TLR4. *Rationale.* An *in vitro* system will be employed because it will allow us to directly asses the role of LPS and TLR4 on insulin resistance in human muscle. Testing the effect of sera in cultured cells while blocking the immune mediator of interest is a commonly employed method to assess the independent biological effects of the numerous immune mediators (antibodies, cytokines, etc.) which are altered in patients with immune-mediated diseases (lupus, rheumatoid arthritis) (59). In this Aim we will apply the same concept in view that obesity and T2DM are considered inflammatory (and metabolic) diseases.

Experimental model. We will use primary human myotubes developed from the muscle samples obtained in the lean subjects enrolled during Aim 1, as described by our group (38). To avoid experimental variation, myoblasts from all lean subjects will be pooled, aliquoted, and frozen. Prior to each experiment an aliquot will be thawed, cells plated, and differentiated into myotubes. We will use cells from ealry (2-3) passages.

Experiment 1. **Does sera from lean high fat fed, obese, and T2DM induce inflammation and insulin resistance and are these effects ameliorated by *in vivo* treatment with the synbiotic and sevelamer?** Human myotubes are treated with sera from (i) lean subjects studied in Aim 1 who ingested a low and high fat diet and received the synbiotic, sevelamer, and placebo; and (ii) lean, obese and T2DM subjects who received the synbiotic, sevelamer, and placebo (Aim 2). Treatments are conducted in a time-dependent (24h and 48h) and dose-dependent (10%, 20% and 40% serum in MEM culture media) manner. After these incubations, cells are collected for measurement of TLR4 signaling (TLR4-MyD88 association, IKK/NFκB, MAPK, inflammatory genes) (38, 48). To assess the effect on insulin action, after treatment with the subjects' serum, cells are stimulated with insulin for 20 min. Cells are then collected in lysis buffer for insulin signaling studies (49) or subjected to a glucose transport assay (60). Increasing concentrations of insulin (0, 1, 10 and 100 nM) will be employed to determine whether the sera affects insulin responsiveness (shifting of the curve).

Experiment 2. **Does elevated LPS in the circulation mediate the inflammatory state and insulin resistance observed in muscle from lean high fat fed, obese, and T2DM subjects?** Serum samples from these subjects will be treated with polymyxin B which sequesters LPS and specifically blocks its biological

activity (61). Polymyxin B cannot be administered to human subjects due to its nephrotoxicity. However, because of its capacity to sequester LPS, ongoing multicenter clinical trials are being conducted assessing the effect of extracorporeal hemoperfusion with polymyxin B absorption columns in patients with sepsis (62). Assays of TLR4 signaling, inflammatory gene expression, insulin signaling, and glucose transport will be conducted in the presence and absence of polymyxin B (20 μg/ml).

Experiment 3. **Does TLR4 mediate the pro-inflammatory state and insulin resistance observed in lean high fat fed, obese, and T2DM subjects?** Myotubes from lean subjects will be treated with sera from lean high fat fed, obese and T2DM subjects, and the same experiments described in Experiment 1 will be conducted (TLR4 signaling, inflammatory genes, insulin signaling, glucose transport). To test whether the effects from the sera are TLR4-mediated, TLR4 will be blocked/inhibited using two different approaches:
a) TAK242: This agent (Takeda Pharmaceuticals) binds specifically to TLR4 and inhibits signaling by preventing its interaction with the adapter proteins TRAM and TIRAP (63).
b) shRNA: To knock down TLR4 we will use adenoviral-driven shRNA (Vector Biolabs, Philadelphia, PA) as described by our group (38). As negative control we will use a scrambled non-mammalian sequence (Ad-scrambled). We will perform a dose-response study to determine the minimal amount of virus that will achieve maximal knock down efficient. Based on our own experience, we anticipate that this dose will be in the $1x10^2$ to $1x10^8$ PFU/ml range. Fig. 17 shows images of typical human myotubes developed in our lab infected with adenovirus tagged with green fluorescent protein. Figs.18a and 18b show that our method is highly effective to knock down TLR4.

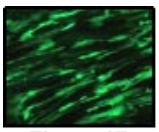


Figure 17.

Fig. 18a

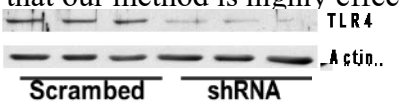
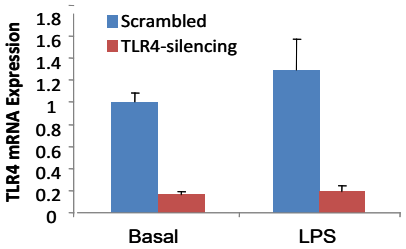


Fig. 18b



Interpretation and limitations. Based on our preliminary studies (Fig. 13), we anticipate that sera from lean high fat fed, obese, and T2DM will induce an inflammatory response and insulin resistance as evidence by elevated IKK/NFκB and MAPK signaling and inflammatory gene expression, decreased insulin signaling, and decreased glucose transport, as compared with myotubes treated with sera from lean subjects. If our hypothesis is correct, sera from high fat fed, obese and T2DM subjects, collected after treatment with the synbiotic and sevelamer, will induce a less severe inflammatory response and insulin resistance, and the changes in LPS level after *in vivo* treatment will predict the *in vitro* response.

One limitation of this approach is that numerous systemic factors could be responsible for the inflammatory response and insulin resistance caused by sera from high fat fed, obese and T2DM subjects. If these effects are mediated by LPS, its neutralization with polymyxin B should ameliorate them. In addition, if the effect is mediated through TLR4, the inflammatory response and insulin resistance caused by the sera will be reduced/prevented by blocking/downregulating TLR4 with TAK242 and shRNA.

TLR4 regulates numerous signaling cascades, including IKK/NFκB, JNK, ERK, p38 and SOCS. One or more branches downstream of TLR4 might be responsible for the negative effect of LPS on glucose metabolism. Dissecting the role of each of these branches in LPS-induced insulin resistance is beyond the scope of this grant application. Nonetheless, using our primary cell culture system and shRNA, in the future we could perform an in-depth analysis about the contribution of each of these signaling branches.

Statistical analysis. Experimental results will be expressed as means ± SE. Comparisons of means between all the groups will be done by ANOVA for repeated measures (67). Associations between measures of LPS concentration vs.TLR4 signaling and vs. insulin signaling/sensitivity, within a group, will be determined by Pearson's correlation. For tests of correlation coefficients between groups we will use the Fisher's Z transformation (64). We will also determine the relationship between plasma LPS concentration vs. TLR4 signaling and LPS vs. insulin signaling/sensitivity, by using multiple regression analysis. Scatter plots will be done to look for outliers and to verify linearity. Prof. Alex McMahan is an experienced biostatistician who will direct the statistical analysis of this study. Based on our preliminary results and data from the literature (36, 65) regarding the variability of these measurements, we calculated that an n=12 subjects/group is required to obtain power (1-β) of 0.80 with a 2-sided test at α= 0.05 significance level. Assuming a ~20% dropout rate, 15

subjects/group would be sufficient to achieve the required group sizes. The following data and power analysis (2-tailed) were obtained from an ongoing human study (PI: Musi).

Variable	Mean±SD Lean	N	Mean±SD Obese	n	Power
LPS Concentration	0.049±0.055	12	0.119±0.014	12	0.99
Insulin Sensitivity (M)	11.3±2.8	12	8.1±2.4	12	0.91
Ins Stim IRS1-P-Y	4.0±1.1	12	0.9±0.6	12	0.99
Variable	**Mean±SD Lean**	**N**	**Mean±SD T2DM**	**n**	**Power**
LPS Concentration	0.049±0.055	12	0.130±0.071	12	0.99
Insulin Sensitivity (M)	11.3±2.8	12	4.2±1.7	12	0.99
Ins Stim IRS-1 P-Y	4.0±1.1	12	1.5 ±1.0	12	0.99

For the following variables, data were obtained from published human studies (36, 40, 65).

Variable	Mean±SD Pre-Sevel	N	Mean±SD Post-Sevel	n	Power
LPS Concentration	3.6±3.0	12	1.2±2.3	12	0.99
CRP Concentration	4.8±2.4	12	0.44±0.24	12	0.99
Variable	**Mean±SD Placebo**	**N**	**Mean±SD Synbiotic**	**n**	**Power**
LPS Concentration	87±14	12	54±10	12	0.99

We performed the following calculations (two-tailed) using data from our preliminary *in vitro* experiments:

Variable	Mean±SD Lean Serum	n	Mean±SD Obese Serum	n	Power
MCP-1 mRNA	1.18±0.18	12	2.3±0.6	12	0.88
IL-6 mRNA	0.6±0.06	12	1.2±0.06	12	0.99

Other specific methods: ***Intestinal permeability test.*** Five g lactulose and two g mannitol dissolved in water are ingested, hourly urine and plasma samples are collected for 8 hours, and lactulose and mannitol are analyzed by gas chromatography (66, 67). Lactulose and mannitol permeate only in minimal amounts across the normal intestinal wall and are not metabolized by the body. In disease state, the intestine becomes preferentially permeable to lactulose than mannitol. Dr. Fanti (Co-I) has previously conducted this test (68).

Metagenomics. The metagenomics experiments will be carried out by Dr. Qunfeng Dong (Co-I), as described by his group (69-71). DNA is extracted using a kit from MoBio (Carslbad, CA) and following the protocol established by the Human Microbiome Project Consortium (72). PCR-amplified 16S rDNA is sequenced using the Illumina HiSeq2000 platform, following the sequencing and data processing protocol designed by Caporaso (73). Taxonomy is assigned to each high-quality read based on the best matching Greengenes database sequence (74). Operational Taxonomic Units are clustered by using the QIIME toolkit (75). For Aim 1, ANOVA and t-test (and their non-parametric versions) and Chi-square/Fisher's exact test are applied to detect taxa with significantly different relative abundance either within the same individuals *(i.e*., low fat vs. high fat) or between treatments (placebo, synbiotic, sevelamer). Shannon, UniFrac, and Bray-Curtis indices are compared to quantify the community-level variability among the samples within and between individuals. Multiple test corrections (*e.g.,* Benjamini-Hochberg-Yekutieli) are applied to control for false discovery rate. The response variable (microbial composition) is analyzed with the explanatory variables (insulin sensitivity, LPS level, etc.) to evaluate for associations by Generalized linear models. Models also are used for analyzing the influence of explanatory variables on the relative abundance of significantly changing bacterial taxa (or) communities with treatments. For Aim 2, the data analysis and comparisons between groups (obese, T2DM, and lean) and treatments (placebo, synbiotic, sevelamer) are done similarly as in Aim 1.

Summary/Timetable. We are very excited about this proposal which will study the role that the microbiome and endotoxemia play a key role in the pathogenesis of insulin resistance. The studies proposed are innovative because the role of the micobiome on insulin resistance is unclear and relevant because they could lead to the development of novel therapies for T2DM. This application describes a 3 year program:
Year 1: Start Aim 1 Year 2: Complete Aim 1; Start Aims 2 and 3 Year 3: Complete Aims 2 and 3

Risks: a) Muscle biopsy: Local hematomas occur in <2% of subjects. b) Sevelamer: This drug is FDA approved for use as a phosphate binder. Sevelamer does not cause hypophosphatemia in subjects with normal kidney function (71, 76). As a precaution, we will only enroll subjects with normal phosphorus plasma concentration, which will be monitored during treatment. Sevelamer is a very safe drug; its main side effects are gastrointestinal. Subjects with a history of gastrointestinal obstruction and/or surgery within 2 years will be excluded. c) Oligofructose and Bididobacteria administration is very safe. Rarely these can cause bloating.

2. RELEVANT PUBLICATION ABSTRACTS: N/A

3. REFERENCES

1. Neish AS. Microbes in gastrointestinal health and disease. Gastroenterology. 2009 Jan;136(1):65-80.
2. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, et al. Metagenomic analysis of the human distal gut microbiome. Science. 2006 Jun 2;312(5778):1355-9.
3. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nature. 2007 Oct 18;449(7164):804-10.
4. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nature. 2006 Dec 21;444(7122):1022-3.
5. Wu X, Ma C, Han L, Nawaz M, Gao F, Zhang X, et al. Molecular characterisation of the faecal microbiota in patients with type II diabetes. Curr Microbiol. 2010 Jul;61(1):69-78.
6. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med. 2009 Nov 11;1(6):6ra14.
7. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes. 2008 Jun;57(6):1470-81.
8. Alhamoruni A, Lee AC, Wright KL, Larvin M, O'Sullivan SE. Pharmacological effects of cannabinoids on the Caco-2 cell culture model of intestinal permeability. J Pharmacol Exp Ther. 2010 Oct;335(1):92-102.
9. Ferraris RP, Vinnakota RR. Intestinal nutrient transport in genetically obese mice. Am J Clin Nutr. 1995 Sep;62(3):540-6.
10. Brown AJ, Goldsworthy SM, Barnes AA, Eilert MM, Tcheang L, Daniels D, et al. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem. 2003 Mar 28;278(13):11312-9.
11. Le Poul E, Loison C, Struyf S, Springael JY, Lannoy V, Decobecq ME, et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J Biol Chem. 2003 Jul 11;278(28):25481-9.
12. Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, et al. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci U S A. 2008 Oct 28;105(43):16767-72.
13. Zaibi MS, Stocker CJ, O'Dowd J, Davies A, Bellahcene M, Cawthorne MA, et al. Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. FEBS Lett. 2010 Jun 3;584(11):2381-6.
14. Vijay-Kumar M, Aitken JD, Carvalho FA, Cullender TC, Mwangi S, Srinivasan S, et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science. 2010 Apr 9;328(5975):228-31.
15. Tamrakar AK, Schertzer JD, Chiu TT, Foley KP, Bilan PJ, Philpott DJ, et al. NOD2 activation induces muscle cell-autonomous innate immune responses and insulin resistance. Endocrinology. 2010 Dec;151(12):5624-37.
16. Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol. 2008 Feb;6(2):121-31.
17. Xu J, Bjursell MK, Himrod J, Deng S, Carmichael LK, Chiang HC, et al. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. Science. 2003 Mar 28;299(5615):2074-6.
18. Sonnenburg JL, Xu J, Leip DD, Chen CH, Westover BP, Weatherford J, et al. Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science. 2005 Mar 25;307(5717):1955-9.
19. Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: evidence of a novel mechanism of postprandial inflammation. Am J Clin Nutr. 2007 Nov;86(5):1286-92.
20. Moreira AP, Texeira TF, Ferreira AB, do Carmo Gouveia Peluzio M, de Cassia Goncalves Alfenas R. Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. Br J Nutr. 2012 Apr 16:1-9.

21. Opal SM, Scannon PJ, Vincent JL, White M, Carroll SF, Palardy JE, et al. Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. J Infect Dis. 1999 Nov;180(5):1584-9.
22. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes. 2007 Jul;56(7):1761-72.
23. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, et al. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007 Nov;50(11):2374-83.
24. Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut. 2009 Aug;58(8):1091-103.
25. Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R. Improvement of glucose tolerance and hepatic insulin sensitivity by oligofructose requires a functional glucagon-like peptide 1 receptor. Diabetes. 2006 May;55(5):1484-90.
26. Membrez M, Blancher F, Jaquet M, Bibiloni R, Cani PD, Burcelin RG, et al. Gut microbiota modulation with norfloxacin and ampicillin enhances glucose tolerance in mice. FASEB J. 2008 Jul;22(7):2416-26.
27. Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. J Lipid Res. 2009 Jan;50(1):90-7.
28. Mehta NN, McGillicuddy FC, Anderson PD, Hinkle CC, Shah R, Pruscino L, et al. Experimental endotoxemia induces adipose inflammation and insulin resistance in humans. Diabetes. 2010 Jan;59(1):172-81.
29. Ghanim H, Abuaysheh S, Sia CL, Korzeniewski K, Chaudhuri A, Fernandez-Real JM, et al. Increase in plasma endotoxin concentrations and the expression of Toll-like receptors and suppressor of cytokine signaling-3 in mononuclear cells after a high-fat, high-carbohydrate meal: implications for insulin resistance. Diabetes Care. 2009 Dec;32(12):2281-7.
30. Deopurkar R, Ghanim H, Friedman J, Abuaysheh S, Sia CL, Mohanty P, et al. Differential effects of cream, glucose, and orange juice on inflammation, endotoxin, and the expression of Toll-like receptor-4 and suppressor of cytokine signaling-3. Diabetes Care. 2010 May;33(5):991-7.
31. Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, et al. Energy intake is associated with endotoxemia in apparently healthy men. Am J Clin Nutr. 2008 May;87(5):1219-23.
32. Szeto CC, Kwan BC, Chow KM, Lau MF, Law MC, Chung KY, et al. Coagulase negative staphylococcal peritonitis in peritoneal dialysis patients: review of 232 consecutive cases. Clin J Am Soc Nephrol. 2008 Jan;3(1):91-7.
33. Dandona P, Ghanim H, Bandyopadhyay A, Korzeniewski K, Ling Sia C, Dhindsa S, et al. Insulin suppresses endotoxin-induced oxidative, nitrosative, and inflammatory stress in humans. Diabetes Care. 2010 Nov;33(11):2416-23.
34. Harte AL, da Silva NF, Creely SJ, McGee KC, Billyard T, Youssef-Elabd EM, et al. Elevated endotoxin levels in non-alcoholic fatty liver disease. J Inflamm (Lond). 2010;7:15.
35. Sun PP, Perianayagam MC, Jaber BL. Sevelamer hydrochloride use and circulating endotoxin in hemodialysis patients: a pilot cross-sectional study. J Ren Nutr. 2009 Sep;19(5):432-8.
36. Stinghen AE, Goncalves SM, Bucharles S, Branco FS, Gruber B, Hauser AB, et al. Sevelamer decreases systemic inflammation in parallel to a reduction in endotoxemia. Blood Purif. 2010;29(4):352-6.
37. Navarro-Gonzalez JF, Mora-Fernandez C, Muros de Fuentes M, Donate-Correa J, Cazana-Perez V, Garcia-Perez J. Effect of phosphate binders on serum inflammatory profile, soluble CD14, and endotoxin levels in hemodialysis patients. Clin J Am Soc Nephrol. 2011 Sep;6(9):2272-9.
38. Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, et al. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 2008 Oct;57(10):2595-602.
39. Wang Z, Xiao G, Yao Y, Guo S, Lu K, Sheng Z. The role of bifidobacteria in gut barrier function after thermal injury in rats. J Trauma. 2006 Sep;61(3):650-7.
40. Malaguarnera M, Vacante M, Antic T, Giordano M, Chisari G, Acquaviva R, et al. Bifidobacterium longum with fructo-oligosaccharides in patients with non alcoholic steatohepatitis. Dig Dis Sci. 2012 Feb;57(2):545-53.

41. Perianayagam MC, Jaber BL. Endotoxin-binding affinity of sevelamer hydrochloride. Am J Nephrol. 2008;28(5):802-7.
42. Heinrich T, Heidt H, Hafner V, Schmidt-Gayk H, Jenetzky E, Walter-Sack I, et al. Calcium load during administration of calcium carbonate or sevelamer in individuals with normal renal function. Nephrol Dial Transplant. 2008 Sep;23(9):2861-7.
43. Goldberg DI, Dillon MA, Slatopolsky EA, Garrett B, Gray JR, Marbury T, et al. Effect of RenaGel, a non-absorbed, calcium- and aluminium-free phosphate binder, on serum phosphorus, calcium, and intact parathyroid hormone in end-stage renal disease patients. Nephrol Dial Transplant. 1998 Sep;13(9):2303-10.
44. Defronzo RA. Glucose intolerance and aging: evidence for tissue insensitivity to insulin. Diabetes. 1979 Dec;28(12):1095-101.
45. Simonson DC, DeFronzo RA. Indirect calorimetry: methodological and interpretative problems. Am J Physiol. 1990 Mar;258(3 Pt 1):E399-412.
46. Rood J, Smith SR. Triglyceride concentrations and endotoxemia. Am J Clin Nutr. 2008 Jul;88(1):248-9; author reply 9-50.
47. Sriwijitkamol A, Christ-Roberts C, Berria R, Eagan P, Pratipanawatr T, DeFronzo RA, et al. Reduced skeletal muscle inhibitor of kappaB beta content is associated with insulin resistance in subjects with type 2 diabetes: reversal by exercise training. Diabetes. 2006 Mar;55(3):760-7.
48. Tantiwong P, Shanmugasundaram K, Monroy A, Ghosh S, Li M, DeFronzo RA, et al. NF-kappaB activity in muscle from obese and type 2 diabetic subjects under basal and exercise-stimulated conditions. Am J Physiol Endocrinol Metab. 2010 Nov;299(5):E794-801.
49. Chavez AO, Lopez-Alvarenga JC, Tejero ME, Triplitt C, Bastarrachea RA, Sriwijitkamol A, et al. Physiological and molecular determinants of insulin action in the baboon. Diabetes. 2008 Apr;57(4):899-908.
50. Pehleman TL, Peters SJ, Heigenhauser GJ, Spriet LL. Enzymatic regulation of glucose disposal in human skeletal muscle after a high-fat, low-carbohydrate diet. J Appl Physiol. 2005 Jan;98(1):100-7.
51. Bachmann OP, Dahl DB, Brechtel K, Machann J, Haap M, Maier T, et al. Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans. Diabetes. 2001 Nov;50(11):2579-84.
52. Borkman M, Campbell LV, Chisholm DJ, Storlien LH. Comparison of the effects on insulin sensitivity of high carbohydrate and high fat diets in normal subjects. J Clin Endocrinol Metab. 1991 Feb;72(2):432-7.
53. Musso G, Gambino R, Cassader M. Obesity, diabetes, and gut microbiota: the hygiene hypothesis expanded? Diabetes Care. 2010 Oct;33(10):2277-84.
54. Pussinen PJ, Havulinna AS, Lehto M, Sundvall J, Salomaa V. Endotoxemia is associated with an increased risk of incident diabetes. Diabetes Care. 2011 Feb;34(2):392-7.
55. Lassenius MI, Pietilainen KH, Kaartinen K, Pussinen PJ, Syrjanen J, Forsblom C, et al. Bacterial endotoxin activity in human serum is associated with dyslipidemia, insulin resistance, obesity, and chronic inflammation. Diabetes Care. 2011 Aug;34(8):1809-15.
56. Dasu MR, Devaraj S, Park S, Jialal I. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. Diabetes Care. 2010 Apr;33(4):861-8.
57. Spruss A, Kanuri G, Wagnerberger S, Haub S, Bischoff SC, Bergheim I. Toll-like receptor 4 is involved in the development of fructose-induced hepatic steatosis in mice. Hepatology. 2009 Oct;50(4):1094-104.
58. Ohno I, Yamaguchi Y, Saikawa H, Uetake D, Hikita M, Okabe H, et al. Sevelamer decreases serum uric acid concentration through adsorption of uric acid in maintenance hemodialysis patients. Intern Med. 2009;48(6):415-20.
59. Zhang R, Xing M, Ji X, Gu L, Yang X, Wang H, et al. Interferon-alpha and interleukin-6 in SLE serum induce the differentiation and maturation of dendritic cells derived from CD34+ hematopoietic precursor cells. Cytokine. 2010 May;50(2):195-203.
60. Guigas B, Sakamoto K, Taleux N, Reyna SM, Musi N, Viollet B, et al. Beyond AICA riboside: in search of new specific AMP-activated protein kinase activators. IUBMB Life. 2009 Jan;61(1):18-26.
61. Radin MS, Sinha S, Bhatt BA, Dedousis N, O'Doherty RM. Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle. Diabetologia. 2008 Feb;51(2):336-46.

62. Vesentini S, Soncini M, Fiore GB, Redaelli A. Mechanisms of polymyxin B endotoxin removal from extracorporeal blood flow: molecular interactions. Contrib Nephrol. 2010;167:45-54.
63. Matsunaga N, Tsuchimori N, Matsumoto T, Ii M. TAK-242 (resatorvid), a small-molecule inhibitor of Toll-like receptor (TLR) 4 signaling, binds selectively to TLR4 and interferes with interactions between TLR4 and its adaptor molecules. Mol Pharmacol. 2011 Jan;79(1):34-41.
64. Snedecor GW C, W. Statistical Methods. Ames: Iowa State University Press
1967.
65. Vlachogiannakos J, Saveriadis AS, Viazis N, Theodoropoulos I, Foudoulis K, Manolakopoulos S, et al. Intestinal decontamination improves liver haemodynamics in patients with alcohol-related decompensated cirrhosis. Aliment Pharmacol Ther. 2009 May 1;29(9):992-9.
66. Farhadi A, Keshavarzian A, Holmes EW, Fields J, Zhang L, Banan A. Gas chromatographic method for detection of urinary sucralose: application to the assessment of intestinal permeability. J Chromatogr B Analyt Technol Biomed Life Sci. 2003 Jan 25;784(1):145-54.
67. Farhadi A, Keshavarzian A, Fields JZ, Sheikh M, Banan A. Resolution of common dietary sugars from probe sugars for test of intestinal permeability using capillary column gas chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2006 May 19;836(1-2):63-8.
68. Odunsi A, Flood K, Wells S, Kempton C, Al-Shahrouri HZ, Forsyth C, et al. Increased Permeability of the Small Intestine Wall in Type 2 Diabetic Patients with Nephropathy and Laboratory Evidence of Systemic Inflammation J Invest Medicine. 2011;59(532).
69. Dong Q, Nelson DE, Toh E, Diao L, Gao X, Fortenberry JD, et al. The microbial communities in male first catch urine are highly similar to those in paired urethral swab specimens. PLoS One. 2011;6(5):e19709.
70. Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, et al. The ecoresponsive genome of Daphnia pulex. Science. 2011 Feb 4;331(6017):555-61.
71. Nelson DE, Dong Q, Van der Pol B, Toh E, Fan B, Katz BP, et al. Bacterial communities of the coronal sulcus and distal urethra of adolescent males. PLoS One. 2012;7(5):e36298.
72. A framework for human microbiome research. Nature. 2012 Jun 14;486(7402):215-21.
73. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 2012 Aug;6(8):1621-4.
74. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006 Jul;72(7):5069-72.
75. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010 May;7(5):335-6.

4. MANUSCRIPTS: N/A