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ROLE OF MICROBIOME IN IMPACTING TREATMENT OF OBESITY AND TYPE 2 DIABETES

Aneseh Adeshirlarijaney

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Under the Direction of Andrew T. Gewirtz, PhD

ABSTRACT

Metabolic Syndrome is a constellation of metabolic abnormalities associated with insulin resistance and obesity, including hyperglycemia and hypertension. Metabolic syndrome often progresses to type 2 diabetes, hypertension, cardiovascular disease, and liver disease. Metabolic syndrome is increasingly appreciated to be an inflammatory disease in that is associated with increased expression of pro-inflammatory genes and markers, remodeling of adipose tissue, and markedly increased incidence in the last 50 years. Additionally, both diseases alter the microbiota, specifically with alteration in gut microbiota composition. Metabolic syndrome requires a microbiota in that disease is not observed in germ- free mice, and some aspects of the disease can be transferred by fecal transplant. There is a probable correlation between metabolic disorder and

gut microbiota. It's also been shown that the efficacy of systemic medications can be affected by the gut microbiota and that some medications can alter the microbiota. Metformin is believed to be one of those medications. Accordingly, the results of the present study could be employed to develop novel methods for treating metabolic syndrome using medications such as metformin. Furthermore, this study can set the stage for further research towards the application of fecal transplantation as a treatment strategy for individuals with conditions like metabolic syndrome. The overall goal of my studies was to investigate this hypothesis.

First, I comprehensively examined the existing gut microbiota literature to discern the range of treatment of type 2 diabetes mellitus that have been associated with, or attributed to, changes in microbiota composition. Chapter 1 outlines findings from this effort.

Next, I performed experiments to investigate the extent to which metformin attenuates metabolic syndrome and inflammation by alternation of intestinal microbiota. My results support the notion that metformin induces changes in gut microbiota composition. However, such changes were not necessary for metformin to alleviate parameters of metabolic syndrome indicating that metformin can, at least in part act independently of gut microbiota. Rather Metformin reduced indices of inflammation in both conventional germ-free conditions. These results support a role for metformin's anti-inflammatory effects rather than its direct action on microbiota for its beneficial metabolic impacts.

INDEX WORDS: Intestinal microbiota, Obesity, Inflammation, Metabolic Syndrome, type 2 diabetes, treatment

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ANESEH ADESHIRLARIJANEY

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by

ANESEH ADESHIRLARIJANEY

Committee Chair: Andrew T. Gewirtz

Committee: Benoit Chassaing

Didier Merlin

Jun Zou

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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DEDICATION

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **V**

LIST OF FIGURES ..... **IX**

LIST OF ABBREVIATIONS ..... **X**

1 INTRODUCTION **1**

1.1 Relationship between Obesity and Diabetes **2**

1.2 A Complex Mechanism based on Inflammation and Microbiome Links Obesity to Diabetes **2**

1.3 Effect of Metformin on Inflammation and Microbiome **6**

1.4 Intestinal Microbiota may Lead to new therapies for Metabolic Syndrome **7**

2 CONSIDERING GUT MICROBIOTA IN TREATMENT OF TYPE 2 DIABETES MELLITUS **9**

2.1 Abstract **9**

2.2 Introduction: Rationale for considering gut microbiota in diabetes **9**

2.3 Alteration of gut microbiota composition in humans with T2D **11**

2.4 Interventions that deliberately target microbiota in T2D **12**

2.5 Probiotics in diabetes **14**

2.6 T2D pharmaceutical agents that impact microbiota **15**

2.7 Metformin **15**

2.8 Other drugs proven to benefit T2D **17**

2.9 Herbal agents used to treat T2D and gut microbiota 19

2.10 Microbiota-metabolizable carbohydrates (fermentable fiber) 21

2.11 Conclusions & Perspective 23

3 AMELIORATION OF METABOLIC SYNDROME BY METFORMIN ASSOCIATES WITH REDUCED INDICES OF LOW-GRADE INFLAMMATION INDEPENDENTLY OF THE GUT MICROBIOTA 24

3.1 Abstract 24

3.2 Introduction 25

3.3 Experimental Procedures 27

3.3.1 Mice and high-fat diet administration 27

3.3.2 Metformin administration 27

3.3.3 Microbiota reduction 28

3.3.4 Fasting Blood Glucose Measurement 29

3.3.5 Magnetic resonance imaging (MRI) 29

3.3.6 Bacteria localization by FISH Staining 29

3.3.7 Microbiota analysis by 16S rRNA gene sequencing 29

3.3.8 RNA Isolation and QRT-PCR 29

3.3.9 Quantitation of microbiota by qPCR 30

3.3.10 Western blot 31

3.3.11 Histology 31

3.3.12 ELISAs *31*

3.3.13 Quantification and Statistical Analysis *31*

3.4 Results **32**

3.4.1 Metformin's amelioration of diet-induced metabolic syndrome is associated with beneficial impacts on gut microbiota *32*

3.4.2 Metformin ameliorates metabolic syndrome in the absence of a microbiota *34*

3.5 Discussion **38**

3.6 Figures and legends **43**

3.7 Supplemental Figures and Legends **48**

3.8 Disclosures **56**

3.8.1 Funding *56*

3.8.2 Authors' contributions *56*

4 CONCLUSION **57**

REFERENCES **61**

LIST OF FIGURES

Figure 1 Metformin ameliorates HFD-induced metabolic syndrome. 43

Figure 2 Metformin impacts microbiota composition and reduces microbiota encroachment. ... 44

Figure 3 Amelioration of HFD-induced metabolic syndrome in antibiotic-treated mice..... 45

Figure 4 Efficacy of oral metformin amidst antibiotics. 46

Figure 5 Maintained efficacy of metformin in HFD-fed germfree mice. 47

Figure 6(relates to Figs 1, 3, and 4). 48

Figure 7(relates to Fig 2)..... 49

Figure 8(relates to Fig 4)..... 51

Figure 9(related to Fig 4). 52

Figure 10 (relates to Fig 4)..... 53

Figure 11 (relates to Fig 4)..... 54

LIST OF ABBREVIATIONS

IDF	International Diabetes Federation
MCP-1	Monocyte chemotactic protein 1
MIF	Macrophage migratory inhibitory factor
TNF-a	Tumor necrosis factor alpha
IL-6	Interleukin 6
IL-1	Interleukin-1
DM2	Type 2 diabetes mellitus
AMP	Activated protein kinase
AMPK	AMP-activated protein kinase
T2DM	type 2 diabetes mellitus
ATP	Adenosine triphosphate
HFD	High-fat diet
LPS	Lipopolysaccharide
GABA	Gamma-aminobutyric acid
IBD	Inflammatory bowel disease
TLR5	Toll like receptor 5
FMT	Fecal microbiota transplant
SCFAs	Short-chain fatty acids
GLP-1	Glucagon-like peptide-1
PPAR-γ	proliferator-activated receptor gamma
IL-22	Interleukin-22
GBC	Grain-based chow

IP	Intraperitoneal
CXCL1	Chemokine (C-X-C motif) ligand 1
DIO	Diet-induced obesity
ELISA	Enzyme-linked immunosorbent assay
MRI	Magnetic resonance imaging
IR-HFD	Irradiated HFD
Lcn-2	Lipocalin-2
LEfSE	Linear discriminant analysis effect size
PCoA	Principal coordinate analysis
NS	Normal Saline
ASF	Altered Schaedler flora

1 INTRODUCTION

While many consider obesity as a major health concern of the current century, in fact, existing artwork, even those from the prehistoric and paleothic times, prove obesity is not a new problem. The statues of Venus of Willendorf from Stone Age show obese silhouettes from 25 thousand years ago. Obesity can also be seen in the Venus of Catalhoyuk (Turkey), representing a female figure from Neolithic times sitting on a leopard throne. Even the statues of the Mayan men demonstrate the existence of protuberant abdomen and central adiposity during the Atlantic Ocean Mesoamerican times (in Central America) (1).

Yet, indeed, prevalence of obesity is rising throughout the world, and thus it is considered as a global epidemic. According to the International Diabetes Federation (IDF) in 2017, there were 451 million (age 18-99 years) people with diabetes worldwide (2). This is expected to reach 693 million by 2045. It is also estimated that half of the individuals living with diabetes (49.7%) remain undiagnosed. In 2017, approximately 5 million adult deaths worldwide were attributed to diabetes. The global healthcare expenditure on diabetes was estimated to be 850 billion USD in the same year (3)

Obesity affects health in various aspects, ranging from depression and anxiety to respiratory problems like sleep apnea and asthma, type 2 diabetes, heart disease, increased risk of cancer, liver disease, stroke, musculoskeletal complications such as osteoarthritis and back pain and infertility. Obesity is more common amongst people from more deprived areas of developed countries, although, in less developed countries, the wealthier class with an easier access to food are more prone to the condition. It also affects a range of minority ethnic groups disproportionately.

1.1 Relationship between Obesity and Diabetes

The ongoing rise in the prevalence of obesity in the United States has been driven, at least in part, by the availability of cheap, appetizing, and high-calorie foods along with reduced fiber impacted on gut microbiota (4-5). On the other hand, the close relationship between diabetes and obesity has been clear and well-documented for the last 50 years. Prevalence of obesity in people with diabetes varies between 30% and 80%, depending on the ethnicity and geographical region. While the opposite is not true, as diabetes is not commonly reported among obese individuals. Statistics show that approximately one in four US adults suffer from obesity. This is while only one in every 20 of them are diagnosed with diabetes. In other words, approximately 10% of the obese individuals have diabetes (6).

1.2 A Complex Mechanism based on Inflammation and Microbiome Links Obesity to Diabetes

Many hypotheses such as 'lipid overflow', 'inflammation', 'adipokine', 'microbiome', and 'brown fat' are reported to explain the mechanism linking obesity to diabetes (7-10). It is important to highlight that these mechanisms are not necessarily exclusive, as multiple mechanisms may co-exist and interact, causing obesity in diabetic individuals. Considering the objectives of the current project, inflammation and microbiome will be discussed in more detail. The **inflammation hypothesis** suggests that the accumulation of dysfunctional adipose tissue leads to 'retained inflammation,' or chronic low-level inflammation in response to positive energy balance. Many believe white adipose tissue is the main culprit in obesity-related diabetes. The white adipose tissue is considered as an organ, given its distinct functions such as long-term energy storage and regulation of metabolism. However, unlike most organs, the white adipose

tissue can be found in different parts of the body. Different depots of white adipose tissue appear to have different immuno-phenotypes, metabolic profiles, and differentiation potentials. The two most significant depots of white adipose tissue are subcutaneous and visceral adipose tissues. Dysfunctional adipose tissue is mainly found in the visceral fat depots where subcutaneous adipose tissue is less inflammatory. Adipocyte hypertrophy leads to infiltration and accumulation of adipose tissue macrophages, which are the critical cells mediating the inflammatory response. Two main molecules involved in the recruitment of macrophages are monocyte chemotactic protein 1 (MCP1) and macrophage migratory inhibitory factor (MIF), which respectively attract macrophages and retain them in the adipose tissue; and thus, are responsible for the accumulation of high numbers of adipose tissue macrophages, leading to the secretion of pro-inflammatory cytokines (11).

One of the critical pro-inflammatory cytokines produced in dysfunctional adipose tissue is tumor necrosis factor-alpha (TNF-α,), a well-known cytokine produced by the adipose tissue macrophages and responsible for an increase insulin resistance in adipose tissue, muscle, and liver (12). The adipocytes themselves produce interleukin 6 (IL-6), resulting in an increase in insulin resistance, lipolysis, and thus, a surge in free-fatty acid levels and hepatic glucose output; these all lead to hyperglycemia (13). Interleukin-1 (IL-1), similar to TNF-α, causes insulin resistance. There appears to be a link between these interleukins as they inhibit the downstream signal transduction from the insulin receptor, thus, inducing insulin resistance in target cells (14). Therefore, the induction of lipolysis by IL-6 can amplify the insulin resistance response by releasing fatty acids.

MCP-1, as previously noted, is expressed at high levels in obesity. Hyperinsulinemia increases adipocyte expression of MCP-1 in case of insulin resistance, e.g., during diet-induced obesity.

Moreover, this attracts macrophages towards adipose tissue, initiating and perpetuating inflammation. This results in increased insulin resistance in adipocytes, skeletal muscles, and liver, leading to impaired glucose disposal. Inflammatory cytokines, at the same time, increase the lipolytic release of free-fatty acids and blood glucose levels via insulin resistance, causing glucolipotoxicity at the beta-cell level. This is associated with beta-cell apoptosis and impaired insulin secretion. The combination of insulin resistance and failure to secrete insulin, is obviously the recipe for diabetes (11).

Over the last few years, mounting evidence has been produced indicating that there exist bacteria in the gastrointestinal tract known as microbiota, which have a profound effect on health. The term **"microbiota**" is used to describe the bacteria residing in the gastrointestinal lumen, whereas, "microbiome," is used to describe a collection of genes existing in this bacterial population. Currently, the bacteria species and their relative proportions in microbiota is defined through DNA isolation from a stool sample and analyzing the 16s RNA sequences of the various types of bacteria obtained from the lumen.

Microbiota can influence metabolism through different means. They regulate appetite by controlling hormones and other mediators, regulating glucose and lipid production in the gastrointestinal lumen. This changes the patterns of digestion and absorption of nutrients, regulating inflammatory responses in the body.

The 'microbiome hypothesis' postulates that in some people, the existence of pro-obesity microbiota causes obesity and diabetes through different mechanisms such as reducing satiety feedback signals to the brain, increasing free-fatty acid levels, increasing pro-inflammatory changes in the liver and adipose tissue, causing alterations in skeletal muscle metabolism, and finaly degrading the barrier function of the intestinal epithelium. The latter mechanism results in

the excessive entrance of mediators including pro-inflammatory toxins into the portal circulation. This inflammatory state also alters the absorption of different nutrients from the food consumed (15).

There exists solid evidence indicating that obesity is associated with various changes in the characteristics of the microbiota, such as reduced diversity and altered relative proportions of different species. It is even demonstrated that obesity is also responsible for some alternations in the microbiota population, including an increase in the ratio of Firmicutes to Bacteroidetes and in relative abundance of Proteobacteria (16). One of the main practical applications of the research carried out in this field, is the provision of new horizons for transplantation of metabolic phenotypes. For example, in a randomized controlled trial, when transplanted with lean persons' feces, obese volunteers demonstrated a statistically significant improvement in insulin sensitivity. It should be noted that despite the promising results, fecal transplantation is not yet clinically available to patients who may benefit from the procedure (17-18).

In summary, several pieces of evidence have emerged to link obesity to diabetes. Firstly, both obesity and diabetes affect the same organs such as adipose tissue, pancreas, liver, muscles, and the brain. Secondly, chronic positive energy balance leads to accumulation of fat in the adipose (particularly in the visceral fat depot) and peripheral tissues such as liver, muscle, and pancreas. Thirdly, the chronic low-level inflammatory state caused by obesity leads to insulin resistance, hyperlipidemia, and accumulation of fat and lipotoxicity in the periphery. Finally, when present, brown adipose tissue that may play a role in insulin sensitivity can further highlight the relationship between obesity and diabetes. It should be stated, nonetheless, that all mentioned mechanisms may coexist, interact, and influence individuals in different proportions.

1.3 Effect of Metformin on Inflammation and Microbiome

Biguanides, notably metformin, have been used extensively as the first line medication for treating type 2 diabetes mellitus (DM2) over the past 50 years (19). However, the exact mechanism(s) of action of metformin, especially those responsible for its numerous reported health benefits and anti-hyperglycemic properties, are still unknown (19, 20). The UK Prospective Diabetes Study Group in 1998 reported the survival benefit and cardiovascular protection of metformin compared to other conventional DM2 treatments, including diet, sulfonylurea, and insulin (21). In a clinical trial, the consumption of metformin was found to be advantageous even in patients suffering from renal insufficiency or heart failure who were historically thought to be at higher risk for the side effects of biguanides, notably lactic acidosis (22,23).Moreover, several reports recommend metformin over phenformin, the first generation biguanide, as the former is considered a safe medication with no evidence of lactic acidosis in non-diabetics, even at old ages (24-26). It is widely accepted that the anti-hyperglycemic properties of metformin are mainly because of a mild and transient inhibition of the mitochondrial respiratory-chain complex 1 (27). This increases the level of intracellular activated protein kinase (AMP) compared to Adenosine triphosphate (ATP). The increase in AMP to ATP ratio activates AMP-activated protein kinase (AMPK), which switches cells from an anabolic to a catabolic state. Consequently, glucose, lipid, and protein synthesis are inhibited while the oxidation of fatty acids and glucose uptake are stimulated (19). This pathway has been confirmed in several cell types involved directly in metabolism and energy expenditure such as hepatocytes (28), skeletal muscle cells (29), and pancreatic cells (30). Moreover, there is some evidence from

clinical studies revealing that metformin improves gut microbiota in obese and type 2 diabetes mellitus (T2DM) patients; the underlying mechanisms however remain unknown (31).

The metformin-induced increase in AMPK plays an essential role in maintaining energy balance and controlling glucose metabolism; the cellular AMP/ATP ratio is maintained by increasing ATP consumption and decreasing ATP production, which is associated with AMPK activation (24). Recent studies also reported that metformin regulates hepatic gluconeogenesis and improves hyperglycemia independently of the AMPK pathway (25), suggesting that metformin-induced improvement of metabolic disorders can be mediated by other mechanisms. Moreover, it has been shown that metformin can induce changes in the gut microbiota; however, the relationship between metformin treatment and the gut microbiota remains unclear (24). In this study, the composition of the gut microbiota, as well as several metabolic parameters, will be investigated in a mouse model with high fat diet (HFD)-induced metabolic syndrome receiving metformin treatment or not. According to recent studies, HFD can affect the intestinal environment, and thus results in dysbiosis of the microbiome population (32). This causes a decrease in their distance from the intestinal epithelial cells and thus increased intestinal permeability and easy passage of lipopolysaccharide (LPS) into the portal blood circulation (33). The disruption of the intestinal barrier causes gut microbiota-derived endotoxemia and is believed to contribute to obesity, insulin resistance, and T2DM (34-40). This study further examines the effects of metformin on reversing obesity and T2DM.

1.4 Intestinal Microbiota may Lead to new therapies for Metabolic Syndrome

In recent years, there has been an enormous interest in the role of microorganisms in the gut, both bacteria and viruses, in influencing body mass; for instance, a recent study has suggested

that adenovirus residing in the gut of chickens can cause obesity. It is essential to define the mechanistic paradigm by which "environmental" (i.e., non-genetic) factors might alter the microbiota-intestinal interactions, leading to chronic intestinal inflammation, which can manifest as metabolic syndrome. The epithelium of a healthy gut does not display any signs of transformation. The change observed in both obese and T2DM patients arises from the interactions between diet antimicrobials and the often-inadvertent colonization of the gut with new biota. An inflamed gut shows abnormal structures, which cause thickening of the mucosa and protrusion of the immune cells, depletion of gamma-aminobutyric acid (GABA) cells, disruption of the brush border and surface epithelial cells. Only some bacteria can adapt to this environment. The others, which mainly are responsible for good health in humans, become scarce or disappear. The excessive use of antibiotics during early childhood, which is already known to contribute to allergic disorders, inflammatory bowel disease (IBD), and metabolic problems such as obesity, is another reason for microbiota damage. Moreover, diet can also cause rapid changes in the microbiota. Thus, a better understanding of the causes of mismanagement of gut microbiota are of paramount importance.

As previously described, there is a probable correlation between metabolic disorder and intestinal microbiota. On the other hand, it has been elucidated that the effects of certain medications on the intestinal microbiota and its bacterial pattern determines their specific properties. Metformin is believed to be one of these medications. Accordingly, the results of the present study could be used to develop novel treatments for metabolic syndrome. Furthermore, this study can set the stage for further research towards the application of fecal transplantation as a treatment strategy for individuals with conditions such as metabolic syndrome. Using the later

technique, optimizing the existing treatments would be sufficient to overcome obesity and no new drugs would be needed.

2 CONSIDERING GUT MICROBIOTA IN TREATMENT OF TYPE 2 DIABETES MELLITUS

2.1 Abstract

Advances in the understanding of the pathogenesis of type 2 diabetes mellitus has revealed a role for gut microbiota dysbiosis in driving this disease. This suggests the possibility that approaches to restore a healthy host-microbiota relationship might be a means of ameliorating this disorder. Indeed, recent studies indicate that many currently used treatments for type 2 diabetes are reported to impact gut microbiota composition. Such changes in gut microbiota may mediate and/or reflect the efficacy of these interventions. This article outlines the rationale for considering the microbiota as a central determent of development of type 2 diabetes and reviews evidence that amelioration of this disorder in response to therapeutic interventions based on pharmaceutical reagents and diet involves changes in gut microbiota.

2.2 Introduction: Rationale for considering gut microbiota in diabetes

Type 2 diabetes (T2D) is characterized by loss of glycemic control resulting in hyperglycemia, especially post-prandially, due to hyporesponsiveness to insulin; i.e. insulin resistance. The notion that gut microbiota might play a role in this disorder stems largely from the general appreciation, stemming from work of Jeff Gordon and colleagues, that gut microbiota contribute broadly to energy balance (41) and the realization of Patrice Cani that microbiota products such as LPS can drive low-grade inflammation (42), which had long been recognized as a potential cause of insulin resistance. Re the former, briefly, mice completely lacking

microbiota (i.e. germfree mice) exhibit reduced energy harvest from ingested food and increased energy expenditure, associated with increased activation of AMP-activated protein kinase (AMPK), which plats a central role in energy homeostasis. Such activation of AMPK has been suggested to protect germfree mice from diet-induced diabetes. While germfree mice can be considered an extreme state, interpolating their phenotype suggests that differences in microbiota composition can, more subtly but nonetheless broadly, influence metabolic phenotype and thereby be a determinant of diabetes and its inter-related metabolic diseases states, namely obesity and metabolic syndrome. In accord with this notion, obesity in mice and humans is associated with alterations in microbiota composition, and transfer of microbiota from obese hosts to germfree mice leads to increased adiposity, relative to germfree mice receiving microbiotas from lean hosts (43).

The hypothesis that low-grade inflammation drives the insulin resistance that characterizes T2D type 2 diabetes originated from Hotamisligil and colleagues, who demonstrated that increases in adipose tissue characteristic of obesity is typically accompanied by increased expression of pro-inflammatory cytokines, which are produced by adipocytes themselves and macrophages that are recruited into adipose tissue as obesity develops (44). While Hotamisligil hypothesized that such pro-inflammatory gene expression resulted from intracellular stress of adipocytes being overloaded with lipids, Cani found that such inflammation, and subsequently insulin resistance could result from translocation of lipopolysaccharide from the gut lumen into portal circulation resulting in activation of pro-inflammatory gene expression via Toll-like receptor 4 (45). This scenario suggests a variety of means by which alterations in microbiota composition could impact T2D, including altering abundance of species that produce LPS and/or other microbial products with strong pro-

inflammatory potential. It also underscores a key role for epithelial barrier function in restricting microbial products to the gut lumen. In this context, gut barrier function includes not only intercellular junctions that directly impede passage of bacterial products but also host systems of mucus deployment and innate immunity that keep bacteria, themselves, at a safe distance from the epithelium and help maintain stable microbiota composition. These latter points are shown by our study of mice that with a discrete defect in innate immunity, namely absence of the flagellin receptor toll-like receptor 5 (TLR5). TLR5-deficient mice fail to manage their microbiota, resulting in altered composition, including elevated γ-Proteobacteria and, moreover, exhibit microbiota encroachment, which is defined as a decrease in bacterial-epithelial distance (46, 47). Such alterations result in TLR5-deficient mice developing insulin resistance, which can be transferred to WT germ free mice via microbiota transplant. Such studies provide a rational basis for targeting microbiota as a means of preventing and/or treating T2D.

2.3 Alteration of gut microbiota composition in humans with T2D

While much of our understanding of mechanisms whereby microbiota can impact glucose homeostasis comes from mouse studies, alterations in microbiota composition have also been observed in a range of human cohorts T2D (48-50). Larsen et. al observed that the Bacteroidetes to Firmicutes ratio and the ratio of *Bacteroides-Prevotella* group to C. *coccoides-E. rectale* group associated positively with plasma glucose concentration (49). Other differences observed included decreased abundance of butyrate-producing bacteria, including *Clostridiales* spp., *E. rectale*, *Faecalibacterium prausnitzii*, *Roseburia intestinalis* and *Roseburia inulinivorans*, and increased abundance of *Lactobacillus* species. Increased prevalence of Bacteroidetes and Proteobacteria phyla were also observed. Proteobacteria contain many pathobionts, which can be envisaged to have a role in inducing low-grade inflammation in diabetic patients through their

LPS, flagella, and/or other surface components (49). Analysis of microbiota composition in a cohort of Chinese T2D patients and healthy control subjects observed increased abundance of opportunistic pathogens including *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum*, *Eggerthella lenta* and *E. coli in T2D* patients (50). In a longitudinal cohort on monozygotic Korean twins, it is suggested that decreased *Akkermansia muciniphila* could be used as a biomarker for the early diagnosis of type 2 diabetes (51). Moreover, *Acidaminococcus*, *Aggregatibacter*, *Anaerostipes*, *Blautia*, *Desulfovibrio*, *Dorea*, and *Faecalibacterium* have been identified as being associated with type 2 diabetes in a Mendelian randomization study and these genera should be investigated more in future research (52). While the wide variety of specific differences can be envisioned to have a variety of functional consequences, we have observed that TD2 patients exhibit microbiota encroachment (53). Thus, these human observational studies are in accord with the concept that targeting microbiota is a logical target to treat insulin resistance and, consequently T2D.

2.4 Interventions that deliberately target microbiota in T2D

An array of studies, some of which are outlined above, indicate that dysregulated or improperly managed microbiota promotes insulin resistance, leading to the suggestion that broadly suppressing levels of microbiota might be a means of ameliorating this disorder. While, relatively short-term studies in mice support this concept, it is unlikely to prove a therapeutic option to manage this chronic disease in humans due to the general negative impacts of antibiotics on gut health, especially relating to risk of serious infection by antibiotic resistant bacteria. Moreover, numerous studies have associated frequent use of antibiotics with T2D, thus further supporting the role of a stable microbiota in preventing T2D but arguing against the notion that broad-based ablation of microbiota can be a practical approach to treat T2D in

humans. Rather, direct deliberate attempts to influence microbiota composition to promote insulin sensitivity and thus ameliorate T2D have utilized fecal microbiota transplant (FMT), probiotics or prebiotic. The latter will be discussed below under dietary fiber, since such studies were often initiated prior to appreciation of the role of microbiota. Here, we discuss FMT and use of specific probiotics.

The logic of FMT is relatively straight forward, namely to replace a dysbiotic microbiota with a healthy one, which will have the needed diversity to stably persist in its new host. Proof of concept generally comes from mouse studies wherein transplanted communities persist in their new hosts for extended periods and the highly effective use of FMT to prevent recurrence of *C. difficile* infection. Use of FMT to ameliorate insulin resistance in humans is largely the work of Nieuwdorp and colleagues whom have performed well-controlled randomized clinical trials studying this approach. Such trials have shown that, following colonoscopy in which the preparation of the colon removes a considerable portion of the total microbial mass in the intestine, FMT from healthy subjects, or persons with good glycemic control as result of bariatric surgery, can improve insulin sensitivity relative to FMT with one's own feces (i.e. placebo control) (54, 55). However, the beneficial impacts are transient as is the engraftment of the donor microbiota. Moreover, a variety of poorly understand factors in recipient's microbiota influence both engraftment and any beneficial metabolic impacts (56). The transient nature of these effects may reflect that, unlike germfree mice, the microbiota of a host with an established microbiota is more difficult to permanently replace and/or that whatever causes had led to an unhealthy microbiota in the first place, for an example, an unhealthy diet, have not changed and will result in failure to maintain the engraft microbiota. In any case, collectively, results from

FMT studies support the notion that changing microbiota can positively impact diabetes but underscores that doing so in a lasting manner is not yet be easily achieved.

2.5 Probiotics in diabetes

One potential approach to attaining a healthy microbiota is to directly administer beneficial bacteria; i.e. probiotics, which the International Scientific Association for Probiotics and Prebiotics defines as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (49). Experimental studies and clinical trials support the hypothesis that the modulation of the intestinal microbiota by probiotics could be effective in diabetes management (50) in which the most widely studied probiotics with respect to diabetes are *Bifidobacterium* and *Lactobacillus* strains. Specific strains of *L. rhamnosus*, *L. acidophilus*, *L. gasseri* and *L. casei* have been demonstrated to exert anti-diabetic effects (51-55). Moreover, several strains of *L. plantarum* species have been reported to improve the glycemic control in obese and diabetic patients by their carbohydrate-utilizing genes (56, 57). *Bifidobacterium animalis*, *B. breve* and *B. longum* could also be helpful in improvement of glucose intolerance (58, 10, 19). While results individual studies are variable, a recent meta-analysis has shown that these probiotics improve glycemic control and significantly decrease the risk of gestational diabetes mellitus in pregnant women (60). Underlying mechanisms by which probiotics might have impacted host metabolism have not been well defined but may include favorable changes of the composition and/or activity of the microbiota, inhibition of α-glucosidase activity, production of anti-microbial lactic acid, improvement of intestinal barrier function, immune modulation, short-chain fatty acid production, and regulation of bile acid metabolism (58,50,61). Other candidate probiotics include gut bacteria including *Akkermansia muciniphila* and *Faecalibacterium prausnitzii*, which are negatively associated with overweight and

hyperglycemia, may be potential candidates for next generation probiotics; further studies are needed in this field (62). A recent placebo-controlled trial supported this concept in that direct administration of Akkermansia muciniphilia improved glycemic control in persons with metabolic syndrome although, unexpectedly, the impact of heat-killed Akkermansia appeared more significant than that of the live organism highlighting that further development is needed before deploying this strategy on a large scale.

2.6 T2D pharmaceutical agents that impact microbiota

While deliberate targeting of the microbiota to ameliorate T2D is clearly in early stages of development, it is increasingly being appreciated that several drugs that have long been used to treat T2D result in impacts on gut microbiota in a manner that might contribute to their efficacy

2.7 Metformin

Metformin (dimethyldiguanide), discovered in 1922 based on study of the plant Galega officinalis (Goat's Rue) to lower blood glucose, is a very common treatment for T2D, especially T2D associated with obesity. Yet, its mechanism of action remains unclear but may include inhibition of mitochondrial function via respiratory chain complex I or glycerophosphate dehydrogenase, activation of 5' AMP-activated protein kinase (AMPK), and amelioration of glucagon-induced cAMP. Moreover, there is evidence to suggest a role for gut microbiota in mediating metformin's ability to improve glycemic control. In contrast to oral metformin, intravenous administration of metformin lacks does not control hyperglycemia thus suggesting the intestine as an important site of metformin action (57). Furthermore, in both mice and humans, metformin alters microbiota composition to make it more resembling of microbiotas of healthy hosts (58-60). Some of these changes were observed amidst healthy persons who don't

exhibit changes in glycemic control in response to this agent thus suggesting the changes in the microbiota result from metformin itself rather than simply reflect improved glycemic control. Yet specific associations have been variable amongst different studies and different states of health in part reflecting the difficulty of dissociating impacts on microbiota due to drugs and/or disease. Overall, in healthy subjects, metformin impacted relative abundance of several phyla including a reduced abundance of Intestinibacter spp. and Clostridium spp., as well as an increased abundance of Escherichia/Shigella spp. and Bilophila wadsworthia. Some of these changes appear reminiscent of changes associated with disease and thus irrespective of potential impacts on glycemic control such change may contribute to gastrointestinal distress, which is the leading cause of metformin intolerance. Indeed, prevalent gastrointestinal side-effects after metformin intake including diarrhea, nausea, vomiting, and bloating have been attributed to increased abundance of *Escherichia* (61).

Regarding how such changes might impact glycemic control, metagenomic analysis of microbiota suggests a range of functional categories of microbial genes that are impacted, including those related to oxidative stress and metal transport. Additionally, metformin-induced changes in microbiota are proposed to impact production of butyrate and propionate activating intestinal gluconeogenesis (62, 63). Stimulated gluconeogenesis in gut has beneficial effects on hepatic glucose production and appetite suppression, contributing to weight reduction and glycemic control (64). On the other hand, expression of microbial genes involved in the degradation of glycine and tryptophan was higher in the untreated diabetic patients compared to metformin-treated patients. Since glycine has been reported to improved insulin sensitivity, this pathway could be nominated as another underlying mechanism (65).

One approach that suggests the overall impact of metformin-induced changes in microbiota are beneficial is that transfer of microbiota from metformin-treated mice was observed to improve metabolic parameters in aged mice suggesting changes in microbiota play a functional role in its beneficial metabolic effects (59). However, this approach does not address the extent to which changes in microbiota are necessary for its impact. We recently investigated this question in mice. We found that the ability of metformin to beneficially impact metabolic syndrome in mice was not impacted by ablation of gut microbiota achieved by use of antibiotics or germfree mice. Rather, while microbiota ablation itself suppressed diet-induced dysglycemia, other features of metabolic syndrome including obesity, hepatic steatosis, and low-grade inflammation were similarly suppressed by metformin in the presence or absence of gut microbiota. While this approach did not directly informative re the role of microbiota in metformin-induced improvement in glycemic control, it suggests a potential role for metformin's anti-inflammatory activity, irrespective of gut microbiota, in driving some of this drug's beneficial impacts.

2.8 Other drugs proven to benefit T2D

Acarbose, α-glucosidase inhibitor, lowers postprandial blood glucose concentration via inhibiting conversion of oligosaccharides into mono- and di-saccharides and delaying intestinal glucose absorption. However, acarbose has also been recently appreciated to impact microbiota composition. For example, assessing gut microbiota alteration after acarbose treatment in type 2 diabetic patients showed increased abundance of *Bifidobacterium longum* and decreased concentration of lipopolysaccharides (66). In another clinical trial in patients with prediabetes, *Butyricicoccus, Phascolarctobacterium,* and *Ruminococcus* decreased while *Lactobacillus, Faecalibacterium* and *Dialister* increased after acarbose intake (67). These compositional shifts

of gut microbiota after acarbose intake suggested microbial mediation of the therapeutic effects of acarbose in part. The extent to which these changes may contrinute to acarbose's impacts on glycemic control are not known.

Stimulation of glucagon-like peptide-1 release has been declared as a potential mediating mechanism for the effects of SCFAs on glucose homeostasis. Glucagon-like peptide-1 as a gut hormone is involved in appetite control and gastric emptying. GLP-1 receptor agonists like liraglutide slow gastric emptying, stimulate satiety, enhance insulin secretion, and suppress glucagon (68). In animal models of obesity, liraglutide induced a reduction of Proteobacteria and an increase of *Akkermansia muciniphila* in gut microbiota (69). Another study on diabetic male rats reported enhancement of SCFA-producing bacteria, including *Bacteroides*, *Lachnospiraceae*, and *Bifidobacterium* after injection of liraglutide (70). Liraglutide substantially altered the overall composition of the gut microbiota, consistent with its weight-lowering effect (71). Genera including *Allobaculum*, *Turicibacter* , *Anaerostipes*, *Blautia*, *Lactobacillus*, *Butyricimonas* and *Desulfovibrio* enhanced and the orders including *Clostridiales* and *Bacteroidales* diminished after intervention, consistent with changes reported in gut microbial composition after body weight control (71). Further investigations are needed to elucidate the role pf microbial mediation in the therapeutic effects of GLP-1 receptor agonists.

Another agent used in treatment of T2D is Pioglitazone, which is a member of of the thiazolidinedione class with hypoglycemic effects thought to result from stimulating activity of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ). Such stimulation results in reducing insulin resistance and decreasing liver gluconeogenesis (72). In animal models of T2D, Pioglitazone decreases diversity of gut microbiota and shift beta diversity in C57BL/6J mice (73). This suggests a possible involvement of the microbiota although human

studies re hos this drug impacts microbiota in T2D and other diseases states have not been reported

It has been found that Sitagliptin and Vildagliptin, DPP-4 inhibitors, altered gut microbial composition in diabetic rats (74, 75). These drugs reduced the diversity of microbiota, increased the abundances of short-chain fatty acids-producing bacteria including *Blautia, Roseburia, Clostridium, Baceroides* and *Erysipelotrichaeae* in gut microbiota and corrected the Bacteroidetes/Firmicutes ratio (74, 75). Therefore, it has been suggested that these drugs may have beneficial effects on blood glucose partly through maintaining the gut barrier integrity and correcting the dysbiosis of intestinal microbiota in diabetes although human studies are needed in this regard.

2.9 Herbal agents used to treat T2D and gut microbiota

Many societies have long treated T2D with a variety of plant-based products and extracts. In some cases, active ingredient(s) have been isolated with results suggesting a potential rolefor microbiota. Additionally, a range of herbal-derived products including berberine, resveratrol, alliin, capsaicin, betacyanins, and cranberry proanthocyanidins have bioactions and antidiabetic effects potentially mediated by modulation of gut microbiota (76-83). Galactomannan, pectin, capsaicin, and red pitaya betacyanins altered the proportion of Firmicutes to Bacteroidetes (78-80). Increased fecal butyrate concentration and *Roseburia* abundance and decreased *Bacteroides* and *Parabacteroides* abundances have been reported after intervention by capsaicin in obese diabetic ob/ob mice (80). Alliin from garlic caused decrease in *Lachnospiraceae* abundance and increase in *Ruminococcaceae* abundance which enhanced glucose homeostasis and insulin sensitivity, but has no effect on adiposity (81). Berberine decreased the relative abundance of branched-chain amino acids-producing bacteria, including *Streptococcus* and *Prevotella* whereas

increased the relative abundance of short-chain fatty acids-producing bacteria, including *Blautia* and *Allobaculum* (82, 84). It has been shown that berberine exerts comparable effects to metformin in altering the microbial diversity and overall structure of the gut microbiota in high-fat diet-induced obese rats (85).

For some herbal extracts, active ingredients are not well defined, nor is their efficacy, nor mechanism of action, although there is increasing effort being applied to fill these gaps of knowledge. Herbs common in traditional Chinese medicine for glycemic control of diabetic patients includes *Folium Mori*, *Dendrobium candidum*, *Rhizoma Dioscoreae*, *Coptis chinensis*, *Fructus MoriL* (86, 87). For example, a multicenter randomized clinical trial on type 2 diabetic patients revealed that both metformin and a traditional Chinese herbal formula significantly alleviated hyperglycemia and dyslipidemia. Such effects correlated with impacts on gut microbiota wherein the herbal mixture had larger effect on this. Changes in microbiota induced by these herbal formulations included enrichment of *Blautia* and *Faecalibacterium* spp., which are thought to be benefici herbal preparations impact gut microbiota composition, suggesting it as a possible contributor to their effects. A range of individual herbal extracts that have been used to treat T2D impact microbiota. *Alpinia oxyphylla Miq*. extract was found to improve glycemic control and renal function in diabetic mice in a manner that associated with increased abundance of *Akkermansia* and increasing the ratio of Bacteroidetes-to-Firmicutes (88) Increased Bacteroidetes to Firmicutes has also shown after intervention with Qijian mixture (*Astragalus membranaceus*, *Ramulus euonymi*, *Coptis chinensis* and *Pueraria lobata*) and extract of *Dendrobium loddigesii* (49, 50). Increased abundance of *Akkermansia* was also associated with glucose-lowering properties of polyphenol-rich extracts of cranberry. Water-ethanol extract of green macroalgae *Enteromorpha prolifera* and Oil tea (green tea and ginger)

enriched *Lachnospiraceae* has been reported after intake of in animal studies (91, 92), and was suggested to underlie its beneficial impact on glycemic control. Extracts of cinnamon bark and grape pomace induced a decrease in *Peptococcus*, *Desulfovibrio*, *Lactococcus* abundances and an increase in *Allobaculum* and *Roseburia* abundances (93), which associated with decreased fat mass, reduced adipose inflammation, and improved glucose tolerance (93). The notion that beneficial impacts of herbal extracts are mediated by reduced inflammation is supported by a study that found *Dendrobium loddigesii* and *Houttuynia cordata,* which are traditional Chinese treatment for T2D results decreased abundance of Gram negative bacteria including *Escherichia coli* and *Bacteriodetes fragilis* that was suggested to improve T2D by reducing exposure to LPS absorption and subsequently inflammatory (90, 94). Moreover, *Dendrobium loddigesii* was reported to improve the gut barrier integrity, which can also reduce metabolic endotexemia and insulin resistance (83, 90). In summary, changes in gut microbiota in response to herbal extracts include increasing microbial diversity, reducing the Firmicutes/Bacteroidetes ratio, increasing the abundances of anti-inflammatory bacteria such as *Bifidobacterium*, *Lactobacillus*, *Akkermansia*, and *Faecalibacterium*, and decreasing the pathogenic bacteria such as *Escherichia coli* and *Enterococcus*, which might alleviate low-grade inflammation subsequently improving glycemic control.

2.10 Microbiota-metabolizable carbohydrates (fermentable fiber)

In addition to specialized, plants and extracts, one major type of macronutrient that has long been recognized as important for metabolic health in general, and thus potentially promoting good glycemic control is dietary fiber. While mechanisms by which fiber might promote metabolic health are complex, it has recently been appreciated that such beneficial impacts are mediated, at least in part by gut microbiota (95). Briefly, complex carbohydrates that

reach the colon that can be fermented by gut bacteria are the major fuel source of the microbiota and has such will have a major impact on total levels of bacteria, composition and its functional activity. Re the former, in mice, lack of fiber decimates microbiota density, which slows enterocyte proliferation, deteriorates mucus, and alters microbiota composition, which together result in microbiota encroachment that promotes low-grade inflammation and insulin resistance (95, 96). Hence, enriching a "western-style" low-fiber high-fat diet with fermentable but not insoluble fiber restores gut health and prevents these consequences. The notion that changes in microbiota are pivotal to such impacts of fiber on glycemic control include lack of effects of such fiber in germfree conditions and that addition of some of the specific bacteria enriched by fiber, namely bifidobacterial could provide beneficial metabolic impacts. A range of other fermentable fibers, including pectin and glucomannan, and resistant corn starches, which can be considered functionally fibers, also impact microbiota and improve glycemic control. The caveats of such studies include that it is difficult to disentangle impacts on glycemic control from other interrelated parameters of metabolic syndrome and that they are largely restricted to mice. In contrast, study of one highly fermentable fiber, inulin, has revealed this fiber can increase levels of Akkermansia muciniphilia in both mice and humans (97). Levels of this microbes are reduced in T2D and direct administration of this microbe to mice improves glycemic control and has shown promise in a recent human trial. In terms of mechanisms, by far the most studied aspects of how nourishing microbiota might improve glycemic control involves the major product of fiber fermentation, short-chain fatty acids (SCFA). SCFA have a variety of direct metabolic benefits that can improve insulin-resistance irrespective and have a variety of anti-inflammatory actions that can be expected to indirectly. However, ability of fermentable fiber, while fully microbiota-dependent, does not absolutely require SCFA per se in that blocking

fermentation only moderately reduced impact of such fibers. Rather, nourishing microbiota with fermentable fiber inulin led to host IL-22 production that was necessary to improve glycemic control and restore gut health, which is impaired by western-style diet. Yet, another means by which inulin improves glycemic control involves its enrichment of Akkermansia muciniphilia, which restores mucus robustness resulting in protection against low-grade inflammation (98). While the notion that a bacteria that feeds on mucus results in more robust mucus is somewhat counter-intuitive, it can be viewed as analogous to the notion that cutting a lawn of grass encourages dense growth. Thus, to some extent microbiota mediated approaches to treating preventing T2D can be viewed in terms of ameliorating inflammation.

2.11 Conclusions & Perspective

TD2 is currently, and seems likely to remain for some time, one of humanities major public health problems. As such humanity needs new approaches to treat and prevent this disorder. While most treatments currently in use, especially pharmaceutical agents with proven effects, have generally focused on agents designed to directly impacts signaling pathways that directly regulate glucose, or work by unknown mechanisms, better understanding of root causes of T2D suggest targeting the gut microbiota might be a logical approach to treating this disorder. As reviewed herein, studies, especially those in animal models, support this notion. Moreover, investigation of currently used pharmaceutical reagents suggest their beneficial effects may be, in part, mediated by impacts on gut microbiota. Given that dysglycemia itself impacts microbiota, disentangling cause and effect is a major confounder this area of research. Yet, we submit that, even if such changes in microbiota are, initially, a consequence of improved

glycemic control, they may still be part of maintaining good glycemic control. Thus, examining how, in humans. current and future treatments of T2D is important to understanding impacts of these agents on health, regardless of whether these agents directly impact microbiota. Similar logic applies to diet-type approaches to ameliorate T2D. Indeed, approaches like caloric restriction to prevent T2D have long preceded appreciation of the microbiota but recent studies that this approach impacts microbiota suggest it as a possible mediator of its prevention of this disorder. In this regard, we suggest that further study and consideration of microbiota, in humans, in response to both pharmaceutical and dietary interventions should pave the way for better approaches to treat and prevent T2D.

3 AMELIORATION OF METABOLIC SYNDROME BY METFORMIN ASSOCIATES WITH REDUCED INDICES OF LOW-GRADE INFLAMMATION INDEPENDENTLY OF THE GUT MICROBIOTA

3.1 Abstract

Metformin beneficially impacts several aspects of metabolic syndrome including dysglycemia, obesity and liver dysfunction, thus making it a widely used frontline treatment for early-stage type 2 diabetes, which is associated with these disorders. Several mechanisms of action for metformin have been proposed, including that it acts as anti-inflammatory agent, possibly as a result of its impact on intestinal microbiota. In accord with this possibility, we observed herein that, in mice with diet-induced metabolic syndrome, metformin is impacting the gut microbiota by preventing its encroachment upon the host, a feature of metabolic syndrome in mice and

humans. However, the ability of metformin to beneficially impact metabolic syndrome in mice was not markedly altered by reduction or elimination of gut microbiota achieved by use of antibiotics or germfree mice. Rather, while reducing or eliminating microbiota by itself suppressed diet-induced dysglycemia, other features of metabolic syndrome including obesity, hepatic steatosis, and low-grade inflammation remained suppressed by metformin in the presence or absence of gut microbiota. These results support a role for metformin's anti-inflammatory activity, irrespective of gut microbiota, in driving some of this drug's beneficial impacts on metabolic syndrome.

3.2 Introduction

The marked worldwide increased prevalence of type 2 diabetes (T2D) is thought to be driven, in large part, by increased prevalence of obesity (108). Obesity promotes an array of metabolic disorders collectively referred to as metabolic syndrome whose features include hypertension, hyperlipidemia, and, most importantly, insulin resistance, which is the central and defining cause of T2D. Metformin (dimethyldiguanide), discovered in 1922 via investigating the ability of the herb Galega officinalis (Goat's Rue) to lower blood glucose, is widely used as a frontline treatment for T2D, especially T2D associated with obesity, in part due its ability to beneficially impact multiple parameters of metabolic syndrome including obesity and liver dysfunction (99). Several mechanisms by which metformin alleviates metabolic syndrome have been proposed, including inhibition of mitochondrial function via respiratory chain complex I or glycerophosphate dehydrogenase, activation of 5' AMP-activated protein kinase (AMPK), and amelioration of glucagon-induced cAMP. Moreover, several studies suggest a central role for gut microbiota in mediating metformin's amelioration of metabolic syndrome.

The notion that metformin's action is mediated by impacts on gut microbiota is supported by reports that, in both mice and humans, metformin alters microbiota composition to make it more resembling of microbiotas of healthy hosts (111). Furthermore, transfer of microbiota from metformin-treated mice was observed to improve metabolic parameters in aged mice suggesting changes in microbiota play a functional role in its beneficial metabolic effects (124). A range of potential means by which select alterations in microbiota by metformin might ameliorate metabolic syndrome have been proposed including impacting sensing of glucose in the small intestine (102), direct alteration of metabolism of ingested food so as to increase bacterial fermentation (109), and alteration of bile acids (113).

More generally, we hypothesize that metformin's restoration of a healthy microbiota composition might reduce the microbiota's capacity to induce host inflammation, which occurs in metabolic syndrome and is known to promote this disorder (104). Our observations that, in both mice and humans, dysglycemia is associated with reduced bacterial-epithelial distance, a state referred to as "microbiota encroachment", suggests that alterations in microbiota composition and/or gene expression can influence microbiota localization, which may influence low-grade inflammation and consequently metabolic syndrome (106,107). Hence, we hypothesized that metformin's impact on microbiota composition might reduce microbiota encroachment, consequently ameliorating metabolic syndrome. In accord with this hypothesis, together with previous observations reporting that metformin results in beneficial impacts on gut microbiota (124,127), we observed herein that metformin-induced changes in microbiota composition that associated with alleviation of microbiota encroachment, low-grade inflammation and metabolic syndrome. However, in contrast to our hypothesis, we failed to demonstrate that the ability of metformin to alleviate metabolic syndrome required the presence

of a microbiota. In part, this observation may reflect that one of the central parameters of the metabolic syndrome that is treated by metformin, namely dysglycemia, is itself ameliorated by reducing or eliminating microbiota thus obscuring metformin's potential to improve glycemic control (103). Nonetheless, our results argue that metformin may ameliorate metabolic syndrome by reducing adiposity and/or low-grade inflammation per se rather than acting directly on gut microbiota.

3.3 Experimental Procedures

3.3.1 Mice and high-fat diet administration

Male, 4-6 week old C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained at Georgia State University, Atlanta, Georgia, USA under institutionally approved protocols (IACUC # A18006), housed 5 mice per cage with ALPHA-dri bedding (Shepherd Specialty Papers) and nestlets (Ancare) and fed *ad libitum*. Mice were fed standard grain-based chow (GBC), Purina/Lab Diet 5001, which is comprised of relatively unrefined ingredients and given 2 weeks to allow for microbiota stabilization. Where indicated, mice were then administered a diet composed of 60% kcal from fat (Research Diet, D12492) for 8-12 weeks. For germfree experiments, we utilized an irradiated version of this diet (10-20 KGy) that achieves sterilization.

3.3.2 Metformin administration

Metformin, 1,1-dimethyl biguanide hydrochloride (Sigma-Aldrich) was administered via intraperitoneal (IP) injection or orally concurrent with HFD exposure. For IP route, 100 μl of saline (NS, vehicle control) or NS containing an amount of metformin that resulted in 300 mg metformin/kg weight, which equates to a 20g mouse receiving 6 mg per day. For oral administration, metformin was added to drinking water at a concentration of 3 or 10 mg/ml or

which was calculated to result in ingestion of 9 or 30 mg per day based on 3 ml water consumption per day. These doses were selected as being typical from reading several papers (101, 125,126). While higher than used in humans, they are approximately similar to that used in other studies in mice.

3.3.3 Microbiota reduction

Antibiotics**:** Mice were administered drinking water containing ampicillin (1.0 g/l) and neomycin (0.5 g/l) upon HFD feeding and continued until mice were euthanized.

Germfree mice: Germ-free wild-type C57BL/6 male mice were purchased from Taconic Inc. and maintained in Isocages (Techniplast USA, West Chester, PA), using procedures which we've shown maintain a germfree state (31). To assure sterility of metformin, after dilution it was filtered through Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES Membrane of 0.2 μm units, protect against bacterial contamination. Similarly-filtered water was used for the control group. GF mice were fed autoclaved chow or γ-irradiation (10-20KGy) HFD. To verify that HFD was truly sterilized by the irradiation, we measured levels of bacterial DNA in feces before and after feeding of this diet as previously described (129). After 8 weeks of metformin treatment, mice were fasted overnight, removed from isocages and immediately euthanized. Lastly, we note that the germfree experiment was done in parallel with another study focused on comparing chow-fed and HFD-fed mice and thus those mice served as control groups for metformin-treated mice (Tran et. Al. CMGH, manuscript in revision).

3.3.4 Fasting Blood Glucose Measurement

Mice were placed in clean cage, fasted for 5 h or overnight as indicated, and blood glucose was measured by Nova Max Glucose Meter and expressed in mg/dL.

3.3.5 Magnetic resonance imaging (MRI)

Bruker MiniSpec MRI Body Composition Measurement was used for MRI fat % using open source MiniSpec software. Mice were weighed, placed into restrainer, inserted into assay tube and data collected for 2 min. Data is expressed as % fat.

3.3.6 Bacteria localization by FISH Staining

Microbiota localization was performed as previously described (Chassaing et al., 2015) and (Jun Zou et al, 2017)

3.3.7 Microbiota analysis by 16S rRNA gene sequencing

Microbiota composition was performed as previously described (Chassaing et al., 2015). Unprocessed sequencing data are deposited in the European Nucleotide Archive under accession number ERP117168.

3.3.8 RNA Isolation and QRT-PCR

Total RNA was extracted from transverse colonic tissues, liver and adiposity using TRIzol (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. Quantitative RT–PCR was performed using the iScript One-Step RT–PCR Kit with SYBR Green (Bio-Rad, Hercules, California) in a CFX96 apparatus (Bio-Rad, Hercules, California) with the primers

listed are listed below in transcript levels were quantified by normalization of each amplicon to housekeeping gene 36B4.

(36B4: 5’ TCCAGGCTTTGGGCATCA-3’ and 5’CTTTATTCAGCTGCACATCACTCAGA-3’ from Invitrogen)

(CXCL1: 5’ TTGTGCGAAAAGAAGTGCAG-3’ and 5’TACAAACACAGCCTCCCACA-3’ from Invitrogen)

(IL-6: 5’ GTGGCTAAGGACCAAGACCA-3’ and 5’GGTTTGCCGAGTAGACCTCA-3’ from Invitrogen)

(TNF-α: 5’ CGAGTGACAAGCCTGTAGCC-3’ and 5’CATGCCGTTGGCCAGGA-3’from Invitrogen)

3.3.9 Quantitation of microbiota by qPCR

Total DNA was isolated via QIAamp DNA Stool Mini Kit (Qiagen). DNA was then subjected to quantitative PCR using QuantiFast SYBR Green PCR kit (Biorad) with universal 16S rRNA primers (515 F GTGCCAGCMGCCGCGGTAA) and (806 R GGACTACHVGGGTWTCTAA) to quantitate total bacteria. Results are expressed as bacteria number per mg of stool, using a standard curve generated from dilutions of a purified culture of E. coli. Proteobacteria analysis used the following primers purchased from Invitrogen: Gamma 877F: GCTAACGCATTAAGTRYCCCG and Gamma 1066R GCCATGCRGCAAATGTCT, Beta 979F: AACGCGAAAAACCTTACCTACC and TGCCCTTTCGTAGCAACTAGTG, Epsilon 940F: TAGGCTTGACATTGATAGAATC and CTTACGAAGGCAGTCTCCTTA from Invitrogen)

3.3.10 Western blot

Due to the high lipid content of livers from HFD-fed mice, Sample were extracted using a high volume of lysis buffer (500 μl per 100 mg) this permitting easy separation of lipid and proteinaceous fractions thus allowing protein quantification and SDS-PAGE immunoblotting to proceed using anti-Phospho-AMPKα monoclonal and anti-AMPKa rabbit polyclonal antibodies from Cell Signaling at 1:200 dilutions.

3.3.11 Histology

Mouse colon, adipose tissue, and liver were fixed in 10% buffered formalin at room temperature before embedding in paraffin. Tissues were sectioned at 5 mm thickness and stained with hematoxylin and eosin (H&E). The picture capture by Image by software OLYMPUS VS-ASW virtual slide system from H&E. Steatosis was graded 1-5 wherein a score of 1 was assigned for no discernable steatosis (relative to chow-fed mice), and scores of 2-5, on a 0.5 interval used to grade modest to severe steatosis.

3.3.12 ELISAs

Serum from overnight-fasted mice was assayed for MCP-1 via a Duoset ELISA purchased from R&D Systems. Serum insulin was measured with an ELISA kit (Cat: EZRMI-13K) purchased from Millipore, inc. Fecal Lipocalin-2 was measured as previously described.

3.3.13 Quantification and Statistical Analysis

Data were expressed as mean ± SEM. Statistical significance was analyzed by unpaired Student's t test (GraphPad Prism 7). Differences between experimental groups were considered significant at $P < 0.05$

3.4 Results

3.4.1 Metformin's amelioration of diet-induced metabolic syndrome is associated with beneficial impacts on gut microbiota

While, in humans, Metformin is generally administered orally, most studies of this drug in mice have relied upon systemic administration, typically intraperitoneal (IP) administration, in which relatively moderate doses of this drug are able to ameliorate various aspects of metabolic syndrome (115,116). Hence, our initial approach was to examine the extent to which IP administration of metformin ameliorated metabolic syndrome and microbiota composition in mice fed an obesogenic western-style high-fat diet (HFD), which is high in saturated fats and low in fiber. Mice (C57BL/6 males) were maintained on standard grain-based rodent chow or, at 6 weeks of age, administered HFD ad libitum and 2 weeks later, given daily injections of vehicle (normal saline, NS) or metformin. Such metformin treatment ameliorated various other aspects of metabolic syndrome including obesity, as assessed by body weight and adiposity (Fig 3.6.1A-C and 3.7.1), dysglycemia (Fig 3.6.1 D), and hepatic steatosis (Fig 3.6.1G&H). Moreover, in accord with the general notion that metabolic syndrome is characterized low-grade inflammation, which might be impacted by metformin (directly and/or via metformin's impacts on gut microbiota), we observed that metformin ameliorated indices of HFD-induced low-grade inflammation, including colon shortening (Fig 3.6.1E) and increases in levels of serum macrophage chemotactic protein-1 (MCP-1), (Fig 3.6.1F), which associates with diet-induced obesity (121,129). Together, these results indicate that, in accord with previous studies, IP administration of metformin ameliorated HFD-induced low-grade inflammation and metabolic syndrome.

Next, we considered the extent to which metformin impacted gut microbiota composition, envisaging that metformin administered IP might reach the intestinal lumen via bile trafficked by enterohepatic circulation and/or that metformin might indirectly impact the microbiota. Feces were collected from HFD-fed mice immediately prior to and 10 weeks following IP administration of vehicle or metformin. We utilized 2 cages of mice (5 per cage) in order to reduce chances of observing differences that primarily reflected the tendency of mice to have microbiomes that resemble that of their cagemates, due to their coprophagic habits. Fecal microbiota composition was assessed via sequencing of 16S ribosomal RNA genes. Overall, untargeted assessment of microbiota composition was assessed by plotting of principal coordinates of Uni-Frac values. Prior to treatment, overall microbiota composition did not differ significantly between mice that had been assigned to the metformin and vehicle groups (Fig 3.6.2A, week 2). In contrast, at 10 weeks following metformin treatment, there was a marked distinction in overall microbiota composition between metformin and vehicle-treated mice (Fig 3.6.2A, week 12). The extent of this change was such that it could be visualized even amidst the large change in microbiota composition that resulted from prolonged exposure to HFD. Such changes in microbiota composition reflected enrichment and depletion of a variety of taxa (Figs 3.6.2 and 3.7.2), without an impact on species richness (i.e. α-diversity) (Fig 3.6.2C). One general consequence of alterations in the host-microbiota relationship in diabetic patients and mice with metabolic syndrome is encroachment of gut microbiota into the inner mucus layer, which might result in such bacteria promoting low-grade inflammation (107). Moreover, metformin increases goblet cells, which increases mucus thickness (120). Hence, we measured by confocal microscopy the extent to which metformin impacted bacterial-epithelial distance. We observed that metformin treatment resulted in greater bacterial-epithelial distance (Fig 3.6.2

D&E). Together, these results indicate that metformin treatment of HFD-fed mice resulted in changes in microbiota composition and localization that may reduce low-grade inflammation and are thus in accord with previous studies that suggest that the beneficial actions of this drug may be mediated, in part, by gut microbiota.

3.4.2 Metformin ameliorates metabolic syndrome in the absence of a microbiota

The above-described and previously reported (124,127) impacts of metformin on gut microbiota can be envisaged to contribute to, and/or reflect, metformin's ability to ameliorate metabolic syndrome. Hence, we next examined if reducing levels of gut microbiota via antibiotic treatment would impact ability of this drug to ameliorate HFD-induced metabolic syndrome. Metformin or vehicle was administered to HFD-fed mice, as described above or under conditions wherein vehicle- and metformin-treated mice were subjected to a mixture of antibiotics, namely ampicillin and neomycin, administered via their drinking water. As expected antibiotics quickly resulted in a more than 3-log reduction in the total amount of bacteria per mg of feces that was maintained, irrespective of metformin treatment, throughout the course of the 12-week period of administration utilized (Fig 3.6.3A). While the reduction of metformin on HFD-induced weight gain was not seen under conditions of antibiotic administration (Fig 3.6.3B), this may have reflected that the weight gain itself was reduced by antibiotics. In any case, the extent to which metformin suppressed adiposity, as measure by MRI (Fig 3.6.3C) or post-euthanasia measured fat pad mass (Fig 3.6.3D) was not markedly impacted by antibiotic treatment, which itself had only a minor impact on increased adiposity. Nor did the antibiotics prevent metformin from ameliorating hepatic steatosis (Fig 3.6.3E and S3.7.3). Moreover, while antibiotics moderately reduced fasting blood glucose in HFD-fed mice, particularly at later time points following HFD

exposure, metformin still lowered this parameter particularly at these later time points (Fig 3.6.3F). Nor did antibiotic administration prevent metformin from ameliorating indices of low-grade inflammation that are known to result from exposure to HFD. Specifically, metformin reduced serum MCP-1 (Fig 3.6.3G) and adipose levels of MCP-1 and TNFα mRNA (Fig 3.6.3H&I), irrespective of antibiotic treatment. Additionally, while the extent to which metformin protected against colon shortening, which is an indicator of inflammation, was variable, it was significant under conditions of antibiotics (Fig 3.6.3J). Lastly, use of PCoA analysis to holistically considering all of these metabolic and inflammatory parameters (Fig 3.6.3K) indicated that metformin-treated mice had similar overall metaboinflammatory phenotypes irrespective of antibiotic treatment. Thus, overall, use of antibiotics to markedly reduce gut bacterial loads did not support our original hypothesis that the ability of metformin to ameliorate metabolic syndrome was mediated by its actions on gut microbiota.

One caveat to the above-described studies is that administering metformin via the IP route may have reduced interaction of this drug with the intestinal mucosa and luminal microbiota. One the one hand, that IP metformin altered gut microbiota and that metformin administered IP might reach the intestinal lumen via bile trafficked by enterohepatic circulation argues against the importance of this caveat. On the other hand, given that several studies, albeit largely-association based, have implicated an important role for microbiota in mediating metformin's impact, we sought to develop a means of orally delivering metformin that would ameliorate HFD-induced metabolic syndrome. Hence, we sought to develop an oral dosing regimen in which metformin markedly and reliably ameliorated HFD-induced metabolic syndrome. First, based on published work, we administered metformin in drinking water to deliver a daily dose of 6 mg per day per mouse (300 mg/kg body weight) (110). This dose of

metformin moderately reduced HFD-metabolic syndrome in Swiss-Webster mice (Fig S3.7.4) but had only slight impacts in C57BL/6 mice (Fig S3.7.5), in which the above studies and most work in this model has been performed. Hence, we selected a higher dose of oral metformin, 30 mg per day per mouse, which was well tolerated and clearly reduced many aspects of HFD-induced metabolic syndrome, including weight gain, increased adiposity, and hyperglycemia (Fig 3.6.4). Antibiotics did not reduce such ability of metformin to reduce HFD-induced increases in weight gain, adiposity, and hepatic steatosis (Fig 3.6.4A-D and S3.7.6). In accord with the notion that HFD-induced dysglycemia is itself mediated in part by microbiota, administration of antibiotics ameliorated, by themselves, HFD-induced dysglycemia as assessed by measure of fasting glucose, thus obscuring our ability to measure if metformin would prevent HFD-induced dysglycemia in absence of microbiota (Fig 3.6.4E). Nonetheless, under antibiotic treatment, metformin still improved glucose tolerance following 3 weeks and 8-weeks of HFD exposure (Fig 3.6.4F&G). Ability of metformin to reduce glucose levels was not, irrespective of antibiotics, associated with any adverse effects or general signs of ill health, which can itself lower blood glucose. Nor did metformin result in any markers of inflammation that can occur from drug toxicity, nor did antibiotics appear to impact the extent to which metformin reduced low-grade inflammation in that metformin treatment modestly but broadly reduced expression of a panel of indices of low-grade inflammation (Fig 3.6.4H-J). Overall, a similar pattern was seen in a distinct strain of mice (Swiss-Webster), in that while metformin had only modest reduced HFD-induced metabolic syndrome in these mice, antibiotics did not reduce that effect (Fig S3.7.4). Hence, analogous to the case for metformin administered by the IP route, use of antibiotics to reduce levels of microbiota did not yield data to support the notion that microbiota plays a key role in this drug's ability to ameliorate metabolic syndrome.

One potential explanation for inability of antibiotics to suppress metformin's amelioration of HFD-induced metabolic syndrome is that, despite broad suppression of total bacterial loads, perhaps select bacteria can bloom. We explored this possibility focusing on Proteobacteria, a phyla in which we (Fig 3.6.2) and others have observed contains much of the taxa enriched by metformin treatment (111, 124). We observed that while antibiotics suppressed increases in β-Proteobacteria, increased γ and ε proteobacteria were observed in metformin-treated mice amidst antibiotic treatment (Fig S3.7.7). While it is difficult to discern if such changes contributed to metformin's action in these conditions, this result serves as an example of the general limitation of investigating the role of the microbiota via antibiotics. Indeed, antibiotics reduce but do not eliminate gut bacteria and can select for gut bacteria, such as those that populate inner mucus that may have an outsize impact on low-grade inflammation and hence metabolic syndrome. Hence, we next used germfree mice as a means to examine the extent to which metformin might ameliorate HFD-induced metabolic syndrome in a host completely lacking gut bacteria. A technical limitation to germfree mice is that it is very difficult to track/monitor metabolic parameters without compromising the germfree state. Hence, groups of age-matched germ-free mice were split into 3 groups, visually assessed to be of similar body mass and administered autoclaved grain-based chow, irradiated HFD (IR-HFD), which we've shown does not compromise germfree status, or IR-HFD and oral metformin (filter sterilized) via drinking water (129). In accord with the notion that microbiota may be more important for HFD-induced dysglycemia than obesity per se, under germfree conditions, IR-HFD still induced clear increases in weight and adiposity but induced only minimal dysglycemia (Fig 3.6.5), in accord with our recent work (123). While such minimal dysglycemia precluded ability of metformin to correct it, metformin still clearly diminished the extent of adiposity induced by IR-HFD (Fig

3.6.5 and S3.7.5) albeit not to the extent observed in conventional mice (Fig 3.6.4C). Metformin also continued to reduce hepatic steatosis in germfree mice (Fig 3.6.5 and S3.7.8 D). Additionally, metformin-treated mice showed a trend to a trend , albeit not statistically significant, toward reduced insulin levels, which, together with the slight trend toward reduced glucose, in accord with notion that metformin's ability to sensitize response to insulin remained intact. Metformin's sensitization of insulin signaling is proposed to be related to its activation of AMPK. Hence, we analyzed metformin's impact on levels of Phospho-AMPK in livers of HFD-fed mice in conventional and GF mice. In accord with widely reported notion that metformin activates AMPK (112), and that AMPK activity is elevated in GF mice (100), we observed induction, albeit variable, of phospho-AMPK in conventional mice and elevated basal phospho-AMPK in GF mice (Fig S3.7.8 C). We did not observe any additional induction of phospho-AMPK in metformin-treated GF mice. We view these results as being in accord with the notion that activation of phosphor-AMPK might be an important even in conventional mice but that additional actions of metformin drive its impacts in GF mice. More generally, metformin continued to broadly ameliorate an array of indices of low-grade inflammation that were induced by IR-HFD. Specifically, metformin ameliorated colon shortening and reduced expression of MCP-1, IL-6, CXL-1, and TNFα in colon, liver and adipose tissue (Fig 3.6.4). Together, these results support the notion that metformin has anti-inflammatory activity, irrespective of gut microbiota, which may not be required for this drug to ameliorate metabolic syndrome.

3.5 Discussion

Metformin is amongst the world's most widely prescribed drugs, largely due to its ability to effectively treat early-stage type 2 diabetes and, moreover, ameliorate many of the metabolic abnormalities collectively referred to as metabolic syndrome, that are associated with

dysglycemia (128). Limitations of metformin include limited potency and some gastrointestinal dysfunction including nausea and diarrhea in some patients. Hence, there has long been interest in understanding mechanisms by which metformin acts, as such knowledge might allow design of better treatments for metabolic syndrome. While an array of potential mechanisms that contribute to metformin's action have been proposed, the extent to which such mechanism are crucial contributors to this drug's beneficial effects are less clear, nor have direct targets of metformin been convincingly defined.

Considering the recent extensive appreciation that gut microbiota contribute mightily to numerous aspects of host health, including the view promulgated by ourselves and others that microbiota dysbiosis drives low-grade inflammation that promotes metabolic syndrome (104), it is not surprising that multiple research groups hypothesized that metformin might impact gut microbiota in a manner that might contribute to metformin's beneficial effects (118). That, in both rodents and humans, metformin's beneficial effects were associated with alterations in the microbiome supported this notion (116, 124, 127). However, since the conditions that metformin treats are, themselves, associated with alterations in gut microbiota composition, such results could also reflect metformin ameliorating metabolic syndrome resulting in changes in gut microbiota. That transplant of microbiota from metformin-treated hosts to mice not exposed to this drug conferred beneficial metabolic effects suggests metformin-induced changes in microbiome are not purely a consequence of treatment benefits (124), but is also consistent with the possibility that such changes are both a cause and consequence of amelioration of metabolic syndrome. Our observation that metformin impacted the microbiota in a manner that reduced its encroachment into the mucus is in accord with this paradigm, but yet none of these findings

address the extent to which presence of a gut microbiota is necessary for metformin's beneficial effects.

A central tenet in the microbiota field is that if the microbiota plays a crucial role in the process by which a particular intervention causes a phenotype, that intervention should not induce that phenotype in the absence of microbiota. For example, many interventions that cause colitis in conventional conditions are without effect in germfree conditions (114). Another example of this tenet is our work that synthetic dietary emulsifiers that cause mucus thinning and low-grade inflammation and metabolic syndrome in conventional conditions, cause none of these in germfree mice indicating that these events are fully microbiota dependent (105). Such appreciation of the importance of the microbiota in low-grade inflammation and metabolic syndrome led us to hypothesize that metformin would fail to impact metabolic syndrome in the absence of microbiota. However, our data did not support this hypothesis. Rather, neither antibiotics or germfree approaches prevented the ability of metformin to ameliorate metabolic syndrome. In particular, irrespective of whether microbiota were present, metformin attenuated HFD-induced low-grade inflammation, glucose intolerance, adiposity, and hepatic steatosis. While many of the impacts of metformin in micro-ablated mice could be the result of reduced adiposity, the observation that it also prevented indices of low-grade inflammation in the gut are in accord with suggestions that metformin's anti-inflammatory activity is central to its beneficial effects. Indeed, metformin is now recognized to have fairly broad anti-inflammatory effects (119), and our results herein are consistent with the possibility that such effects contribute to metformin's protection against diet-induced metabolic syndrome.

A major caveat of our results is that one of the central features of metabolic syndrome, namely dysglycemia, was itself clearly reduced by reducing/eliminating microbiota, thus

obscuring our ability to examine how metformin impacted it in these conditions. Another caveat of our findings is that effective doses of metformin in mice are higher than that used in humans, and thus might possibly be mediated by distinct mechanisms. Indeed, although mice exhibited no behavioral, histopathologic, or other indicators of any ill effects from oral or intraperitoneal metformin, we cannot rule out the possibility that some type of subtle toxicity reduced adiposity, which led to the reductions in the metabolic/inflammatory parameters we measured. Lastly, we note that the extent to which metformin ameliorated HFD-induced adiposity was less in GF mice relative to antibiotic-treated mice suggesting that metformin-induced changes in microbiota in antibiotic-treated mice might contribute to its efficacy therein. Such caveats notwithstanding, we submit that the most reasonable and simplest explanation for existing data, and our results herein, is a scenario wherein metformin's ability to ameliorate metabolic syndrome results in alterations in gut microbiota that alleviate low-grade inflammation and subsequently further alleviates, and maintains alleviation of, metabolic syndrome. In support of this possibility, we note that other means of inducing dysglycemia, for example islet toxin streptozotocin, also induce microbiota dysbiosis and promotes microbial breach of the gut barrier and interventions that ameliorate such dysglycemia will reduce the associated dysbiosis (117, 122, 127).

The notion that the beneficial impact of metformin on gut microbiota might be indirect does not preclude the possibility that such effects are important to the efficacy of the drug in its real usage wherein a microbiota is present. Indeed, amelioration of dysbiosis and microbiota encroachment may be critical to the lasting efficacy of this compound. By analogy, a weight loss intervention might facilitate the ability of a person to exercise, which is then critical for sustained efficacy of the intervention. Extending the analogy, transfer of exercise phenotype by itself may, like a fecal microbiota transplant, recapitulate many of the benefits of the original intervention.

Thus, we do not question the conclusion of the array of studies that indicate that metformin ameliorates microbial dysbiosis and that such effects are important to the drug's beneficial metabolic effects. Yet, we suggest the gut microbiota is not germane to identifying the direct targets and primary mechanism of action of metformin.

3.6 Figures and legends

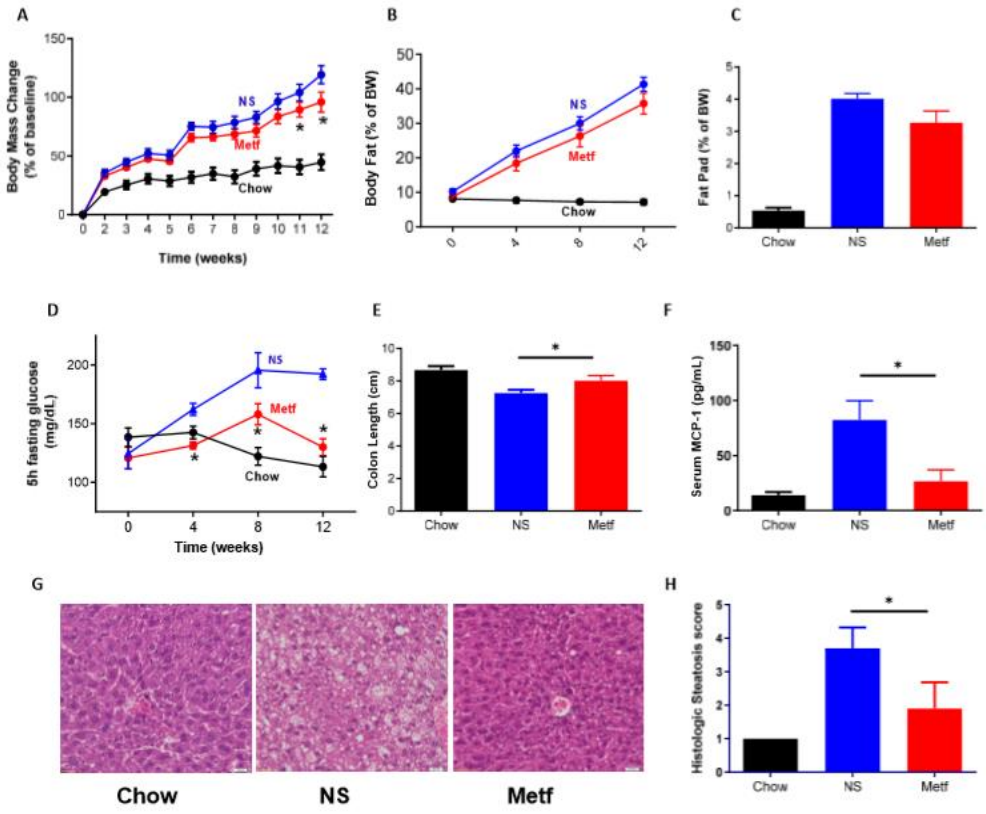

Figure 1 Metformin ameliorates HFD-induced metabolic syndrome.
6-week old male C57 BL/6 mice (n=10 per condition) were maintained on a standard grain-based chow diet or administered a western style low-fiber high-fat diet (HFD) for 12 weeks. HFD-fed mice were injected IP with saline (NS) or metformin (metf) daily from 2 weeks post-diet exposure until being euthanized. Weight (**A**) and Body composition **(B)** was monitored. **(C)** Epididymal fat pad pass measured post-euthanasia. **(D)** Mice were fasted for 5h every 4 weeks and blood glucose measured. **(E)** Colon length measured post-euthanasia. **(F)** Serum MCP-1 measured by ELISA following 8 weeks HFD exposure and overnight fasting. **(G)** Representative H&E stained liver sections. **(H)** Histologic scoring of liver sections for extent lipid accumulation. Data are shown as mean +/- SEM; * indicates statistical significance ($p < 0.05$) by Student's t-test for HFD/metformin vs HFD/NS.

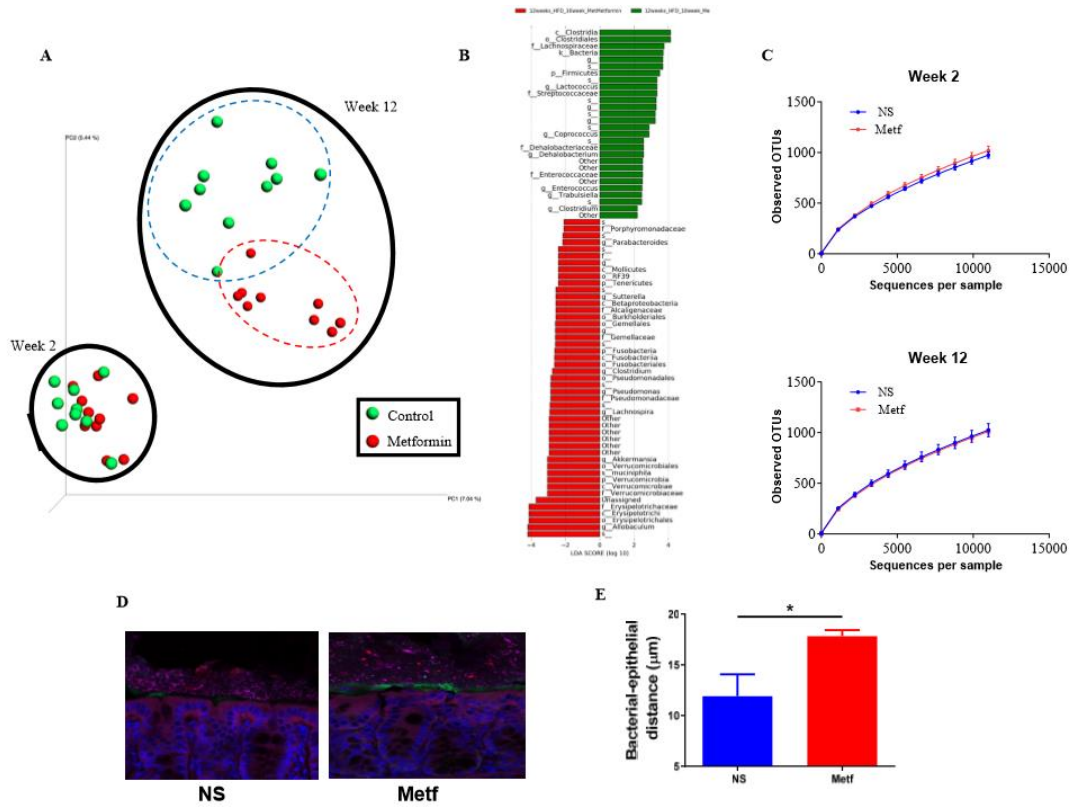

Figure 2 Metformin impacts microbiota composition and reduces microbiota encroachment.

6-week old male C57 BL/6 mice (n=10 per condition) were HFD for 12 weeks. HFD-fed mice were injected IP with saline (NS) or metformin (metf) daily from 2 weeks post diet exposure until being euthanized**. (A)** Comparison of fecal microbiome composition via Principal coordinate analysis (PCoA) of the UniFrac coordinates following week 2 and 12 weeks; 0 and 10 weeks post-metformin treatment. **(B)**The histogram shows Linear discriminant analysis (LDA). **(C)** Rarefaction curves of number of observed OTUs based on sequence similarities for control and treatment group at week 2 and week 12. **(D)** Confocal microscopy analysis of microbiota localization; Muc2 (green), actin(purple), bacteria (red), and DNA (Blue). Bar= 20µm. **(E)** Distance of closest bacteria to intestinal epithelial cells (IEC) per condition over 5 high-powered field per mouse. Pictures are representatives of 10 biological replicates.

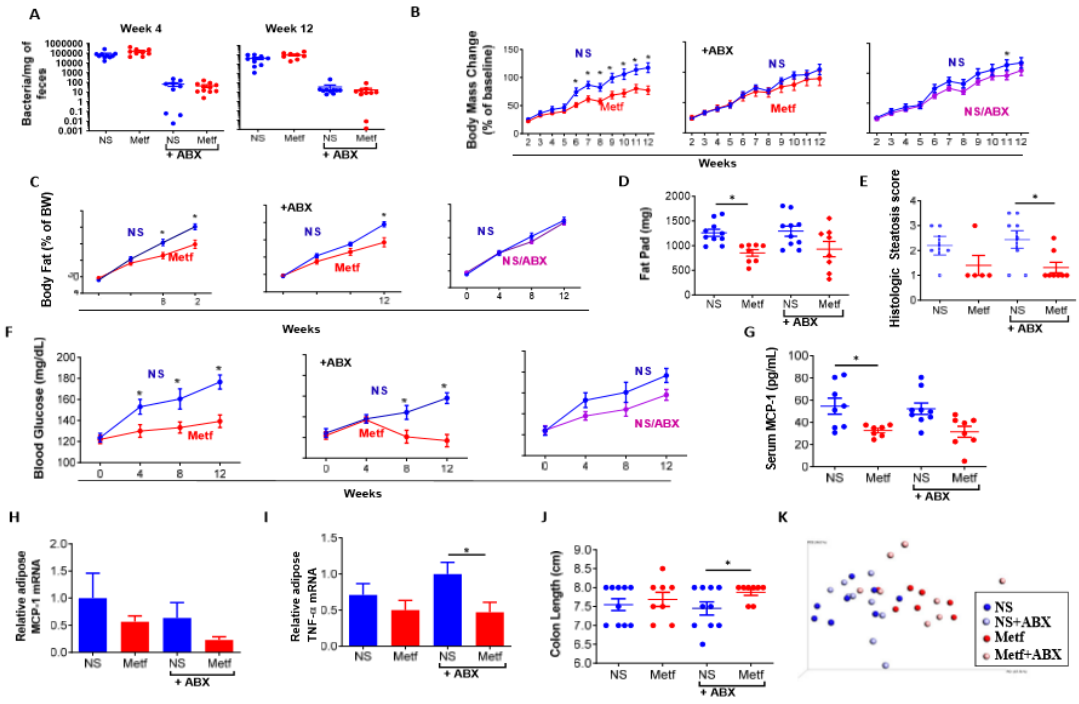

Figure 3 Amelioration of HFD-induced metabolic syndrome in antibiotic-treated mice.

6-week old male C57 BL/6 mice (n=10 per condition) were fed HFD for 12 weeks. HFD-fed mice were injected IP with saline (NS) or metformin (metf) daily from 2 weeks post diet exposure until being euthanized, with or without antibiotic (Ab) in drinking. (**A**) PCR- based quantification of bacterial load adhered to feces on week 4 and 12. **(B)** Body weight **(C)** MRI and analyzing body composition every 4 weeks. **(D)** Epididymal fat pad pass measured post-euthanasia. **(E)** Histologic scoring of section of liver **(F)** Mice were fasted for 5h every 4 weeks and blood glucose measured. **(G)** Serum MCP-1 measured by ELISA following 12 weeks HFD exposure and overnight fasting. **(H-I)** The mRNA was extracted from fat and expression level of MCP-1 and TNFα was analyzed by RT-PCR **(J)** Colon length measured post-euthanasia**.** Data are shown as mean +/- SEM; * indicates statistical significance ($p < 0.05$) by Student's t-test for HFD/metformin vs HFD/NS. **(K)** PCoA plot combining terminal parameters of weight, fat composition, fat pad mass, blood glucose, MCP-1, and colon length.

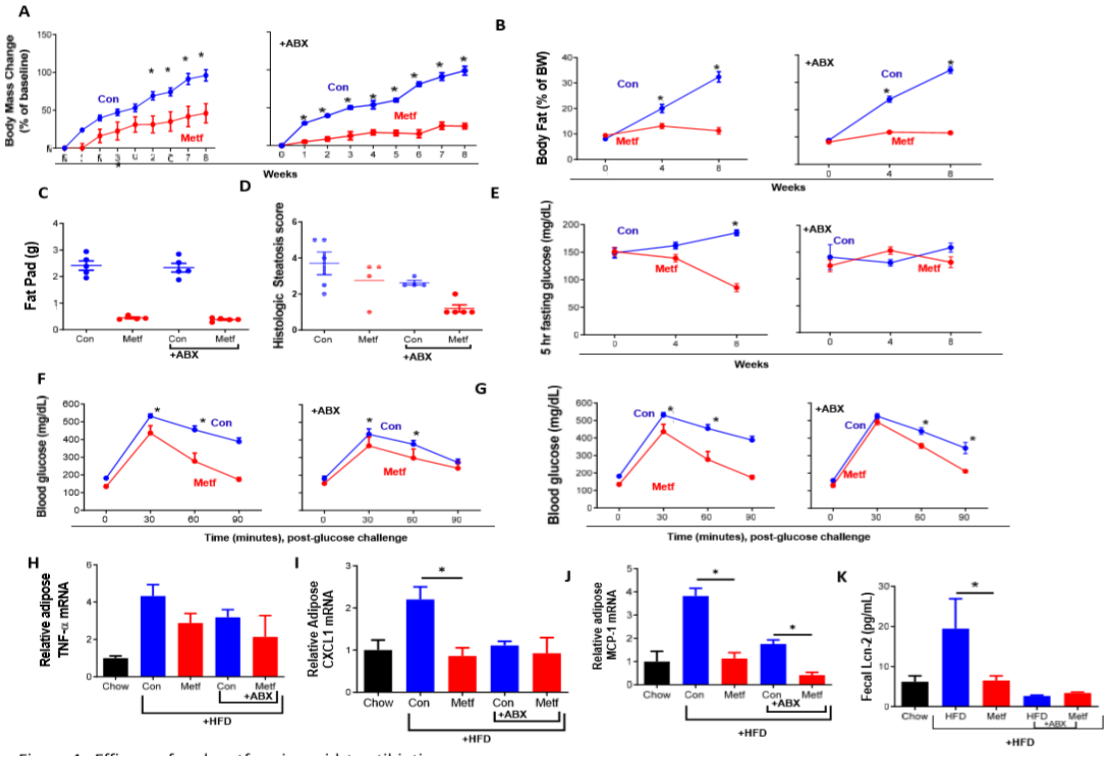

Figure 4 Efficacy of oral metformin amidst antibiotics.

6-week old male C57 BL/6 mice (n=5 per condition) were maintained on a western style low-fiber high-fat diet (HFD) for 8 weeks. HFD-fed mice were administered drinking water containing metformin (10 mg/ml) or vehicle control from first week of study, with or without antibiotics (ABX) in drinking water. **(A)** Body weight **(B)** MRI and analyzing body composition **(C)** Epididymal fat pad pass measured post-euthanasia **(D)** Histologic scoring of section of liver **(E)** Mice were fasted for 5h every 4 weeks and glucose was measured. **(F-G)** Glucose measured after 0, 30, 60, and 90 min after interperitoneally injected with glucose at week 3 **(F)** and week 8 **(G)**. **(H)** The mRNA was extracted from adipose and expression level of MCP-1. **(I)** CXCL1 **(J)** and TNFα was measured by RT-PCR. **(K)** Fecal lipocalin-2 measured by ELISA at 8-week timepoint. Data are shown as mean +/- SEM; * indicates statistical significance ($p < 0.05$) by Student's t-test for HFD/metformin vs HFD Control.

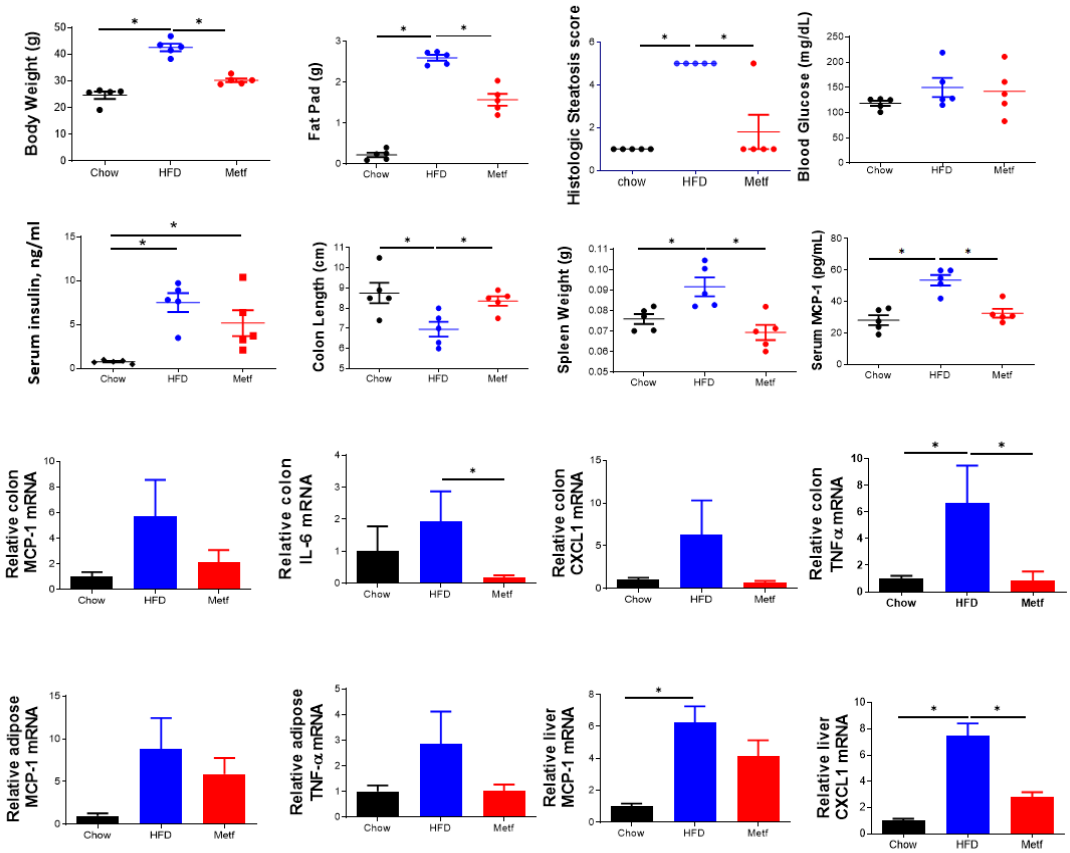

Figure 5 Maintained efficacy of metformin in HFD-fed germfree mice.
6-week old male C57 BL/6 mice (n=5 per condition) were maintained on autoclaved chow or irradiated HFD (IR-HFD) for 8 weeks while being administered drinking water containing metformin (10 mg/ml) or vehicle. All parameters were measured following euthanasia. Body weight and epididymal fat pad. Histologic scoring of section of liver. Mice were fasted overnight and blood glucose and insulin was measured. The colon length and spleen weight measured. Serum MCP-1 measured by ELISA and overnight fasting. The mRNA was extracted from colon and expression level of MCP-1, IL-6, CXCL1 and TNFα was analyzed by RT-PCR. The mRNA was extracted from adipose and expression level of MCP-1 and TNFα was analyzed by RT-PCR. The mRNA was extracted from liver and expression level of MCP-1 and CXCL1 was analyzed by RT-PCR. The mRNA was extracted from adipose and expression level of MCP-1 and TNFα was analyzed by RT-PCR. Data are shown as mean +/- SEM; * indicates statistical significance ($p < 0.05$) by Student's t-test for HFD/metformin vs. HFD.

3.7 Supplemental Figures and Legends

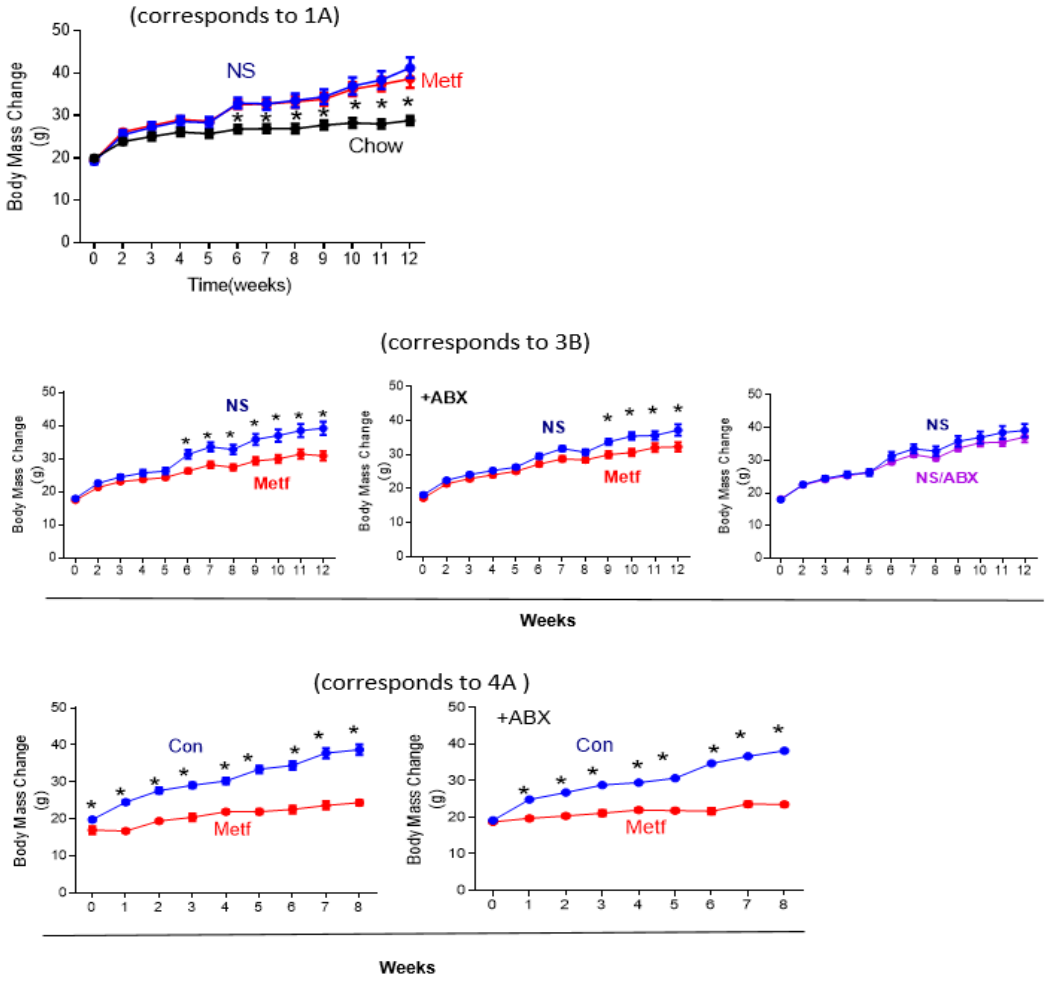

Figure 6(relates to Figs 1, 3, and 4).
Data are raw unnormalized mouse weights used to generate corresponding figures showing relative changes in weight in main figures.

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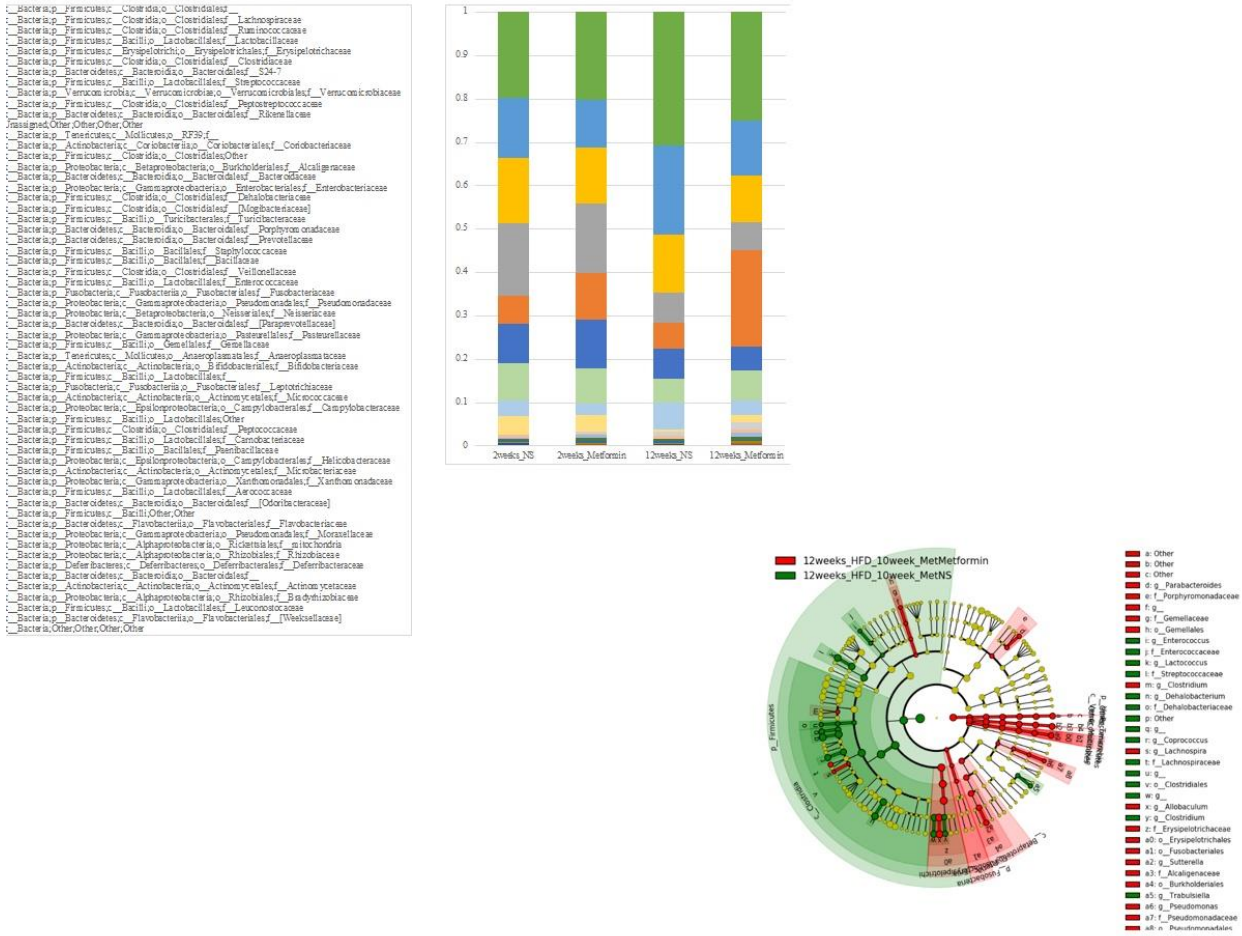
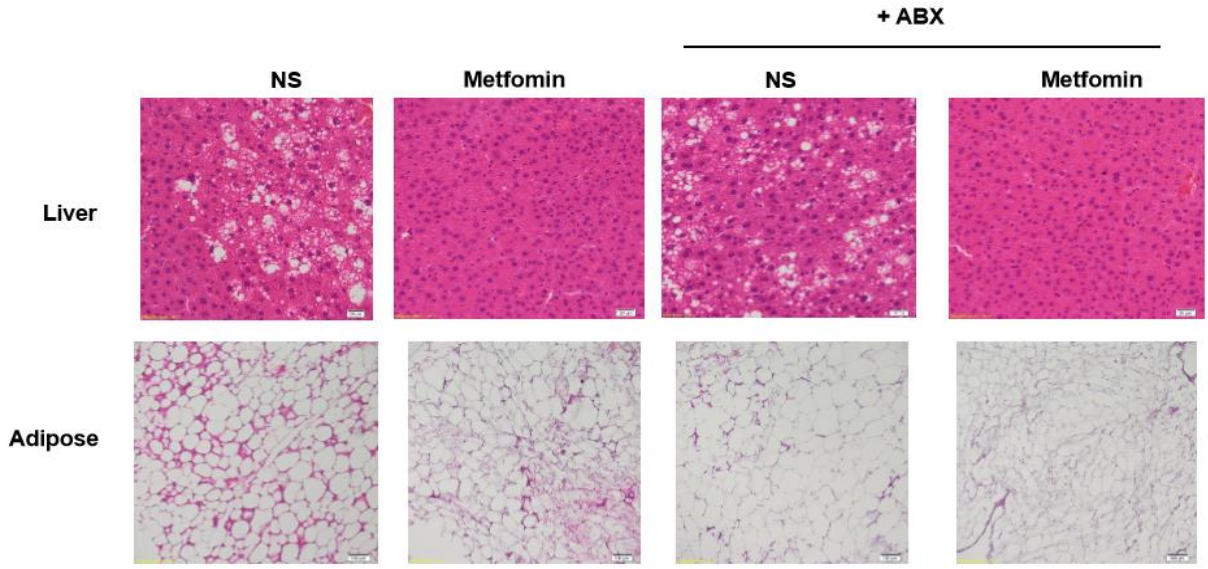


Figure 7(relates to Fig 2).

Impact of metformin on gut microbiota composition. Additional analysis on fecal microbiome sequencing data from Fig 2. The bar graph represents relative abundance of bacteria at the phylum level at post treatment and terminal point. Linear discriminant analysis Effect Size (LEfSe) of gut microbiota changes following consumption of HFD vs HFD/NS. The phylogenetic tree shows LDA scores calculated for different microbiome abundance between treated mice with metformin versus saline.



Tissues samples from mice described in Fig 3 were subjected to histopathologic analysis. Liver and adiposity were H&E stained. Picture are representatives of 10 biological replicates.

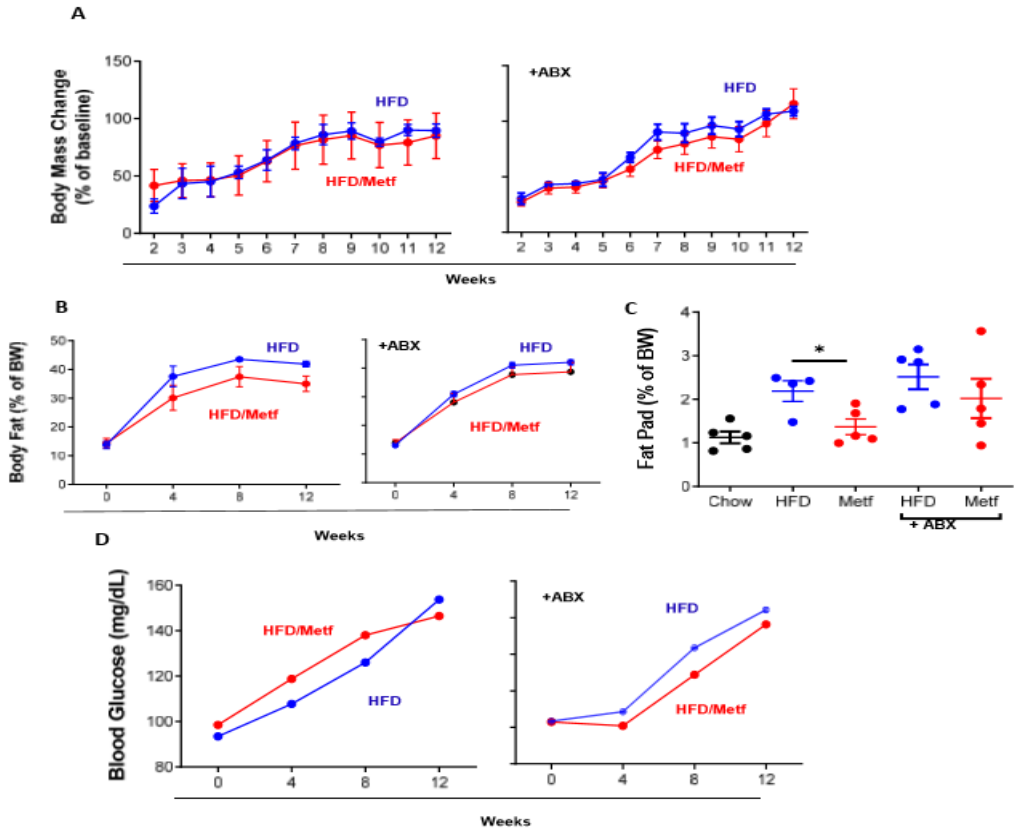


Figure 8(relates to Fig 4).

Efficacy of oral metformin amidst antibiotics in Swiss-Webster mic. 6-week old male Swiss Webster mice (n=5 per condition) were maintained on a western style low-fiber high-fat diet (HFD) for 12 weeks. HFD-fed mice were administered drinking water containing metformin (3 mg/ml) or vehicle control from first week of study, with or without antibiotics (ABX) in drinking water. **(A)** Body weight **(B)** MRI and analyzing body composition **(C)** Epididymal fat pad pass measured post-euthanasia (**D)** Mice were fasted for 5h every 4 weeks glucose was measured Data are shown as mean +/- SEM; * indicates statistical significance ($p < 0.05$) by Student's t-test for HFD/metformin vs HFD.

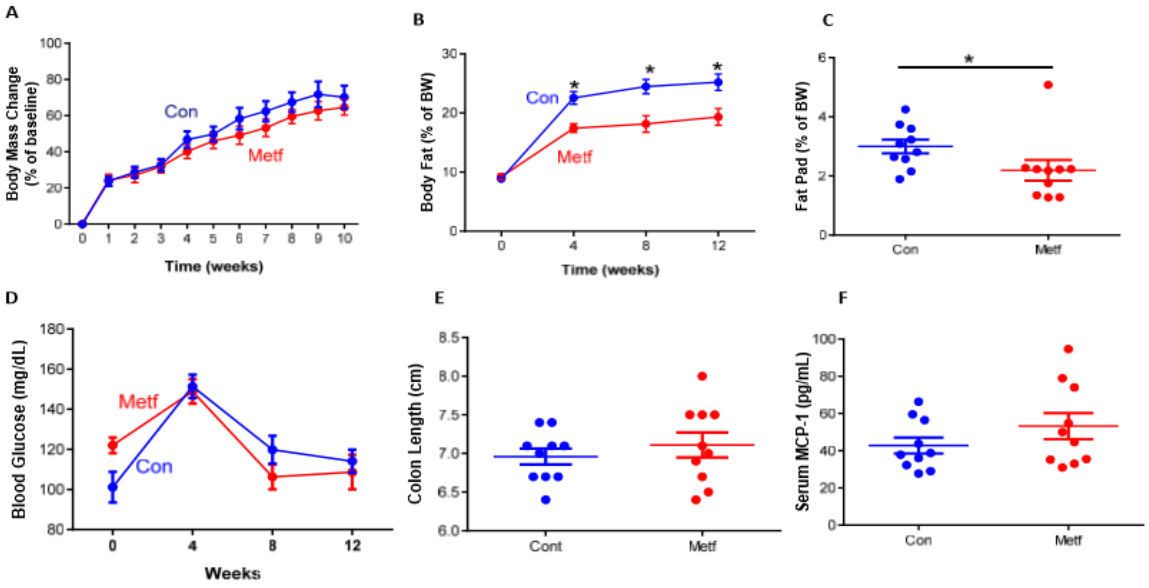


Figure 9(related to Fig 4).
Effect of lower dose oral metformin in C57BL/6 mice. 6-week old male C57 BL/6 mice (n=10 per condition) were maintained on a western style low-fiber high-fat diet (HFD) for 12 weeks. HFD-fed mice were administered drinking water containing metformin (3 mg/ml) or vehicle control from first week of study, with or without antibiotics (ABX) in drinking water. **(A)** Body weight **(B)** MRI and analyzing body composition **(C)** Epididymal fat pad pass measured post-euthanasia (**D)** Mice were fasted for 5h every 4 weeks glucose was measured. **(E)** Colon length. **(F)** Serum MCP-1. Data are shown as mean +/- SEM; * indicates statistical significance ($p < 0.05$) by Student's t-test for HFD/metformin vs HFD.

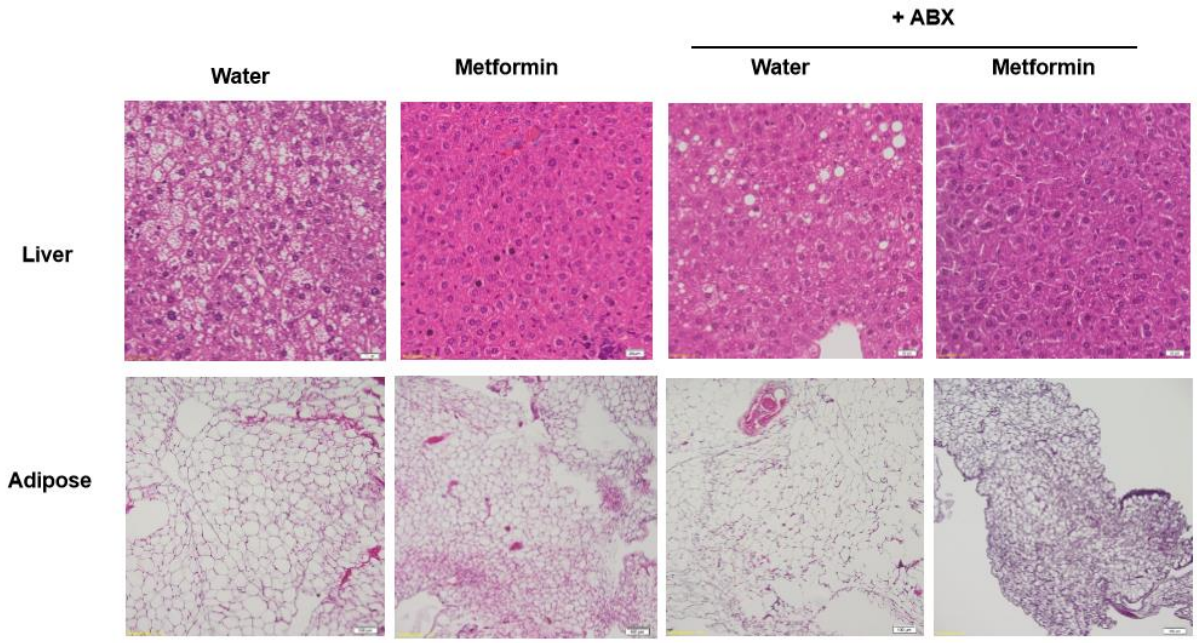


Figure 10 (relates to Fig 4).
Efficacy of oral metformin amidst antibiotics. Tissues samples from mice described in Fig 4 were subjected to histopathologic analysis. Liver and adiposity were H&E stained. Picture are representatives of 10 biological replicates.

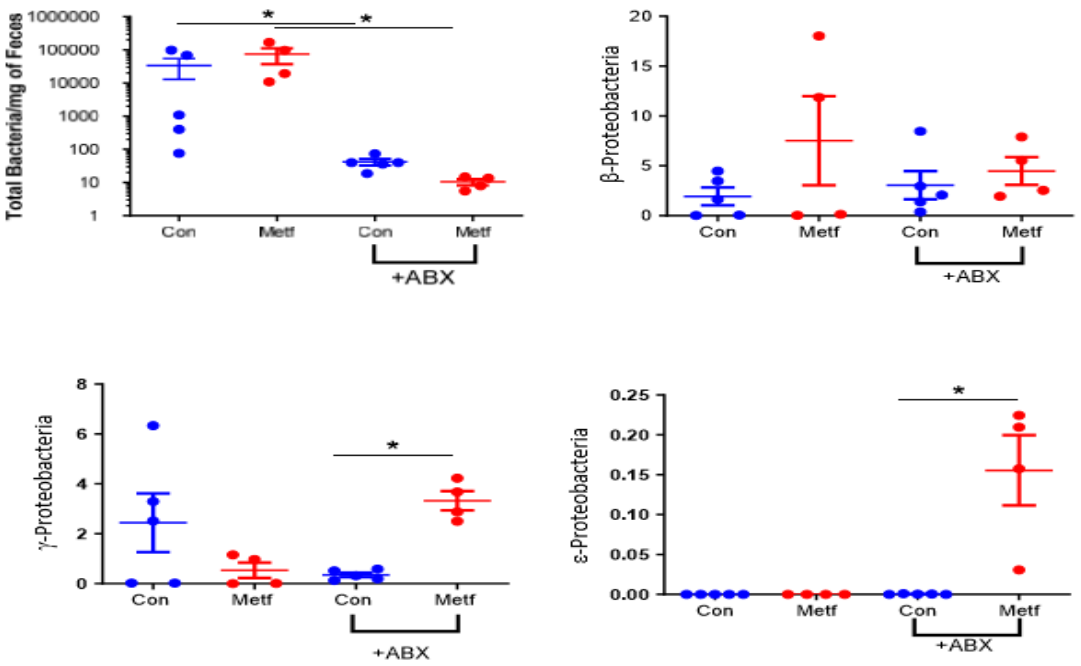
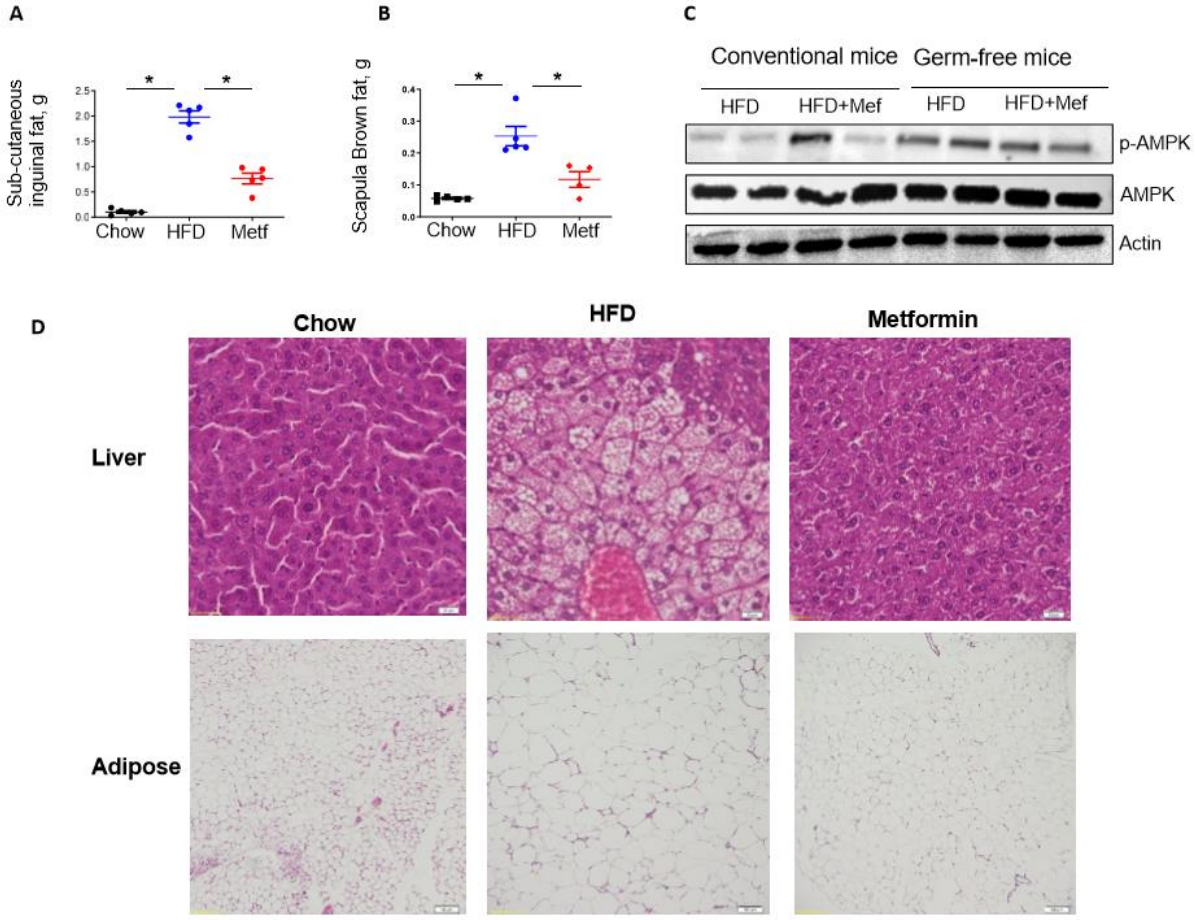


Figure 11 (relates to Fig 4).
Impact of metformin on Proteobacteria amidst antibiotic treatment. Fecal samples were collected from mice in figure 4, at 8-week timepoint, DNA extracted and subjected to qPCR using universal 16S primers or primers specific for indicated family of Proteobacteria. Family-specific PCR values expressed as relative to total level of bacteria. Data are shown as mean +/- SEM; * indicates statistical significance ($p < 0.05$) by Student's t-test for HFD/metformin vs HFD.



Tissues samples from mice described in Fig 5 were subjected to further analysis. (A) Inguinal subcutaneous fat mass. (B) Scapula brown fat mass. (C) Liver extracts were analyzed by SDS-PAGE immunoblotting. (D) Liver and adiposity were H&E stained. Picture are representatives of 10 biological replicates.

3.8 Disclosures

3.8.1 Funding

This work was supported by National Institute of Diabetes and Digestive and Kidney. Diseases Grants DK099071 (ATG) and DK083890 (ATG). B. Chassaing is supported by a Career Development Award from the Crohn's and Colitis Foundation of America.

3.8.2 Authors' contributions

Conception and design: AA, BC, ATG. Development of methodology: AA, HQT, AATG. Writing, review, and/or revision of the manuscript. None of the authors have any conflicts TG. Acquisition of data: AA, HQT, BC, JZ. Analysis and interpretation of data: AA, BC, of interest with this work.

4 CONCLUSION

The murine intestine normally contains about 10^{14} microorganisms, mainly residing in the colon. The gram-negative *Bacteriodetes* and the gram-positive *Firmicutes* are the most common phyla in human and mouse (131). The gram-negative Verrucomicrobia, Proteobacteria, Spirochaetes, and Tenericutes, as well as the gram-positive Actinobacteria, are also found in the gut of mammals. In the upper parts of the intestine, such as the ileum, Firmicutes are the dominant species; however, a considerable amount of the bacteriodetes fraction and some Actinobacteria could also be found. In the caecum, on the other hand, the Proteobacteria, including the Enterobacteriaceae are present and thus, the most diverse microbiota with different types of phyla could be found in the feces.

Microbiota may be removed by two means, namely the use of antibiotics and germfree mice. Antibiotic approaches provide only a partial removal of the microbiota but offer logistical ease and the advantage that the mice are generally immunologically normal. In contrast, germ free approaches are laborious and yield mice with a poorly developed immune system but provide complete and total absence of microbiota.

The Gordon group first reported that the germfree mice gained weight after colonization with bacteria from the lower gut of conventionally raised mice (133). They also reported that C57BL/6J mice in GF environment are resistant to obesity; therefore, HFD induced obesity is, itself dependent upon the presence of microbiota. (100-132)

My advisor's lab found that HFD in the bacterial milieu, reduces mucus thickness and decreases the bacterial-epithelial distance that, together, drive host inflammatory signaling. Thus, I expected that, unlike conventional mice, germ-free mice that consumed metformin would not

show a reduced risk of HFD-induced metabolic syndrome. However, we observed completely opposite results.

To better investigate treatments for obesity and type 2 diabetes, metabolic profiling will help us study obesity mechanisms by investigating the involved pathways or processes. Consequently, we know inflammation is associated with both chronic and acute diseases and so is obesity. Moreover, this is the changes that people see about inflammation regarding the metabolites and proteins and the omic interactions, often referred to as the inflammasome. I envision that future studies can use mass spectrometry to perform a targeted quantitative analysis of metabolites, as the microbiota generates secondary chemicals from not absorbed food or body secretions such as bile salts. These are absorbed by the gut and can be detected in blood and urine. The alteration of the microbiota is associated with changes in the metabolome; this profile can be applied agnostically to figure out which metabolites are associated with which conditions regarding obesity.

Studying these metabolic profiles and signatures earlier than other conventional techniques can help us identify the risk of obesity or obesity-related diseases or even pre-obesity. Alternatively, it can help us realize which treatment works better for different groups of patients. In other words, metformin is reported in the current study as a concept from the larger perception, known as pharmacometabonomics. Using these findings, one can predict from a pre-dose or pre-intervention baseline metabolic signature how a person will react either to disease or drugs or therapy. Finally, it is important to point out that variation in human populations can accommodate. Obesity does not have a single cause and variability, although it is a distraction by many people, can actually identify individuals or groups of individuals who have a slightly

different metabolic response and we can use this variation to try and really home in on pathways that may be important in obesity and move towards the concept of personalized medicine.

Also, other approaches specific to what we utilized in investigating metformin are include gnotobiotic mice with limited microbiota such as mice with an altered Schaedler flora (ASF). The advantages of utilizing ASF mice instead of Germ-Free animals is their better developed immune system and metabolic capabilities. Another alternative is depleting of the microbiota with antibiotics post-weaning. However, these alternatives have their own disadvantages which do not allow us to fully understand the effects of metformin on HFD. For instance, the limited microbial diversity in ASF mice will not fully recapitulate the milieu in a human microbiota perturbed by a high-fat low-fiber diet.

Another potential follow up approach would be to utilize in ex-vivo to determine if metformin promotes the growth of bacteria in our gut directly via providing energy or another substrate beneficial to certain taxa. While also elucidating the interactions with host physiology and immune system development and response. Further, transfer the ex-vivo microbiome to Germ-Free hosts with the intention of studying the effects a metformin perturbed microbiota may have on a host. In our study we did not see significant changes in germ-free mice. However, this does not mean that metformin perturbed microbiota transferred to germ free host will not induce distinctive effects. Transfer of feces, ex-vivo and small intestinal material to hosts will best illuminate the role microbiota may play in metformin's protective effects.

In conclusion, much work is needed to fill gaps of knowledge. Better understanding of these pathways will allow better treatments for the prevention and cure of obesity. I hope the

information uncovered by this project will constitute one small step towards the bettering of human health.

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