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VITAMIN D AND PREBIOTICS MAY BENEFIT THE INTESTINAL MICROBACTERIA AND IMPROVE GLUCOSE HOMEOSTASIS IN PREDIABETES AND TYPE 2 DIABETES

Running title: Microbiota and vitamin D in diabetes

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ABSTRACT

Objective: To review the role of human large bowel microbacteria (microbiota) in the glucose homeostasis, to address vitamin D (VD) and prebiotics interactions with microbiota, and to summarize recent randomized clinical trials (RCT) of VD and prebiotics supplementation in prediabetes and T2DM.

Methods: Primary literature was reviewed in the following areas: composition and activity of human microbiota associated with prediabetes and T2DM, the mechanisms of the interactions between microbiota and glucose homeostasis, the interaction of microbiota with VD/prebiotics, and RCT of VD/prebiotics use in prediabetes and T2DM.

Results: The human microbiota comprise 100 trillion bacteria with the genome that is 150 fold larger than the human genome. Data from the animal models and human studies reveal that "obesogenic" diet results into the initial event of microbiota transformation from symbiosis to dysbiosis. The microbial antigens [e.g. Gram(-) bacteria and lipopolysaccharide] translocate to the host interior and trigger increased energy harvesting and Toll-like receptor activation with subsequent inflammatory pathways signaling. The "double hit" of steatosis (ectopic fat accumulation) and "–itis" (inflammation) and contribution of "co-risks" (e.g. vitamin D deficiency) are required to activate molecular signaling including impaired insulin signaling and secretion that ends with diabetes and diseases associated with T2DM. Dietary changes (e.g. prebiotics, vitamin D supplementation) may ameliorate this process if started at the time prior to the process becoming irreversible.

Conclusion: The emerging evidence suggests the important role of microbiota for glucose homeostasis. The vitamin D supplementation and prebiotics use may be considered for improving prediabetes and T2DM management.

Key words: Microbiota, Vitamin D, Glucose metabolism, Prebiotics, Prediabetes,

Abbreviations:

1,25D = 1,25-dixydroxyvitamin D; **25-hydroxyvitamin D** = 25(OH)D; **FSIVGTT** = intravenous; **GutM** = gut (large bowel) microbiota; **LPS** = lipopolysaccharide; **NGT** = normal glucose tolerance; **OGTT** = oral glucose tolerance test; **PreDM** = prediabetes; **RCT** = randomized clinical trials; **SCFA** = short chain fatty acids; **TLR** = toll-like receptors; **VD** = vitamin D; **VDD** = vitamin D deficiency; **VDR** = vitamin D receptor

INTRODUCTION

The process of deterioration of normal glucose tolerance (NGT) into prediabetes (PreDM) and type 2 diabetes mellitus (T2DM) includes contribution of "nature", i.e. internal, non-modifiable factors (genetic background, age, and gender) and "nurture", external, modifiable factors. Among the external risk factors the western (high fat, obesity-prone) diet and vitamin D (VD) deficiency (VDD) are important (1,2). The emerging evidence suggests that another important external influence on the pathophysiology of glucose homeostasis may be the large bowel (gut) microbial community (microbiota) (3-10). Over the past ten years there is an explosion of knowledge, the number of published papers increased exponentially from 121 (2002) to 1,965 (2012) under "microbiota" and from 74 (2002) to 956 (2012) under "human microbiota" in Pubmed search. The complete composition and function of the gut microbiota (GutM) are still unknown, but it is proposed to contribute to the low grade inflammation that plays substantial role in the dysregulation of normal glucose tolerance into PreDM and T2DM (5-10). The complexity of the process notwithstanding, there may be significant interactions between GutM, dietary components (such as prebiotics and vitamin D) and glucose homeostasis

suggesting that diet enrichment or supplementation with prebiotics and vitamin D may improve glucose homeostasis.

This article will review the role of GutM in the pathogenesis of prediabetes and T2DM, address the mechanisms of vitamin D and prebiotics interaction with GutM, and summarize the recent randomized clinical trials (RCT) of VD and prebiotics supplementation in PreDM and T2DM.

GUT MICROBIOTA COMPOSITION AND FUNCTION IN PREDIABETES AND T2DM.

The recent studies demonstrate that the diverse microorganisms, which colonize the body at birth, live on the mucosal surfaces and in the lumen of the human large bowel and exist in beneficial symbiotic relationship with their host (3,4). Bacteria make up most of the flora and up to 60% of the dry mass of the feces (7). The GutM comprise 100 trillion bacteria that is 10-fold the number of cells in the human body, and the genome of these bacteria (microbiome) has 150 times more genes than the human genome (3,4). Multiple molecular methods, including quantitative PCR (qPCR), barcoded pyrosequencing, and phylogenetic microarrays of 16S rRNA helped to generate comprehensive microbial community profile. This profile shows that more than 99% of microbiota are anaerobes and 98% contain several families (phyla) including *Firmicutes* (64%), *Bacteroidetes* (23%), *Proteobacteria* (8%) and *Actinobacteria* (3%) (3,4,7). The human host provides nutrient-rich habitat and impacts GutM survival by dietary composition (5,9,10) and the use of prebiotics (11-13), probiotics (13,14) and antibiotics (14). Reciprocally, GutM enzyme activities are important for facilitating nutrient absorption, metabolizing vitamins, drugs, and endogenous hormones and carcinogen detoxification (3,4,7,15,16), suggesting that GutM may be a major player in human disease (infection, inflammation, allergy, cancer, obesity,

and diabetes) and possibly longevity (3-5,7-17).

There is multiple convincing evidence of GutM involvement in glucose homeostasis (Table 1) (18-33). The data from in vitro experiments and in vivo animal models (knockout, germ-free and humanized gnotobiotic mice) helped to understand the mechanisms of glucose homeostasis regulation by GutM (Table 1 and 2) (34-45) and reciprocal changes produced by Western "Obesogenic" diet altering nutrients available to GutM and resulting in detrimental qualitative and quantitative dysbiosis of GutM ecosystem with preferential increased in Gram negative bacteria (5,9,10,18,21,22).

Particularly revealing in elucidating contribution of GutM to human biology and pathobiology was studying the humanized gnotobiotic mouse model, a well-defined, representative animal model of the human gut ecosystem created by transplanting human fecal microbial communities into germ-free wild mice (10). In this model switching from a low-fat, plant polysaccharide-rich "Healthy" to a high-fat, high-sugar "Obesogenic" diet shifted GutM ecosystem, metabolic pathways, and gene expression within a single day and after two weeks "Obesogenic" diet fed mice almost doubled their body fat (10) suggesting that "Obesogenic GutM" had increased capacity of harvesting ene rgy (9,10). Furthermore, this trait was transmissible by GutM: the germ-free mice colonized with an "Obesogenic GutM" doubled their body fat compared to mice colonized with a "Lean GutM" despite of consuming exactly the same diets (9,10). Similarly, transplantation of GutM from mice with metabolic syndrome and insulin resistance to the wild-type germ-free mice resulted in the transmission of the insulin resistance to the recipient mice (8).

GutM dysbiosis resulted in increased production of lipopolysaccharide (LPS), a molecule from the outer membrane of Gram negative [Gram(-)] bacteria that disrupted mucosal immunity,

braked mucosal barrier, acquired access to the interior of the host and activated inflammation and systemic immunity (Table 1 and 2). In a mouse model LPS was identified as a causative triggering factor of the onset of obesity, insulin resistance, and diabetes (5). In this model usual diet increased or decreased circulating LPS during the fed or fasted state, respectively. In contrast, a high-fat diet chronically increased LPS two to three times, a threshold that was defined by the authors as metabolic endotoxemia (for comparison, sepsis and other infections would increase circulating LPS 10-50 times). Importantly, a high-fat diet increased the proportion of an LPS-generating GutM as well as endogenous LPS production proportionally to the amount of dietary fat and enhanced the absorption of exogenous orally administered LPS (5). When metabolic endotoxemia was induced through continuous subcutaneous infusion of LPS, fasted glycemia and insulinemia and whole-body, liver, and adipose tissue weight gain increased to a similar extent as in high-fat-diet mice. Both, endogenous and exogenous LPS caused insulin resistance acting via CD14 (a protein from the Cluster of Differentiation group that acts as a co-receptor for LPS along with TLR-4, LPS binding protein and MD2 protein). The macrophage infiltration was higher in adipose tissue of mice fed a high-fat diet and in mice infused with LPS, and this effect was not observed in the absence of CD14 (5). The authors suggested that LPS might be a newly identified inflammatory factor from GutM, which upon binding to its receptor served as a vector for the triggering of 'inflammation-insulin resistance-diabetes' cascade induced by high-fat feeding.

In healthy humans circulating LPS was associated with both prevalent and incident T2DM (46); with fat and energy intakes (47); and circulating CD14 correlated with insulin resistance (HOMA-IR), visceral adiposity and markers of inflammation and endothelial dysfunction (48). In patients with T2DM genetic polymorphism of CD14 correlated with insulin

sensitivity index (ISI) and endothelial dysfunction (48). Healthy subjects had higher fasting LPS after a fat-enriched compared to a control meal (5). Recently intravenous LPS infusion to healthy subjects was used to establish a human model of endotoxemia and subclinical inflammation (19). In a double blind, placebo-controlled, random sequence crossover study low-dose LPS infusion caused a rapid and transient increase of plasma TNF-α (25-fold) and IL-6 (100-fold) followed by modest inflammation in adipose tissue manifested by induction of mediators of insulin receptor signaling (IL-6, TNF-α, MCP-1, fractalkine, SOCS-1 and SOCS-3). Most importantly, LPS caused significant induction of systemic insulin resistance measured by IVGTT (insulin sensitivity declined by 21%) without evidence of pancreatic beta-cell dysfunction (no change in the first phase of insulin secretion AIRg index). Consistent with the IVGTT data, insulin resistance by HOMA-IR increase by 32% while HOMA-B was unchanged (19). Bariatric surgery in morbidly obese patients with and without diabetes resulted in significant changes in GutM after 3- and 6-months and improved metabolic profile including blood glucose, A1C, insulinemia, HOMA-IR, leptin, and adiponectin (33). In these patients *Faecalibacterium prausnitzii*, a conserved and dominant species of the healthy GutM, was directly linked to the reduction in low-grade inflammation state independently of calorie intake and was negatively associated with HOMA-IR (33). Finally, transplantation of GutM from lean healthy donors to recipients with metabolic syndrome improved insulin sensitivity of recipients (median rate of glucose disappearance increased from 26.2 to 45.3 μmol/kg/min; $P < .05$) and increased abundance of butyrate-producing GutM (49).

The investigation of molecular mechanisms of the effects of LPS on pancreatic beta-cell function showed that LPS inhibited insulin gene expression and decreased glucose-induced insulin secretion (40). These deleterious effects of LPS were mediated through TLR-4 (a protein

from Toll-like receptor family abundantly present in pancreatic beta-cells) and via NF-kB signaling in pancreatic islets, suggesting a novel mechanism by which GutM might affect pancreatic beta-cell function (40). All these data support but do not prove the emerging view that GutM might play a causal role in pathogenesis of insulin resistance, prediabetes and diabetes.

One of the links in microbiota – glycemia interaction is the enteroendocrine system and the related hormones, including glucagon like peptide-1 and -2 (GLP-1, GLP-2) and peptide YY (PYY) secreted by the intestinal L-cells; ghrelin, secreted by the stomach; amylin and pancreatic polypeptide (PP) secreted by the pancreas; and adiponectin and leptin secreted by the adipose tissue (Table 2). These hormones are well known modulators of appetite, food intake and glucose and fat metabolism and some of them (e.g. GLP-2) are involved in regulation of gut barrier function through the expression of tight junction proteins (50).

Various models were utilized to establish the role of enteroendocrine system in microbiota-glycemia connection through manipulation of GutM including genetically obese and high-fat-diet-induced obese and diabetic mice and fermentable carbohydrate-enriched (prebiotic) diets. In the study of genetically obese and high-fat-diet-induced obese and diabetic mice both obesity and prebiotic supplementation caused changes in GutM and in enteroendocrine system (34). The researchers used multiple molecular methods to generate comprehensive microbial community profiles and showed that prebiotic intervention resulted in a decrease of *Firmicutes* and an increase of *Bacteroidetes* phyla and a change of 102 distinct taxa, 16 of which displayed a >10-fold change in abundance, and these changes ameliorated obesity-induced gut permeability and metabolic endotoxemia (34). Interestingly, some specific bacteria were identified as possibly playing particularly important role. For example, there was a very strong correlation between gut permeability and the abundance of *Streptococcus intermedius*, known to produce a specific

cytolysin (intermedilysin) (39) altering intestinal cell tight-junction architecture (34). Also, *Akkermansia muciniphila* (*A. muciniphila*) associated with healthy colon mucosa, strongly and positively correlated with the enteroendocrine L-cell number (34). Of note, in humans *A. muciniphila* inversely correlated with body weight, increasing after the gastric bypass-induced weight loss (51). Moreover, prebiotic-induced change in mice GutM was accompanied by increased colon L-cell number (~50%), enhanced intestinal proglucagon mRNA expression (~50-70%), increased plasma GLP-1 levels (~50-200%), and improved leptin sensitivity (~30-50%). The prebiotic use counteracted high-fat-induced body weight gain and fat mass development and resulted in improved glucose tolerance, decreased plasma triglycerides and muscle lipid content (total, triglycerides, and phospholipids), reduced expression of oxidative stress (measured by NADPH oxidase) and inflammatory (IL-1 mRNA) markers in the colon (34). In the same mouse model, similar beneficial changes in GutM (specifically increase in bifidobacteria and decrease in lactobacilli) were accompanied by improved gut barrier integrity, improved metabolic endotoxemia, inflammation, weight, and glucose tolerance (50). Multiple enteroendocrine and other hormones were studied (GLP-1, PYY, PP, amylin, leptin, ghrelin) with significant changes observed in GLP-1, PYY and leptin. Analysis of the colon gene expression profile showed up-regulation of PYY, PPAR-γ, TLR-4 and endocannabinoid system receptor CB2 genes. The authors postulated that the observed effects could be related to modulation of GutM such as higher content of bifidobacteria in the caecum (50).

In healthy humans a randomized, double-blind, parallel, placebo-controlled trial of fermentable carbohydrates (prebiotic) showed a 3-times increase in gut microbial fermentation, increased satiety, decreased hunger and energy intake, whereas postprandial plasma glucose responses decreased after the standardized meal. These changes were accompanied by higher

serum GLP-1 and PYY concentrations (12). In overweight and obese adults prebiotic supplementation resulted in lower circulating ghrelin, higher PYY, and improved insulin sensitivity in the intervention group vs. control (52). The mechanism of enteroendocrine system action might involve sensing the presence of bacterial antigens (including LPS) through toll-like receptors (TLR) and neutralizing intestinal bacteria by releasing chemokines and defensins as demonstrated in vitro and in vivo models, and by inducing contraction of the muscular tunica, favoring the emptying of the distal small intestine (53).

The other systems important in obesity-dysglycemia-dyslipidemia and implicated in the crosstalk between GutM and metabolism are the endocannabinoid and apelinergic systems, the former involving a group of neuromodulatory lipids and their receptors and the latter involving a peptide apelin and its G-protein-coupled APJ receptors (Table 2) (12,34-36,50-53). In vitro and in vivo studies using endocannabinoid system agonists and antagonists showed that this system linked microbiota with adipogenesis and glucose metabolism by modulating gut permeability, tight junction proteins, expression of inflammatory markers, markers of adipocyte differentiation, adipocyte size and number and acting via colon and adipose tissue cannabinoid receptors with LPS being a master switch (54). Although causality would remain to be proven, these experiments highlighted the essential role of the enteroendocrine and endocannabinoid systems in microbial-related modulation of systemic inflammation and glucose metabolism.

VITAMIN D INTERACTION WITH GUT MICROBIOTA AND VITAMIN D USE IN RANDOMIZED CLINICAL TRIALS FOR IMPROVING GLUCOSE HOMEOSTASIS IN PREDIABETES (Table 3 and 4)

In humans, active 1,25-dixydroxyvitamin D (1,25D) can be produced and function in endocrine, paracrine and autocrine manner. Vitamin D (VD) system directly or indirectly

regulates about 3% of mouse and human genome (55) including genes involved in glucose homeostasis. Vitamin D role in glucose homeostasis is one of the well-established non-classical roles of this multi-potential vitamin (2). All components of VD system are present in the mucosal cells lining the large bowel (colonocytes) including vitamin D receptor (VDR), VD response elements (VDRE), and the enzymes involved in synthesis and catabolism of active 1,25D (55-58).

Vitamin D interaction with GutM may become yet another non-classical function of VD (Table 3) (59-72). There are several pathways for possible VD and GutM relationships in the large bowel. Vitamin D may contribute to acquisition and maintenance of symbiotic gut microflora. Both, VD and GutM regulate calcium absorption, the cellular tight junctions, mucosal and systemic immunity, and colonocyte activity (Table 2 and 3).

Vitamin D-regulated intestinal calcium absorption, the classical action of vitamin D, occurs predominantly in duodenum and jejunum and involves active intracellular and passive paracellular absorption. Similar mechanisms are involved in about 10% of calcium absorption that occurs in the large bowel (57,67), and this process is enhanced by GutM-produced propionic acid (44,45). Calcium plays crucial role in all physiological functions, and it is possible that VD and GutM act synergistically to maintain calcium balance. The tight junctions between the intestinal cells play an important role in the paracellular calcium absorption and are also an integral component of the mucosal immunity. Both VD and GutM are involved in the regulation of the tight junctions that establish mucosal barrier of defense against intestinal pathogens (Table 2 and 3). The intestinal pathogens may have developed ability of penetrating mucosal barrier to exploit host's physiological pathway for nutrients' absorption (e.g. calcium) as a crucial step in gaining access to the host's internal resources. Both VD and GutM play a role in the regulation

and signaling pathways of all three components (mucosal, innate and adaptive) of the immune system (Table 2 and 3) and act to develop and maintain self-tolerance by down regulating the adaptive immune responses while enhancing the protective innate immune responses. Similarly, both VD and GutM have trophic effects on colonocytes (Tables 2 and 3) shown in vitro, in animal models and in human studies of inflammatory bowel disease and colorectal cancer including genetic data (77,78).

A few studies suggested possible interdependence of microbiota and vitamin D and its role in glycemia. In human monocytic cells LPS stimulated secretion of 1,25D via up-regulation of 1α-hydroxylase, a transcription factor (STAT1α), a coactivator (p300), and chromatin remodeling (79). Similarly, LPS activated 1α-hydroxylase in the kidney (80) and in endothelial cells (81). In a human colon cancer cells butyrate (metabolic product of GutM) significantly increased number and activity of VDR (250% vs. control). Both butyrate (640% vs. control) and 1,25D (350% vs. control) significantly stimulated differentiation of cells, whereas combined treatment with butyrate and 1,25D resulted in a synergistic amplification of cell differentiation (1400% vs. control). The responsiveness to LPS of monocytes modulated by vitamin D (1,25D) differed between T2DM patients and controls (82,83). The direct evidence of the interaction between GutM, vitamin D and glucose metabolism came from ApoE deficient mice ($ApoE^{-/-}$), a widely used model of obesity with accelerated atherosclerosis. A recent work showed that $ApoE^{-/-}$ mice had impaired glucose tolerance and insulin signaling; increased intestinal permeability, inflammatory mediators (TNF-α, RANTES, ICAM-1, and MIP-1α in the colon, liver, and adipose tissue) and scavenger cells among circulating macrophages as well as a significant increase in the intestinal expression of VDR. The probiotic bacterial combination efficiently

prevented insulin resistance in these ApoE$^{-/-}$ mice likely acting via transactivation of PPAR-γ, Farnesoid-X-receptors (FXR) and VDR (84).

The vitamin D deficiency (VDD) models highlighted importance of vitamin D in GutM-glycemia relationship. In the VDD rats oral vitamin D supplementation improved metabolic efficiency likely acting via modulation of GutM, there was an increase in food intake (1.8 times), in caecum size (1.4-times), in short chain fatty acid (SCFA) production and in metabolic rate while body mass was maintained (85). In a diet-induced VDD mouse model 27 genes were up- or down-regulated (more than 2-fold) and VDD mice had elevated levels (50-fold) of bacteria in the colonic tissue suggesting that VDD dysregulated colonic antimicrobial activity and impaired GutM ecosystem (59). The activated genes were involved in containment of enteric bacteria (an angiogenin-4 gene coding for an antimicrobial protein) and in glucose homeostasis. For example, the carbonyl reductase 3 (CBR3) gene was down-regulated 3-fold in VDD mice and genetic variation in CBR3 conferred risk of type 2 diabetes and insulin resistance most likely via regulation of adipogenesis (86). Some other genes included Apolipoprotein C-II (a modulator of cardiovascular disease in T2DM) (87), TF3 (an adaptive-response gene) induced by various stress signals relevant to T2DM and involved in insulin gene transcription (88), and pleiomorphic adenoma gene like 1 (Plagl1), involved in insulin secretion and possibly important in beta-cell exhaustion in insulin-resistant states (89).

These in vitro and in vivo animal models may be indicative, however, they cannot be translated into human clinical setting, and further studies are needed to elucidate causal relationship between GutM, glucose metabolism and vitamin D. Based on the available data the proposed hypothesis may be that VDD may act as a predisposing or/and precipitating factor among others. The VDD may contribute (among other mechanisms) to destabilization of GutM,

decreased colon cell antimicrobial activity and increased mucosal permeability. These events may allow the intact bacteria and/or bioactive bacterial products (e.g. LPS) to translocate from the intestinal lumen to the body interior and induce (after activation of multiple complex pathways) low-grade inflammation, a suggested mechanism linking GutM with metabolic disorders including prediabetes and T2DM.

Several randomized clinical trials in subjects with prediabetes and T2DM are underway to prove causality of vitamin D role in these conditions (90). The results from a few published clinical trials are controversial (Table 4) (91-97) and do not clearly establish the benefits of vitamin D supplementation for improving glucose homeostasis proving Thomas Henry Huxley in saying "The great tragedy of science — the slaying of a beautiful hypothesis by an ugly fact".

PREBIOTICS INTERACTION WITH GUT MICROBIOTA AND PREBIOTIC USE IN RANDOMIZED CLINICAL TRIALS FOR IMPROVING GLUCOSE HOMEOSTASIS (Table 3 and 4).

Prebiotics (11) are non-digestible fermentable food ingredients that are able to change the gut microflora composition and/or activity in a way beneficial to the host (Table 3 and 4) (98-101). Prebiotics are not digested and reach the large bowel where they are fermented by GutM to produce short chain fatty acids (SCFA, acetate, propionate, and butyrate), L-lactate, CO_2, hydrogen, methane and other metabolites that regulate downstream metabolic processes in multiple ways. The beneficial properties of prebiotics include their contribution to reducing constipation, weight loss, improving level of glucose and lipids and anticarcinogenic effect (11). The most studied prebiotics are complex carbohydrates, i.e. fructans and arabinoxylans.

Fructans are polymers of fructose molecules (99). Fructans are composed of linear chains of fructose units linked by β glycosidic bonds and typically terminating in a glucose unit. There

are short chain (oligofructose) and longer chain (polyfructose, i.e. inulin and levan) fructans. Fructans are typically found in the roots and serve as energy reservoir instead of starch for many plants. An arabinoxylan (AX) is a hemicellulose, a copolymer of two pentose sugars, arabinose and xylose. It is found in cell walls of plants including cereal grain and chiefly serves a structural role (99). Fructans belong to soluble and arabinoxylans belong to soluble and insoluble dietary fibers. Both, fructans and arabinoxylans, change GutM composition, e.g. promote proliferation of beneficial Bifidobacteria and Lactobacilli (Table 3).

The examples of raw natural food particularly rich in prebiotics (percent content by weight) include chicory root (~65%), Jerusalem artichoke (~32%), barley (22%), garlic (~18%), onion (~10%), globe artichoke (~7%), rye bran or grain (~7%), wheat bran (~5%), asparagus (4%), and examples of cooked food are chocolate (9%) and white bread (~3%) (100,101).

Since Marcel Roberfroid introduced the concept prebiotics in 1995 (11) in vitro and in vivo models have produced convincing evidence of profound prebiotics' effects on energy and glucose homeostasis (Table 3 and 4). Some of the mechanisms of these effects, which still need to be further clarified, include the change of GutM composition (e.g. decrease *Firmicutes* and increase *Bacteroidetes*), increase of satietogenic gut peptides, decrease of systemic inflammation and improved glucose tolerance (Table 3). These data prompted initiation of RCT that produced promising but variable results (Table 4) requiring further confirmation.

CONCLUSION

The role of GutM in pathogenesis of prediabetes and diabetes as well as contribution of vitamin D and prebiotics to this process are inadequately understood. Available data suggest that "obesogenic" diet results into the initial event of microbiota transformation from symbiosis to dysbiosis. The microbial antigens [e.g. Gram(-) bacteria and LPS] translocate to the host interior

and trigger TLR complex activation with subsequent macrophage tissue infiltration and inflammatory pathways signaling. The "double hit" of steatosis (ectopic fat accumulation) and "–itis" (inflammation) and contribution of "co-risks" (e.g. vitamin D deficiency) are required to activate molecular signaling including impaired insulin signaling and secretion that ends with diabetes and diseases associated with T2DM (Figure 1). The awareness that vitamin D deficiency may play a role only as a contributing factor among others helps to understand the failure of recent randomized trials of vitamin D supplementation in prediabetes and indicates that the search for an appropriate intervention or intervention combination (e.g. vitamin D and prebiotics) and an appropriate time (prior to the process becoming irreversible) should continue.

The complexity of multidirectional effects and mechanisms of diabetes pathogenesis remains to be elucidated. In the interim, the current professional society guidelines can be used for recommending vitamin D and dietary fiber daily intake. For vitamin D the Institute of Medicine (IOM), the Endocrine Society and the American Association of Clinical Endocrinologists (AACE) Recommended Daily Allowances (RDA) are 600 and 800 IU/day for ages 19-70 years and 70+ years, respectively, and 4,000 IU/day is recommended for obese adults, while to raise the blood level of 25-hydroxyvitamin D [25(OH)D] above 30 ng/ml may require 1500 - 2,000 IU/day for all adults and 10,000 IU/day for obese adults (102). For fiber (endorsed by the IOM, the American Diabetes Association, and other groups) the Recommended Adequate Intake (RAI) is 25-38 g/day (14 g/1,000 kcal/day) for all adults (103) and 25 - 50 g/day for T2DM (104) and these suggestions correspond to the AACE recommended 7 - 10 servings/day of "healthful" carbohydrates (1). The average intakes of vitamin D and fiber are reported as 152 - 220 IU/day (105) and 12.5 -18 g/day (106), respectively, both lower than recommended. Interestingly, the mean serum 25(OH)D concentration in traditionally living

populations in East Africa is 46 ng/ml (107) and the estimated (from stable carbon isotope analysis of human skeletons) consumption of inulin-type fibers by the prehistoric hunter-foragers is 135 g/day (108), both higher than presently recommended. The recommendation to increase consumption of food rich in vitamin D (salmon, mackerel, sardines, herring, and cod liver oil) and prebiotics (artichokes, onion, garlic, etc) is expected to provide multiple health benefits and ensure the delivery of the best medical care.

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Table 1.
Human studies of the major interactions between the gut microbiota (GutM) and glucose metabolism: data from the normal weight, obese and diabetic patients*

Popu-lation	Major changes in human microbiota composition and activity
Healthy, normal weight	□Firmicutes (~20%) and □Bacteroidetes (~20%) after □calorie intake (from 2400 to 3400 kcal/d and □absorption ~150 kcal), whereas □calorie intake (from 3400 to 2400 kcal/d and □absorption ~150 kcal) □ □Bacteroidetes (~20%) and □Firmicutes (~20%) (18) □inflammatory markers (IL-6, TNF-α, MCP-1, CX3CL1) in adipose tissue after LPS IV (19) LPS: correlated with INS (20)
Obese	□GutM diversity, □□Bacteroidetes, □Firmicutes, □Bifidobacteria, □□Archaea (methanogens) in OB vs CON (9,21-24) □Bifidobacteria in neonates of OW mothers and associated with obesity later in childhood (25) □Bacteroides, □Firmicutes, □Proteobacteria with wt loss diet (24,26) or bariatric surgery (27) GutM profile correlated with INS sensitivity (28) LPS: □LPS after a high-fat meal (29); predicted by fat and energy intakes (18); +correlated with INS (20,30) and with TG (30) and −correlated with HDL (30), and ≠ with BG (20,30)
T2DM	□GutM diversity, □Firmicutes, □Bifidobacteria, □Bacteroides in T2DM vs CON (31,32) GutM profile correlated with BG (31) and inflammatory markers (33) LPS: □LPS in T2DM vs OB sex-, and age-matched CON (20); +correlated with INS (20,30) and TG and −correlated with HDL (30)

□=decreased, □=increased, □□=variable, □=results into, +correlated=positively correlated, −correlated =negatively correlated, ≠ =did not correlate
*Abbreviations: LPS=circulating lipopolysaccharide, INS=circulating insulin, BG=blood glucose, wt=weight, TG=triglycerides, OW=overweight, OB=obese, CON=controls, IV=intravenous infusion, IL-6= interleukin-6, TNF-α=tumor necrosis factor-α, MCP-1=monocyte chemotactic protein-1, CX3CL1=fractalkine.

Table 2.
The changes induced in the host's systems due to the interactions of the gut microbiota (GutM) with the nutrients*

System	Major characteristic changes and related mechanisms
Energy and glucose	□extraction of energy from diet □ obesity, INS resistance and T2DM (5,21,24,34) +correlation between GutM and glucose intolerance, TG, and muscle lipids (34)
Adipose tissue (AT)	AT mass: □AT mass in various models (e.g. humanized gnotobiotic mice[a]) (5,9-11,21,34) AT metabolism: regulated by LPS[b] and SCFA that act via GPR (GPR43), PPAR-γ (35,36) and intestinal Fiaf (37) AT inflammation: via □LPS □□TLR-2 and TLR-4 □□NF-κB pathway □ □IL-6 and TNF-α in AT from OB and T2DM patients vs lean CON (20)
Entero-endocrine	□□regulation of GI peptides involved in food intake (GLP-1, GLP-2, PYY, PP, ghrelin, amylin) (12,34,35) and GI endocannabinoid[c] and apelinergic[d] systems (34,36)
Gut permeability, inflammation	□TJ function □□gut permeiability (5,10-12,38) □□absorption of GutM toxins □ □systemic inflammation (e.g. Streptococcus intermedius produced a specific cytolysin, intermedilysin) (39)
LPS-related mechanisms, inflammation, and immunity	□INS secretion via TLR4 and NF-κB signaling in pancreatic islets in vitro (40) □INS resistance during infection (41) and LPS IV in men (19) and in vivo models (5)
Trophic effect on mucosa	□obesity- and T2DM-associated systemic immunity that is activated via the inflammatory cascade components (TLRs, NF-κB, cytokines, etc) (5,38,19,34,42) □total volume, thickness, and crypt branching in colon mucosa (43) □protection against gut injury and associated colonocyte apoptosis (42)
Ca absorp	□entero-endocrine cell number (43) □GutM-produced propionic acid □□calcium absorption (44,45)

□=decreased, □=increased, □□=variable, □=results into, +correlation=positive correlation
*Abbreviations: AT=adipose tissue, LPS=circulating lipopolysaccharide, INS=circulating insulin, BG=blood glucose, TG=triglycerides, OW=overweight, OB=obese, CON=controls, IV=intravenous infusion, TJ=tight junctions, GI=gastrointestinal, SCFA=short chain fatty acids (acetate, butyrate, propionate), IL-6= interleukin-6, TNF-α=tumor necrosis factor-α, GPR=G-protein-coupled receptors, TLR=Toll-like receptors, PPAR-γ=peroxisome proliferator-activated receptor-γ, NF-κB=nuclear factor kappa-light-chain-enhancer of activated B cells, Fiaf= fasting-induced adipose factor (a circulating lipoprotein lipase inhibitor), GLP= Glucagon-like peptide, PYY=peptide YY, PP=pancreatic polypeptide, Ca absorp=Calcium absorption.
[a]Humanized gnotobiotic mice is the mouse model of the human gut ecosystem of germ-free mice colonized with the human gut microbiota. [b]LPS is a molecule from the outer membrane of Gram negative bacteria that disrupts mucosal and systemic immunity. [c]The endocannabinoid

system refers to a group of neuromodulatory lipids and their receptors that are involved in a variety of physiological processes including appetite, pain-sensation, and mood. [d]The apelinergic systmen refers to a peptide Apelin and its G-protein-coupled APJ receptors that are involved in a variety of physiological processes including glucose metabolism.

Table 3.
The changes induced in the host's systems due to the interactions of GutM with vitamin D (VD) and prebiotics*

System	Major characteristic changes and related mechanisms
Vitamin D-related changes and mechanisms	
GutM composition	□infiltration of mucosa by intestinal pathogens (61) VDR signaling modulates GutM composition (59,60)
Gut permeability, inflammation, and immunity	□TJ proteins (62,63) □ gut permeability (63) □bacterial translocation to mesenteric lymph nodes (63) □ proinflammatory cytokines (e.g. IL-1, IL-6, IL-8, INF-γ and TNF-α) (59,63) □histologic features of colon inflammation (63) Improved systemic immunity: innate (via TLR- and NOD-systems' and downstream NF-κB pathway) (60,62,64), and adaptive (via T- and B-lymphocyte function) (65) □mucosal immunity (63,65) □secretion and function of antimicrobial proteins, e.g. cathelicidin (64), angiogenin-4 (59), and defensin (64)
Trophic effect on mucosa	□colonic epithelium proliferation and differentiation (63,65)
Calcium	□calcium absorption in the large bowel (67)
Reciprocal effects of GutM on VD	GutM metabolizes VD analogs (68) and GutM affects VDR expression, distribution, transcriptional activity, and target gene expression in colonocytes (60)
Vitamin D deficiency-related changes and mechanisms	
GutM composition	□bacterial infiltration of the colon (50-fold) (59)
Trophic effect on mucosa	□hyperplasia and hyperproliferation of colonocytes (66) □benign and □malignant neoplasms in the colon (66)
Prebiotic-related changes and mechanisms	
GutM composition	□Proteobacteria, □Firmicutes, □Bacteroidetes, □Bifidobacteria (11,12,34,38,69) □□102 distinct taxa, 16 of which displayed a 10-fold change in abundance (34)
Gut	

permeability, inflammation, and immunity	□TJ proteins (zonula occludens-1 and claudin-3) (69) □TJ function (69) □macrophage infiltration in the adipose tissue (34,69) □systemic inflammation via the endocannabinoid system modulation (34,38,69)
Trophic effect on mucosa	Improved regulation of the immune system (e.g. □TLR-4) (11,35,38) □crypt branching and release of mucins (70)
Calcium	Improved intestinal mucosal morphometry (70)
Entero-endocrine	□calcium absorption via mechanism involving SCFA (propionate) (44,45) □differentiation of stem cells into entero-endocrine L-cells (34,38) □GLP-1, GLP-2, PYY (34,38,69,71)
Metabolism	□leptin sensitivity (34)
Reciprocal effects of GutM on prebiotics	□adiposity, body weight, serum and hepatic cholesterol and insulin resistance (69) □serum SCFA, breath hydrogen and breath methane (metabolic products of prebiotics) in healthy and hyperinsulinaemic men (72) and in vitro (11,71) and in vivo animal models (11,69,71)

□=decreased, □=increased, □□=variable, □=results into
*Abbreviations: VD=vitamin D, VDD=vitamin D deficiency, VDR=vitamin D receptor, INF-γ=interferon-γ, NOD=nucleotide-binding oligomerization domain (NOD)-containing proteins, and as in Table 2.

Table 4.
Randomized clinical trials (RCT) of Vitamin D or prebiotic supplementation in subjects at risk or with prediabetes (PreDM) and T2DM*

Study	N	Popu-lation	Dur, wks	Treatment and test	Main glycemic outcome	Other outcomes
RCT of Vitamin D doses ≥1,000 IU and duration ≥12 weeks						

Jorde et al. (91,92)	330	OW&OB	52	40,000 D3/w OGTT	□FBG, FINS	□Iipids, IL, MCP-1, INF-γ □CRP
Harris et al. (93)	89	PreDM & eT2DM	12	4,000 D3 OGTT	□INS-S, □A1c □INS-Sec, □DI	□C-peptide, HOMA-IR
Mitri et al. (94)	92	PreDM & eT2DM	16	2,000 D3 IVGTT	□DI, □INS-Sec □A1c	□INS-S, □FBG
von Hurst et al. (95)	81	INS-R >1.93	24	4,000 D3 HOMA	□INS-S □INS-R	□C-peptide, CRP, lipids
Jorde et al. (96)	36	T2DM	24	40,000 D3/w FBG, FINS	□FBG, FINS □A1c	□C-peptide
Davidson et al. (97)**	99	PreDM & eT2DM	99	~90,000 D3/w OGTT	□% new T2DM	□FBG, FINS; □A1c □INS-S, INS-Sec, DI
Prebiotics (OF=oligofructose): duration ≥12 weeks						
Parnell et al. (52)	48	OW/OB	12	21g OF MTT	□FBG, FINS □post-MTT BG □post-MTT INS	□BW, GIP, □ghrelin □ post-MTT: ghrelin, PYY, leptin □post-MTT: GIP & GLP-1
Malagua-rnera et al. (98)	66	OB & NASH	24	OF&PRO HOMA	□HOMA-IR	□AST, LDL, CRP, TNF-α, LPS, steatosis, NASH activity index

□=decreased, □=increased, □no change

*RCT duration ≥12 weeks are included. Vitamin D RCT: D supplement doses are daily doses in IU unless specified, only RCT using ≥1,000 IU/day are included. ** Vitamin D3 dose was calculated based on body weight and baseline 25(OH)D level and raged 64,731–134,446 IU/week. Insulin sensitivity and secretion and disposition index were calculated by multiple methods based on OGTT.

Abbreviations: D3=cholecalciferol, OF=oligofructose, PRO=probiotics (Bifidobacterium longum), Dur=Duration, w=week, OGTT= oral glucose tolerance test, MTT=meal tolerance test; IVGTT= intravenous glucose tolerance test, HOMA=homeostatic model assessment, OW=overweight, OB=obese, eT2DM=early T2DM, NASH= non alcoholic steatohepatitis, F=fasting, BG=blood glucose, INS=insulin, FINS=fasting insulin, INS-S=INS sensitivity, INS-R=insulin resistance, INS-Sec=INS secretion, DI=disposition index, CRP=C-reactive protein,

BW=body weight, GIP=glucose-dependent insulinotropic peptide, AST=aspartate aminotransferase and other as in Tables 2 and 3.

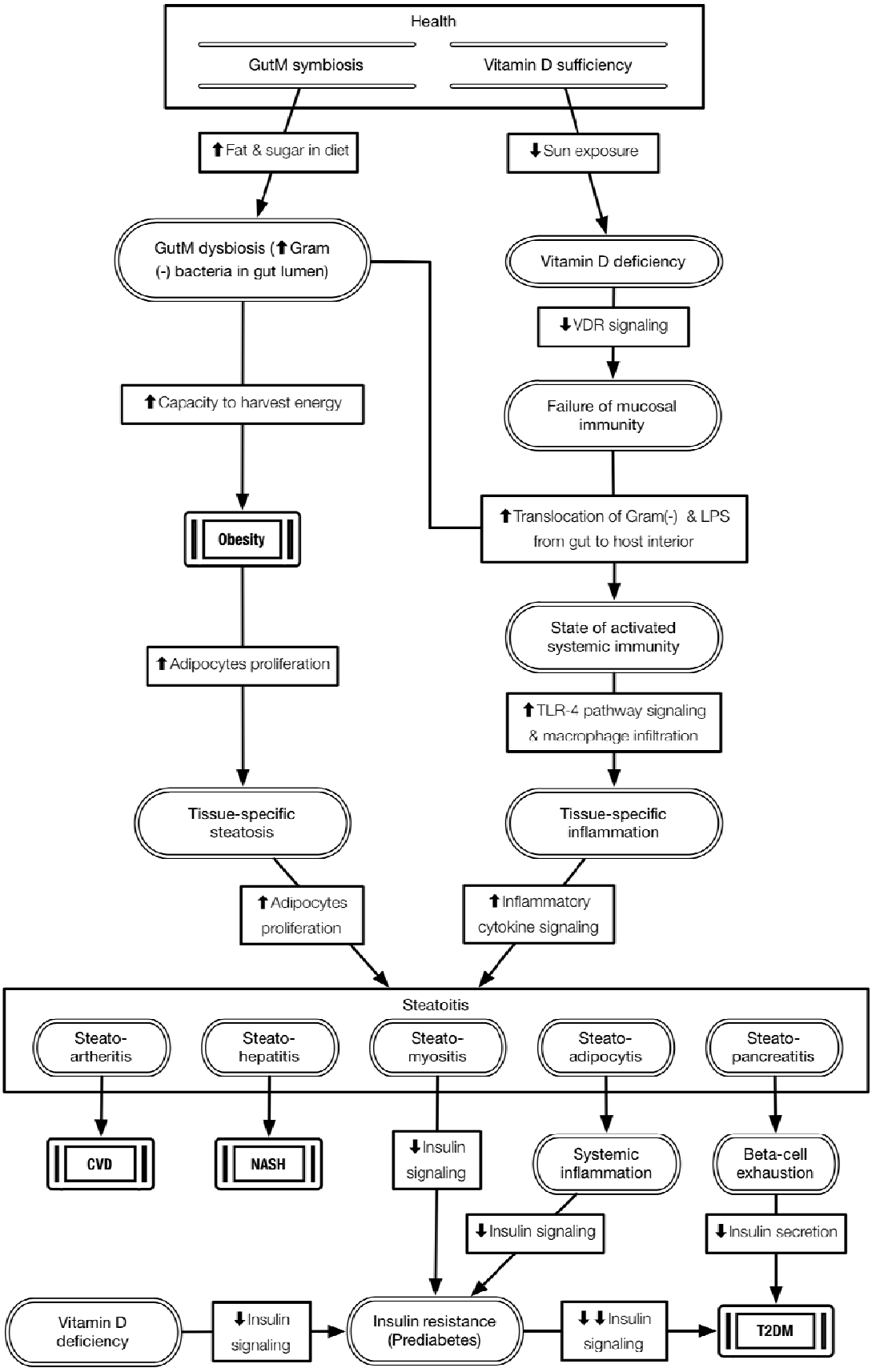
Health
GutM symbiosis
Vitamin D sufficiency
⬆Fat & sugar in diet
⬇Sun exposure
GutM dysbiosis (⬆Gram (-) bacteria in gut lumen)
Vitamin D deficiency
⬇VDR signaling
⬆Capacity to harvest energy
Failure of mucosal immunity
Obesity
⬆Translocation of Gram(-) & LPS from gut to host interior
State of activated systemic immunity
⬆Adipocytes proliferation
⬆TLR-4 pathway signaling & macrophage infiltration
Tissue-specific steatosis
Tissue-specific inflammation
⬆Adipocytes proliferation
⬆Inflammatory cytokine signaling
Steatoitis
Steato-artheritis
Steato-hepatitis
Steato-myositis
Steato-adipocytis
Steato-pancreatitis
CVD
NASH
⬇Insulin signaling
Systemic inflammation
Beta-cell exhaustion
⬇Insulin signaling
⬇Insulin secretion
Vitamin D deficiency
⬇Insulin signaling
Insulin resistance (Prediabetes)
⬇⬇Insulin signaling
T2DM

Figure 1. The microbiota and T2DM and related diseases. The "obesogenic" diet results into detrimental change in microbiota, the initial event of pathologic metabolic processes. The "double hit" of steatosis (ectopic fat accumulation) and "–itis" (inflammation) and "co-risks" (e.g. vitamin D deficiency) are required for the progression to the disease, T2DM, CVD, and NASH. Dietary changes (e.g. prebiotics, vitamin D supplementation) may prevent or ameliorate the process if started at the appropriate time prior to the process becoming irreversible. The ovals represent the "states" and the rectangles represent the "processes", the double lines highlight the outcomes.
Abbreviations: VDR = vitamin D receptor, LPS = lipopolysaccharide, TLR = Toll like receptor, CVD = cardiovascular disease, NASH = non-alcoholic steatohepatitis.