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Resistant starch: implications in kidney health and vitamin D homeostasis in diabetes mellitus

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**Resistant starch: Implications in kidney health and vitamin D homeostasis
in diabetes mellitus**

by

Gar Yee Koh

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Nutritional Sciences

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TABLE OF CONTENTS

	Page
LIST OF FIGURES	iv
LIST OF TABLES	vi
ACKNOWLEDGMENTS	vii
ABSTRACT	viii
CHAPTER 1 GENERAL INTRODUCTION	1
Introduction	1
Dissertation Organization	2
References.....	3
CHAPTER 2 LITERATURE REVIEW	5
Diabetes Mellitus	5
Type 1 diabetes (T1D)	5
Type 2 diabetes (T2D)	6
Insulin resistance and adipose tissue inflammation	7
Insulin resistance and metabolic endotoxemia	8
Macrovascular and Microvascular Diabetic Complications	9
Atherosclerosis.....	10
Diabetic retinopathy.....	11
Diabetic neuropathy	11
Diabetic nephropathy	12
Impacts of nephropathy on vitamin D homeostasis.....	12
Impacts of nephropathy on the renin-angiotensin system.....	13
Vitamin D Status.....	15
Vitamin D Biosynthesis and Metabolism	16
Molecular Action of Vitamin D.....	19
Vitamin D and calcium homeostasis.....	20
Vitamin D and diabetes mellitus.....	21
Resistant Starch.....	23
Resistant starch and glucose homeostasis.....	25
Short chain fatty acids.....	34
Intestinal gluconeogenesis	35
Resistant starch and kidney health.....	35
Conclusion	37
References.....	38

CHAPTER 3 DIETARY RESISTANT STARCH PREVENTS URINARY EXCRETION OF VITAMIN D METABOLITES AND MAINTAINS CIRCULATING 25-HYDROXYCHOLECALCIFEROL CONCENTRATIONS IN ZUCKER DIABETIC FATTY RAT	61
Abstract	61
Introduction.....	62
Materials and Methods.....	63
Results.....	65
Discussion.....	67
References	71
CHAPTER 4 CONSUMPTION OF DIETARY RESISTANT FOLLOWING THE ONSET OF DIABETES DOES NOT PREVENT HYPERGLYCEMIA AND COMPROMISED KIDNEY FUNCTION.....	80
Abstract	80
Introduction.....	81
Materials and Methods.....	83
Results.....	84
Discussion.....	86
References.....	89
CHAPTER 5 DIFFERENTIAL EFFECTS OF HIGH- AND LOW-DOSE DIETARY RESISTANT STARCH ON VITAMIN D HOMEOSTASIS AND RENAL FUNCTION IN ZUCKER DIABETIC FATTY RATS	99
Abstract	99
Introduction.....	100
Materials and Methods.....	102
Results.....	104
Discussion.....	106
References	110
CHAPTER 6 GENERAL CONCLUSIONS	120
Overall Summary and Conclusions	120
Strengths and Limitations	122
Future Research	123
References.....	124
APPENDIX A LIST OF ABBREVIATIONS.....	125
APPENDIX B CHAPTER 3 SUPPLEMENTAL DATA	128
APPENDIX C CHAPTER 4 SUPPLEMENTAL DATA	131
APPENDIX D CHAPTER 5 SUPPLEMENTAL DATA	133

LIST OF FIGURES

	Page
CHAPTER 2 LITERATURE REVIEW	5
Figure 1. Vitamin D metabolism	17
Figure 2. Endocytic uptake of vitamin D-DBP complex in the proximal tubule cells	19
CHAPTER 3 DIETARY RESISTANT STARCH PREVENTS URINARY EXCRETION OF VITAMIN D METABOLITES AND MAINTAINS CIRCULATING 25-HYDROXYCHOLECALCIFEROL CONCENTRATIONS IN ZUCKER DIABETIC FATTY RAT	61
Figure 1. Dietary resistant starch prevented growth stunting of ZDF rats.....	76
Figure 2. Dietary resistant starch improved the renal histopathology scoring in ZDF rats	77
Figure 3. Dietary resistant starch maintained circulating 25D concentrations and prevented urinary excretion of 25D, 1,25D, and DBP in ZDF rats	78
Figure 4. Renal megalin expression was reduced and DAB2 expression was enhanced by dietary resistant starch in the kidneys of ZDF rats	79
CHAPTER 4 CONSUMPTION OF DIETARY RESISTANT FOLLOWING THE ONSET OF DIABETES DOES NOT PREVENT HYPERGLYCEMIA AND COMPROMISED KIDNEY FUNCTION	80
Figure 1. Dietary resistant starch attenuated body weight loss at a dose-dependent manner in T1D rats	95
Figure 2. Dietary resistant starch attenuated urinary DBP, but not 25D excretions in T1D rats	96
Figure 3. Dietary resistant starch elevated circulating IL-6, but not TNF- α in T1D rats	97
Figure 4. Circulating IL-6 concentrations were strongly correlated to fasting blood glucose concentrations.....	98
CHAPTER 5 DIFFERENTIAL EFFECTS OF HIGH- AND LOW-DOSE DIETARY RESISTANT STARCH ON VITAMIN D HOMEOSTASIS AND RENAL FUNCTION IN ZUCKER DIABETIC FATTY RATS	99
Figure 1. HRS attenuated excessive urinary excretion of 25-hydroxycholecalciferol (25D) and 1,25-dihydroxycholecalciferol (1,25D) and maintained circulating 25D in Zucker diabetic fatty rats (ZDF)	116
Figure 2. HRS enhanced serum adiponectin concentrations in Zucker diabetic fatty rats (ZDF) despite no significant change in visceral fat content	117
Figure 3. Serum adiponectin strongly correlated with improved kidney function and serum 25D concentrations	118

Figure 4. HRS reduced serum angiotensin II concentrations and increased renal mRNA expression of nephrin in Zucker diabetic fatty rats (ZDF).....	119
CHAPTER 6 GENERAL CONCLUSIONS.....	120
Figure 1. Proposed renoprotective mechanism of dietary resistant starch in type 2 diabetes mediated by renal renin-angiotensin system.....	121
APPENDIX B CHAPTER 3 SUPPLEMENTAL DATA	128
Supplemental Figure 1. Renal megalin and disable-2 mRNA expression was not affected by diabetes or dietary resistant starch in ZDF rats.....	129
Supplemental Figure 2. Renal CYP27B1 and CYP24A1 expression was not affected by diabetes or dietary resistant starch in ZDF rats.....	130
APPENDIX C CHAPTER 4 SUPPLEMENTAL DATA	131
Supplemental Figure 1. Urinary creatinine in T1D rats was not affected by RS.....	131
Supplemental Figure 2. Urinary albumin in T1D was not affected by RS	132
APPENDIX D CHAPTER 5 SUPPLEMENTAL DATA	133
Supplemental Figure 1. RS did not improve renal histopathological scoring in ZDF rats	134

LIST OF TABLES

	Page
CHAPTER 2 LITERATURE REVIEW	5
Table 1. Vitamin D status based on serum 25D concentrations	15
Table 2. Human interventional studies investigating the effect of resistant starch against glucose metabolism.....	27
 CHAPTER 3 DIETARY RESISTANT STARCH PREVENTS URINARY EXCRETION OF VITAMIN D METABOLITES AND MAINTAINS CIRCULATING 25-HYDROXYCHOLECALCIFEROL CONCENTRATIONS IN ZUCKER DIABETIC FATTY RAT	 61
Table 1. Biochemical measurements of LCs, DCs, and DRSs	75
 CHAPTER 4 CONSUMPTION OF DIETARY RESISTANT FOLLOWING THE ONSET OF DIABETES DOES NOT PREVENT HYPERGLYCEMIA AND COMPROMISED KIDNEY FUNCTION	 80
Table 1. Glucose homeostasis and tissue weight in NDC, CS, LRS, MRS, and HRS following 4 wks of treatment	94
 CHAPTER 5 DIFFERENTIAL EFFECTS OF HIGH- AND LOW-DOSE DIETARY RESISTANT STARCH ON VITAMIN D HOMEOSTASIS AND RENAL FUNCTION IN ZUCKER DIABETIC FATTY RATS	 99
Table 1. Biochemical measurements of LC, DC, MRS, and HRS	114
Table 2. Assessment of renal function in LC, DC, MRS, and HRS following 6 wks of treatment.....	115
 APPENDIX B CHAPTER 3 SUPPLEMENTAL DATA	 128
Supplemental Table 1. Composition of the control (C) and resistant starch (RS) diet.....	128
 APPENDIX C CHAPTER 5 SUPPLEMENTAL DATA	 133
Supplemental Table 1. Primer sequences used in real time-PCR assays.....	133

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ABSTRACT

Diabetic nephropathy has a significant impact on vitamin D status partially due to the role of kidney in maintaining renal uptake of 25-hydroxycholecalciferol (25D) and its subsequent activation to 1,25-dihydroxycholecalciferol (1,25D). We previously reported that feeding high-amylose maize (HAM), a rich source of resistant starch (RS), prevented excretion of 25D-vitamin D-binding protein (DBP) and albumin in Streptozotocin (STZ)-induced type 1 diabetic (T1D) rats. The objectives of the studies described in this dissertation were to: 1) determine if dietary RS could prevent excessive excretion of vitamin D metabolites and maintain serum 25D levels in Zucker diabetic fatty (ZDF) rats, a well characterized animal model of type 2 diabetes (T2D); 2) to conduct dose-response studies to evaluate the renoprotective effect of RS following the induction of T1D; and 3) to determine the impact of RS on cytokines and hormones involved in the inflammatory process and whether the renoprotective actions of RS can be achieved at a lower dose in ZDF rats.

In the first study described in this dissertation, Lean Zucker ($n = 8$) rats were fed a control diet (LC; AIN-93G diet with 550 g/kg of corn starch) and ZDF rats ($n = 8$ /group) were fed either the control diet (DC) or RS diet (DRS; AIN-93G diet with 550 g/kg of HAM) for 6 weeks. RS attenuated hyperglycemia by 41% and prevented albuminuria. Additionally, urinary 25D and 1,25D in DRS were 90% and 97% lower, respectively, resulting in 41% greater serum 25D concentrations ($P < 0.001$) compared to DC rats. Along with the improved renal histopathologic scoring, our data suggest that dietary RS maintained vitamin D balance through its protection against diabetes-induced kidney damage.

In the second study described in this dissertation, we explored the possibility that RS would have a nephroprotective effect following T1D onset. Sprague Dawley (SD) rats ($n = 8$ /group), after induction of T1D with STZ, were fed with AIN-93G diet containing 550g/kg of corn starch (CS), 550 g/kg of high-amylose maize (HRS), 275 g/kg of high-amylose maize + 275 g/kg of corn starch (MRS), or 138 g/kg of high-amylose maize + 412 g/kg of corn starch (LRS) for 4 weeks. Vehicle-treated SD rats ($n = 5$) fed AIN-93G diet containing 550 g/kg of corn starch were served as non-diabetic control (NDC). Our results showed that RS, regardless of dose, did not improve fasting blood glucose levels in T1D rats compared to NDC rats. The overall growth rate in NDC rats was 1.7- to 3.3-fold greater than in T1D rats.

In comparison with CS rats, MRS- and HRS-rats gained 52% and 72%, respectively, but body weight did not differ between LRS and CS rats. Though RS normalized growth pattern in T1D rats, no differences were observed in urinary albumin or 25D concentrations in these rats regardless of treatments. Despite the improvement of vitamin D status (~15 – 20% greater compared to NDC rats) in T1D rats, interleukin-6 (IL-6) was 9 – 31% greater compared to CS rats which was strongly correlated to hyperglycemia ($r = 0.472$, $P = 0.02$). Hence, the potential mechanism by which RS promotes kidney health and reduces inflammation could be contingent on glycemic status and thus future work should focus on a preventative approach with RS to maximize its beneficial effects in diabetes.

The objective of the third study included in this dissertation was to examine the mechanism underlying the RS-mediated effects on vitamin D balance of and whether lower intake of dietary RS would promote kidney health and vitamin D balance in ZDF rats. Here, lean Zucker rats ($n=5$) were used as our control group (LC) and fed a control diet (AIN-93G). For comparison to LC rats, ZDF rats ($n = 5/\text{group}$) were fed the control diet (DC), RS diet (HRS), or a diet containing 275 g/kg of high-amylose maize and 275 g/kg of corn starch (MRS) for 6 weeks. Fasting blood glucose concentrations and hemoglobin A1c% were not affected by HRS or MRS diet. Yet, insulin concentrations were 1.5-fold greater and HOMA- $\beta\%$ was 2-fold greater in HRS rats compared to DC rats, whereas these improvements were not observed in MRS-fed ZDF rats. Additionally, HRS rats, when compared to DC rats, exhibited a 20% increase in serum 25D concentrations and excretion of vitamin D metabolites was blunted. No differences were detected between MRS and DC rats with respect to vitamin D balance. Serum triglycerides were 50% lower and liver triglycerides were 2-fold greater in HRS rats when compared to DC rats. Circulating adiponectin concentrations were 77% greater and serum angiotensin II concentrations were 44% lower in HRS rats than in DC rats. No differences in circulating adiponectin and angiotensin II concentrations were observed in MRS compared to DC rats. Moreover, adiponectin concentrations were highly correlated with vitamin D status ($r = 0.815$, $P < 0.001$) and urinary creatinine output ($r = 0.818$, $P < 0.001$) and inversely correlated with urinary protein ($r = -0.583$, $P = 0.02$).

Collectively, though we showed that the effect of 20% RS on promoting kidney function and vitamin D homeostasis in ZDF rats may be influenced by circulating

adiponectin concentrations, our studies indicate that a 50% reduction in dietary RS does not produce the same results. Nevertheless, the studies described in this dissertation indicate that HAM could be a part of dietary intervention strategies aiming to prevent or attenuate symptoms of diabetic nephropathy that are independent of blood glucose management. Because the level of dietary RS used in these studies would be difficult to translate to human feeding studies, future work should consider combination strategies with RS and other dietary compounds or medications for the prevention and/or management of diabetic nephropathy and its associated complications.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

The worldwide prevalence of diabetes is expected to reach 439 million by 2030 [1]. The increasing burden of diabetes highlights the need for the prevention and management of the disease, especially the secondary complications that dictate the morbidity and mortality of diabetic patients. Unfortunately, diabetes is a major risk factor for cardiovascular and kidney diseases. It is also noteworthy that in the United States, approximately 44% of the reported renal failure cases are due to diabetes [2]. Because the kidney plays an essential role in nutrient reabsorption, impaired kidney function could lead to severe nutrient deficiencies, such as vitamin D deficiency [3], which may further accelerate the progression of diabetic complications. In addition to vitamin D reabsorption, the kidney is critical for vitamin D metabolism because it activates 25-hydroxycholecalciferol (25D) to 1,25-dihydroxycholecalciferol (1,25D) for release into circulation [4, 5]. This could partially explain the underlying causes of low vitamin D status observed in diabetic individuals [6, 7]. Indeed, mounting evidence indicates that the beneficial effects of vitamin D extend beyond bone health, and may have a beneficial impact in chronic disease such as cancer, autoimmune diseases, and diabetes [8-12]. Hence, optimizing vitamin D status could be a feasible approach to delay the secondary complications resulting from diabetes.

Previous work in our laboratory demonstrated that dietary resistant starch (RS) attenuated the symptoms of diabetic nephropathy in streptozotocin (STZ)-induced diabetic rats, a widely accepted model of type 1 diabetes (T1D), through the up-regulation of vitamin D endocytic proteins, megalin and disabled-2 (Dab2) [13]. RS used for these studies is a form of fermentable fiber that is partially resistant to digestion in the small intestine. Not only does RS have low glycemic properties, it has been reported that various forms of RS are potential substrates for microbial production of short chain fatty acids (SCFA) in the gut. Studies have also shown that SCFA was able to reduce abdominal fat and improve insulin sensitivity in obese animals [14-16] and that fermentation of RS could lead to improved gut health [14, 17]. However, no prior studies reported a benefit of RS with respect to kidney health and vitamin D metabolism during the progression of diabetes. Our laboratory previously discovered that the loss of vitamin D in the urine is a result of compromised

kidney function in Zucker diabetic fatty (ZDF) rats, a well-characterized type 2 diabetes (T2D) model [3] and that RS was protective against T1D-induced diabetic nephropathy [13]. Hence, the development of my dissertation aimed to investigate the effect of RS on vitamin D homeostasis as well as the underlying mechanism in diabetic animal models. The *objectives* of the present research were to: 1) determine if high-amylose maize (HAM), a source of RS, could maintain vitamin D balance in ZDF rats (T2D model); 2) determine minimal effective dose of RS that could prevent the perturbation of vitamin D metabolism and compromised kidney function in STZ-induced diabetic rats (T1D model); 3) investigate the mechanism by which RS plays a role in the maintenance of kidney health and vitamin D balance in ZDF rats and 4) whether the nephroprotection provided by RS feeding can be achieved at lower dose that we have previously utilized.

Dissertation Organization

This dissertation consists of six chapters with a general introduction, literature review, three manuscripts, and an overall conclusion. The first manuscript entitled “Dietary resistant starch prevents urinary excretion of vitamin D metabolites and maintains circulating 25-hydroxycholecalciferol concentrations in Zucker diabetic fatty rats” has been published in the *Journal of Nutrition*. This manuscript reported the maintenance of vitamin D homeostasis and kidney health by RS in T2D animal model. The work presented in the second manuscript evaluated the renoprotective effect of RS at a dose-dependent manner utilizing STZ-induced diabetic rats and has been prepared for submission to the *Journal of Nutrition*. The work presented in the third manuscript further investigated the mechanisms underlying the nephroprotection of RS in ZDF rats, which will be submitted to the *Journal of Nutrition*. All literature cited is based on the format of *Journal of Nutrition* and listed at the end of each chapter. This dissertation will end with a general conclusion that includes a discussion of overall results and future directions for my research theme.

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CHAPTER 2: LITERATURE REVIEW

Diabetes Mellitus

Diabetes mellitus currently affects ~300 million people in the world [1]. In the United States, the prevalence of diabetes in children and adults has reached ~29 million or 9.3% of the total population [2]. The diagnostic criteria for diabetes includes: fasting blood glucose concentration >126 mg/dL, hemoglobin A1c (HbA1c) $> 6.5\%$, 2-hour plasma glucose > 200 mg/dL following an oral glucose tolerance test, and random plasma glucose > 200 mg/dL on at least two occasions along with classic symptoms of hyperglycemia [3]. Satisfaction of any of the above criteria classifies an individual as a diabetic.

Type 1 diabetes (T1D)

T1D is an autoimmune disorder that is associated with pancreatic beta cell destruction, where the most common age at diagnosis falls between 10 – 14 years old [4]. Some of the common symptoms observed in T1D are hyperglycemia, polydipsia, polyuria, weight loss, and ketonemia [4]. The etiology of T1D is still largely unknown; however, the initiation of immune response against beta cells likely begins with the production of interferons [5]. The up-regulation of interferons and recruitment of other pro-inflammatory cytokines stimulate autoreactive CD8 T-cells to attack beta cells in the pancreas, which will eventually lead to beta cell apoptosis. As the autoreactive CD8 T-cells continue to proliferate and migrate into the pancreas along with CD4 T-cells and B-cells, the progression of beta cell destruction is exacerbated and defective insulin secretion (i.e. T1D onset) occurs [5]. One of the candidates reported to be most associated with T1D is the human leukocyte antigen (HLA) that is known for its role in immune regulation [6, 7]. Compared to general population, children with T1D susceptible HLA alleles have ~20-fold greater risk for developing T1D [8]. The strongest association with T1D has been related to the HLA class II genotype, which may be inherited [9]. It is reported that individuals who have an HLA genotype identical with their diabetic proband sibling have 55% greater chance for developing diabetes by the age of 12 compared to siblings who share one or less HLA haplotype [9]. Unlike the class II HLA genes, evidence of T1D and polymorphisms of class I HLA genes is less prominent. Some of the class I HLA genotypes, such as HLA-B39 [10]

and HLA-A24 [11], were associated with islet destruction and progression of T1D. However, the interactions between class I and class II HLA alleles in T1D are still not well defined and how environmental risk factors may contribute to the risk of T1D remain to be determined.

Immune cell regulation by the gut microbiota has recently raised a significant amount of interest regarding the risk of developing T1D. Evidence from T1D animal models, mostly with the utilization of BioBreeding diabetes-prone (BB-DP) and non-obese diabetic (NOD) animal models, have suggested that even before the onset of diabetes, the composition of the gut microflora in rodents that eventually developed diabetes differed from those that remained healthy [12, 13]. Compared to BioBreeding diabetes-resistant (BB-DR) rats, the BB-DP rats exhibited lower abundance of *Bifidobacterium*, *Lactobacillus*, and *Pseudobutyrvibrio*, which are all known to promote gut health [13]. Wen et al [14] has reported that systemic immune responses in the NOD mice were mediated by gut microbiota through the MyD88-dependent pathway. MyD88 is an adaptor protein that is capable of initiating an innate immune response when coupled with multiple toll-like receptors (TLR). Compared to wild-type NOD mice, MyD88-knockout (KO) NOD mice housed in a conventional specific-pathogen-free environment were protected from T1D and this was correlated with decreased T-cell proliferation in pancreatic lymph nodes. Interestingly, Wen et al [14] further demonstrated that MyD88-KO mice developed diabetes when housed under germ-free conditions; whereas diabetic incidence and pancreatic islet infiltration in these animals was markedly reduced when colonized with commensal bacteria [14]. This suggests that gut bacteria could have promoted pancreatic health in NOD mice via a mechanism that is independent of MyD88. Although further studies are needed to elucidate and identify the regulatory role of gut microflora in the innate immune system and T-cell mediated beta cell destruction, these new perspectives could provide alternative options for those who are genetically susceptible to T1D.

Type 2 diabetes (T2D)

T2D constitutes 95% of the total documented cases of diabetes worldwide. It is estimated that almost 387 million people are at risk for the development of T2D due to adult onset weight gain and a sedentary lifestyle [1]. The risk factors for T2D may include physical inactivity, family history of T2D, aging, obesity, and ethnicity. For example, African

Americans, Hispanics, and Asians, are at higher risk for T2D development [2]. T2D is generally characterized by hyperglycemia and hyperinsulinemia due to insulin resistance, in contrast to T1D where insulin production is diminished. The common symptoms of T2D are mostly attributed to insulin resistance. Under normal circumstances, insulin secretion is initiated in the postprandial state when glucose levels are elevated. An increase in blood glucose stimulates the entry of glucose into the beta cells of the pancreas via glucose transporter-2 (GLUT2) where the glucose is oxidized for ATP production. Intermediates of the TCA cycle, such as NADPH, malonyl-CoA, and glutamate can then serve as signaling molecules for insulin secretion. The insulin-signaling cascade begins with the binding of insulin to the insulin receptor, which activates the tyrosine kinase domain of the insulin receptor beta subunit [15]. This action subsequently promotes tyrosine phosphorylation of several substrates, including the insulin receptor substrate (IRS) and phosphatidylinositol 3-kinase (PI3). Upon the stimulation of PI3, glucose transporter-4 (GLUT4) in the peripheral tissues translocates from the cytosol to the plasma membrane to facilitate glucose uptake [15]. Although the exact mechanism is not well established, studies have suggested that serine phosphorylation of IRS-1, and thus disruption of downstream insulin signaling and GLUT4 translocation could be a potential cause of insulin resistance [15]. As insulin resistance develops, beta cells continue to proliferate in order to compensate for the demand of insulin as a result of hyperglycemia. Due to excessive production of insulin, the ability of endoplasmic reticulum (ER) to process pro-insulin into insulin is compromised, which leads to the generation of excess misfolded or unfolded proteins that cause ER stress in beta cells [16]. It is believed that ER stress response due to prolong exposure to glucose is a critical factor that leads to beta cell death in T2D and thus insulin-dependent diabetes [16].

Insulin resistance and adipose tissue inflammation

The underlying mechanism responsible for insulin resistance and T2D remains unclear. It is estimated that 36% of the population in the United States is obese, a condition that has been postulated to increase the risk of being diagnosed with T2D by seven times [17, 18]. Because low grade inflammation is closely associated with the development of insulin resistance in obese individuals [19], it is reasonable to suspect that insulin resistance could be a consequence of adipose tissue inflammation as the presence of pro-inflammatory cytokines,

such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6) may disrupt insulin receptor signaling in peripheral tissues as described above [17, 18]. In support of this concept, it was found that manifestation of hypertrophic adipocytes in obese adults elevated the expression of pro-inflammatory factors and enhanced immune cells infiltration into the adipocytes [20, 21]. The subsequent recruitment of pro-inflammatory cytokines following macrophage infiltration is thought to be a critical action that amplifies the inflammatory signal in adipocytes that later contribute to systemic low-grade inflammation [17, 18, 20]. It was also reported that expansion of adipocytes was limited by pro-inflammatory signals via the suppression of peroxisome proliferator-activated receptor- γ (PPAR- γ) and CCAAT/enhancer-binding protein- α (C/EBP- α) activity [22, 23], critical proteins involved in adipogenesis and body weight homeostasis. As storage of lipid is limited in the adipocytes, ectopic lipid storage and hypertriglyceridemia may occur to augment systemic inflammation and insulin resistance if calorie intake becomes excessive [22]. Adiponectin, a predominant adipokine primarily secreted by white adipocytes, has been suggested to serve as a starvation signal that favorably promotes storage of triglycerides in the adipose tissue. In fact, it was observed in transgenic *ob/ob* mice overexpressing adiponectin that despite an increase in total fat mass, these mice exhibited lower liver and serum triglycerides, as well as enhanced insulin sensitivity compared to the wild-type *ob/ob* mice [22]. Because transgenic *ob/ob* mice with overexpressed adiponectin also demonstrated increased expression of PPAR- γ in adipose tissue, it was suggested that elevated adiponectin levels enhanced the local activity of PPAR- γ , which lead to lipid redistribution to subcutaneous adipose tissue and not ectopic lipid storage [22]. Thus, as adiponectin levels are inversely related to adiposity [24], the associated insulin resistance commonly observed in obesity-related diabetes may be related to ectopic lipid-induced local inflammation resulting from adiponectin deficiency.

Insulin resistance and metabolic endotoxemia

Another possible mechanism that leads to insulin resistance could be related to the presence of lipopolysaccharide (LPS), an endotoxin found on the outer membrane of Gram-negative bacteria. This condition is commonly referred to as metabolic endotoxemia, which is characterized by increased concentrations of plasma LPS [25, 26]. Metabolic endotoxemia

is highly dependent on feeding status, where LPS levels usually spike during the feeding period and remain low during the fasting state [25]. However, this cycle was disrupted in mice fed a high-fat diet (HFD) as plasma LPS remained elevated throughout the day. Moreover, LPS-infused mice established hyperglycemia, insulin resistance, low-grade inflammation, and a marked increase in hepatic triglycerides that mimicked the effect of a HFD referred to above [25]. The mechanism underlying metabolic endotoxemia is not entirely clear. Because LPS concentrations were inversely correlated with *Bifidobacterium* spp., a known gut barrier protection factor, and the presence of *Bifidobacterium* in the cecal content was strongly associated with improved glucose tolerance and insulin secretion in HFD-fed mice, it is reasonable to postulate that excessive caloric intake may have disrupted the gut barrier integrity by altering gut microbiota composition. This, in turn, could have enhanced LPS absorption in the gut and triggered systemic inflammation in these HFD-fed mice [26].

Macrovascular and Microvascular Diabetic Complications

Uncontrolled diabetes can lead to severe chronic microvascular and macrovascular complications such as retinopathy, neuropathy, nephropathy, and atherosclerosis [19, 27, 28]. It has been shown that increased oxidative stress, due in part to hyperglycemia, can contribute to the pathogenesis of diabetic complications, such as those resulting from the formation of free radicals, production of advanced glycation end products (AGE), and activation of the polyol pathway [29-31]. Mitochondria are one of the primary sources of reactive oxygen species (ROS) during an incomplete electron transfer [32, 33]. Overproduction or an inability to remove these free radicals produces oxidative stress, a physiological state that can lead to cellular dysfunction, cell death, and chronic diseases such as diabetes, aging, and cancer [34-36]. Indeed, production of AGEs due to prolonged exposure to high glucose has been linked to enhanced inflammation and oxidative stress in diabetes [31, 37-39]. It is believed that the vascular damage by AGEs is partially mediated by the structure and functional modification of intracellular proteins and extracellular matrix molecules [37, 38, 40]. Binding of AGEs to the AGE receptor (RAGE) has also been shown to promote an inflammatory response via the activation of nuclear receptor NF- κ B [37, 39].

Another pathway that could be centrally involved in the development of diabetic complications is the polyol pathway. The polyol pathway can be activated in response to hyperglycemia, which in turn can induce local osmotic stress in locations including the kidney and retina, as glucose can freely enter insulin-independent organs [41-44]. Under a normal glycemic state, glucose has low affinity for aldose reductase, an enzyme responsible for catalyzing glucose to sorbitol. In contrast, glucose's affinity for aldose reductase increased during hyperglycemia and glucose was further catalyzed into sorbitol, a reaction that oxidizes NADPH to NADP⁺ [41-43]. Because NADPH is a precursor for glutathione production, the formation of sorbitol by aldose reductase could thus reduce the availability of glutathione for antioxidant activity indirectly. Simultaneously, sorbitol is able to bind to other functional proteins for the production of AGEs, which exacerbates the progression of oxidative stress in local tissues such as the kidney and retina.

Atherosclerosis

Cardiovascular disease is the leading cause of death in diabetic patients [1]. The underlying mechanism in atherosclerosis caused by a diabetic condition may be partially attributed by the accumulation of AGEs [45, 46]. It has been reported that glycated low-density lipoprotein (LDL) may accelerate atherogenesis in diabetes through the promotion of a chemotactic response in macrophages [45, 47], possibly via the TLR4 signaling pathway [45]. Monocytes that migrate into the artery wall can be activated by glycated LDL and differentiate into macrophages [45-49]. This is a critical aspect of atherogenesis because internalization of modified LDL by macrophages catalyzes the formation of foam cells and "fatty streaks" within the artery wall. This process is followed by collagen synthesis, attraction and accumulation of platelets, and the eventual formation of fibrous plaques, which impede blood flow due to narrowing of the arterial lumen. Fibrous plaques can then become complicated, a condition generally characterized by plaque calcification, hemorrhaging into the plaque, and thrombosis. Due to these factors, fibrous plaques often rupture to cause acute coronary events such as myocardial infarction and ischemia [50].

Diabetic retinopathy

The polyol pathway is one of the major contributors to diabetic retinopathy because aldose reductase, the rate-limiting enzyme for the polyol pathway, is abundant in retinal ganglion cells, Muller glia, pericytes, and the retina pigment epithelial cells. Hence, these cells are more susceptible to damage during the progression of diabetes, when the polyol pathway tends to be highly active [44]. This is further supported by the use of aldose reductase inhibitors to prevent accumulation of sorbitol and fructose in the retina of diabetic rats [51-53], suggesting that the polyol pathway is central to the development of diabetic retinopathy. Other key factors involved in the progression of retinopathy are vascular endothelial growth factors (VEGF), which have been linked to activation of the hypoxia-inducible factor, [54, 55], the key regulator of angiogenesis. Studies have reported that increased VEGF expression was present during hyperglycemia and enhanced neovascularization, vascular permeability, and activation of pro-inflammatory proteins in retinal vessels [55-57]. In fact, VEGF production was markedly higher in patients with diabetic retinopathy [58, 59], while attenuation of VEGF delayed the progression of retinopathy in diabetes [60-62], which indicated that VEGF is highly involved in the formation of retinopathy during chronic hyperglycemia.

Diabetic neuropathy

It is estimated that 60-70% of diabetic patients are affected by neuropathy [63]. Peripheral neuropathy is the most common form of neuropathy in diabetic patients. Symptoms of peripheral neuropathy include pain and tingling in the arms, hands, feet, or toes [63]. Autonomic neuropathy, another form of neuropathy found in many diabetics, manifests in various organ systems and can lead to constipation, diarrhea, and cardiovascular dysfunction [63-65]. While the etiology underlying diabetic neuropathy is not well-known, it is believed that the cause of neural damage is related to polyol pathway activation and production of AGEs [66-68]. Juranek et al. [69] demonstrated that RAGE was expressed in 30% of healthy peripheral nerves, whereas it was observed in more than 40% of diabetic peripheral nerves. Furthermore, RAGE deletion in T1D mice improved axon regeneration and endoneurial vessels after a sciatic nerve crush, a common procedure to induce peripheral nerve injury via mechanical compression of the nerve [70]. Notably, it appeared that the

repair of nerve fiber regeneration was highly dependent on glycemic stage as no difference was detected in non-diabetic RAGE-null mice compared to diabetic RAGE-null mice [70]. Though detailed mechanisms by which AGEs and RAGE signaling may contribute to diabetic neuropathy requires further investigation, evidence suggests that RAGE plays a critical role in the process of neurodegeneration. Thus, strategies that target RAGEs are promising in the prevention of diabetic neuropathy.

Diabetic nephropathy

T2D is the most common cause of kidney disease, constituting 44% of the documented renal failure cases in the U.S. [71]. In diabetic kidney disease, the process of kidney damage is believed to begin in the glomerulus, where the integrity of the basement membrane is compromised, which leads to glomerular leakage of albumin [72-74]. In cultured human proximal tubular cells, glucose has been shown to increase the synthesis of collagen and fibronectin. Furthermore, thickening of the tubular basement membrane was closely correlated with HbA1c levels and AGEs [37, 75]. Accumulation of such extracellular matrices in the kidney has been associated with increased fibroblast proliferation and renal inflammation [76-78], which may partly explain the common symptoms of diabetic nephropathy, such as elevated glomerular filtration rate, proteinuria, and glomerulosclerosis. Zucker diabetic fatty rat (ZDF), a well-established T2D animal model, exhibits glomerular hypertrophy and hyperfiltration as early as 5 months of age due to the damage of podocytes, which has been linked to the formation of an abnormal glomerular basement membrane structure [74]. In *in vitro* studies, glucose-treated kidney cells were shown to be hypertrophic and under high levels of oxidative stress due to increased activity of p27, a cell cycle inhibitor protein [74]. Upregulation of p27 activity has been associated with glomerulonephritis and renal damage, which was characterized by proteinuria, hyperfiltration, and renal hypertrophy. Hence, it is believed that the increased expression of p27 was a compensatory mechanism to stabilize the podocyte cell [74].

Impacts of nephropathy on vitamin D homeostasis

One of the more common nutritional deficiencies often seen in T2D patients is vitamin D deficiency. As the kidney plays a major role in maintaining vitamin D status and

activation of 25-hydroxycholecalciferol (25D) to 1,25-dihydroxycholecalciferol (1,25D), it is not surprising that damage of podocytes has been shown to accelerate the excretion of vitamin D metabolites in ZDF [79] and streptozotocin (STZ)-induced T1D rats [80]. These observations can be explained, at least in part, by increased filtration of vitamin D binding protein complex and compromised reabsorption by the proximal tubule [79, 80].

Furthermore, in vitamin D receptor (VDR)-null mice, accumulation of extracellular matrix proteins and pro-inflammatory as well as fibrogenic factors, was observed in the kidney [81, 82], indicating that VDR signaling could be protective against renal fibrogenesis that is mediated by pro-inflammatory cytokines [82]. The evidence of VDR as a renoprotective factor was strengthened by additional studies in which VDR agonists, such as paricalcitol and calcitriol, reduced proteinuria in chronic kidney disease patients [83-85]. Moreover, in cultured podocytes, paricalcitol reduced renal inflammation by decreasing the expression of pro-inflammatory cytokines through the inhibition of NF- κ B activity [83, 85]. Though the relationship between vitamin D and nephropathy has not been fully elucidated, links between the pathophysiology of diabetic nephropathy and vitamin D imbalance in T2D are becoming more numerous. Hence, this research area may prove to be critical in the development of dietary strategies that can prevent complications associated with diabetes.

Impacts of nephropathy on the renin-angiotensin system

The renin-angiotensin system (RAS) is a hormonal system that regulates arterial pressure, fluid balance, and electrolyte homeostasis of the cardiovascular, renal, and endocrine systems. Evidence suggests that the renoprotective effect of VDR could be mediated by RAS suppression, which seems plausible since the promoter region of renin is target of VDR [83, 86, 87]. When treated with an angiotensin II antagonist, interstitial fibrosis was prevented in VDR-null mice while paricalcitol, a VDR agonist, halted intrarenal RAS activation in an animal model of chronic kidney disease [88]. Collectively, these studies suggest that RAS plays a critical role in the prevention of nephropathy through VDR signaling, where low levels of circulating vitamin D may enhance RAS activity, which in turn could augment the progression of renal injury. Chronic hyperglycemia has been shown to activate the intrarenal RAS, independent of systemic RAS activity, by stimulating the synthesis of intrarenal renin and angiotensin II, which are the pro-inflammatory factors that

promote vascular dysfunction, insulin resistance, and oxidative stress in experimental models of kidney disease [86, 89-92]. In support of these observations, Sharma et al [93] showed that chronic elevation of intrarenal angiotensin II caused proteinuria and glomerulosclerosis whereas treatment with Captopril, an angiotensin-converting enzyme inhibitor (ACEi), reversed the symptoms of diabetic nephropathy [93]. The mechanism responsible for these findings could involve the up-regulation of nephrin activity following the reduction of angiotensin II in diabetic nephropathy [94]. Nephrin is a slit diaphragm-associated protein that is necessary for maintenance of the renal filtration barrier. A reduction in nephrin was associated with proteinuria in patients with nephrotic syndrome [95-97] and treatment with angiotensin II antagonists preserved glomerular nephrin expression and prevented renal injury [94, 96, 98]. Therefore, changes in nephrin expression during the progression of diabetes suggest that podocyte structure alteration could be an early event in the development of diabetic nephropathy. Yet, not only was it observed that a blockade of the RAS restored nephrin expression, but down-regulation of the RAS was also associated with an increase in plasma adiponectin in patients with metabolic syndrome and type 2 diabetes [99-101]. Adiponectin is an adipokine primarily secreted by the adipocytes. In addition to being an insulin sensitizer, adiponectin is also known to have anti-inflammatory properties [102, 103]. Adiponectin has been shown to inhibit NF- κ B activity and suppress production of both TNF- α and IL-6, which are pro-inflammatory cytokines in adipocytes that tend to be elevated in obesity and insulin resistance [22]. Because the use of ACEi has been shown to improve insulin sensitivity and attenuate the development diabetic nephropathy [104-107], this suggests that adiponectin can play a major part in the regulation of RAS activity. In fact, adiponectin-knockout mice exhibited albuminuria and reduced podocyte permeability [108]. When adiponectin was administered exogenously, it delayed the progression of nephropathy in diabetic mice and attenuated renal oxidative stress via an angiotensin II-mediated pathway [109-111], suggesting that suppression of angiotensin II and key components of RAS may be signaled by adiponectin.

Vitamin D Status

The biosynthesis and bioavailability of vitamin D can be affected by several factors. These factors include exposure to UVB radiation, obesity, gastrointestinal disease, and kidney disease [112]. Vitamin D status, as classified by the Institute of Medicine (IOM), is based on circulating 25D concentrations. Individuals can fall into four categories with respect to vitamin D status: deficiency, insufficiency, adequacy, and toxicity (Table 1) [113]. The dietary reference intake (DRI) requirement for vitamin D, which was set at 600 IU/d, was established for healthy populations based on bone health outcome because poor vitamin D status can lead to rickets, osteomalacia, calcium malabsorption, reduced bone mineral density, impaired muscle function, and elevated parathyroid hormone (PTH) levels [114-

Table 1. Vitamin D status based on serum 25D concentrations*

Serum 25D concentrations		Vitamin D Status
nmol/L	ng/mL	
< 30	< 12	Deficiency
30 – 50	12 – 20	Insufficiency or inadequate for bone and overall health
>50	>20	Adequate for bone and overall health
>125	>50	Toxicity

*Adapted from Institute of Medicine (IOM) [113]

116]. Thus the goal of the DRI for vitamin D was that this intake would help ensure that serum 25D levels would be maintained at 40 nmol/L, which in theory would prevent such complications. Yet, the recommended intake may vary based on the individual. Factors such as age, pregnancy, and lactation also need to be considered [113]. According to the Center for Disease Control, at least 32% of the US population aged one year of age and older are either vitamin D insufficient or deficient (serum 25D between 25 – 49.9 nmol/L and less than 25 nmol/L, respectively) [117]. Compromised vitamin D status is not limited to the United States, as similar observations have been found across numerous populations [115, 116, 118-120]. There is emerging evidence suggesting that suboptimal serum levels of 25D increases the risk of chronic diseases unrelated to bone health, such as diabetes [119, 121], cancer [122-124], and autoimmune diseases [125, 126]. In order to reach the optimal level of 25D (50 nmol/L or above), an average adult needs to consume between 2000-5000 IU

vitamin D/day [127, 128], which is 5-fold higher than the recommended daily allowance (RDA). In addition, the RDA for vitamin D does not account for vitamin D requirements in specific groups that exhibit a higher prevalence of vitamin D deficiency and/or insufficiency such as those with chronic kidney disease or other forms of nephropathy. Thus, re-evaluation of the RDA for vitamin D may be needed to ensure that requirements meet the needs for all individuals, due to the many factors that can influence vitamin D status mentioned above. The issue of vitamin D toxicity is currently a topic of debate. Studies [129, 130] have reported that 10,000 IU/d of vitamin D for 4 months did not produce any toxicity. Others have also indicated that consumption of 10,000 IU of vitamin D per week was associated with a reduction in falls and fractures in the elderly [131], and monthly dosing of 50,000 IU of vitamin D corrected vitamin D deficiency in elderly patients [132]. While many have suggested that there is a need to increase the RDA of vitamin D for the prevention of chronic disease, research on vitamin D supplementation and its impact on health is controversial. Even at 4800 IU/day of vitamin D₃ supplementation, calcium absorption as well as serum 25D and 1, 25D concentrations in post-menopausal women were not significantly improved compared to the placebo, unless they were severely vitamin D deficient [133]. The contradictory results yielded from the aforementioned vitamin D studies may be due to lack of consistency in their experimental design because long-term effects and repeated dosing regimens were not accounted for. Considering the potential purported benefits of vitamin D, optimization of vitamin D status for the prevention of chronic diseases is an area that is under intense investigation.

Vitamin D Biosynthesis and Metabolism

Vitamin D is unique among nutrients because it is readily synthesized by the skin when 7-dehydrocholesterol is exposed to UVB radiation (280 – 315 nm). This followed by the generation of pre-vitamin D₃, which is further isomerized to vitamin D₃. Once vitamin D₃ is formed in the epidermis, it is quickly absorbed in the dermal capillary bed and released into circulation, which then is converted into active metabolites or stored in the adipose tissue. [134]. The serum half-life of vitamin D₃ is approximately 15 days, but storage in the adipocytes can prolong the total body half-life to about two months [135, 136]. In addition to sunlight exposure, vitamin D₂ and vitamin D₃ can be acquired through the diet. Examples of

vitamin D₃-rich foods are cod liver oil, salmon, and egg yolks. Vitamin D₂ is found in plant sources such as mushrooms. Hereafter, both vitamin D₃ and D₂ will be referred to collectively as vitamin D, unless otherwise specified. Upon consumption, dietary vitamin D is handled along with dietary lipids and incorporated into micelles prior to absorption by the intestinal mucosa via passive diffusion. Once absorbed by mucosal cells, vitamin D is packaged into chylomicrons along with other lipids and lipid-soluble vitamins, and released into the lymphatic system and then the blood. Circulating vitamin D, acquired either through endogenous synthesis or from the diet, is then transported to the liver via DBP and

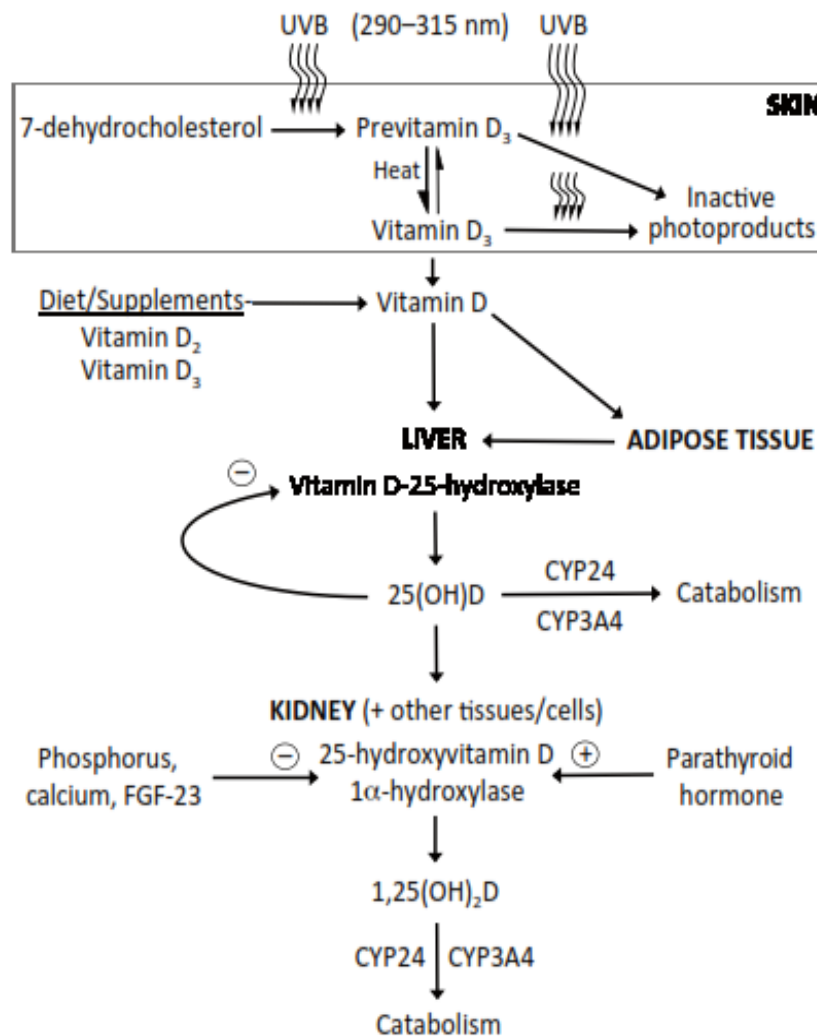


Fig. 1. Vitamin D Metabolism. Diagram adapted from Tsiaras et al [106].

hydroxylated at the C-25 side chain by the vitamin D-25-hydroxylase (CYP27A1) for production of 25D, which again binds to DBP and circulates to the kidney [112, 137] (Fig. 1). Kidney is the major synthesis site of the active vitamin D hormone, 1,25D. Before 25D can be activated in the kidney, it must enter renal proximal tubules by receptor-mediated endocytosis, which requires the endocytic partners, megalin and cubilin (Fig. 2). Megalin and cubilin bind to 25D-DBP complex at high affinity with the help of intracellular adaptor proteins, such as disabled-2 (Dab2), and internalize it into the proximal tubule to form endosomal vesicles [138, 139]. Here, 25D can then either be converted to its active form 1,25D by the CYP27B1 enzyme (25-hydroxycholecalciferol-1 α -hydroxylase) or reabsorbed into circulation. The role of megalin, DBP, and CYP27B1 in vitamin D activation has been defined in studies involving megalin- and CYP27B1-deficient mice [138, 140-142], which developed severe vitamin D deficiency and bone malformations. Moreover, DBP-null mice exhibit decreased serum 1,25D levels [143]. With respect to the metabolism of vitamin D in the kidney, not only is the kidney crucial for the production of 1,25D, it is also responsible for vitamin D catabolism, which is mediated by another mitochondrial P450 enzyme, CYP24A1 (25-hydroxycholecalciferol-24-hydroxylase). CYP24A1, as opposed to CYP27B1, facilitates the catabolism of 25D or 1,25D to calcitronic acid via series of hydroxylation reactions at C-24 [144] for vitamin D inactivation and excretion. The process also decreases the availability of 25D to CYP27B1, and hence suppresses its activation [145]. Due to its extremely high potency, production of 1,25D promotes its own catabolism by transcriptionally regulating CYP24A1. 1,25D concentrations are also influenced by several additional factors, such as PTH, serum calcium and phosphorus concentrations, and fibroblast growth factor 23 (FGF-23), a phosphaturic factor synthesized in the osteoblastic cells that is known to regulate phosphate homeostasis in response to 1,25D [146, 147]. When 1,25D levels are elevated, transcription of FGF-23 in the kidney is enhanced to stimulate the expression of CYP24A1 and simultaneously inhibit the activity of CYP27B1. On the other hand, low levels of 1,25D repress CYP24A1 and activate CYP27B1 expression to enhance the production of 1,25D, which subsequently provides a feedback inhibition of FGF-23 and PTH production [147].

Molecular Action of Vitamin D

The actions of 1,25D are mediated by the membrane-bound vitamin D receptor (VDR), a nuclear receptor that belongs to a subclass of nuclear transcription factors. Upon the binding of 1,25D, VDR translocates from the cytosol to the nucleus where it is phosphorylated and forms a heterodimer with the retinoid X receptor (RXR). The VDR-RXR heterodimer complex can then binds to vitamin D response elements (VDRE) and induce transcription of genes [146, 148]. There are two major functional units of the human VDR, the N-terminal zinc finger DNA-binding domain and the C-terminal ligand-binding domain. Gene expression mediated by 1,25D and VDR is initiated by the RXR heterodimerization. The presence of liganded VDR changes the position of H12 at the C-terminus of VDR for coactivator binding, which further promotes histone acetylation and chromatin remodeling [146]. Transcriptional regulation by 1,25D-bound VDR involves a number of steps at the level of the gene. Many factors are recruited to the transcription site upon the formation of the VDR-RXR heterodimer such as TATA binding protein associated factors, D-receptor interacting proteins (DRIP), and when coupled with RNA polymerase II as well as other nuclear receptor coactivators, this complex will facilitate RNA processing during target gene transcription [137, 146]. VDR can also down-regulate the transcription of various genes. Repression of gene transcription by VDR-RXR is introduced by the docking of the VDR-

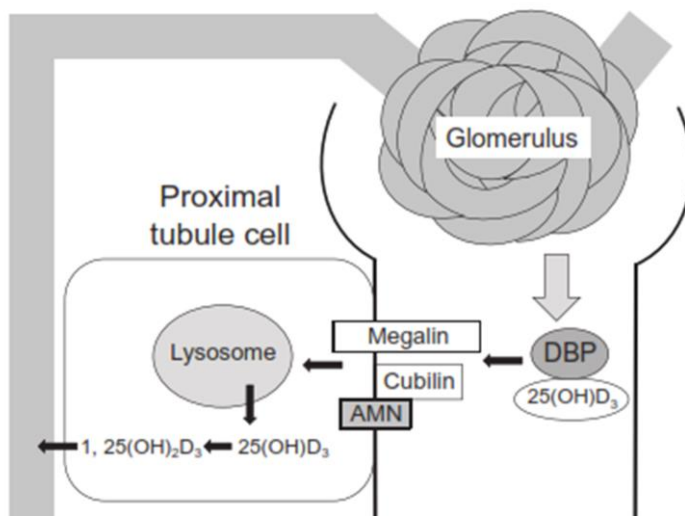


Fig. 2 Endocytic uptake of vitamin D-DBP complex in the proximal tubule cells. Diagram adapted from Kaseda et al [133].

RXR complex on a negative VDRE that binds to a co-repressor and alters the chromatin structure via histone deacetylation and demethylation of histones [137, 146]. In addition, non-genomic actions of VDR have been identified and they are stimulated by the activation of plasma membrane receptors and second messengers such as MAPK, cAMP, and phospholipase C [137]. The discovery of non-genomic

activity of 1,25D has also led to the extension of research on vitamin D and its metabolites beyond bone health, especially after CYP27B1 was discovered in extra-renal sites such as skin, macrophages, colon, breast, prostate, pancreas, and vascular epithelial cells [149-151]. On that note, low circulating 25D concentrations have been associated with diseases such as cancer, diabetes, and autoimmune diseases, in sites that express CYP27B1.

Vitamin D and calcium homeostasis

The role of vitamin D in calcium and phosphorus homeostasis is well established and thus vitamin D status must be maintained for optimal bone mineralization. Studies have shown that VDR null mice developed hypocalcemia, secondary hyperparathyroidism, hyperphosphatemia, all which can contribute to the development of osteopenia [152, 153]. However, these phenotypic characteristics can be prevented in these mice by feeding a high calcium diet in combination with lactose, which enhances the solubility of calcium [137, 144]. The efficiency of calcium absorption is dependent on the serum calcium concentrations. In times of high calcium intake, the majority of calcium absorption involves the transport of calcium ions between intestinal tight junctions in the duodenum and jejunum, a process called paracellular diffusion [144, 154]. In times of low calcium intake, the majority of calcium ions are transported transcellularly through mucosal cells via a vitamin D- and energy-dependent pathway. The transcellular transport of calcium across the epithelial cell is mediated by several vitamin D-dependent transporters including the apical calcium channel, TRPV6, which binds luminal calcium ions and transfers them to calbindin for translocation to the basolateral membrane for release into circulation. Calcium homeostasis is tightly coordinated by the actions of several hormones, which include PTH, 1,25D, and calcitonin [144]. Under hypocalcemic condition, the parathyroid gland is stimulated to secrete PTH. PTH in turn travels to multiple sites simultaneously to increase calcium concentrations by stimulating renal calcium reabsorption, intestinal absorption of calcium phosphate, as well as calcium and phosphate release via bone resorption [144]. During hypercalcemia, 1,25D signals its own catabolism via CYP24A1 and suppresses the renal hydroxylation of 25D [144, 155].

Vitamin D and diabetes mellitus

Current literature has provided rather convincing evidence that vitamin D plays a pivotal role in the prevention of pancreatic beta cell destruction and maintenance of glucose metabolism. Based on cross-sectional studies, low vitamin D status has been closely associated with impaired insulin sensitivity, which characterizes T2D [118, 120, 156-158]. However, more work is needed to further elucidate mechanisms by which vitamin D exerts its actions with respect to glucose metabolism. Compared to populations with lower BMI, those who are overweight and obese are more prone to vitamin D deficiency or insufficiency [119, 120, 159], which may place them at higher risk for T2D due to the potential role of vitamin D in regulating glucose homeostasis. Our laboratory is among those that have demonstrated that hyperglycemia was associated with excessive excretion of vitamin D metabolites [79, 80] and shedding of megalin into the urine [160]. Not surprisingly, a disruption of vitamin D homeostasis during the progression of diabetes was reported in these studies. This is further supported by a clinical study that showed that urinary loss of DBP contributed to vitamin D deficiency in T1D patients [161]. Hence, observations such as these may explain, at least in part, why low vitamin D status is common in diabetic patients.

In a study with pre-diabetic patients, vitamin D deficiency exacerbated insulin resistance [156]. This suggests that vitamin D might have a functional role in modulating glucose homeostasis. In line with this concept, numerous observational studies reported that improvement of vitamin D status via supplementation enhanced insulin sensitivity and reduced fasting blood glucose levels in type 2 diabetic patients [159, 162, 163]. Additionally, Mitri et al. [163] reported that vitamin D supplementation with up to 2000 IU/d improved beta cell function and attenuated hyperglycemia in type 2 diabetics. Furthermore, supplementing diabetic patients with 4000 IU/day of vitamin D improved insulin sensitivity in obese individuals [159] and high dose vitamin D supplementation (50,000 IU/wk) improved fasting plasma glucose and insulin sensitivity in T2D patients [162, 164].

The reasoning behind the investigation of the role of vitamin D in the promotion of insulin secretion includes the presence of VDR [165, 166] and 1- α hydroxylase [167] in beta cells. In mice lacking VDR, mRNA expression of insulin as well as serum insulin concentrations were lower and response to an oral glucose tolerance test was less robust compared to wild-type mice [166]. Similar observations have been reported in studies

involving rats fed a vitamin D deficient diet. In these studies, insulin secretion and glucose tolerance were impaired by vitamin D deficiency and vitamin D repletion normalized insulin secretion [168-170]. The underlying mechanism by which vitamin D promotes insulin secretion by beta cells is still lacking. Gysemans et al. [169] reported that treatment with 1,25D prevented the incidence of diabetes in NOD mice, a model of T1D, through the suppression of pro-inflammatory cytokine expression, such as IL-1beta and IL-15, in beta cells, along with the amelioration of insulinitis. However, when insulinitis was already present (late intervention at 14 wk of age), treatment with 1,25D failed to prevent diabetes in NOD mice. This further indicates that the protective effect of 1,25D against beta cell destruction is limited when they are already infiltrated with immune cells. As mentioned earlier, this is in contrast to its preventative effect during the early stage of insulinitis. Similarly, others have reported that insulin signaling was modulated by VDR via its action on the glutamate receptor or the AMPA receptor [170] and pancreatic RAS signaling [171], as these signaling pathways were altered under hyperglycemic state. However, supplementation with vitamin D normalized the expression of the pancreatic AMPA receptor and RAS components, as well as restored calcium-mediated insulin secretion independent of hyperglycemia [170, 171].

Despite the reported benefits of vitamin D with respect to regulation of glucose homeostasis, the specific relationship between vitamin D deficiency and T2D remains unclear. In children (birth to 6 years of age) and adolescents (~18 years of age) with T1D, the presence of vitamin D deficiency or insufficiency did not correlate with the progression of diabetes [172, 173]. Moreover, in recent reviews, it was concluded that current available randomized controlled and longitudinal studies have not provided sufficient evidence to support the notion that vitamin D supplementation improves hyperglycemia and insulin sensitivity in patients with type 2 diabetes [174, 175]. Although high dose vitamin D supplementation corrected vitamin D status in prediabetes and diabetes, increased vitamin D status was not associated with insulin sensitivity or circulating glucose concentrations [176, 177]. Because long-term interventions are difficult to conduct with vitamin D, since it is believed to have the strongest impact on long-latency diseases, the role of vitamin D in improving glucose homeostasis over the long term remains unclear. Furthermore, because most clinical trials that investigated vitamin D's impact on diabetes involved patients with

pre-existing diabetes, understanding the role of vitamin D in the prevention of diabetes or related complications remains elusive.

Resistant Starch

Dietary fiber is a class of non-digestible carbohydrates that includes lignin, cellulose, oligosaccharides, inulin, and various forms of resistant starch. The various physiological functions of different fibers depend on their structural properties. Viscous or soluble fiber, for example, can delay gastric emptying and lower postprandial glucose concentrations, while insoluble fibers can help to improve gastric motility and usually have a laxative effect [178]. It is recommended that total fiber intake (adequate intake, AI), according to the DRIs, should be 25g/ d for women and 38 g/d for men ages 19 – 50 based on median energy intake (14g of fiber/1000 kcal) [178]. The consumption of fiber in the U.S. is a concern, since Americans, regardless of gender, only consume an average of 15g/d of fiber, which is well below the AI [179]. Because low glycemic index diets and increased consumption of fiber-containing foods have been shown to improve symptoms of obesity-related complications, such as diabetes and cardiovascular diseases [180-182], it has been generally accepted that replacing highly digestible starch with dietary fiber is beneficial with respect to weight and blood glucose management. Though many dietary fibers have received significant attention, with respect to their health benefits, resistant starch (RS) in particular has recently received a great deal of interest. RS is a non-viscous fermentable fiber that is partially indigestible and is classified into four categories [183]:

1. Type 1 (RS1): Physically inaccessible starch

RS1 is found in whole or partly milled grains and seeds. The intact cell walls that surround starch granules retard enzymatic hydrolysis of amylose and amylopectin in these products.

2. Type 2 (RS2): Resistant starch granules

The natural form of RS2 is usually found in raw starch, such as unripe banana and potato starch. However, RS2 found in these sources can be gelatinized after heating and thus become digestible due to increased access of amylases to starch. In contrast,

high-amylose maize (HAM), which is high in RS2 due to a mutated gene on the starch branching enzyme to produce high amylose content, possesses higher gelatinization temperature due to a strong interaction between amylose chains [184]. Because of these properties, RS2 from HAM is able to maintain its resistance against enzymatic hydrolysis even after heating.

3. *Type 3 (RS3): Retrograded starch*

Retrograded starch is mostly found in starchy food products that have been cooked then cooled, such as potatoes, sweet potatoes, and beans. This is mainly due to the dissociation of crystalline structures in both amylose and amylopectin upon heating of a native starch. On cooling, the crystalline structures rearrange through the formation of double helices to yield a much more stable structure (RS3). RS3 has a gelatinization temperature that is highly resistant to heat and enzymes [185]. However, unlike RS2, the retrogradation process may be reversible as reheating the starch may again promote gelation and reduce crystallinity of starch [185].

4. *Type 4 (RS4): Chemically modified starch*

RS4 is formed by the chemical modification of starch with molecules such as acetyl group, octenyl group, or hydroxylpropyl groups to prevent starch granules from enzymatic hydrolysis [186, 187]. Cross-linkage is another common modification method in which the hydrogen bonds in starch molecules are strengthened by a cross-linking agent. This cross-linking prevents starch granules in RS4 from swelling and hydrolysis [188].

5. *Type 5 (RS5): Amylose-lipid complex starch*

Production of RS5 involves the addition of free fatty acids to high-amylose maize. The amylose-lipid interaction leads to the formation of helical complex structure that are resistant to swelling and enzyme hydrolysis [189].

Resistant starch and glucose homeostasis

The number of metabolic syndrome cases across the world has grown at an alarming rate. While treatment options are available to better control metabolic syndrome, the most effective long-term interventions appear to be lifestyle modifications such as dietary changes and increased physical activity. The acceptable macronutrient distribution range for carbohydrate is between 45 – 65% of the total daily energy intake [178]. However, the type of carbohydrate consumed has a profound impact on glycemic response. Silva et al [180] demonstrated that high glycemic index and low fiber breakfast markedly increased postprandial plasma glucose and insulin concentrations in T2D patients. Similarly, animal studies demonstrated that glucose- and amylopectin-fed animals developed insulin resistance at an earlier age compared to rats receiving amylose, which has a lower glycemic index [190, 191]. Thus, consumption of non-digestible fibers such as resistant starch (RS) and oligosaccharides, due to their lower glycemic properties, is recommended for better management of T2D. With respect to RS, studies involving feeding of RS-containing products, such as high-amylose rice and cornstarch, to diabetic animals have consistently shown that these products lower the postprandial glycemic response as well as circulating triglyceride, and cholesterol levels [192, 193]. Interestingly, it was recently shown that administration of RS to diabetic rats optimized lipid oxidation pathways, glycogen synthesis, and insulin sensitivity while the expression of genes central to fatty acid and triglyceride synthesis, such as the sterol regulatory element-binding protein-1c (SREBP-1c), an insulin-regulated protein that is essential for transcriptional regulation of glycolytic and lipogenic genes, was attenuated [194]. This suggests that the improved postprandial insulin and glucose response by RS is driven by the regulation of glucose and lipid metabolism-related genes. Another putative mechanism may involve the secretion of gut hormones associated with the regulation of postprandial satiety, such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), which were secreted in response to administration of short chain fatty acids (SCFA), fermentation products of RS [195, 196]. Studies by Keenan et al. [195] demonstrated that RS lowered abdominal fat and energy intake in rats, which correlated with higher levels of plasma GLP-1 and PYY, as well as an elevation of PYY gene expression in the cecum and large intestine. These observations are supported by other studies that have shown that RS reduced adipocyte size [197] and weight gain in obese prone rats [198]. When evaluating the

literature with respect to the use of RS for management of adiposity, the source of RS appears to be critical when interpreting results from both animal and human studies. In a 24-week feeding study with high-amylose RS2 starch and RS4 starch containing hydroxypropylated distarch phosphate, obese mice fed the RS4 diet gained less weight and exhibited lower adiposity accumulation as well as increased energy expenditure compared to the RS2-fed obese mice [199]. It is not well understood why the phenotypic responses were different between RS2- and RS4-treated mice since the bioavailability of both starches were equivalent [199]. It is possible that RS4 may have affected endogenous nutrient transport differently than RS2 as these starches may alter gastric emptying to a different degree. This is further supported by the observation where the transport of fatty acids from the lumen to the jejunum was significantly lower in HFD-induced obese mice treated with RS4 compared to RS2-fed obese mice, which indicates that RS4 may have reduced intestinal lipid absorption [199].

While solid evidence with respect to the beneficial effect of RS has been reported in animal models of obesity and diabetes, results from human studies have been inconsistent (Table 2). This could be due to the high variability of RS dosage used for these studies. Estimation of resistant starch intake based on 1999 – 2002 NHANES 24-hour dietary recall data has demonstrated that the average consumption of RS among Americans aged 1 year and older is approximately 5 g/d [200]. However, according to the aforementioned studies, the minimal effective daily intake of RS for the prevention of obesity-related diabetes appears to be greater than 5 g/d [181, 201-203]. Moreover, most dietary interventions that yielded positive metabolic outcomes have included RS at a dosage (7 g – 48 g/d of RS) that exceeds what is consumed by the US population (Table 2). Nonetheless, clinical studies with healthy subjects have generally demonstrated that RS feeding resulted in an improved postprandial glycemic response [202-206] compared to obese or diabetic individuals [182, 207, 208]. The differences observed in these studies could be due to the variability in dosage and type of fiber used. Moreover, as most of this work was done by feeding RS or RS-containing diets acutely, contradictory results and lack of long-term studies using dietary RS indicates that the role of RS in benefiting health in overweight and obese individuals requires further investigation.

Table 2. Human interventional studies investigating the effect of resistant starch against glucose metabolism.

Author, Country	Baseline Characteristics	Study Design	Dose	Impact on glucose and insulin outcomes	Other observed outcomes
De Roos et al., 1995 (The Netherlands) [229]	n = 24, male, healthy individuals	-Single-blind, randomized crossover study receiving either glucose, high-amylose corn starch (RS2), or extruded and retrograded high-amylose corn starch (RS3) for 1 wk	RS2 or RS3 at 30 g/day	-RS2 lowered appetite score compared to RS3 -RS3, but not RS2, reduced insulin secretion	
Noakes et al., 1996 (Australia) [230]	n = 23 (10 female, 13 male), overweight (BMI 29 ± 2), aged 51 ± 7	-12-wk dietary trial, 3 phases, each phase consisted of 4 wks (no washout period) -3 treatments (high-amylose diet, oat bran diet, and low-amylose diet)	-High-amylose starch at 50 g/d for female and 74g/d for male -Oat bran at 87 g/d for female and 121 g/d for male	-Fasting glucose was higher with oat bran compared to high- or low-amylose diet -Postprandial insulin was lowered with high-amylose diet compared to low-amylose diet	-Plasma triacylglycerol was lowered with oat bran diet but not with high- or low-amylose diet
Robertson et al., 2003 (UK) [231]	n = 10 (6 female, 4 male), aged 47.2 yrs, BMI 26.9, healthy subjects	-Single-blind crossover study -High RS diet or Low RS diet for 24 h	-High RS diet containing 100 g of Hi-Maize 260® (60 g of RS and 40 g of rapidly digestible starch) -Low RS diet	-High RS, but not Low RS, lowered postprandial insulin concentrations	-No effect on plasma triacylglycerol

Table 2. Continued

Author	Baseline characteristics	Study Design	RS Dose	Impact on glucose and insulin outcomes	Other observed outcomes
... Robertson et al., 2003 (UK) [203]			containing 40 g of waxy-maize starch (40 g of rapidly digestible starch)		
Higgins et al., 2004 (USA) [231]	n = 12 (7 male, 5 female), aged 33 ± 5 yrs, normal glucose tolerance, BMI 24.7 ± 2.4	-Subjects received 4 meals at various doses of RS at 4 wks apart	0% , 2.7%, 5.4%, and 10.7% RS2 in the form of high-amylose maize starch	-No effect on fasting or postprandial insulin and glucose	-5.4% RS increased lipid oxidation compared to controls
Robertson et al., 2005 (UK) [202]	n = 10 (6 female, 4 male), aged 48.5 ± 3.4 yrs, BMI 23.4 ± 1.4	-Single-blind, crossover study for 12 wks -RS or corn starch for 4 wks, with 4 wks of washout period	Hi-Maize 260 ® at 50g/day (30 g of RS2 and 20g of rapidly digestible starch)	-Improved insulin sensitivity -Increased muscle glucose tolerance	-Lowered NEFA and glycerol in subcutaneous abdominal adipose tissue
Behall et al., 2006 (US) [232]	n = 20 (10 overweight women with BMI 30.4; 10 normal weight women with BMI 22), healthy, aged 43.3 yrs	-High-amylose corn starch (HAM) with or without beta glucan	HAM with 5.06 g of RS/100 g of muffin with either 0.26, 0.68, or 2.3 g of beta-glucan/100 g muffin	-Combination of high-amylose starch and beta-glucan improved postprandial plasma glucose and insulin in both normal and overweight women	

Table 2. Continued

Author	Baseline characteristics	Study Design	RS Dose	Impact on glucose and insulin outcomes	Other observed outcomes
Al-Tamimi et al., 2009 (USA) [204]	n = 13, healthy individuals (7 females, 6 males), aged 27±5 yrs, BMI 25± 3	-Randomized crossover design -3 trials to consume cross-linked RS4 bar, glucose beverage, and wheat-containing bar; washout period for 7 d	80 g cross-linked RS4/d (~14 g RS/d)	-RS4 attenuated postprandial glucose and insulin response	
Bodinham et al., 2010 (UK) [233]	n = 20, healthy individuals, aged 19-31 yrs, BMI 23.2 ± 0.65	-Single-blind, randomized crossover study -RS or placebo supplement within the test breakfast and lunch (washout period of 7 d)	80 g Hi-Maize 260® providing 48 g of RS (24 g RS with lunch, 24 g RS with dinner)	-No changes in plasma glucose -Post-prandial insulin response was lowered	-Reduced energy intake -No difference in subjective appetite scores
Johnston et al., 2010 (UK) [206]	n = 20 (8 female, 12 male), aged 50.1 ± 4.05 (placebo) and 45.2 ± 3.55 (RS), BMI 30.4 ± 1.15 (Placebo), and 31.3 ± 1.7 (RS)	-Single-blind, randomized parallel study for 12 wks	Hi-Maize 260® providing 40 g/d RS2 and 27 g/d of rapidly digestible starch	-Improved insulin sensitivity but not fasting insulin and beta cell function -Insulin sensitivity was inversely correlated with waist circumference	-No changes in body weight, fat storage in muscle, liver, visceral fat, vascular function, or inflammatory markers

Table 2. Continued

Author	Baseline characteristics	Study Design	RS Dose	Impact on glucose and insulin outcomes	Other observed outcomes
Ble-Castillo et al., 2010 (Mexico) [234]	n = 28 with T2D (4 male, 24 female), aged 51.7 ± 5.6, BMI 34.89 ± 2.32, fasting glucose 145.94 ± 104.17 mg/dL, fasting insulin 14.1 uU/mL	Crossover design with 2 4-wk experimental periods receiving native banana starch (NBS) and soya milk	24 g of NBS (8.16 g of RS) dissolved in 240 mL water	-Reduced body weight and BMI -Reduced fasting insulin levels -Insulin sensitivity was improved compared to baseline, but not when compared to the control group	
Penn-Marshall et al., 2010 (USA) [201]	n = 17 (8 male, 9 female), aged 36.6 ± 1.55 yrs, African American, T2D, BMI 37.7 ± 2, fasting glucose 99.86 ± 2.63 mg/dL	-Double-blind, crossover study with 2 wks of washout period -6-wk feeding trial	12.39g of Hi-Maize 260® (in the form of bread loaf) with 10.17 g of RS/100 g of bread	-No change in blood glucose, insulin, or CRP levels -HOMA-IR was normalized by RS but was not significantly different from control	
Bodinham et al., 2012 (UK) [205]	n = 12 (8 male, 4 female), aged 37 ± 4 yrs, BMI 28.2 ± 0.4 (overweight), without T2D or insulin resistance	-Single-blind, randomized crossover study for 4 wks with a 4 wk washout period between interventions	67 g Hi-Maize 260® providing 40 g RS2 from maize (HAM-RS2)	-Improved first-phase insulin response	

Table 2. Continued

Author	Baseline characteristics	Study Design	RS Dose	Impact on glucose and insulin outcomes	Other observed outcomes
Maki et al., 2012 (USA) [181]	n = 33 (11 male, 22 female), aged 49.5 ± 1.6, BMI 30.6 ± 0.5	-Double-blind, randomized crossover study for 4 wks, with 3 wks of washout periods between RS or corn starch diet intervention	Hi-Maize 260® at 60% RS (HAM-RS2) given at 15g/d or 30g/d	-RS improved insulin sensitivity with both dosages, but was only observed in men, not women	
Kwak et al., 2012 (Korea) [235]	n =85, 47 male, 38 female, aged 49.4 ± 1.74 yrs (placebo) and 51.7 ± 2.03 yrs (RS group), patients with impaired fasting glucose, impaired glucose tolerance, or newly diagnosed T2D	First phase: 2-wk usual diet Second phase: 4-wk intervention with refined rice or RS-containing rice	Rice containing 6.5 g/d of RS	-Reduced fasting insulin and insulin resistance, postprandial glucose, and improved insulin sensitivity	-Improved endothelial function with increased total nitric oxide and superoxide dismutase activity
Klosterbuer et al., 2012 (USA) [236]	n = 20 (10 male, 10 female), aged 29 ± 8 yrs, BMI 23 ± 2	-Double-blind, randomized crossover study receiving low-(control) or high-fiber diet	25 g of fiber from soluble corn fiber or resistant starch alone or in combination with 5 g of pullulan	-RS + pullulan reduced glucose and insulin	-No effect on satiety or energy intake

Table 2. Continued

Author	Baseline characteristics	Study Design	RS Dose	Impact on glucose and insulin outcomes	Other observed outcomes
Lobley et al., 2013 (UK) [208]	n = 14, male, at least met three symptoms of metabolic syndrome	4 periods of dietary intervention without washing period	<u>Period 1 (7 d):</u> Maintenance diet with 5 g/d of RS, 27 d/g of NSP <u>Period 2 and 3 (21 d):</u> Randomized RS (25 g/d of RS, 16 d/d of NSP) or NSP (41 g/d of NSP, 2.5 g/d of RS) diet <u>Period 4 (21 d):</u> Weight loss high-protein diet with 25 g/d NSP and 2.9 g/d of RS	-Addition of RS or NSP did not affect the glycemic control	
Bodinham et al., 2014 (UK) [207]	n = 17 (12 male, 5 female), well-controlled T2D on anti-diabetic agents, aged 55 ± 2.4, BMI 30.6 ± 1.3, HbA1c levels of 46.6 ± 2	-Single-blind, randomized crossover study for 12 wks with a 12-wks washout period between interventions	67 g Hi-Maize 260®providing 40 g RS2 from maize (HAM-RS2)	-No differences in fasting glucose, insulin, HOMA-B, and HbA1c	-Lower NEFA concentrations but no differences in blood pressure and vascular functions -Lower fasting TNF-α concentrations

Table 2. Continued

Author	Baseline characteristics	Study Design	RS Dose	Impact on glucose and insulin outcomes	Other observed outcomes
Dodevska et al., 2015 (Serbia) [182]	n = 47, overweight and obese men and women, aged 45-74, moderate physical activity for a minimum of 4h/week	-12 months under free-living condition (lifestyle intervention) -Subjects were instructed to follow a diet either high in fiber (Fiber group) or resistant starch (RS group)	<u>RS group:</u> -Total fiber = 27.36 g/d - Total RS = 14.72 g/d <u>Fiber group:</u> -Total fiber = 27.44 g/d -Total RS = 7.52 g/d	-Fiber-rich diet, but not RS-rich diet, improved glucose tolerance in overweight and obese individuals	-Both treatments reduced body weight, BMI, and waist circumference -RS decreased total cholesterol and LDL, no change in HDL -Fiber increased HDL, no change in LDL or total cholesterol

BMI: Body mass index; CRP: C-reactive protein; HAM: High-amylose maize; HbA1c: Hemoglobin A1c; HDL: High density lipoprotein; HOMA-IR: Homeostatic model assessment-insulin resistance; LDL: Low density lipoprotein; NBS; Native banana starch; NEFA: Non-esterified fatty acids; NSP: Non-starch polysaccharides; RS: Resistant starch; T2D: Type 2 diabetes; TNF- α : Tumor necrosis factor- α .

Short chain fatty acids

It is known that SCFAs resulting from colonic microbial fermentation can serve as a local energy source for the host. The predominant SCFAs (detected at the highest concentration in mammalian distal small intestine and colon) produced by bacteria include butyrate, acetate, and propionate [209]. While butyrate is readily absorbed and utilized by colonocytes for energy, propionate and acetate that are transported into circulation can enter the TCA cycle and serve as a precursor for hepatic gluconeogenesis and lipogenesis [210]. SCFAs may also have a therapeutic value as they alleviated symptoms of type 2 diabetes in HFD-induced obese animals. Butyrate, for example, has been shown to enhance insulin sensitivity, glucose tolerance, and attenuate beta cell apoptosis in obese and diabetic animals when administered either intraperitoneally or orally ingested [211-213], possibly due to its role as a histone deacetylase inhibitor [211]. However, the mechanism by which SCFAs promote normal glucose homeostasis is not entirely clear. In *in vitro* studies, it was demonstrated that free fatty acid receptor-2 (FFAR2) is an endogenous transmembrane receptor for acetate and propionate; while the free fatty acid receptor-3 (FFAR3) is a membrane receptor for butyrate and acetate *in vitro* [214, 215]. Because FFAR2 and FFAR3 are abundant in the intestinal L-cells, it is possible that SCFAs regulate intestinal secretion of GLP-1 and PYY and subsequently the regulation of glucose homeostasis through the activation of specific FFAR signaling to modulate L-cell activity [209, 216]. This is supported by a study conducted by Tolhurst et al. [209], in which FFAR2- and FFAR3-deficient mice developed impaired glucose tolerance and decreased plasma insulin levels, both of which correlated with reduced circulating GLP-1. The role of FFAR2 and FFAR3 in energy and glucose homeostasis was further characterized in KO mouse models [216, 217]. Compared to wild type mice, FFAR3-KO mice presented marked weight loss and lower epididymal fat pad adiposity when raised conventionally or co-colonized with *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithii*, prominent bacteria that are known to promote polysaccharide fermentation. However, the improvements on growth rate and adiposity were not observed when mice were housed under germ-free condition [216]. Along with these responses, the FFAR3-KO gnotobiotic mouse model exhibited both increased circulating levels of PYY and intestinal transit rate, which led to the assumption that SCFA-mediated FFAR3 signaling is crucial for modulating host adiposity via reduced intestinal

lipid absorption and suppression of energy harvest capacity [216]. On the other hand, Kimura et al [217] has reported that FFAR2 activation suppressed insulin signaling in white adipose tissue, and thus altered glucose and lipid metabolism in FFAR2-KO mice fed a HFD.

Additionally, FFAR2-KO mice, when fed a HFD, contained a higher proportion of Firmicutes and a lower level of proteobacteria and actinobacteria, a bacterial profile that has been associated with increased energy harvest capacity and obesity; however, treatment with antibiotics prevented the changes mentioned above and fecal SCFAs were similar to mice raised in a germ-free environment [217]. Taken together, the utilization of the FFAR-KO mouse models has shed a significant amount of light on role of FFAR signaling in SCFA-mediated alterations in glucose and energy homeostasis.

Intestinal gluconeogenesis

In addition to FFAR signaling, the metabolic benefits of dietary fiber and RS could be attributed to intestinal gluconeogenesis upon stimulation by SCFAs. Interestingly, induction of intestinal gluconeogenesis has been associated with reduced food intake and hunger sensation, as well as suppression of hepatic gluconeogenesis and promotion of glycogen storage [218]. Furthermore, mice lacking intestinal gluconeogenic genes exhibited impaired glucose and insulin tolerance, which were accompanied with increased body weight [219]. It is also interesting to note that propionate and butyrate may regulate intestinal gluconeogenesis via different mechanisms. For instance, butyrate is able to up-regulate intestinal gluconeogenic gene expression in enterocytes via a cAMP-dependent pathway, independent of FFAR2 signaling. In contrast, propionate can up-regulate the gut-brain neural circuit via the activation of FFAR3 to induce intestinal gluconeogenesis [219]. The proposed mechanism of intestinal gluconeogenesis may explain, at least in part, the beneficial effects of SCFA on glucose and energy metabolism that could be beyond the energy harvest capacity of gut microbes.

Resistant starch and kidney health

The prevalence of chronic kidney disease (CKD) is growing rapidly in the elderly population. According to the latest statistics published by the National Institute of Diabetes and Digestive and Kidney Disease, about 24.5% of the elderly U.S. population exhibit CKD

[220]. This alarming figure illustrates that the need to develop dietary interventions to prevent the incidence and progression of CKD is urgent. Because of the many links that investigators have made between the gut microbiome and obesity, the use of RS and other dietary fibers as substrates for microbial fermentation has been an area of increasing interest. While most studies involving the feeding of RS and other fermentable fibers have focused on obesity-related diabetes as discussed above, evidence has suggested that a number of dietary fibers promote kidney health [80, 221-224]. In patients with T2D, dietary fiber intake was strongly correlated with glycemic control and inversely correlated with the prevalence of metabolic syndrome [224]. Moreover, T2D patients who had a greater intake of dietary fiber had a lower risk for developing CKD [222, 223], which has been attributed to the anti-inflammatory properties of certain types of fiber [224]. Xie and colleagues [222] also reported that the addition of 10 – 20 g /d of soluble fiber to the diet of hemodialysis patients for six weeks improved lipid profiles and reduced renal inflammation, which suggests that consumption of dietary fiber is critical for maintaining kidney health. Additionally, gum arabic and cellulose, when coupled with a low-protein diet, increased fecal nitrogen excretion [225, 226]. This suggests that consumption of these fibers restricted the growth of urease-producing bacteria, which are known to damage epithelial tight junctions in CKD [227, 228]. Studies involving animal models of chronic kidney disease have also supported the beneficial effect of fermentable fibers in the attenuation of kidney inflammation and oxidative stress by reducing macrophage infiltration and apoptosis in the kidney [221, 223]. In addition, feeding of HAM, a source of RS2, delayed tubular damage and interstitial fibrosis in adenine-induced CKD mice, which could be related to SCFA-mediated normalization of gut epithelial tight junction protein expression [223]. Consistent with these findings, our laboratory has demonstrated that dietary RS delayed the progression of diabetic nephropathy in STZ-induced T1D rats, though fasting blood glucose concentrations were not affected [80]. Intriguingly, expression of megalin and Dab2, which are critical for the uptake of the 25D-DBP complex, was normalized by RS. In addition, urinary excretion of vitamin D metabolites was markedly reduced in RS-treated rats [80]. These findings suggest that RS is a dietary component that can potently influence vitamin D balance, which is disrupted in diabetes-related kidney disease. Furthermore, because the mechanisms responsible for the

beneficial effects of RS on kidney health are largely undefined, much more work is needed in this area.

Conclusions

Obesity-related diabetes is a growing epidemic that warrants the need for preventive therapies. As extensive literature reviews have consistently mentioned, the most effective preventative approach has been to maintain normal level of blood glucose in order to delay the progression of secondary complications that are a result of sustained hyperglycemia. Because maintaining kidney health is critical for vitamin D metabolism, we speculate that optimization of glycemic control could alleviate hyperglycemia-mediated kidney disease and restore vitamin D balance, which can ultimately prevent diabetic complications that are associated with vitamin D insufficiency, such as osteoporosis, cancer, and heart disease. To date, effective dietary interventions that target diabetic complications are still lacking. RS as a low glycemic fermentable fiber could be a potential candidate for the prevention of diabetes complications. The effect of RS on kidney health and vitamin D metabolism has been explored in our previous studies with T1D animal models. However, the effect of RS in T2D and T2D-induced kidney damage was still largely unknown until the studies described in future chapters of this dissertation were conducted. We based our hypotheses on the evidence that RS can have an impact on the development and progression of chronic kidney disease and metabolic control as presented in this literature review. Therefore, the goal of the studies we conducted was to evaluate the effect of RS on T2D-mediated kidney complications, including vitamin D homeostasis, and to explore the possible mechanisms that drive the renoprotective effect of RS in diabetes. This novel approach could lay down the foundation for the development of dietary strategies that can positively impact vitamin D balance and prevent diabetic complications that are also associated with aberrant vitamin D metabolism.

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**CHAPTER 3: DIETARY RESISTANT STARCH PREVENTS URINARY
EXCRETION OF VITAMIN D METABOLITES AND MAINTAINS CIRCULATING
25-HYDROXYCHOLECALCIFEROL CONCENTRATIONS IN ZUCKER
DIABETIC FATTY RAT**

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Abstract

BACKGROUND: Type 2 diabetes (T2D) is the leading cause of nephropathy in the United States. Renal complications of T2D diabetes include proteinuria and suboptimal serum 25-hydroxycholecalciferol (25D) concentrations. 25D is the major circulating form of vitamin D and renal reabsorption of the 25D-vitamin D-binding protein (DBP) complex via megalin-mediated endocytosis is believed to determine whether 25D can be activated to 1,25-dihydroxycholecalciferol (1,25D) or returned to circulation. We previously demonstrated that excessive urinary excretion of 25D-DBP and albuminuria occurred in type 1 (T1D) - and T2D rats. Moreover, feeding T1D rats high-amylose maize partially resistant to digestion (resistant starch; RS) prevented excretion of 25D-DBP, without significantly impacting hyperglycemia.

METHODS: We utilized Zucker diabetic fatty rats (ZDF), a model of obesity-related T2D, to determine whether feeding RS could similarly prevent loss of vitamin D and maintain serum 25D concentrations. Lean control Zucker (n=8) rats were fed a standard semi-purified diet (AIN-93G) and ZDF rats were fed either the AIN-93G diet (n=8) or the AIN-93G diet in which cornstarch was replaced with RS (550 g/kg diet; 35% resistant to digestion, n=8) for 6 wk.

RESULTS: RS attenuated hyperglycemia by 41% and prevented urinary DBP excretion and albuminuria, which were elevated 3.0- and 3.6-fold, respectively, in control diet-fed ZDF rats. Additionally, urinary excretion of 25D and 1,25D was higher (89 And 97%,

respectively), whereas serum 25D concentrations were 31% lower in ZDF rats fed the control diet compared to RS-fed ZDF rats. Histopathological scoring of the kidney revealed that RS attenuated diabetes-mediated damage by 21% despite a ~50% decrease in megalin protein abundance.

CONCLUSIONS: Taken together, these data provide evidence that suggests vitamin D balance can be maintained by dietary RS through nephroprotective actions in T2D, which are independent of vitamin D supplementation and renal expression of megalin.

KEYWORDS: *Resistant starch, high-amylose maize, diabetes, diabetic nephropathy, Zucker diabetic fatty rats, vitamin D, 25-hydroxycholecalciferol, megalin*

Introduction

Type 2 diabetes (T2D) is an epidemic that has facilitated the need to develop feasible dietary strategies that can minimize the risk of developing secondary complications. Perhaps the most significant factor that must be considered when evaluating nutritional strategies for diabetics is kidney health, due in part to the role of the kidney in the reabsorption of nutrients [1]. It is estimated that out of all documented cases of renal failure in the United States, ~44% are due to diabetes [2], which has implications in the development of nutrition-related complications. In the kidney, the major circulating form of vitamin D [25-hydroxycholecalciferol (25D)] and the subsequent production of the active vitamin D hormone, 1,25-dihydroxycholecalciferol (1,25D) are dependent on receptor-mediated endocytosis of the 25D-vitamin D binding protein (DBP) complex by the proximal tubule [1, 3-5]. Here, the endocytic proteins megalin and cubilin internalize the 25D-DBP complex from renal filtrate via endocytosis in concert with the intracellular adaptor protein disabled-2 (Dab2) [3]. 25D is either hydroxylated by CYP27B1 to generate its active derivative, 1,25D, or returned to the circulation as 25D-DBP. Because the maintenance of circulating 25D and 1,25D concentrations is dependent on kidney function, it is not surprising that compromised vitamin D status is a concern in diabetic patients [6-9], particularly those that experience symptoms of nephropathy [10-12]. Moreover, with a proclivity to exhibiting suboptimal vitamin D status, diabetics may be at an increased risk for developing complications that are consistent with vitamin D deficiency, such as bone disease, autoimmune disorders, and multiple forms of cancer, as has been comprehensively reviewed [13].

We recently reported that in both type 1 (T1D) and T2D animal models, urinary excretion of the 25D-DBP complex was markedly elevated [14, 15]. Specifically, we observed a marked increase in urinary 25D excretion. Furthermore, reduced expression of megalin and Dab2 was associated with a decline in serum 25D concentrations and loss of 25D-DBP in the urine [14, 16, 17]. We also reported that these observations were prevented in dietary intervention studies with T1D rats, where feeding rats high-amylose maize, which is partially resistant to digestion, as a carbohydrate source, prevented the loss of renal megalin and Dab2 expression, as well as the urinary excretion of 25D and DBP [15].

Resistant starch (RS) is a family of fermentable dietary fibers, some of which have been shown to improve the classic symptoms of obesity-related diabetes [18-20]. However, no published studies have reported a benefit of RS with respect to renal vitamin D metabolism and systemic vitamin D balance in obesity-related diabetes. In the present study, our objective was to determine whether feeding an AIN-93G diet in which the cornstarch was replaced with a carbohydrate source containing RS could protect against the perturbation of vitamin D metabolism in Zucker diabetic fatty (ZDF) rat, a well-characterized animal model of obesity-induced T2D. Specifically, we determined whether feeding ZDF rats high-amylose maize, which was chosen because it is partially resistant (~35%) to digestion [21], would maintain serum vitamin D status through the prevention of excessive urinary excretion of 25D, 1,25D, and DBP.

Materials and Methods

Animals and Diets - All animal studies were approved by the Institutional Animal Care and Use Committee at Iowa State University and were performed according to Iowa State University Laboratory Animal Resources Guidelines. All diet ingredients, with the exception of high-amylose maize (Amylogel®, Cargill), were purchased from Harlan Teklad. Male ZDF and lean Zucker control rats were purchased at 8 wk of age (Charles River Laboratories) and housed individually in plastic cages in a room with a 12 h light-dark cycle and fed ad-libitum. Rats were randomly assigned to a diet (AIN-93G, Supplemental Table 1) containing either cornstarch (550 g/kg diet, C diet) or high-amylose maize (550 g/kg diet) that was ~35% resistant to digestion. Thus rats were divided into 3 groups (n=8): 1) lean control rats on a C diet (LC); 2) ZDF rats on a C diet (DC); 3) and ZDF rats on a RS diet

(DRS). All dietary starches were prepared as described previously [15]. The RS content (%) was verified in all diets over the 1 wk usage timeframe after it was prepared by *in vitro* digestion analysis (AOAC 991.43 Method) to confirm the stability of resistant starch [22]. All rats were provided free access to experimental diets and water for 6 wk. Prior to euthanization at the end of wk 6, rats were placed in metabolic cages for a 12 h fasting period. Subsequent urine samples were then collected and stored at -20°C until analysis. At the time of sacrifice, rats were anesthetized with a ketamine: xylazine cocktail (90:10 mg/kg body weight) via intraperitoneal injection. Whole blood was then collected via cardiac puncture and blood glucose concentrations were measured with a glucometer (Bayer Healthcare) after which tissues were removed and stored at -80°C prior to analysis.

Assessment of urinary creatinine, total protein, albumin, and DBP - Urinary creatinine was measured using a commercial colorimetric kit (Cayman Chemical). Total urinary protein concentrations over 12 h were assessed using a bicinchoninic acid assay (Thermo Scientific Pierce). Urinary albumin and DBP were measured with commercial ELISA kits as described [15]. Urinary excretion of DBP and albumin were expressed as mg excreted/12 h.

Assessment of urinary and serum 25D and 1,25D - Serum and urinary levels of 25D were measured via a commercial enzyme immunoassay kit (Immunodiagnostic Systems) as previously described [15]. Assessment of 1,25D in both serum and urine were measured with a commercially available ELISA kit (My BioSource, Inc.). The total urinary excretion of 25D and 1,25D was calculated and normalized to urinary creatinine as we have reported previously [15].

RNA Isolation and Real-Time PCR - Total kidney RNA was isolated as previously described [15]. Total RNA was then quantified by UV detection and single-strand cDNA synthesis was carried out with a Verso cDNA Synthesis kit (Thermo Scientific). Real-time PCR reactions were performed in duplicate using iScript SYBR Green Detection reagents (Bio-Rad) at 200 ng/well for the detection of *megalyn*, *Dab2*, *CYP27B1*, and *CYP24A1* with an Applied Biosystems Plus® real-time PCR system (Life Technologies). The primers sets specific for *megalyn* (forward primer: AACGGTCAGTGTATTCCGAGCGAA; reverse

primer: TTGGCAGTCGTCATCTCCATCACA), *Dab2* (forward primer: AGGTTGAAGAAGCCAACAAAGCGG; reverse primer: AGTCCTGCTTTACGCCATTCGGTA), *CYP27B1* (forward primer: GAGATCACAGGCGCTGTGAAC; reverse primer: TCCAACATCAACACTTCTTTGATCA), and *CYP24A1* (forward primer: TGGATGAGCTGTGCGATGA; reverse primer: TGCTTTCAAAGGACCACTTGTTTC), were normalized against *18S* (forward primer: ACATCCAAGGAAGGCAGCAG; reverse primer: TTCGTCACCTCCCCGG). Expression of each target gene was determined as mean fold change in gene expression relative to the non-diabetic animals (NDC).

Histology and Immunohistochemistry. Kidneys were fixed in formalin, embedded in paraffin, sectioned at 5 μ M, and stained with hematoxylin and eosin Y for histological assessment of kidney health. Histopathological scoring of kidneys was performed as described [23]. The lesions in the renal cortex that were evaluated included: tubular degeneration, interstitial fibrosis, dilated glomerular space, hydronephrosis, and dilated tubules in renal cortex. We performed Immunohistochemistry for the detection of Dab2 and megalin was done as described previously [14] and data were expressed as intensity per area.

Statistical Analysis. All data were analyzed via the Statistical Analysis System (SAS). Analysis of variance (ANOVA) with repeated measures was performed on the body weight changes and relative daily food intake. All other end-point analyses were evaluated statistically for differences between groups using one-way ANOVA followed by Tukey's post hoc test. A non-parametric analysis was used when normality failed via Kruskal-Wallis one-way ANOVA by ranks followed by Tukey's or Dunn's multiple comparison test for unequal groups. Interactions between treatments and differences between means were considered significant at $P \leq 0.05$.

Results

RS normalized growth pattern despite reducing food intake in ZDF rats. By the beginning of the 3 wk treatment period, DRS rats gained considerably more weight than the LC and DC rats. By the of the 6 wk study, DRS rats gained 24 and 51% more weight than LC and DC

rats, respectively (Fig. 1A). Moreover, LC and DRS rats continued to gain weight throughout the treatment period, whereas the growth of the DC rats leveled off at wk 3 and rats did not gain any additional weight. However, these data do not reflect food intake by ZDF rats. Despite the DRS rats exhibiting the greatest growth rate out of any of the groups, they consumed 35% less diet by day 35 till the end of the treatment period, where they consumed 40% less, when compared to DC rats (Fig. 1B).

RS normalized blood glucose, urinary volume, creatinine excretion, and proteinuria in ZDF rats. Compared to LC rats, fasting blood glucose concentrations were elevated 3-fold greater in the DC rats compared to a 1.4-fold increase in DRS rats and were 41% lower in the DRS rats compared to the DC rats (Table 1). Likewise, the total volume of urine collected during the 12 h fasting period was increased 1.9-fold in DC rats compared to both LC and DRS rats (Table 1). Urinary creatinine concentrations were 91 and 86% higher in LC and DRS rats, respectively, compared to DC rats; however there was no statistical difference between LC and DRS rats (Table 1). Similarly, total urinary protein did not differ between LC and DRS rats, but was 4.2- and 1.6-fold higher in DC compared to LC and DRS rats, respectively (Table 1). Urinary albumin was 3.6-fold greater in DC than in DRS rats, and there were no differences in urinary albumin between LC and DRS rats (Table 1).

Protection of the kidney by dietary RS rescued serum 25D concentrations and prevented urinary loss of 25D, 1,25D, and vitamin D-binding protein. The renal histopathological score of the DC rats was highest among the treatments (48% higher than LC, Fig. 2A) and renal pathological scores of DRS rats did not differ from LC rats. Similarly, kidney weights in DC rats were greater than in LC and DRS rats (16% and 26%, respectively), and we did not detect a difference in kidney weight between the LC and DRS rats (Fig. 2B). Vitamin D status of the DC rats, as indicated by serum 25D concentrations, was 45% lower than in the LC rats and 31% lower than in DRS rats (Fig. 3A). Serum 1,25D did not differ between groups regardless of serum 25D status (Fig. 3B). Consistent with the decline in vitamin D status in DC rats, urinary excretion of DBP was markedly elevated in DC rats compared to LC rats, in which DBP was virtually undetectable, and was 75% greater in DC than in DRS rats (Fig. 3C). Moreover, urinary 25D concentrations were higher in DC rats compared to LC

and DRS rats (92 and 89% higher, respectively,) as were urinary 1,25D concentrations (94 and 97% higher, respectively, Fig. 3D – 3E).

mRNA expression of renal vitamin D transport and metabolism proteins did not change in ZDF rats regardless of diet. We did not detect any statistical differences in *megalyn*, *Dab2* (Supplemental Fig. 1), *CYP27B1*, and *CYP24A1* mRNA expression between any of the treatment groups (Supplemental Fig. 2). Unlike mRNA expression, immunohistochemical staining of the kidney sections revealed that megalin protein abundance in renal proximal tubules was ~50% lower in the DRS rats compared to the LC or DC rats (Fig. 4A). In contrast, expression of Dab2 protein in the renal proximal did not differ between DC and DRS rats, which was ~40- and 50% greater, respectively, than in LC rats (Fig. 4B).

Discussion

We have previously reported that vitamin D metabolism is disrupted in T1 and T2D and that dietary RS attenuated the urinary excretion of 25D in a model of T1D [14, 15]. Here, we demonstrate that the impact of dietary RS on maintaining vitamin D balance and the attenuation of symptoms were markedly more robust in T2D than what we found in our T1D studies. Specifically, our data show that RS virtually prevented proteinuria and the urinary excretion of 25D and 1,25D, as well as maintained serum 25D concentrations. Furthermore, inclusion of dietary RS promoted the growth of DRS rats despite RS-fed rats consuming the lowest volume of diet based on body weight, indicating better overall health of DRS rats.

It is interesting that body weight gain of the DRS rats was significantly higher than both LC and DC rats with no changes in food intake compared to LC rats. A typical symptom of uncontrolled diabetes with renal complications is glucosuria, which can lead to significant fluid and calorie loss [24]. We suspect a reason for our observation is that instead of excreting glucose in the urine, DRS rats likely were able to retain and store or metabolize it due to their better overall renal health. Consistent with this concept, RS attenuated the rise in fasting blood glucose concentrations in ZDF rats, which can explain, at least in part, the absence of osmotic diuresis due to hyperglycemia in RS-fed rats. However, these rats were still clearly diabetic as indicated by a glucose concentration that was over 2 × greater than in LC rats. Though DC rats fed the control diet consumed more overall diet than DRS rats and

thus more vitamin D, serum 25D levels in DRS rats were markedly higher compared to DC rats. These findings, combined with our histology data that demonstrated kidneys from DRS rats did not exhibit the same degree of pathology as DC rats, suggest that RS consumption did not prevent diabetes. Rather, it supports the possibility that dietary RS, due to the nature of its digestibility, attenuated a glycemic insult to the kidney of diabetic rats fed the cornstarch-based control diet. Thus kidney function was protected, which in turn promoted vitamin D balance in hyperglycemic ZDF rats.

Surprisingly, we did not detect a decrease in mRNA expression of *megalyn* and *Dab2* as we have consistently observed previously in diabetic rats [14, 15]. A possibility for this is that the macronutrient compositions are different between the AIN-93G diet we used for this work and the high-energy diabetogenic diet we used previously, which may have more strongly impacted renal health. Moreover, in our earlier studies, rats were fed a diabetogenic diet for 8 wk prior to sacrifice compared to a 6 wk feeding period of the AIN-93G diet for the present study. We also observed decreased megalin protein expression in the renal proximal tubules of DRS. Because our study demonstrated that RS protected kidney health in the ZDF rats, we suspect that the decreased expression of megalin in the proximal tubules of DRS rats may have been a protective action. 25D-DBP is one of many known megalin ligands, which also include pro-inflammatory cytokines, chemokines, and nephrotoxins [25, 26]. Our data also suggest that absorption of the 25D-DBP complex by the kidney can be achieved by different means. Thus, we have not ruled out the possibility that there is more than one mechanism by which the 25D-DBP complex can be internalized by renal proximal tubules. Furthermore, we are currently investigating the possibility that the activity and/or expression of megalin can be regulated at the translational or post-translational levels. Our earlier T2D work also involved dietary restriction of vitamin D to ZDF rats, which may help explain why we did not detect differences in the expression of *CYP27B1* and *CYP24A1* in the present study during which vitamin D intake was not restricted. Because both genes are potently regulated by 1,25D [27-29] a likely reason for no change in their expression is that serum 1,25D concentrations were not different between any of the treatment groups, which was also observed in a recent clinical trial with T1D subjects [30].

With the worldwide obesity-induced diabetes epidemic and the many questions that remain with respect to vitamin D requirements and vitamin D metabolism in diabetes, the

present study is timely as it is a highly translatable advancement in vitamin D research. Consistent with our animal studies, Thrailkill et al. [12] have observed elevated urinary excretion of 25D, 1,25D, and DBP in a clinical study with T1 diabetic subjects whom also exhibited compromised vitamin D status. Collectively, these are new insights into why suboptimal vitamin D status is common in diabetes and could prove to be valuable with respect to further interpretation of observational studies that reported compromised vitamin D status in both types of diabetes [31-34]. However, there is still a lack of controlled clinical trials that show vitamin D intervention can reduce the incidence and severity of diabetes. Yet in part due to the complexity of coordinating long-term trials with supplemental vitamin D, especially in diabetic patients, the knowledge gap with respect to the role of vitamin D in T2D outcomes may be difficult to overcome. Moreover, vitamin D trials in diabetic patients, which have mostly been utilized in the context of glucose metabolism, have not yielded promising results [35-38] and resistance to vitamin D supplementation has been reported in diabetic patients with suboptimal vitamin D status [39]. Elevated vitamin D excretion in both types of diabetes, as we have observed, could therefore be a significant limitation for such studies and thus a vitamin D supplement and/or UV exposure may not be effective for the maintenance of circulating vitamin D. Hence, alternative dietary strategies that can reduce the excretion of vitamin D may be more viable with respect to improving vitamin D status in diabetes.

Here, we have demonstrated that independent of vitamin D supplementation, vitamin D balance can be protected from the effects of obesity-induced diabetes through the inclusion of RS in the diet. Because the ZDF rat is an extreme model of T2D compared to what is typical of a human with T2D, it is reasonable to hypothesize that the beneficial effects of RS on vitamin D balance could be produced in a clinical setting by a diet containing less RS than we utilized for this study. In support of this concept, we have recently found that reducing the RS content in the diet by half of what was utilized in the present study normalized growth of T1D rats (Koh and Rowling, unpublished observations). Additionally, our future work will focus on whether other types of dietary fiber could provide similar benefit with respect to vitamin D balance and the prevention of vitamin D-related secondary complications.

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Authors' Contributions

G.K. performed all aspects of animal maintenance, preparation of experimental diets, and laboratory experiments as well as drafted the original version of this manuscript. E.M.W assisted with paraffin embedding of tissues and slide preparation, as well as histological and immunohistochemistry analyses. K.L.S. assisted with the study design. K.M., Y-T.L., K.G., and E.B. assisted in animal maintenance and laboratory procedures. M.J.R. was the principal investigator and prepared the final draft of the manuscript. All authors read and approved the final version of this manuscript.

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Table 1. Biochemical measurements LCs, DCs, and DRSs.¹

	LC	DC	DRS	P value
12h Urinary volume, mL	5.3 ± 0.1 ^b	15.3 ± 2.4 ^a	5.4 ± 0.6 ^b	<i>P</i> < 0.001
Fasting blood glucose, mg/dL	149 ± 16.4 ^c	594 ± 53.9 ^a	351 ± 46.7 ^b	<i>P</i> < 0.0001
Urinary total protein, mg/12 h	35.2 ± 7.7 ^b	183 ± 32.7 ^a	70.0 ± 14.5 ^a	<i>P</i> < 0.001
Urinary albumin, mg/12 h	0.1 ± 0.0 ^b	45.7 ± 16.9 ^a	3.1 ± 1.8 ^b	<i>P</i> < 0.01
Urinary creatinine, mg/dL	169 ± 47.5 ^a	15.3 ± 4.1 ^b	107 ± 13.1 ^{ab}	<i>P</i> < 0.01

¹Values are means ± SEM (n = 7-8). Mean values within a row without a common letter differ, *P* < 0.05. DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS: Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet.

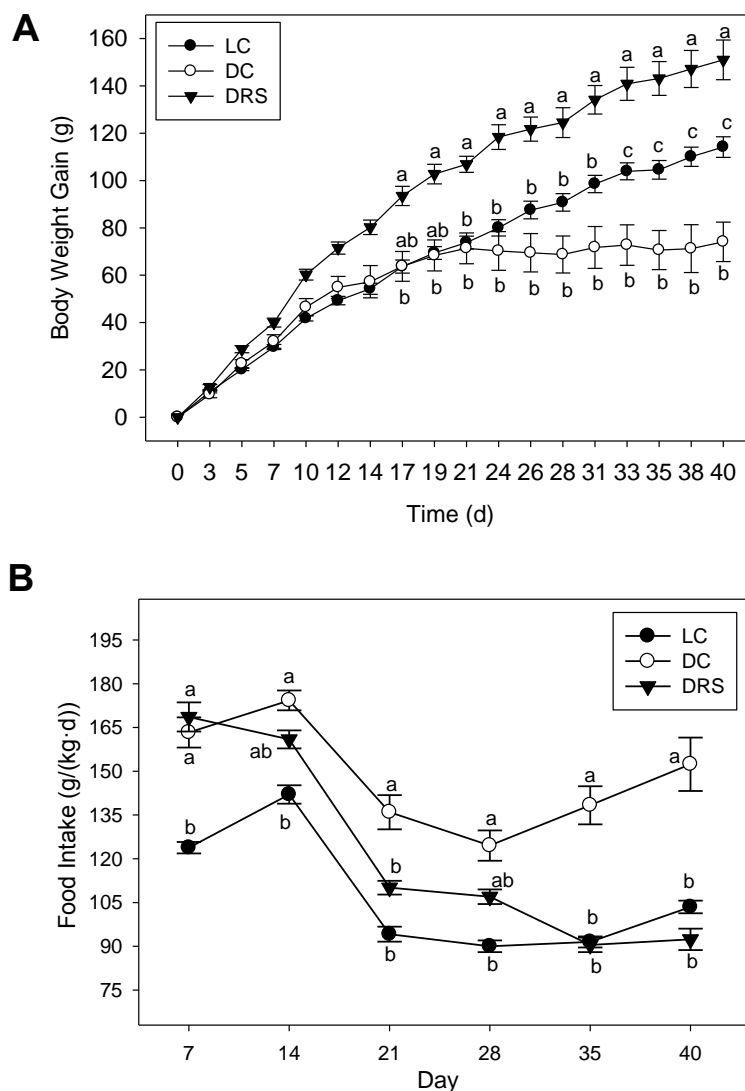


Fig. 1. Dietary resistant starch prevented growth stunting of ZDF rats. A) Cumulative body weight gain of LCs, DCs, and DRSs. Rats were fed experimental diets for 6 wk. **B)** Daily food intake (in g) of LCs, DCs, and DRSs consumed per kilogram body weight per day. Data are expressed as mean \pm SEM ($n = 8$). At each time point, values with different letters differ, $P < 0.05$. DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS: Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet; ZDF, Zucker diabetic fatty.

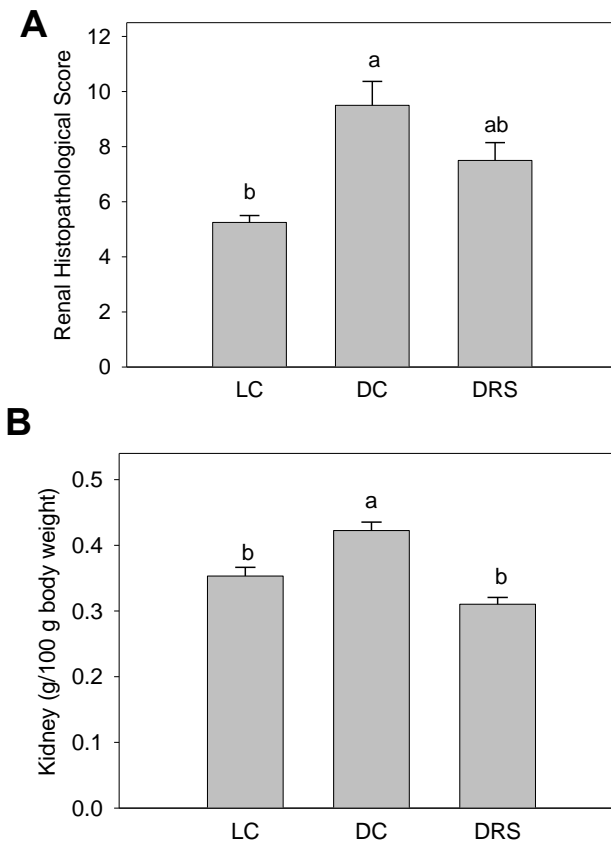


Fig. 2. Dietary resistant starch improved the renal histopathology scoring in ZDF rats. *A)* Renal histopathological scores of LCs, DCs, and DRSs. *B)* Kidney weight of LCs, DCs, and DRSs. Data are expressed as means \pm SEM ($n = 4$). Bars with different letters differ, $P < 0.05$. DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS: Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet; ZDF, Zucker diabetic fatty.

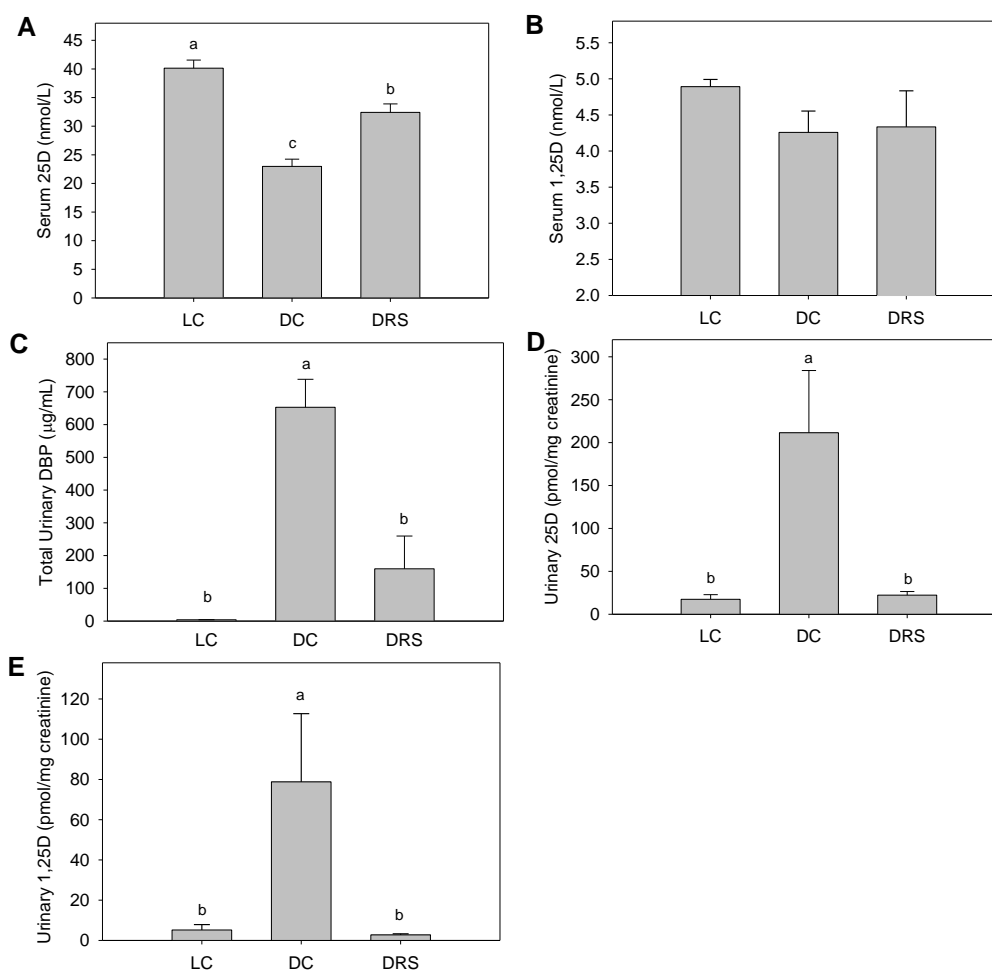


Fig. 3. Dietary resistant starch maintained circulating 25D concentrations and prevented urinary excretion of 25D, 1,25D, and DBP in ZDF rats. *A)* Serum 25D concentrations in LCs, DCs, and DRSS. *B)* Serum 1,25D concentrations in LCs, DCs, and DRSS. *C)* Urinary DBP excretion by LCs, DCs, and DRSS. *D)* Urinary 25D excretion by LCs, DCs, and DRSS. *E)* Urinary 1,25D excretion by LCs, DCs, and DRSS. Data are expressed as means \pm SEM ($n = 5 - 8$). Bars with different letters differ, $P < 0.05$. DBP, vitamin D-binding protein; DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS: Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet; ZDF, Zucker diabetic fatty; 1,25D, 1,25-dihydroxycholecalciferol; 25D, 25-hydroxycholecalciferol.

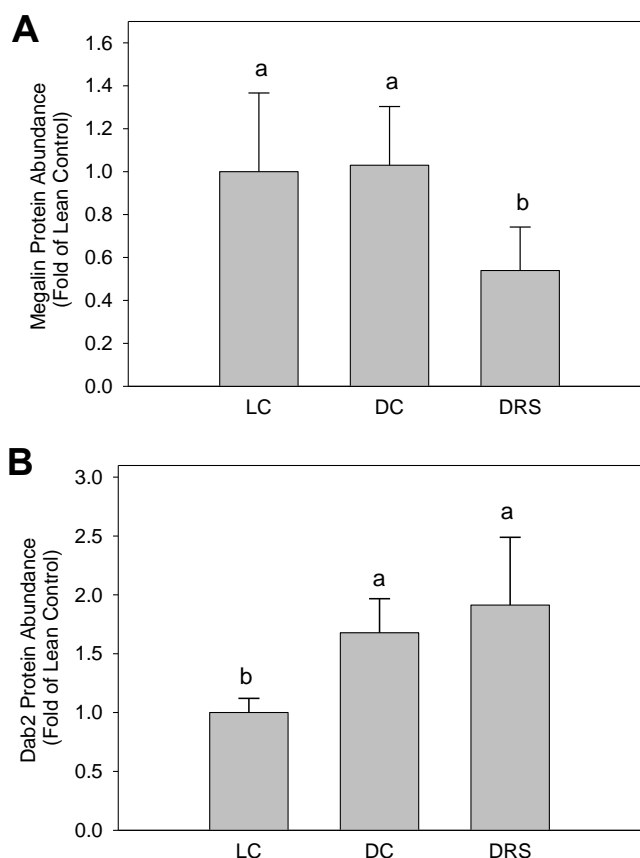


Fig. 4. Renal megalin expression was reduced and DAB2 expression was enhanced by dietary resistant starch in the kidneys of ZDF rats. A) Renal expression of megalin in LCs, DCs, and DRSs. **B)** Renal expression of Dab2 in LCs, DCs, and DRSs. Data are expressed as means \pm SEM ($n = 4$). Bars with different letters differ, $P < 0.05$. DAB2, disabled-2; DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS: Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet; ZDF, Zucker diabetic fatty.

CHAPTER 4: CONSUMPTION OF DIETARY RESISTANT STARCH FOLLOWING THE ONSET OF DIABETES DOES NOT PREVENT HYPERGLYCEMIA AND COMPROMISED KIDNEY FUNCTION

A manuscript prepared for the submission to the *Journal of Nutrition*

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Abstract

BACKGROUND: The morbidity and mortality rates of diabetes are contingent upon the development of secondary complications. Our laboratory has previously demonstrated that feeding of dietary resistant starch (RS) prior to the onset of diabetes delayed the progression of diabetic nephropathy and maintained vitamin D balance in streptozotocin (STZ)-induced T1D diabetic rats. In the present study, we conducted a dose response study with RS and assessed kidney function and vitamin D homeostasis following the onset of diabetes in STZ-treated rats.

METHODS: Male Sprague-Dawley (SD) rats were administered STZ ($n = 8/\text{group}$) and fed either a semi-purified diet (AIN-93G) containing 550 g/kg of corn starch (CS), 550 g/kg of high-amylose maize (HRS), a carbohydrate source rich in type 2 resistant starch, 275 g/kg of resistant starch + 275 g/kg of corn starch (MRS), or 138 g/kg of resistant starch + 412 g/kg of corn starch (LRS). Vehicle-treated rats fed an AIN-93G diet containing 550 g/kg of cornstarch served as a non-diabetic control group (NDC). All rats were maintained on the experimental diet for 4 wk.

RESULTS: T1D rats fed the MRS and HRS diet gained 52% and 73% more weight, respectively, than CS-fed rats. No differences in weight gain were detected between CS and LRS rats. In contrast to our previous RS intervention study, in which rats were fed RS prior to injection with STZ, RS did not modulate renal health, as indicated by urinary creatinine and albumin concentrations in T1D rats. Fasting blood glucose concentrations, hemoglobin A1c%, and fasting insulin concentrations were also not affected by RS. Despite excessive excretion of urinary 25-hydroxycholecalciferol (25D), serum 25D concentrations were significantly higher in RS-fed T1D rats compared to NDC rats. Overall, circulating IL-6 concentrations were 9 – 31% higher in RS-fed T1D rats, compared to CS rats regardless of

improved vitamin D status, and were strongly correlated with hyperglycemia ($r = 0.472$; $P = 0.02$). No changes in serum TNF- α concentrations were detected.

CONCLUSIONS: Dietary RS normalized growth patterns in T1D rats following the onset of diabetes in a dose-dependent manner despite having no effect on blood glucose and serum 25D concentrations.

KEYWORDS: *25-hydroxycholecalciferol; diabetic nephropathy; high-amylose maize; hyperglycemia; interleukin-6; Streptozotocin; type 1 diabetes; resistant starch; vitamin D*

Introduction

Diabetes is an epidemic that affects approximately 23 million Americans [1]. If left uncontrolled, diabetes typically leads to severe macrovascular and microvascular complications such as atherosclerosis, retinopathy, and neuropathy. It is the progression of such secondary complications that determines the morbidity and mortality associated with diabetes. A complication associated with diabetes that has significant implications with respect to nutritional status is nephropathy, which constitutes 44% of documented kidney failure cases in the United States according to the National Institute of Diabetes and Digestive and Kidney Disease [2]. In addition to its role in the filtration of waste and toxins, the kidney is critical for nutrient reabsorption. Thus, it is not surprising that compromised kidney function can lead to vitamin D deficiency because vitamin D, as 25-hydroxycholecalciferol (25D), is reabsorbed or activated to 1,25-dihydroxycholecalciferol (1,25D) in the kidney. Uptake of 25D by the renal proximal tubular cells is dependent on the internalization of the 25D-vitamin D binding protein (DBP) complex by the endocytic receptor proteins, megalin and cubilin, as well as the adaptor protein disabled-2 (Dab2) [3, 4]. We have previously demonstrated that excessive urinary excretion of 25D and DBP due to reduced kidney function led to compromised vitamin D status in type 2 diabetic (T2D) rats [5]. Therefore, it is not surprising that low vitamin D status has been consistently linked with diabetes [6-8]. Furthermore, several studies have demonstrated that like diabetes, suboptimal vitamin D levels are linked to an increased prevalence of bone disease, autoimmune disorders, and various types of cancer [9-11]. Because of the diverse biological actions of vitamin D, maintaining kidney health in diabetes for maintenance of vitamin D balance could

be critical to the prevention of secondary complications that are associated with low vitamin D status.

We have recently reported that the inclusion of high-amylose maize (HAM), which contains ~35% of type 2 resistant starch (RS2), as a substitute for cornstarch in the AIN-93G diet, promoted renal health and vitamin D balance in animal models of type 1 diabetes (T1D) [12] and type 2 diabetes (T2D) [13]. While T2D is characterized by insulin resistance, T1D is usually mediated by an autoimmune response and is characterized by dysfunctional or destroyed pancreatic beta cells. There are multiple reports that suggest vitamin D has an important immunomodulatory role [14-16]. In addition, low vitamin D status was associated with increased incidence of T1D [15, 16] and microalbuminuria in T1D patients [17, 18]. Although the causal relationship between vitamin D and T1D remains largely unknown, our laboratory observed that rats fed RS prior to STZ injection exhibited normal vitamin D balance and attenuated nephropathy [12]. Specifically, we found that RS-feeding reduced urinary excretion of 25D and DBP and normalized renal expression of megalin and Dab2 [12]. These findings suggest that the inclusion of RS into the diet could be an effective means to prevent aberrant vitamin D metabolism.

RS is a class of fermentable carbohydrates that has been shown to improve insulin sensitivity and reduce abdominal adiposity in obesity-related diabetes [19-21]. RS2 in particular is abundant in raw starchy vegetables and fruits such as potato and unripe banana. The source of RS2 in our previous work was high-amylose maize (HAM), which contains an *amylose-extender* mutant that allows the maize to contain a higher concentration of amylose, and thus it is more resistant to enzymatic hydrolysis [22]. RS2 from HAM is highly fermentable by the gut microbiota [23, 24] and has been shown to reduce adiposity and postprandial glycemic response in obese animals [20, 21, 25]. We have previously reported that feeding rats HAM prior to the induction of diabetes by STZ prevented nephropathy and vitamin D imbalance. The objectives of the current study, therefore, were to: 1) determine whether feeding HAM to rats following the induction of T1D could promote renal health and vitamin D balance and 2) whether these effects could be produced by feeding T1D rats lower doses of RS2 than we have utilized previously.

Materials and Methods

Animals and Diets. All procedures and protocols performed were approved by the Institutional Animal Care and Use Committee at Iowa State University and in compliance with Iowa State University Laboratory Animal Resources Guidelines. Male Sprague-Dawley (SD) rats were purchased at 4 wk of age from Harlan and were housed individually in plastic cages with free access to food and water. Diet ingredients (AIN-93G) were purchased from Harlan Teklad, whereas high-amylose maize (Amylogel®) was obtained from Cargill. To induce T1D, streptozotocin (STZ, 60 mg/kg) in 10 mM citrate buffer (pH 4.5) was injected into the intraperitoneal cavity (i.p.) of SD rats at 5 wk of age. Control rats (NDC) (n = 8) were injected with an equal volume of citrate buffer. NDC rats were then fed the AIN-93G diet, which contains cornstarch at a level of 550 g/kg diet. All STZ-treated SD rats were randomly assigned to 4 groups after STZ injection (n = 8/group) and fed AIN-93G diet, which contained either 550 g/kg of corn starch (CS), 550 g/kg of HAM (HRS), 275 g/kg of HAM + 275 g/kg of corn starch (MRS), or 138 g/kg of HAM + 412 g/kg of corn starch (LRS) for 4 weeks. All diets were prepared as previously described and RS content was analyzed as we have previously reported [12, 13]. Our analysis revealed that the HAM used for this study was 35% resistant to digestion. Thus, the final RS content in the LRS, MRS, and HRS diets was 5%, 10%, and 20%, respectively. Prior to the termination of study, all rats were fasted overnight for 12 h in individual metabolic cages and urine was collected then frozen at -20°C until analysis. Prior to the collection of blood and tissues, rats were anesthetized with a ketamine: xylazine cocktail (90: 10 mg/kg BW) that was injected i.p. Whole blood was then collected via cardiac puncture and an aliquot was kept in EDTA-coated vacutainer at 4°C for glycated hemoglobin analysis. The remaining blood was stored in a serum tube and allowed to clot at room temperature for 15 min prior to centrifugation at $2000 \times g$ for 15 min. Serum was transferred into a microcentrifuge tube and kept at -20°C until analysis. Liver and kidneys were removed, weighed, and stored at -80°C until analysis.

Assessment of blood glucose, hemoglobin A1c, and serum insulin. Blood glucose was measured using a glucometer (Bayer Healthcare) at the time of sacrifice. Hemoglobin A1c% (HbA1c%) was measured in whole blood samples within 48 h of sacrifice with a commercial

kit (Stanbio). Serum insulin concentrations were measured with a commercial ELISA kit (Millipore).

Assessment of urinary creatinine, urinary albumin, and vitamin D status. Urinary creatinine, albumin, DBP, and 25D as well as serum 25D were assessed using commercial available kits as we have reported previously [5, 12, 13]. Total urinary excretion of albumin, DBP, and 25D over 12 h was normalized to urinary creatinine as we have previously reported [5, 12, 13].

Assessment of serum pro-inflammatory markers. Serum TNF- α (R&D systems) and serum IL-6 (Thermo Scientific) were analyzed via commercial ELISA kits. Data were expressed as fold changes relative to NDC rats.

Statistical Analysis. Data were analyzed with Statistical Analysis System (SAS 9.4) using one-way ANOVA followed by a Fisher Least Square Difference (LSD) post-hoc test. Outliers were detected by Mixed Model Influential Diagnostics and removed if internal studentized residuals were outside the interval of [-2.5, 2.5]. Correlation analysis was assessed between fasting blood glucose concentrations, serum IL-6 concentrations, and serum TNF- α concentration by a Pearson Product-Moment Correlation test. Significance of all tests was set at $P \leq 0.05$.

Results

Dietary RS partially corrected the growth pattern in STZ-treated rats in a dose-dependent manner. When combined, all T1D rats gained significantly less weight than NDC rats throughout the 4 wk treatment period, (Fig. 1). CS rats gained 65% less weight than the NDC rats, but no differences in weight gain were detected between the CS and LRS. However, MRS- and HRS-fed T1D rats gained approximately 52% and 73% more weight, respectively, compared to CS rats (Fig. 1). Although RS prevented normalized growth patterns in the T1D rats in a dose-dependent manner, fasting blood glucose concentrations, hemoglobin A1c%, and insulin levels were not affected by RS diets (Table 1). Kidney weight in CS rats was 1.7-fold greater than in NDC rats, but did not differ from LRS and MRS rats. Kidney weight in

HRS rats was 20% lower than in CS rats, though it was still 36% greater than in NDC rats (Table 1). With respect to liver weight, we did not detect differences in T1D rats, regardless of RS dose (Table 1). In addition, both urinary creatinine (Supplemental Fig. 1) and albumin (Supplemental Fig. 2) in T1D rats were ~3- to 5.5-fold lower, and ~4- to 6-fold greater compared to NDC, respectively, but did not differ among the RS-fed T1D rats.

Serum 25D concentrations were elevated in T1D rats despite excessive urinary excretion of 25D and DBP. Overall, urinary excretion of DBP was 21- to 32-fold greater in T1D rats compared to NDC rats. LRS, MRS, and HRS diets reduced excretion of DBP by 15%, 36%, 32% in T1D rats, respectively, when compared to CS rats (Fig. 2A). All T1D rats excreted 3.5- to 6-fold higher amount of 25D in the urine compared to control rats, but no differences were detected between the T1D rats regardless of dietary RS content (Fig 2B). Urinary excretion of 25D and DBP had no impact on vitamin D status in T1D rats. Serum 25D concentrations in T1D rats were between 33 - 45% higher than in NDC rats regardless of dietary RS content (Fig 2C). In addition, average daily intake of vitamin D was ~2-fold higher in T1D rats compared to NDC rats (Fig 2D).

Circulating IL-6 concentrations were enhanced by dietary RS in T1D rats and correlated with hyperglycemia. Because a pro-inflammatory state is highly associated with the development of microvascular complications in diabetes, we assessed the effect of RS on the secretion of pro-inflammatory cytokines, TNF- α and IL-6 in T1D animals. Specifically, we tested whether the presence of these cytokines correlated with vitamin D status and progression of diabetic nephropathy in T1D rats. As a whole, serum IL-6 concentrations were higher in RS-fed T1D rats than in NDC and CS rats (Fig. 3A). Specifically, LRS-, MRS-, and HRS-fed T1D rats exhibited 31%, 9%, and 31% higher circulating IL-6 concentrations compared to CS rats (Fig. 3A). On the contrary, we did not detect any differences in circulating TNF- α among all groups (Fig. 3B). Additionally, circulating IL-6, but not TNF- α , correlated with hyperglycemia ($r = 0.472$; $P = 0.02$) (Fig. 4). No correlation was identified between IL-6, vitamin D status, and renal function.

Discussion

In the present study, we demonstrated that inclusion of dietary RS normalized the growth pattern of T1D rats. Because the total weight gain in both MRS- and HRS-fed T1D rats did not differ from the CS rats, we estimate that the minimal effective dose that can impact the growth of T1D rats is 10% dietary RS. Body weight fluctuation is a common symptom of T1D. Because dietary RS attenuated the catabolic nature of uncontrolled T1D in our study, which indicates that though a number of parameters did not differ between RS-treated and CS-fed T1D rats, these results suggest that RS-fed T1D rats exhibited better overall health. Yet, the growth pattern improvement in RS-fed rats was independent of hyperglycemia and vitamin D status, and was not associated with inflammation status as serum IL-6 concentrations in these rats were still significantly higher than in CS rats. However, since our study was limited to the measurement of two pro-inflammatory cytokines, TNF- α and IL-6, we recognize that a full inflammatory panel may reveal that other factors play a larger role with respect to inflammation status in our animal model.

Previously, we reported that feeding rats HAM prior to induction of T1D normalized growth patterns and attenuated urinary excretion of 25D and serum proteins [12]. Based on these observations, we hypothesized that the renoprotective effect of dietary RS may have partially contributed to the overall weight maintenance because the RS-fed T1D rats were less diabetic and therefore likely retained calories from glucose and protein. Interestingly, our results show that though growth stunting was attenuated by RS in a dose-dependent manner, growth rate appeared to be independent of kidney function since no differences were detected in urinary creatinine or urinary albumin between groups (Supplemental Fig. 1 and Fig. 2). One possibility with respect to the normalized growth pattern in T1D rats by RS is that RS influenced the secretion of glucagon-like protein 1 (GLP-1) and peptide YY (PYY), gut hormones that are known to mediate postprandial satiety. Dietary RS has been reported to enhance GLP-1 and PYY secretion *in vivo* [26]. GLP-1 has also been shown to improve beta cell proliferation and insulin secretion. Hence, GLP-1 agonists have been widely used as a co-treatment for T1D and T2D to delay the progression of diabetes and attenuate diabetic symptoms [27-29]. Therefore, it is possible that RS enhanced insulin secretion via a GLP-1-mediated pathway, which in turn could have promoted energy retention and growth in T1D rats.

Here, we reported that kidney function did not appear to be protected by RS when RS feeding was introduced following the induction of T1D. This is in contrast with our previous study where we found that RS, when fed to T1D rats prior to induction of T1D, attenuated urinary loss of vitamin D and maintained renal integrity [12]. Because RS2 is fermentable, we speculate that feeding RS before the induction of diabetes may have altered the microbial composition of the gut. We hypothesize this based on reports that SCFA production or other products of fermentation can provide protection against nephropathy [30-32]. In fact, it has been shown that modulation of the gut microflora through the supplementation of probiotics and fermentable fibers reduced the incidence of T1D [33, 34] and chronic kidney disease [35, 36], suggesting that the composition of the microbiome can, at least in part, modulate the risk of T1D development and complications that occur during its progression.

Vitamin D status, as indicated by serum 25D concentrations, was greater in all diabetic groups compared to control rats, even though T1D rats excreted higher amounts of 25D and DBP. Because we observed hyperphagia in T1D rats, a likely explanation for the increase in serum 25D concentrations is that a higher intake of vitamin D compensated for the urinary excretion of 25D. As vitamin D status has been inversely associated with the incidence of both T1 and T2D [15, 16], we expected to observe at least moderate amelioration of diabetic symptoms in rats with higher serum concentrations of 25D. Our current data showed that vitamin D status in T1D rats had no impact on circulating pro-inflammatory cytokines, fasting blood glucose, or HbA1c%. Similar to the current study, Shih et al. [37] reported that correction of vitamin D deficiency had no effect on inflammation and other diabetic symptoms in adolescents with T1D. It is possible that the onset of nephropathy or other diabetic complications promoted oxidative stress and an inflammatory state in the T1D rats used in our studies, which may have confounded the protective effect of vitamin D. In support of this concept, we showed that RS did not improve glucose metabolism in T1D rats while serum IL-6 concentrations, a pro-inflammatory cytokine, were strongly correlated with hyperglycemia. Consistent with our findings, Jaacks et al [38] reported that dietary fiber intake among youth who were diagnosed with T1D was not associated with systemic inflammation. In contrast, C-reactive protein and fibrinogen concentrations, the positive acute-phase proteins secreted in response to inflammation, were inversely correlated with fiber consumption in non-diabetic adolescents [39], indicating that

the mechanism of by which dietary fiber such as RS impacts inflammation could be dependent on glycemic status.

Taken together, we speculate that the time at which dietary RS is introduced is critical with respect to providing nephroprotection in T1D. This may also partially explain our observation that kidney health, and thus vitamin D balance, was protected in T1D rats when RS was given prior to the onset of diabetes [12]. In line with our observations, others have reported that the protective actions of 1,25D in T1D were most effective when treatment was given at weaning (prior to insulinitis onset) rather than when insulinitis was already present [40-42]. Hence, our future work will focus on the development of preventative strategies with RS that will delay or prevent the onset of diabetic nephropathy and secondary complications associated with suboptimal levels of vitamin D.

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Table 1. Glucose homeostasis and tissue weight in NDC, CS, LRS, MRS, and HRS following 4 wks of treatment¹

	NDC	CS	LRS	MRS	HRS
Fasting blood glucose, mg/dL	108 ± 8.7 ^a	598 ± 134 ^b	636 ± 95 ^b	472 ± 91 ^b	535 ± 103 ^b
Hemoglobin A1c, %	3.84 ± 0.67 ^a	6.08 ± 0.67 ^{ab}	6.51 ± 1.20 ^b	5.62 ± 1.13 ^{ab}	5.87 ± 0.63 ^{ab}
Insulin, ng/mL	1.65 ± 0.33 ^b	0.83 ± 0.07 ^a	0.72 ± 0.02 ^a	0.94 ± 0.08 ^a	0.97 ± 0.06 ^a
Liver, g/kg of body weight	2.77 ± 0.04 ^c	4.84 ± 0.42 ^{ab}	5.33 ± 0.13 ^a	4.27 ± 0.35 ^b	4.21 ± 0.27 ^b
Kidney, g/kg of body weight	0.36 ± 0.01 ^d	0.61 ± 0.05 ^{ab}	0.64 ± 0.03 ^a	0.51 ± 0.04 ^{bc}	0.49 ± 0.03 ^c

¹Data are expressed as mean ± SEM (n = 6 – 8). Mean values across the row with different letters are differ, $P \leq 0.05$. All rats were fed experimental diets for 4 weeks as indicated in Materials and Methods. NDC, non-diabetic rats fed AIN-93G diet; CS, T1D rats fed AIN-93G diet; LRS, T1D rats fed AIN-93G diet containing 5% RS; MRS, T1D rats fed AIN-93G diet containing 10% RS; HRS, T1D rats fed AIN-93G diet containing 20% RS.

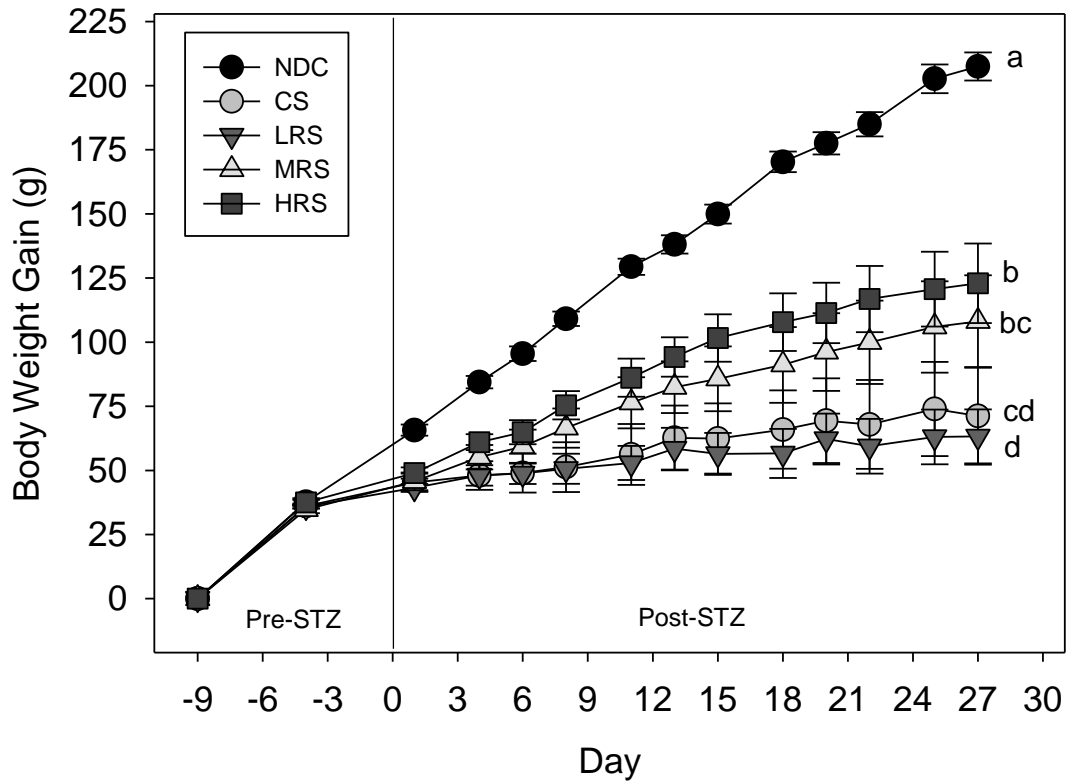


Fig. 1. Dietary resistant starch (RS) attenuated body weight loss at a dose-dependent manner in T1D rats. STZ was injected i.p. at Day 0 and all rats were fed experimental diets for 4 wks starting on Day 1 as indicated in Materials and Methods. NDC, non-diabetic rats fed a AIN-93G diet; CS, T1D rats fed AIN-93G diet; LRS, T1D rats fed AIN-93G diet containing 5% RS; MRS, T1D rats fed AIN-93G diet containing 10% RS; HRS, T1D rats fed AIN-93G diet containing 20% RS. Baseline body weight gain is indicated as pre-streptozotocin (STZ) injection. Data are means \pm SEM ($n = 8/\text{group}$). Groups with different letters differ ($P < 0.05$).

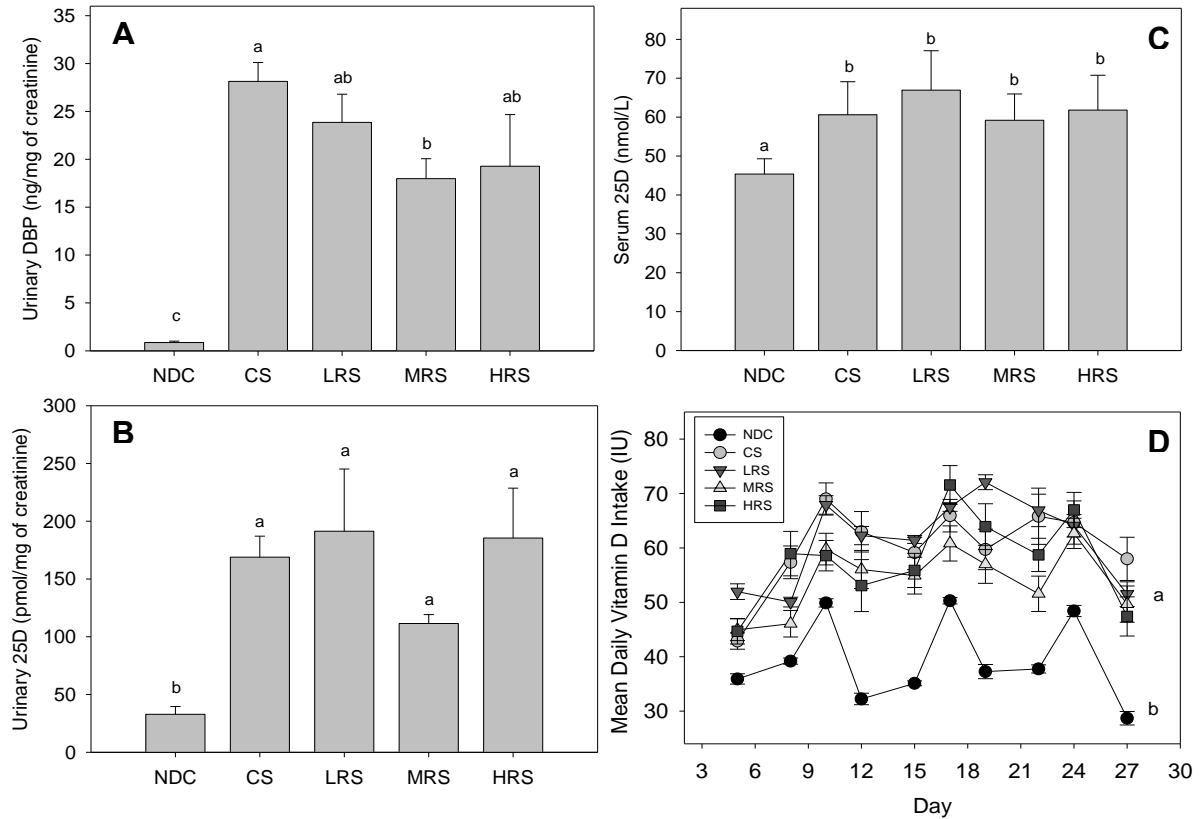


Fig 2. RS attenuated urinary DBP, but not 25D excretions in T1D rats. A) Urinary DBP, B) urinary 25D, C) serum 25D, D) mean daily intake of vitamin D in NDC, CS, LRS, MRS, and HRS rats. All rats were fed experimental diets for 4 weeks as indicated in Materials and Methods. NDC, non-diabetic rats fed AIN-93G diet; CS, T1D rats fed AIN-93G diet; LRS, T1D rats fed AIN-93G diet containing 5% RS; MRS, T1D rats fed AIN-93G diet containing 10% RS; HRS, T1D rats fed AIN-93G diet containing 20% RS. Data are means \pm SEM (n = 6-8/group). Groups with different letters differ ($P < 0.05$).

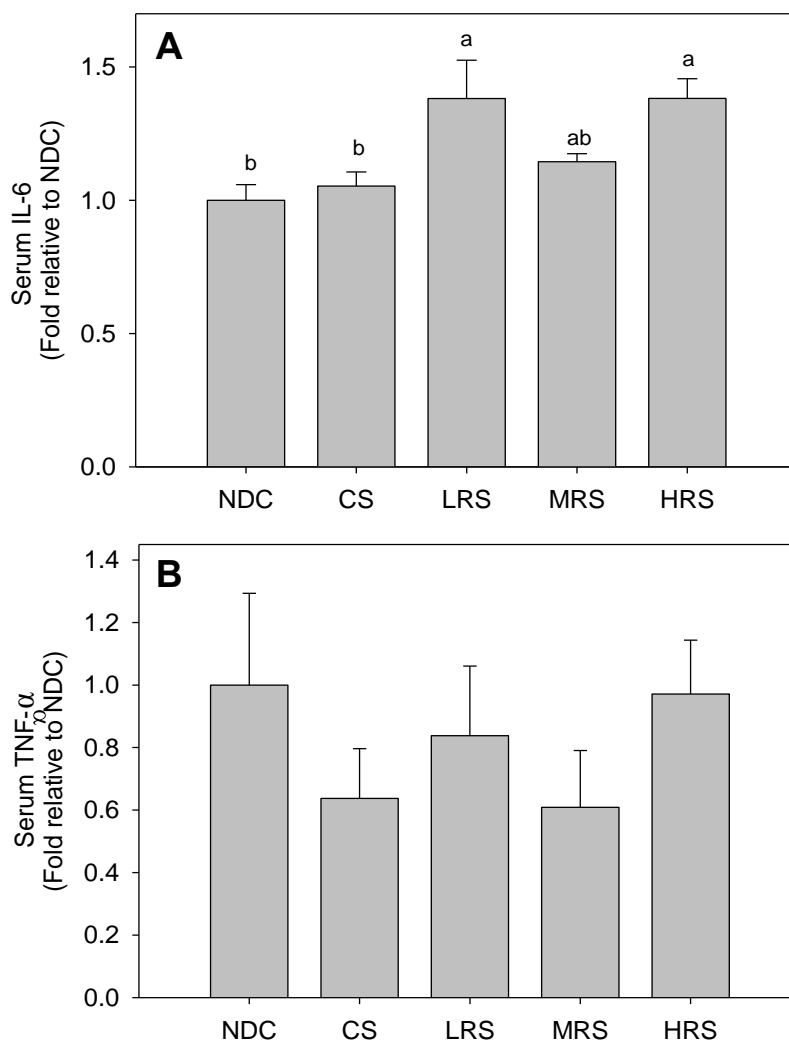


Fig 3. RS elevated circulating IL-6, but not TNF- α in T1D rats. **A)** Serum IL-6 and **B)** serum TNF- α concentration in NDC, CS, LRS, MRS, and HRS rats. All rats were fed experimental diets for 4 weeks as indicated in Materials and Methods. NDC, non-diabetic rats fed AIN-93G diet; CS, T1D rats fed AIN-93G diet; LRS, T1D rats fed AIN-93G diet containing 5% RS; MRS, T1D rats fed AIN-93G diet containing 10% RS; HRS, T1D rats fed AIN-93G diet containing 20% RS. Data are means \pm SEM (n = 5/group). Groups with different letters differ ($P < 0.05$).

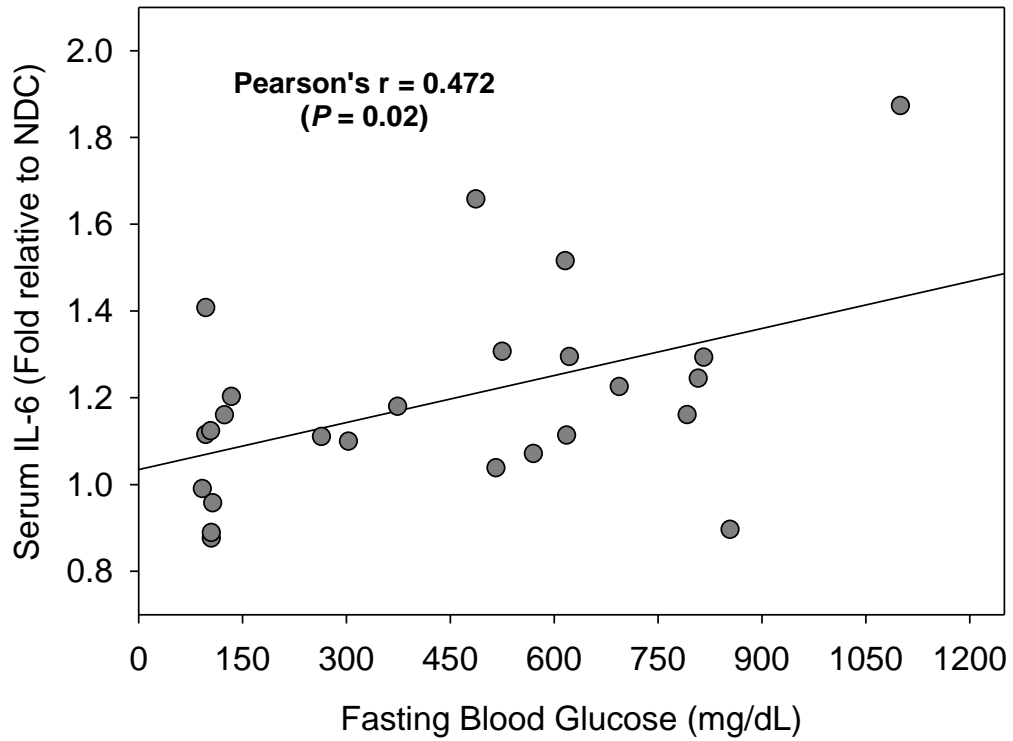


Fig 4. Circulating IL-6 concentrations were strongly correlated to fasting blood glucose concentrations. Each symbol represents data for an individual rat (N = 25).

CHAPTER 5: DIFFERENTIAL EFFECTS OF HIGH- AND LOW-DOSE DIETARY RESISTANT STARCH ON VITAMIN D HOMEOSTASIS AND RENAL FUNCTION IN ZUCKER DIABETIC FATTY RATS

A manuscript prepared for submission to the *Journal of Nutrition*

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Abstract

BACKGROUND: Diabetic nephropathy increases the risk for vitamin D deficiency due in part to the role of kidney in maintaining circulating 25-hydroxycholecalciferol (25D) concentrations. We previously reported that high-amylose maize (HAM), which contains starch that is partially resistant to digestion (RS) protected kidney health and maintained vitamin D status in Zucker diabetic fatty rats (ZDF), an experimental model of type 2 diabetes. Here, our objectives were to determine if a lower dose of RS could similarly promote kidney health and vitamin D balance, as well as to further elucidate the mechanism behind our earlier observations in RS-fed ZDF rats.

METHODS: Lean Zucker rats (n=5) were fed control diet (LC); ZDF rats (n = 5/group) were fed either control diet (DC), diet containing 10% RS (MRS), or diet containing 20% RS (HRS) for 6 wks.

RESULTS: Neither the HRS nor MRS diets attenuated blood glucose levels and hemoglobin A1c % in ZDF rats. However, serum insulin concentrations were 1.5-fold greater and HOMA- β was 2-fold greater in HRS-fed rats compared to DC rats. The increase in insulin secretion was not observed in MRS-fed rats. Additionally, the HRS, but not MRS diet, improved vitamin D status and attenuated urinary loss of vitamin D metabolites in ZDF rats. Serum triglycerides were 50% lower in HRS-fed rats compared to DC rats, despite a 2-fold increase in hepatic triglycerides. Circulating adiponectin concentrations were 77% higher in HRS-fed rats, but no difference was detected in MRS-fed rats, compared to DC rats. Furthermore, adiponectin concentrations were strongly correlated with serum 25D concentrations ($r = 0.815$, $P < 0.001$), urinary protein ($r = -0.583$, $P = 0.02$) and urinary creatinine ($r = 0.818$, $P < 0.001$), respectively. Serum angiotensin II was 44% lower, and a

14-fold increase in renal nephrin expression was observed in HRS-fed rats compared to DC rats, but no changes were detected in MRS-fed rats.

CONCLUSIONS: Here, we provide the first evidence that the maintenance of vitamin D balance and renoprotection we observed in the RS-fed ZDF rats was strongly correlated with serum adiponectin levels and reduced circulating angiotensin II concentrations, which can be achieved with 20% RS, but not at lower intake of RS.

KEYWORDS: *25-hydroxycholecalciferol; adiponectin; angiotensin II; diabetic nephropathy; high-amylose maize; renin-angiotensin system; resistant starch; type 2 diabetes; vitamin D; Zucker diabetic fatty rat*

Introduction

Type 2 diabetes (T2D) is strongly associated with the development of kidney disease and T2D patients account for ~44% of the documented renal failure cases in the United States. Diabetic nephropathy has significant implications with respect to nutrient deficiencies due to the central role of the kidney in maintaining circulating levels of numerous nutrients. We previously reported that compromised kidney function in Zucker Diabetic fatty rats (ZDF), an animal model of T2D, lead to aberrant vitamin D metabolism, mainly due to excessive urinary excretion of vitamin D metabolites [1]. Reabsorption as well as activation of 25-hydroxycholecalciferol (25D) to 1,25-dihydroxycholecalciferol (1,25D) by 1- α hydroxylase is highly dependent on the internalization of the circulating 25D-vitamin D binding protein (DBP) complex in the renal proximal tubule [2, 3]. Hence, it is speculated that diabetic-induced kidney disease may be the underlying factor of vitamin D deficiency in T2D. Moreover, suboptimal vitamin D status is highly associated with the development of secondary complications [4], which may further increase morbidity and mortality in T2D patients. Therefore, comprehensive strategies to optimize nutritional status and attenuate complications associated with diabetic kidney disease in T2D are highly warranted.

One of the common therapeutic interventions for delaying the progression of diabetic nephropathy involves the utilization of angiotensin-converting enzyme inhibitors (ACEi) [5-9], which suggests that the renin-angiotensin system (RAS) is highly involved in the promotion of diabetic complications. The RAS is a multifaceted hormonal system that is essential for regulating arterial pressure, as well as fluid and electrolyte balance. Angiotensin

II, an effector of RAS, has been shown to enhance inflammation and promote vascular dysfunction, insulin resistance, and oxidative stress during the progression of diabetes [10-14]. It is also interesting to note that treatment with ACEi and angiotensin II receptor (AGTR) inhibitors increased plasma adiponectin levels in patients with metabolic syndrome [15, 16]. Adiponectin is an adipokine secreted by adipose tissue that is inversely related to obesity [17]. In addition to the regulation of insulin sensitivity [18, 19], adiponectin has been shown to exert renoprotective effects in animal models of kidney disease [20-22]. The interaction between adiponectin and the RAS was further established when adiponectin treatment attenuated angiotensin II-mediated oxidative stress in cultured proximal tubular cells [23, 24] and delayed the progression of diabetic nephropathy in db/db mice without affecting blood glucose levels [24]. These observations indicate that adiponectin is a suppressor of the RAS. Yet, dietary interventions to target the RAS are still lacking.

We previously demonstrated that high-amylose maize (HAM), a rich source of resistant starch (RS), promoted vitamin D balance in type 1 (T1) [25] and type 2 (T2) diabetic rats [26] by attenuating symptoms of diabetic nephropathy. RS is a class of fermentable fibers with low glycemic indices. Thus, inclusion of RS into the diets of diabetics is a promising strategy. Moreover, RS has been shown to ameliorate the symptoms of obesity-related diabetes [27-29] and retard the progression of chronic kidney disease [30]. Intriguingly, our results indicate that though dietary RS2, provided by feeding high-amylose maize (HAM), protected kidney health of T2D rats, it did not prevent hyperglycemia [25, 26], which lead us to suspect that the renoprotective effect of RS in T2D may occur via suppression of RAS activity. The health benefits of low glycemic carbohydrates with respect to the kidney health and vitamin D metabolism in diabetes have been supported by work in our laboratory that demonstrated that RS attenuated nephropathy and prevented excessive urinary excretion of vitamin D metabolites [25, 26]. Thus, the objective of the present study was to determine whether the prevention of symptoms of nephropathy and maintenance of vitamin D balance in T2D that we have previously observed in RS-feeding studies could be achieved at a lower intake of dietary RS. Moreover, we determined whether RS could impact adiponectin and angiotensin II concentrations as well as downstream targets of angiotensin II in the kidney that have been linked to diabetic nephropathy.

Materials and Methods

Animals and Diets. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at Iowa State University and were conducted under the Iowa State University Laboratory Animal Resources Guidelines. All AIN-93G diet ingredients were purchased from Harland Teklad, whereas HAM (Amylogel®) was purchased from Cargill. 8 wk old male ZDF and lean Zucker rats were procured from Charles River Laboratories and housed individually in plastic cages in a room with a 12-h light-dark cycle with free access to food and water. Lean Zucker rats (n = 5) fed the AIN-93G diet containing 550 g/kg of corn starch served as the lean control group (LC). ZDF rats were randomly assigned to the three following diets (n = 5 rats/group): 1) AIN-93G diet containing 550g/kg of cornstarch (DC), 2) AIN-93G diet containing 275g/kg of cornstarch and 275g/kg of HAM (MRS), or 3) AIN-93G diet where cornstarch was replaced by 550g/kg of HAM (HRS) for 6 wk. Experimental diet preparation and subsequent RS content analyses were conducted as described previously [25, 26]. Our analysis revealed that the HAM used in this study was ~35% resistant to digestion, and thus yielded a final concentrations of 20% RS in the HRS diet and 10% RS in the LRS diet, respectively. All rats were fasted for 12 h in individual metabolic cage prior to sacrifice. Urine and fecal samples were collected during the fasting period and stored at -20°C. Rats were anesthetized with a ketamine: xylazine cocktail (90:10 mg/kg of body weight) via an intraperitoneal injection at the time of sacrifice and whole blood was collected via cardiac puncture. A portion of whole blood was collected in EDTA-coated vacutainer and stored at 4°C. The remaining blood was collected into a serum tube and subjected to centrifugation at 2000 × g for 15 min. Serum was transferred to a microcentrifuge tube and kept at -20°C until analysis. Liver, visceral fat, and whole kidneys were removed, weighed, and stored at -80°C until analysis or fixed in 10% formalin for histopathological examination.

Assessment of blood glucose, hemoglobin A1c, and serum insulin. Blood glucose concentrations were measured with a glucometer (Bayer Healthcare) immediately after blood collection. Whole blood samples were processed within 48 h from the time of sacrifice for hemoglobin A1c (HbA1c) analysis via a commercial kit (Stanbio). Serum insulin was assessed with an ELISA kit (Millipore). Pancreatic beta cell function (%) was calculated

based on the Homeostasis Model Assessment for beta-cell function (HOMA- β):

$$\text{HOMA-}\beta \text{ \%} = [360 \times \text{Insulin (mU/L)}] / \text{Glucose (mg/dL)} - 63$$

Assessment of urinary creatinine, total protein, and albumin. Urinary creatinine was analyzed with a commercial colorimetric kit (Cayman Chemical). Urinary protein was measured using the bicinchoninic acid assay (Thermo Scientific Pierce) and urinary albumin was assessed with an ELISA kit (Innovative Research) as described previously [1, 26]. Total urinary excretion of protein, and albumin over 12 h were calculated and expressed relative to urinary creatinine.

Assessment of urinary and serum 25D and 1,25D. Analysis of urinary and serum 25D were conducted using a commercial enzyme immunoassay kit (Immunodiagnostic Systems). Serum and urinary concentration of 1,25D were assessed via a commercial ELISA kit (My BioSource). Urinary excretion of 25D and 1,25D were normalized to urinary creatinine as described previously [1, 26].

Assessment of hepatic and serum triglycerides. Total hepatic lipids were extracted using the Folch method [31]. Both hepatic and serum triglyceride levels were then determined using a commercially available colorimetric triglyceride assay kit (BioAssay Systems). Hepatic triglyceride concentrations were calculated and expressed as mg triglyceride per g of liver.

Assessment of circulating adiponectin and angiotensin II. Serum angiotensin II (Sigma-aldrich) and serum adiponectin (R&D system) were analyzed with commercially available ELISA kits.

Real-time PCR. Kidney RNA was extracted and quantified by UV detection as described previously [1, 26]. Total RNA was reverse transcribed into cDNA via a Verso cDNA Synthesis Kit (Thermo Scientific). Real-time PCR reactions were conducted in duplicate at 200 ng/well of cDNA with iScript SYBR Green Detection Reagents (Bio-Rad) for the detection of angiotensin II receptor type 1a (AGTR1a), angiotensin II receptor type 2 (AGTR2), angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2),

and nephrin with an Applied Biosystems Plus® real-time PCR system (Life Technologies). Expression of each gene was normalized against 18s or GAPDH. The mRNA expression for each gene was expressed as mean fold change relative to lean control animals. The primer sequences are presented in Supplemental Table 1.

Renal Histology. To assess renal histopathological changes in animals, whole kidneys were fixed in formalin, embedded in paraffin, sectioned at 3 μ M, and subjected to hematoxylin and eosin Y staining. Scoring of kidneys was performed as described previously [26].

Statistical Analysis. Data were analyzed with the Statistical Analysis System (SAS 9.4). Data were analyzed using one-way ANOVA followed by Fisher Least Square Difference (LSD) post-hoc test. If normality failed, non-parametric analysis was used via Kruskal-Wallis one-way ANOVA by ranks. Correlation of serum adiponectin concentrations with serum 25D, urinary creatinine, and urinary protein concentrations were determined by Pearson Product-Moment Correlation. Significance of all tests was set at $P \leq 0.05$.

Results

HRS normalized the growth pattern and lowered serum triglycerides of ZDF rats independent of serum glucose concentrations and the accumulation of hepatic triglycerides. Throughout the 6 wk study, LC and HRS rats exhibited continual weight gain, whereas weight gain of DC and MRS rats plateaued by wk 5. At the end of the 6 wk study, HRS rats gained 48% and 47% more weight than DC and MRS rats, respectively (Table 1). Nonetheless, the difference in weight gain was not affected by food intake (data not shown). In addition to weight gain, fasting blood glucose concentrations in HRS rats were 20% lower and serum insulin concentrations were 1.5-fold higher compared to DC rats, though hemoglobin A1c levels (HbA1c %) were 21% higher (Table 1). To further evaluate beta cell function, HOMA- β % was calculated. HRS rats exhibited the greatest HOMA- β values (2.3- and 4.1-fold greater than in DC and MRS rats, respectively), which did not differ from LC rats (Table 1). No significant changes in blood glucose levels, HbA1c %, serum insulin levels, and HOMA- β were observed between DC and MRS rats (Table 1). Interestingly, serum triglycerides (TG) were 50% lower in HRS-fed ZDF compared to DCs despite a 2-fold

increase in hepatic TGs. In contrast, although the MRS diet attenuated serum TG as we observed in HRS rats, hepatic TG did not differ between MRS and DC rats (Table 1).

HRS improved markers of kidney function and vitamin D status in ZDF rats despite an increase in renal histopathological scoring. Total urine volume was 5-fold higher in DC than in LC rats, and no differences were detected between HRS and LC rats (Table 2). Urine volume of MRS rats was 36% lower compared to DC rats, yet it was not significantly different from DC or LC rats. Urinary creatinine concentrations from HRS rats were 90% and 76% greater than from DC and MRS rats, respectively, but were still significantly lower (55% lower) compared to LC rats (Table 2). Urinary albumin and protein concentrations from HRS rats were markedly lower compared to DC rats (99% and 94% lower, respectively) and these values were not statistically different from LC rats. In contrast, urinary albumin and protein concentrations from MRS rats did not differ from LC or DC rats. Urinary loss of both 25D (Fig. 1A) and 1,25D (Fig. 1B) was greater (40-fold and 24-fold higher, respectively) in DC rats than in LC rats. The HRS diet prevented excessive excretion of vitamin D metabolites in ZDF rats compared to DC rats as indicated by a 94% and 92% reduction in urinary 25D and 1,25D, respectively, and did not differ from LC rats (Figs. 1A and 1B). Unlike the rats fed the HRS diet, urinary 25D loss in MRS rats did not differ from DC or LC rats. Similarly, we did not detect any difference in urinary 1,25D concentrations between MRS and DC rats (Figs. 1A and 1B). Serum 25D concentrations in LC rats were the highest, where they were 36% and 58% greater than in DC and MRS rats, respectively. Serum 25D levels in HRS rats, however, did not differ from LC or DC rats. While no differences were detected in serum 1,25D concentrations between the LC, DC, and HRS rats, they were 4.6-fold lower in MRS compared to LC rats (Fig.1D). Markers of kidney function and vitamin D status were normalized in MRS and HRS rats; however, renal histopathological scores did not differ between ZDF rats regardless of dietary treatment (Supplement Fig. 1).

Circulating adiponectin concentrations were elevated in HRS rats independent of adiposity and correlated with serum 25D concentrations and urinary markers of kidney health. LC rats exhibited the lowest visceral fat content among all the groups (Fig. 2A) and serum

adiponectin concentrations in LC rats were 2.5-fold greater than in DC rats (Fig. 2B). Despite no difference in visceral fat among all ZDF rats, circulating adiponectin concentrations were 77% higher in HRS compared to DC rats, but we did not detect statistical differences between MRS and DC rats (Fig. 2B). Among all animals included in this study, serum adiponectin concentrations correlated with serum 25D levels ($r = 0.815$, $P < 0.001$) (Fig. 3A) and urinary creatinine excretion ($r = 0.818$, $P < 0.001$) (Fig. 3B), and inversely correlated with urinary protein excretion ($r = -0.583$, $P = 0.02$) (Fig. 3C).

HRS-fed ZDF rats exhibited reduced serum angiotensin II concentrations and greater renal nephrin gene expression. Serum angiotensin II concentrations in HRS-fed ZDF rats were 44% lower compared DC rats (Fig 4A). Yet, no differences were observed between LC, DC, and MRS rats. To determine the impact of RS on renal RAS activity, we analyzed renal expression of AGTR1a, an angiotensin II receptor that mediates vascular smooth muscle responses once activated by angiotensin II. MRS and HRS diets attenuated the expression of renal AGTR1a in ZDF rats by 4.5-fold and 3.2-fold, respectively, compared to DC rats (Fig 4B). No changes were detected in all groups with respect to other renal RAS markers, which included the expression of ACE, as well as ACE2 and AGTR2 that are known to counteract the effect of angiotensin II (data not shown). Due to its role in regulating glomerular permeability, we further examined the expression of nephrin. Renal nephrin mRNA expression was 14-fold greater in HRS than in DC rats and we detected no differences in nephrin expression between LC, DC, and MRS rats (Fig. 4C).

Discussion

Dietary interventions targeting T2D have largely focused on the impact of a low glycemic index diet on diabetes incidence and progression, which originally led us to design a diet that included HAM containing ~35% RS, as a strategy to prevent diabetes-associated complications. We reported that RS promoted vitamin D metabolism balance and kidney health in T1 [25] and T2 [26] diabetic rats, though both of these models remained hyperglycemic throughout the experimental period. Here, we found that feeding ZDF rats a diet containing 55% of HAM (HRS) promoted a linear growth pattern, maintained vitamin D balance, and normalized markers of kidney function in HRS-fed ZDF. Yet, we did not

observe the same level of a response, with respect to proteinuria and vitamin D balance in MRS-fed rats. Thus, based on our results it seems likely that the minimal effective dose of RS to produce these results is closer to 20% of the diet.

One possible mechanism by which the reduction of RS dosage by half (MRS) did not provide a benefit equal to the HRS diet in ZDF rats in the current study could be explained by lower production of colonic fermentation products in MRS rats. RS is a fermentable fiber that can serve as a substrate for gut microbiota production of short chain fatty acids (SCFA), such as butyrate. Indeed, intravenous administration of butyrate delayed the progression of kidney failure in rodent models of nephropathy [32, 33]. Hence, it is plausible that the amount of SCFA produced by MRS-fed ZDF rats was insufficient to provide renal protection. Similar to our observations, Robertson et al. [34] reported that consumption of 60 g of RS/d enhanced postprandial insulin sensitivity in healthy subjects but not in those who consumed 40 g of RS/d, and improvements in glucose tolerance were associated with higher plasma SCFA concentrations. Surprisingly, histopathological changes were not observed in the RS-fed rats in this study as we have observed previously [26]. We suspect that this observation can be partially explained by a difference in diabetes onset between this group of rats and the rats used in our previous study. Despite the absence of visually detectable differences in the kidney, a number of parameters that are used to assess kidney function were normalized by RS. Since we have consistently observed an improvement in various markers of kidney function and vitamin D balance in HRS-fed ZDF rats, we postulate that normalized kidney function by RS-feeding maybe attributed by a number of external factors such as systemic regulation of arterial pressure by angiotensin II, oxidative stress, and fibrosis, all of which we are currently investigating.

T2D is typically characterized by hyperinsulinemia due to increased insulin secretion by beta cells to compensate for impaired clearance of blood glucose due to a lack of insulin-mediated GLUT-4 activation. If left untreated, prolonged hyperglycemia in T2D often leads to beta cell apoptosis and an insulin-dependent condition [35]. In the present study, we observed that serum insulin concentrations among all ZDF rats were elevated by 67% - 87% compared to lean Zucker rats. However, insulin levels in HRS rats were the greatest among all treatment groups. Because our data showed that HOMA- β was 2-fold greater and insulin secretion was 1.5-fold greater in HRS rats compared to DC rats, it is possible that

HRS delayed beta cell apoptosis. This could also possibly explain the attenuation of hyperglycemia by the HRS but not the MRS diet. Additionally, we have not ruled out the possibility that reduction of fasting blood glucose in HRS-fed ZDF rats was a result of increased circulating adiponectin concentrations. In support of this concept, dietary RS improved insulin sensitivity in aged mice, which was partially attributed to increased adiponectin secretion from visceral fat [29]. It is also interesting to note that HRS rats exhibited the greatest accumulation of hepatic TGs, though serum TG concentrations were 50% lower in HRS rats compared to DC rats. Others have shown that overexpression of SREBP-1c, a known transcription factor in fatty acid metabolism that is regulated by insulin, induced hepatic lipid synthesis and down-regulated lipid oxidation enzymes [36, 37]. Although the underlying mechanisms are not fully understood, it was postulated that hepatic TG accumulation was due to the up-regulation of SREBP-1c by insulin and the inhibition of hepatic VLDL export, which in theory would result in reduced serum TG concentrations, and be consistent with our observations.

Our data indicate that neither the MRS nor the HRS diet attenuated hyperglycemia, which suggests that the protection of kidney in ZDF was mediated by other means, such as RAS activation. We postulate this because therapeutic approaches with ACEi have yielded promising results with respect to the attenuation of diabetic nephropathy [5-8], which would be consistent with our data that showed that circulating angiotensin II concentrations were suppressed by the HRS diet. Since renal expression of AGTR1a were also lowered by the HRS diet and we did not observe any changes in the expression of other RAS components, it is possible that RS may have protected kidney health in ZDF rats via the suppression of AGTR1a-induced signaling upon binding to angiotensin II. Interestingly, circulating angiotensin II and renal expression of AGTR1a were greater in LC rats compared to HRS-fed ZDF rats, indicating that the phenotypic response resulting from systemic or local suppression of angiotensin II could be dependent upon glycemic status. Moreover, since our data demonstrated that adiponectin concentrations strongly correlated with vitamin D status and inversely correlated with urinary albumin and protein, and that renal AGTR1a expression was reduced in HRS-fed ZDF rats, it is plausible that these parameters could have been modulated by adiponectin, a known suppressor of angiotensin II activity. As opposed to the HRS diet, the MRS diet modestly affected kidney function and vitamin D homeostasis.

Furthermore, circulating adiponectin and angiotensin II concentrations were not affected in MRS-fed ZDF rats, though renal AGTR1a expression was suppressed. Several studies have demonstrated that RAS inhibition was consistent with elevated plasma adiponectin concentrations in patients with metabolic syndrome [15, 16, 38]. Thus, our findings support the idea that RS modulates renal angiotensin II-induced signaling through an adiponectin-dependent mechanism. Besides, adiponectin-knockout mice exhibited increased albuminuria and podocyte injury whereas treatment with adiponectin blunted the symptoms of renal injury and attenuated angiotensin II-induced oxidative stress in an animal model of chronic kidney disease [20, 21, 23, 24]. One potential mechanism by which adiponectin could have promoted renal handling of the 25D-DBP complex in ZDF rats may be the induction of nephrin expression. Nephrin is a slit diaphragm-associated protein that is vital in maintaining renal filtration barrier. Hence, suppression of nephrin expression could be an early event in the progression of diabetic nephropathy [39]. Moreover, RAS antagonists have been shown to preserve nephrin expression and reduce albuminuria independent of the presence of glomerular lesions in diabetic animals [39, 40].

In the current study, nephrin expression was markedly elevated in HRS rats, which was accompanied by a reduction in serum angiotensin II, renal mRNA expression of AGTR1a, albuminuria, and proteinuria. Although the causal relationship remains to be elucidated, we speculate that the HRS diet could have, at least in part, prevented proteinuria and excessive loss of 25D in ZDF rats by maintaining the expression of nephrin, which may have been driven by adiponectin-mediated suppression of angiotensin II. Although the underlying mechanism is still unclear, our future work will focus on the modulation of the RAS system and perhaps an intervention involving a combination of dietary RS and ACEi, the latter which is routinely used for the amelioration of diabetic nephropathy symptoms, with the goal of providing protection against diabetic complications that are associated with suboptimal vitamin D status.

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Table 1. Biochemical measurements of LC, DC, MRS, and HRS¹

	LC	DC	MRS	HRS
Total body weight gain, g	113 ± 4.8 ^b	115 ± 15 ^b	116 ± 21 ^b	167 ± 3.1 ^a
Fasting blood glucose, mg/dL	153.8 ± 8.4 ^b	570 ± 70 ^a	585 ± 130 ^a	407 ± 43 ^{ab}
HbA1c, %	6.76 ± 0.55 ^b	7.45 ± 0.72 ^{ab}	9.05 ± 0.90 ^a	9.55 ± 0.61 ^a
Serum insulin, ng/mL	0.82 ± 0.05 ^b	2.83 ± 0.73 ^{ab}	2.51 ± 1.06 ^{ab}	4.41 ± 1.01 ^a
HOMA-β, %	88.1 ± 12.1 ^{ab}	57.6 ± 19.5 ^b	31.8 ± 10.0 ^b	132 ± 36.5 ^a
Serum triglycerides, mg/dL	63.6 ± 15.4 ^c	800 ± 17 ^a	386 ± 88 ^b	401 ± 51 ^b
Hepatic triglycerides, mg/g of liver	2.22 ± 0.08 ^c	6.98 ± 0.50 ^b	7.71 ± 1.46 ^b	13.4 ± 0.86 ^a

¹Data are expressed as mean ± SEM (n = 3 – 5). Mean values across the row with different letters are differ, $P \leq 0.05$. LC, lean Zucker rats fed the control diet; DC, ZDF rats fed control diet; MRS, ZDF rats fed diet containing 10% resistant starch; HRS, ZDF rats fed diet containing 20% resistant starch. HOMA-β, Homeostasis Model Assessment for beta-cell function; ZDF, Zucker diabetic fatty.

Table 2. Assessment of renal function in LC, DC, MRS, and HRS following 6 wks of treatment¹

	LC	DC	MRS	HRS
12-h urinary volume, mL	4.90 ± 0.68 ^b	14.5 ± 3.10 ^a	9.25 ± 1.92 ^{ab}	5.80 ± 0.72 ^b
Urinary creatinine, mg/dL	140 ± 17 ^a	6.66 ± 1.81 ^c	15.0 ± 4.2 ^c	63.6 ± 5.9 ^b
Urinary albumin, μg/mg of creatinine	0.0 ± 0.0 ^b	3.89 ± 1.74 ^a	1.56 ± 0.29 ^{ab}	0.03 ± 0.01 ^b
Urinary protein, μg/mg of creatinine	0.70 ± 0.01 ^b	20.5 ± 5.0 ^a	8.61 ± 3.54 ^{ab}	1.22 ± 0.41 ^b

¹Data are expressed as mean ± SEM (n = 4 – 5). Mean values across the row with different letters are differ, $P \leq 0.05$. LC, lean Zucker rats fed the control diet; DC, ZDF rats fed control diet; MRS, ZDF rats fed diet containing 10% resistant starch; HRS, ZDF rats fed diet containing 20% resistant starch; ZDF, Zucker diabetic fatty.

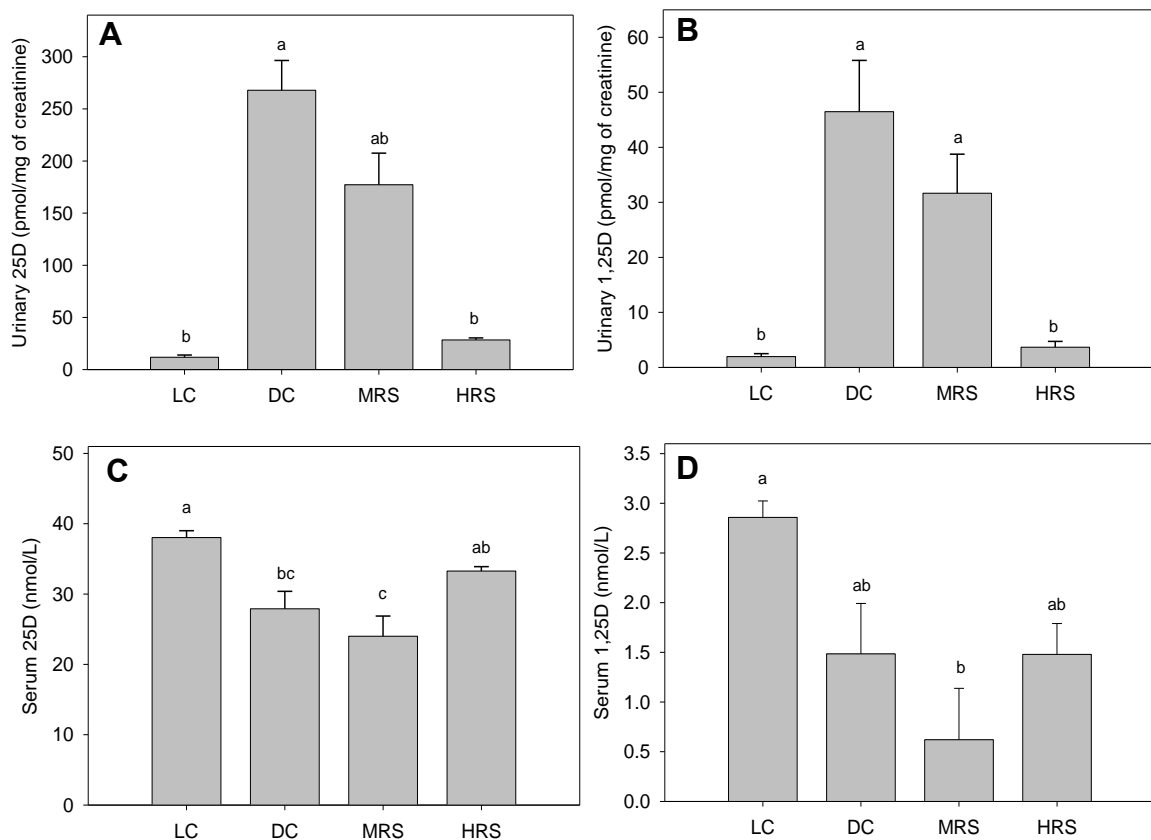


Fig. 1. HRS attenuated excessive urinary excretion of 25-hydroxycholecalciferol (25D) and 1,25-dihydroxycholecalciferol (1,25D) and maintained circulating 25D in Zucker diabetic fatty rats (ZDF). A) Serum 25D concentrations, B) serum 1,25D concentrations, C) urinary excretion of 25D, and D) urinary excretion of 1,25D. Data are means \pm SEM (n = 4) where bars with different letters differ, $P \leq 0.05$. LC, lean Zucker rats fed the control diet; DC, ZDF rats fed control diet; MRS, ZDF rats fed diet containing 10% resistant starch; HRS, ZDF rats fed diet containing 20% resistant starch; ZDF, Zucker diabetic fatty.

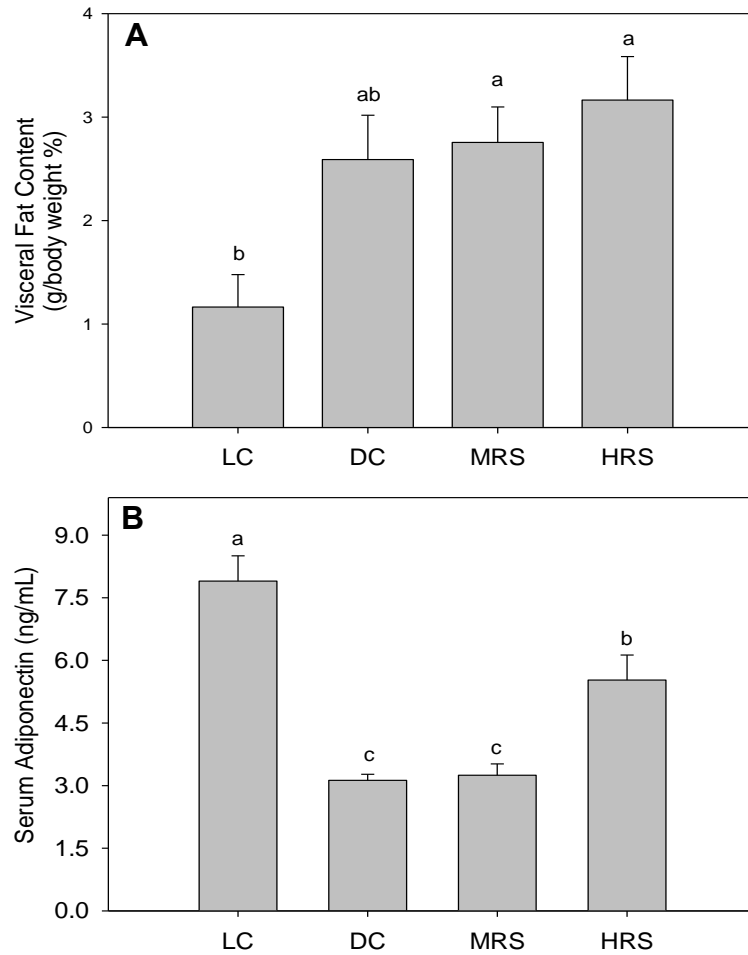


Fig. 2. HRS enhanced serum adiponectin concentrations in Zucker diabetic fatty rats (ZDF) despite no significant change in visceral fat content. A) Visceral fat content and B) serum adiponectin concentrations. Data are means \pm SEM ($n = 4 - 5$) where bars with different letters differ, $P \leq 0.05$. LC, lean Zucker rats fed the control diet; DC, ZDF rats fed control diet; MRS, ZDF rats fed diet containing 10% resistant starch; HRS, ZDF rats fed diet containing 20% resistant starch; ZDF, Zucker diabetic fatty.

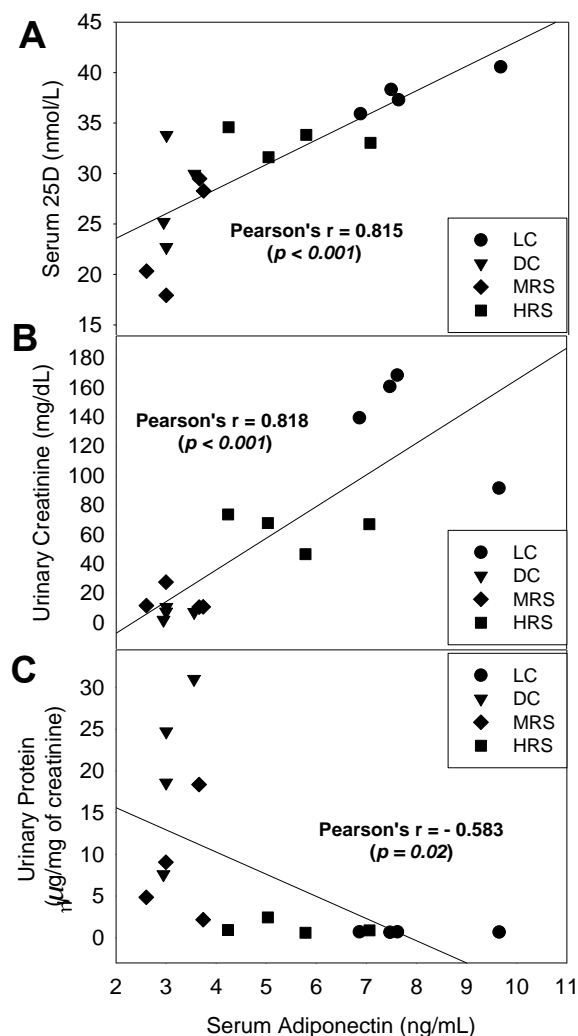


Fig. 3. Serum adiponectin strongly correlated with improved kidney function and serum 25D concentrations. Serum adiponectin concentrations correlated with A) serum 25D concentrations, B) urinary creatinine, and C) urinary protein. Each symbol represents data for an individual rat, where each group is represented by a discrete symbol ($n = 4/\text{group}$). LC, lean Zucker rats fed the control diet; DC, ZDF rats fed control diet; MRS, ZDF rats fed diet containing 10% resistant starch; HRS, ZDF rats fed diet containing 20% resistant starch; ZDF, Zucker diabetic fatty; 25D, 25-hydroxycholecalciferol.

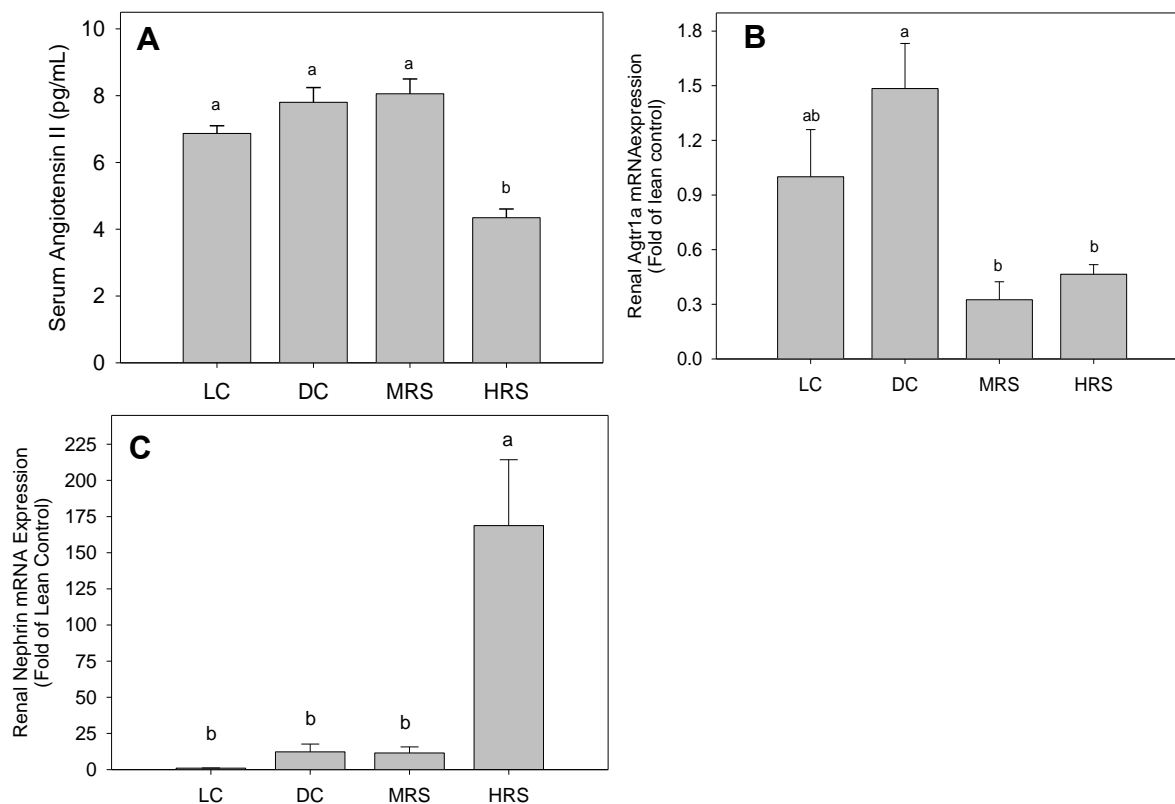


Fig. 4. HRS reduced serum angiotensin II concentrations and increased renal mRNA expression of nephrin in Zucker diabetic fatty rats (ZDF). A) Circulating angiotensin II concentrations, B) renal angiotensin II receptor type 1a (AGTR1a) mRNA expression, and C) renal nephrin mRNA expression in LC, DC, MRS, and HRS. LC, lean Zucker rats fed the control diet; DC, ZDF rats fed control diet; MRS, ZDF rats fed diet containing 10% resistant starch; HRS, ZDF rats fed diet containing 20% resistant starch; ZDF, Zucker diabetic fatty. Data are means \pm SEM (n = 3 - 5) where bars with different letters differ, $P \leq 0.05$.

CHAPTER 6: GENERAL CONCLUSIONS

Overall Summary and Conclusions

The studies presented in this dissertation have demonstrated that dietary resistant starch (RS) has a renoprotective role. In these studies, feeding RS promoted kidney health and vitamin D balance in ZDF rats, a well-established animal model of type 2 diabetes. Specifically, high-amylose maize (HAM), as a source of RS, prevented proteinuria and albuminuria, which together, prevented urinary vitamin D excretion through its protective actions in the kidney of ZDF rats. Results obtained from study two and study three suggest that the minimal effective dose of RS, based on the attenuation of diabetic nephropathy symptoms, would be between 275g/kg to 550 g/kg of HAM in the total diet, translated into 10 – 20% of RS, assuming that HAM is ~35% resistant to digestion. Although dietary RS appeared to improve kidney health, ZDF rats fed RS were still clearly diabetic as indicated by HbA1c% and fasting blood glucose levels. Hence, we believe that the delayed progression of kidney damage in ZDF rats by RS is mediated by local RAS signaling upon the stimulation of adiponectin, a mechanism that promotes the expression of nephrin, a protein central to maintaining the integrity of the renal filtration barrier (Fig. 1). Yet, we cannot exclude the possibility that RS may have a direct effect on renal RAS and nephrin expression. Interestingly, fasting insulin concentrations were significantly higher in HRS-, but not MRS-treated ZDF rats, compared to diabetic control rats. While the underlying mechanism is still uncertain, these reported observations could be a potential indication of improved pancreatic function and insulin sensitivity, which may also partially explain the elevated hepatic TG accumulation and reduced serum TG in ZDF rats.

In study 2, where we utilized STZ-induced T1D diabetic rats, we illustrated that the renoprotective effect of dietary RS is likely dependent on the stage of diabetes when RS feeding is introduced. Our laboratory has previously reported that feeding a HAM-containing diet prior to STZ injection attenuated the symptoms of diabetic nephropathy in rats that later developed diabetes following STZ treatment [1]. However, our study showed that treatment with dietary RS following the onset of diabetes did not exert similar effect on kidney function, though dietary RS promoted growth of T1D rats in a dose-dependent manner. Because RS is a fermentable fiber, it is reasonable to argue that diabetes onset altered the gut

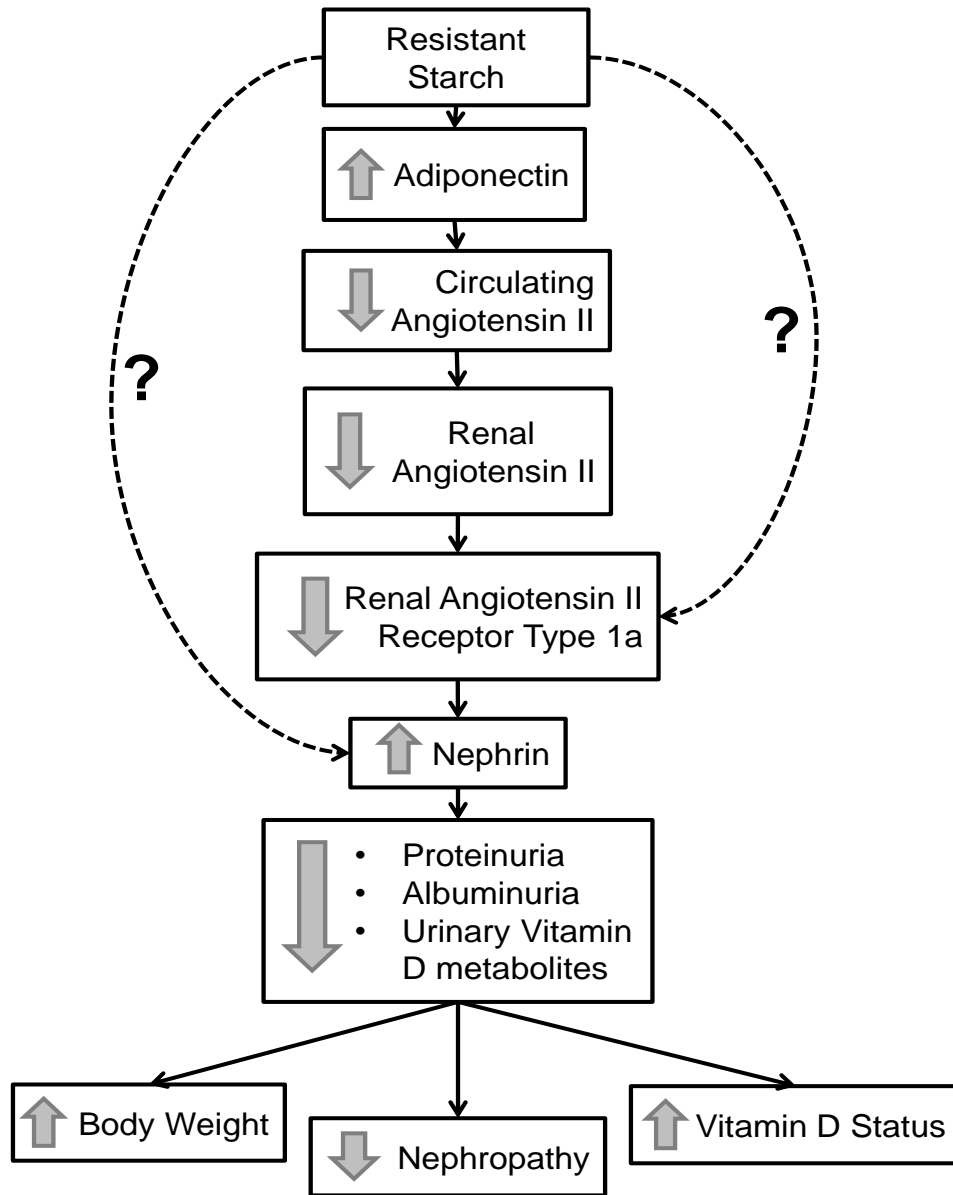


Fig. 1. Proposed renoprotective mechanism of dietary resistant starch in type 2 diabetes mediated by renal renin-angiotensin system. Direct effect of resistant starch on renal angiotensin II receptor type 1a and nephrin remained to be determined (dotted line).

microbiota composition in T1D rats that is reasonable to argue that diabetes onset altered the gut microbiota composition in T1D rats that could diminish the beneficial effect of RS in diabetic rats. In addition, the marked hyperglycemia following STZ injection may have greatly enhanced oxidative stress and inflammation status that potentiated the progression of diabetes in our T1D model. This is supported by our data that indicated that hyperglycemia

was strongly associated with serum IL-6 concentration, a pro-inflammatory cytokine, in STZ rats. We anticipate that optimal glycemic control could potentially augment the effect of RS on the kidney, and thus inclusion of RS in the diet should be introduced during the early onset of diabetes as a preventive intervention to delay diabetic complications.

Strengths and Limitations

Our novel approach in developing a dietary intervention via the utilization of dietary RS to delay the progression of diabetic nephropathy in ZDF rat model was the key strength of this study. More importantly, the ability of dietary RS to ameliorate other diabetic symptoms, such as vitamin D status, insulin sensitivity, hyperglycemia, and serum triglycerides, support its application in diabetes management.

One major limitation in the study was a low sample size that decreased the power to detect a treatment effect. Based on the current data on serum 25D concentrations (study 1), we would expect a sample size greater than 6 animals per group is needed to reach a power of 0.9. However, the sample size estimation via power analysis is limited to detecting a difference in serum 25D levels and does not account for other variables. Due to high inter-individual variation, sample size of each group should be carefully determined by using multiple variables. Another limitation involves the use of ZDF rat, a leptin-deficient model of type 2 diabetes. Because ZDF is an extreme model of T2D and leptin deficiency is rare in humans, we are uncertain whether the differences in T2D pathophysiology in humans are comparable to what we have reported in ZDF rats. Furthermore, the role of leptin signaling towards the renoprotection of RS in ZDF has been excluded in the present studies. Thus, results should be carefully addressed when it comes to translating the beneficial effect of RS. Multiple animal models, such as the use of high-fat diet induced or spontaneous diabetic animal models should be taken into consideration.

Another concern of the studies described in this dissertation was the RS dosage. On average, it has been reported that Americans only consume an average of 5 g of RS per day [2]. Our studies demonstrated that 550 g/kg of HAM exerted a protective effect in ZDF with respect to maintaining vitamin D balance, which is equivalent to the consumption of ~55 g of RS per day in a human diet of 2000 kcal/day. Our studies showed that renal health was not protected to the same degree in both the T1D or T2D animal models when RS dose was

reduced by half (10% RS or 275 g/kg of HAM). Thus, a combination approach with pharmaceutical drugs, such as ACEi or other dietary interventions may be a more effective and viable intervention. Nonetheless, the studies presented in my dissertation are perhaps the only evidence that shows that dietary RS promoted kidney health in diabetes, which in turn, normalized vitamin D metabolism in well-established diabetes models, possibly via an adiponectin-mediated angiotensin II suppression pathway.

Future research

The health benefits of dietary RS has been widely described and confirmed by many researchers, including our most recent studies targeting the renoprotective action of dietary RS and its impact on vitamin D metabolism. However, there are still questions that remain to be answered: 1) What is the mechanism underlies the elevated adiponectin concentration in HRS-fed ZDF? 2) What would be the potential mechanism by which RS enhanced insulin concentrations in ZDF rats and how would that associate with hepatic accumulation and lipid metabolism? 3) What is the potential role of vitamin D in RAS? 4) What is the potential role of short chain fatty acid production by the gut microflora in diabetic nephropathy?

Moreover, to further define the mechanism underlying the renoprotective effect of RS, *in vitro* studies that target specific tissues could be employed to examine the changes of biomarkers relevant to local RAS signaling or other possible pathways. As our current results suggest, the mechanism by which RS promotes kidney health in our diabetic models is unknown and rather complex. Future studies should consider the use of complimentary or a combination approach with RS, such as ACEi, other fiber types, and functional ingredients that have been proven effective against diabetes to optimize the protective effect of RS in diabetes mellitus and its associated complications.

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APPENDIX A: LIST OF ABBREVIATIONS

1,25D	1,25-dihydroxycholecalciferol
25D	25-hydroxycholecalciferol
ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitor
AGE	Advanced glycation end product
AGTR1a	Angiotensin II receptor type 1a
BB-DP	BioBreeding-diabetes prone
BB-DR	BioBreeding-diabetes resistant
C/EBP- α	CCAAT/enhancer binding protein- α
cAMP	Cyclin adenosine monophosphate
CKD	Chronic kidney disease
CYP27A1	Cytochrome p450, family 24, subfamily A, polypeptide 1
CYP27B1	Cytochrome p450, family 24, subfamily B, polypeptide 1
DAB2	Disabled-2
DBP	Vitamin D binding protein
DRI	Dietary reference intake
ER	Endoplasmic reticulum
FFAR	Free fatty acid receptor
FGF23	Fibroblast growth factor 23
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transporter 2

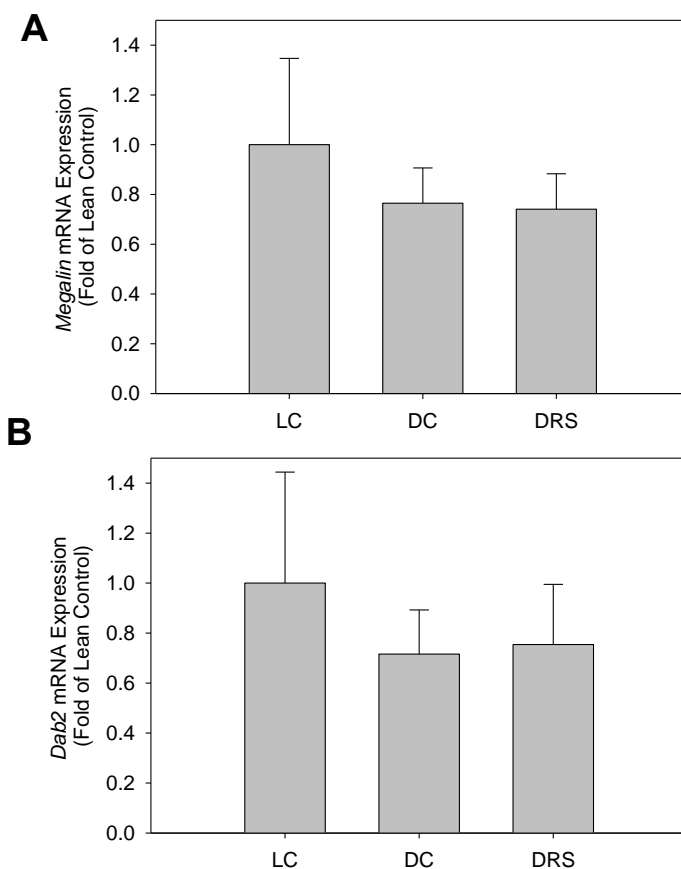
GLUT4	Glucose transporter 4
HAM	High-amylose maize
HbA1c	Hemoglobin A1c or glycated hemoglobin
HFD	High-fat diet
HLA	Human leukocyte antigen
IL-1	Interleukin-1
IL-6	Interleukin-6
KO	Knockout
IOM	Institute of Medicine
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinases
NEFA	Non-esterified fatty acid
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Non-obese diabetic
NSP	Non-starch polysaccharide
OP	Obese prone
OR	Obese resistant
PPAR- γ	Peroxisome proliferator-activated receptor- γ
PTH	Parathyroid hormone
PYY	Peptide YY
RAGE	Receptor for advanced glycation end product
RAS	Renin-angiotensin system

ROS	Reactive oxygen species
RS	Resistant starch
RXR	retinoid X receptor
SREBP-1	Sterol regulatory element-binding transcription factor 1
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
TRPV6	Transient receptor potential cation channel, subfamily V, member 6
VDR	Vitamin D receptor
VDRE	Vitamin D response element
VEGF	Vascular endothelial growth factor
ZDF	Zucker diabetic fatty

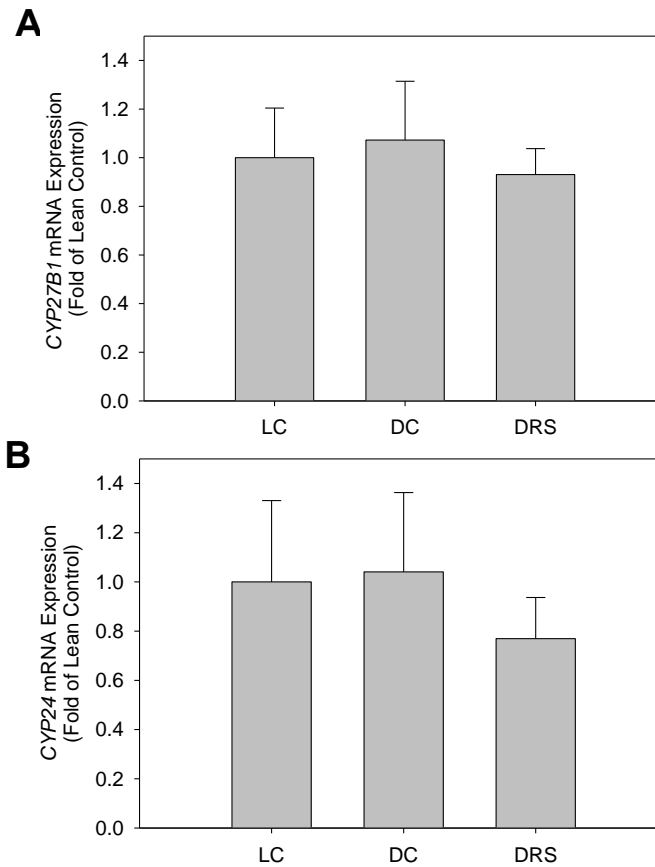
APPENDIX B: CHAPTER 3 SUPPLEMENTAL DATA

Supplemental Table 1. Composition of the control (C) and resistant starch (RS) diets.

Ingredient	C	RS
	<i>g/kg</i>	<i>g/kg</i>
Casein	200	200
Corn Starch	550	0
High-amylose maize	0	550
Glucose	150	150
Mineral mix (AIN-93G)	35	35
Vitamin mix (AIN-93G)	10	10
Corn Oil	50	50
Choline bitartrate	2	2
L-methionine	3	3

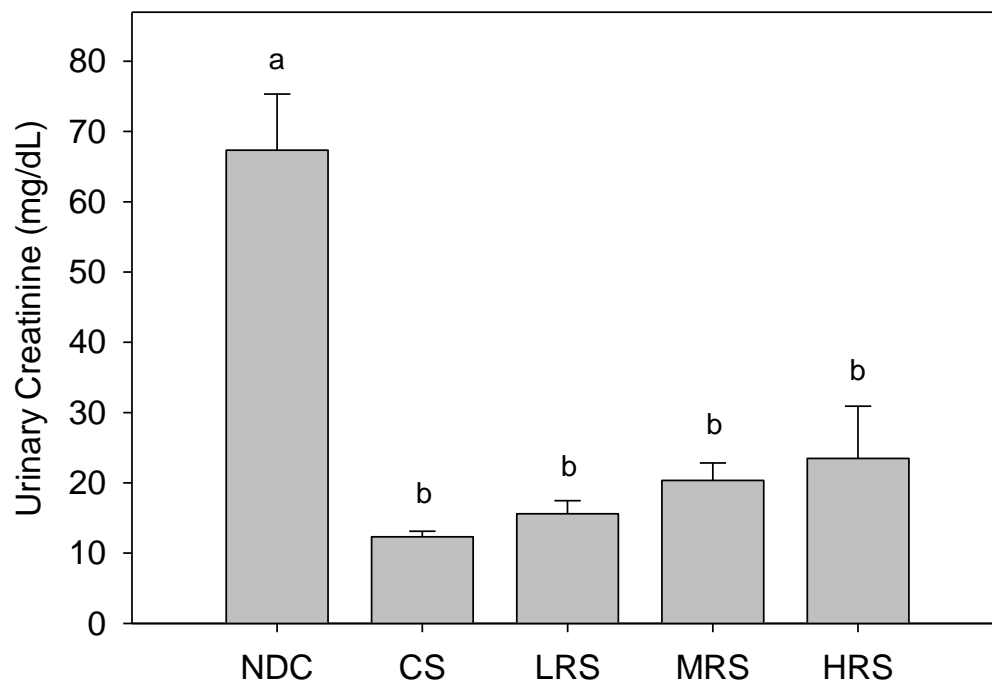


Supplemental Figure 1. Renal *megalín* and *Dab2* mRNA expression was not affected by diabetes or dietary resistant starch (RS) in ZDF. Quantitative real-time PCR analysis from the same rats as described in Figure 1 was conducted as described in Materials and Methods. **A)** Renal megalin expression in LCs, DCs, and DRSs. **B)** Renal *Dab2* in LCs, DCs, and DRSs. Mean values for the 3 treatment groups were subjected to a one-way ANOVA. Data are expressed as means \pm SEM (n = 7-8). *Dab2*, disabled-2; DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS: Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet; ZDF, Zucker diabetic fatty.

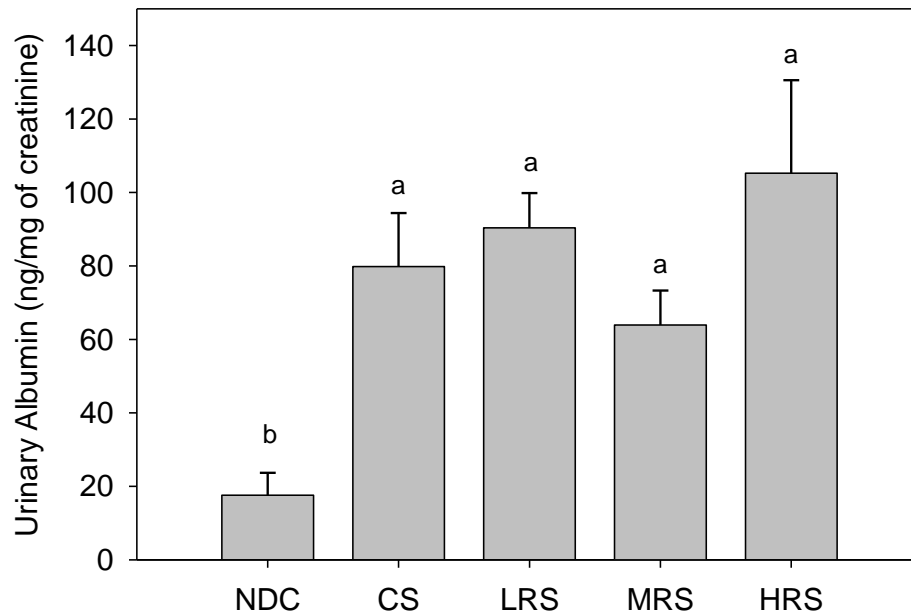


Supplemental Figure 2. Renal *CYP27B1* and *CYP24A1* expression was not affected by diabetes or dietary resistant starch (RS) in Zucker diabetic fatty rats (ZDF). A) Renal *CYP27B1* expression in LCs, DCs, and DRSs. B) Renal *CYP24A1* expression LCs, DCs, and DRSs. Mean values for the 3 treatment groups were subjected to a one-way ANOVA. Data are expressed as means \pm SEM (n = 7-8). DC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet containing 55% cornstarch; DRS: Zucker diabetic fatty rat fed a resistant starch diet in which the cornstarch was replaced with an equivalent amount of high-amylose maize; LC, Zucker diabetic fatty rat fed a standard semi-purified AIN-93G control diet; ZDF, Zucker diabetic fatty.

APPENDIX C: CHAPTER 4 SUPPLEMENTAL DATA



Supplemental Fig. 1. Urinary creatinine in T1D rats was not affected by RS. All rats were fed experimental diets for 4 weeks as indicated in Materials and Methods. NDC, non-diabetic rats fed AIN-93G diet; CS, T1D rats fed AIN-93G diet; LRS, T1D rats fed AIN-93G diet containing 5% RS; MRS, T1D rats fed AIN-93G diet containing 10% RS; HRS, T1D rats fed AIN-93G diet containing 20% RS. Data are means \pm SEM (n = 6/group). Groups with different letters differ ($P < 0.05$).

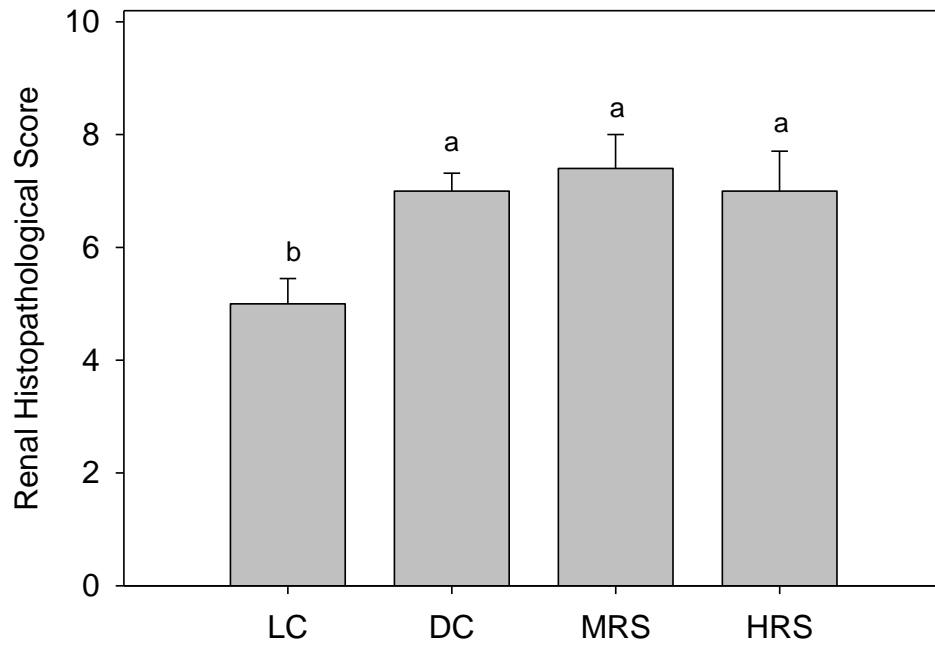


Supplemental Fig. 2. Urinary albumin in T1D rats was not affected by RS. All rats were fed experimental diets for 4 weeks as indicated in Materials and Methods. NDC, non-diabetic rats fed AIN-93G diet; CS, T1D rats fed AIN-93G diet; LRS, T1D rats fed AIN-93G diet containing 5% RS; MRS, T1D rats fed AIN-93G diet containing 10% RS; HRS, T1D rats fed AIN-93G diet containing 20% RS. Data are means \pm SEM (n = 4 – 6/group). Groups with different letters differ ($P < 0.05$).

APPENDIX D. Chapter 5 Supplemental Data

Supplemental Table 1. Primer sequences used in real time-PCR assays.

Gene	Sequence (5' to 3')
Angiotensin-converting enzyme (ACE)	Forward: CTTGACCCTGGATTGCAGCC Reverse: GTTTCGTGAGGAAGCCAGGA
Angiotensin-converting enzyme 2 (ACE2)	Forward: GAATGCGACCATCAAGCGTC Reverse: GGCTCAGTCAGCATGGAGTT
Angiotensin II receptor Type 1a (AGTR1a)	Forward: AGTCCTGTTCCACCCGATCA Reverse: TCCAGACAAAATGCCAGCCA
Angiotensin II receptor Type 2 (AGTR2)	Forward: GTAGGTTGAAGGCTCCCCAG Reverse: ATTCTGTGCTCGCTCCATCC
Nephrin	Forward: GATGAAGTGGAGAGAGCCTATG Reverse: GTGTGTGTGTGTGTGTATTG
18s	Forward: ACATCCAAGGAAGGCAGCAG Reverse: TTCGTC ACTACCTCCCCGG
GAPDH	Forward: CTCTCTGCTCCTCCCTGTTCTA Reverse: GGTAACCAGGCGTCCGATAC



Supplemental Figure 1. RS did not improve renal histopathological scoring in ZDF rats.

Data are expressed as mean \pm SEM (n = 5). LC, lean Zucker rats fed the control diet; DC, ZDF rats fed control diet; MRS, ZDF rats fed diet containing 10% resistant starch; HRS, ZDF rats fed diet containing 20% resistant starch; ZDF, Zucker diabetic fatty.