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The protective effect of resistant starch in type 1 diabetic rats

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The protective effect of resistant starch in type 1 diabetic rats

by

Alysse S. Anderegg

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

MASTER OF SCIENCE

Major: Diet and Exercise

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2014

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# TABLE OF CONTENTS

**LIST OF ABBREVIATIONS** iii

**LIST OF TABLES** viii

**LIST OF FIGURES** ix

**ABSTRACT** x

## CHAPTER 1. LITERATURE REVIEW

1. Introduction to Type 1 Diabetes Mellitus
   - Carbohydrate Metabolism and Insulin Secretion 1
   - Etiology 4
   - Type 1 Diabetes Onset 8
   - Clinical Symptoms and Disease Complications 10

2. Introduction to Vitamin D
   - Vitamin D and its Immunomodulatory Role in the Immune System 15
   - Overview of Vitamin D Metabolism 17
   - Vitamin D and Type 1 Diabetes 17

3. Introduction to Methyl Group Metabolism
   - Transmethylation 19
   - Folate-Dependent Remethylation 21
   - Folate-Independent Remethylation 22
   - Transsulfuration 22
   - Disruption of Methyl Group Metabolism and Pathological Implications 23
   - Methyl Group Metabolism and Type 1 Diabetes 25

4. Resistant Starch
   - Starch Background 27
   - Definition and Classifications of Resistant Starch 29
   - Resistant Starch: Applications in Health and Disease 29
   - Resistant Starch and Glycemic Control in Diabetes 30
   - Resistant Starch and the Gut Microbiome 30

## CHAPTER 2. RESISTANT STARCH PROMOTES REGULATION OF VITAMIN D AND METHYL GROUP METABOLISM

1. Abstract 32

2. Introduction 33

3. Materials and Methods 34

4. Statistical Analysis 37

5. Results 38

6. Discussion 39

7. Tables and Figures 45

8. General Conclusion 53

9. Literature Cited 59
LIST OF ABBREVIATIONS

1,25 (OH)\(_2\)D\(_3\): 1,25-dihydroxyvitamin D or 1,25 dihydroxycholecalciferol

25(OH)D3: 25-hydroxyvitamin D\(_3\) or calcidiol

5-CH\(_3\)-THF: 5-methyltetrahydrofolate

β-cell: beta cell

γ-cystathionase: gamma cystathionase

AB: antibiotic

ADP: adenosine diphosphate

ACAT: acyl-coenzyme A cholesterol acyltransferase

AGEs: advanced glycation end products

APC: antigen presenting cell

APS-1: autoimmune polyendocrine syndrome type 1

ATP: adenosine triphosphate

BHMT: betaine-homocysteine S-methyltransferase

Ca\(^{2+}\): calcium

CBS: cystathionine beta synthase

CD28: cluster of differentiation 28

CKD: chronic kidney disease

CS: cornstarch

CTLA4: cytotoxic T-lymphocyte antigen 4

CVB4: Coxsackievirus B4
CVD: cardiovascular disease
Dab-2: disabled-2
DBP: vitamin d binding protein
DEX: dexamethasone
DKA: diabetic ketoacidosis
DNA: deoxyribonucleic acid
DM: diabetes mellitus
ECM: extracellular matrix
ER: endoplasmic reticulum
ERK: extracellular signal-related kinase
ESRD: end stage renal disease
ETC: electron transport chain
FBG: fasting blood glucose
FFAR2/FFAR3: free fatty acid receptor 2/3
GADA: glutamic acid decarboxylase
GAMT: guanidinoacetate N-methyltransferase
GFR: glomerular filtration rate
GK: glucokinase
GLP1: glucagon like peptide-1
GLUT: glucose transporter
GNMT: glycine N-methyltransferase
GTP: guanosine-5’-triphosphate
Hemoglobin A1C: glycated hemoglobin
HLA: human leukocyte antigen
HR: hazard ratio
HTN: hypertension
IA: islet autoimmunity
IA-2: insulinoma-associated autoantibody 2
IA-2B: insulinoma-associated autoantibody 2B
IAA: islet autoantibodies
IDDM1: insulin-dependent diabetes mellitus locus
IFN: interferon
IL2Ra: interleukin-2 receptor
IPEX syndrome
LC Acyl-CoA: long-chain fatty acid coenzyme A
LDL: low density lipoprotein
LVH: left ventricular hypertrophy
LYP: lymphoid protein tyrosine phosphatase
MAPK: p38 mitogen activated protein kinase
MAT: methionine adenosyl transferase
MHC Complex: major histocompatibility complex
MS: methionine synthase
MT: methyltransferase
MTHFR: methylenetetrahydrofolate reductase
Na⁺: sodium
NADPH: nicotinamide adenine dinucleotide phosphate
NFκβ: nuclear factor kappa beta
NO: nitric oxide
NOD: non-obese model
PBMC: peripheral blood mononuclear cells
PGE₂: prostaglandin E₂
PKC: protein kinase C
PTPN22: protein tyrosine phosphatase 22
Pre-vitamin D₃: 7-dehydrocholesterol
RA: retinoic acid
RAGE: receptor for advanced glycation end products
RNA: ribonucleic acid
ROS: reactive oxygen species
RRP: ready releasable pool
RS: resistant starch
RT-PCR: real time polymerase chain reaction
SAH: S-adenosylhomocysteine
SAHH: S-adenosylhomocysteine hydrolase
SAM: S-adenosylmethionine
SCFA: short chain fatty acid
SKI: Sloan-Kettering Institute
SMAD: similar to mothers against decapentaplegic
SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SnoN: ski-related novel N
STAT-1: signal transducer and activator of transcription 1

STZ: streptozotocin

T-cell: thymus cell

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

TGF-β-1: transforming growth factor-β-1

TGIF: TG-interacting factor

THF: tetrahydrofolate

TNFα: tumor necrosis factor alpha

UAE: urinary albumin excretion

UVB: ultraviolet B

Vitamin D₃: cholecalciferol

VDR: vitamin D receptor

VNTR: variable number tandem repeat
LIST OF TABLES

Table 2.1: Diet composition. 35
Table 2.2: Real-time RT-PCR primers. 45
Table 2.3: RS diet significantly reduces weight loss associated with DM after STZ injection. 49
Table 2.4: RS diet normalizes renal GNMT activity and attenuates increased hepatic GNMT activity associated with the diabetic condition 51
Table 2.5: Hepatic mRNA abundance of one-carbon metabolism enzymes in diabetic rats fed CS and RS diets relative to control rats. 52
LIST OF FIGURES

Figure 1.1: Mechanism of glucose absorption and insulin secretion. 3
Figure 1.2: Factors leading to β cell failure in type 1 diabetes mellitus onset. 7
Figure 1.3: Autoimmune response and β cell apoptosis. 9
Figure 1.4: One carbon methyl group metabolism and folate metabolism. 19

Figure 2.1: Renal fibrosis. 42
Figure 2.2: Urine and serum creatinine concentrations. 46
Figure 2.3: RS has no effect on serum albumin concentrations in DM rats. 47
Figure 2.4: RS significantly reduces hyperglycemia associated with DM. 48
Figure 2.5: Weight gain/loss in diabetic rats fed standard CS and RS diets relative to control rats. 49
Figure 2.7: RS prevents increased urination associated with the diabetic condition and progressive decline in renal function. 50
Figure 2.7: Hepatic and renal GNMT activity. 51
Figure 2.8: Hepatic GNMT and BHMT mRNA abundance relative to the control. 52
Figure 2.9: Chronic kidney disease and hypertension contribute to cardiovascular disease risk. 54
Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by abnormal carbohydrate metabolism, insulin deficiency and subsequent hyperglycemia. Complications arise with poor glycemic control leading to the onset of microvascular and macrovascular diseases. Advanced glycation end products (AGEs) associated with hyperglycemia infiltrate microvascular tissues, ultimately leading to vascular disease of the nervous system, eyes and kidneys. Diabetic nephropathy is the leading cause of chronic kidney disease. The severity of this autoimmune disease is therefore independently and dependent associated with numerous pathologies such as cardiovascular disease, vitamin D deficiency and impaired one-carbon metabolism.

CKD is characterized by structural and functional changes of the glomerulus and renal tubules, which results in impaired filtration and reabsorption of various proteins and nutrients involved in methyl group metabolism and vitamin D metabolism. Declining glomerular filtration associated with renal disease is associated with hyperfiltration of vitamin D binding protein (DBP) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), disrupting vitamin D status and decreased homocysteine clearance and subsequent plasma homocysteine elevation. Understanding the mechanisms that mediate methyl group supply and homocysteine regulation is imperative in the prevention and treatment of these interrelated chronic diseases.

Alterations in key regulatory proteins within one-carbon metabolism have been observed in type 1 diabetes as compensatory mechanisms for disturbed homocysteine levels and methyl group supply. Previous research has demonstrated normalization of glycine N-methyltransferase (GNMT) and other regulatory proteins associated with administration of insulin, glucocorticoids and retinoic acid (RA). These results indicate the potential role of hormonal modulation in
regulating one-carbon pathways.\textsuperscript{6} In addition, we have implemented the use of resistant starch (RS) in our laboratory as a therapeutic dietary agent for glycemic control in diabetes. In these studies, we have demonstrated the ability of RS to prevent and/or alleviate many DM-related complications including weight loss, hyperglycemia and diabetic nephropathy. In addition, RS-treatment normalized gene expression of proteins involved in vitamin D metabolism.

These results have illustrated the protective effect of RS in diabetes, specifically related to diabetic nephropathy and associated perturbations in vitamin D metabolism. The goal of this study was to investigate the effect of dietary RS in preventing/attenuating abnormalities related to diabetes perturbed methyl group metabolism. Furthermore, this study aimed to explore the possibility of RS and glucose as potential hormonal and nutritional modulators in methyl group metabolism using a streptozotocin (STZ)-induced model of T1DM.
CHAPTER 1: LITERATURE REVIEW

Introduction to Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is a complex autoimmune disease that manifests from a combination of genetic, epigenetic and environmental factors. This disease is characterized by abnormal carbohydrate metabolism and subsequent hyperglycemia due to pancreatic beta (β) cell dysfunction and insulin deficiency. Traditionally referred to as juvenile diabetes, the highest incidence of this disease occurs in children between the ages of 5 and 9 years old. While this disease occurs most commonly in adolescents, diagnosis of this autoimmune disease is rising among all age groups. According to the World Health Organization, 180 million individuals are living with diabetes and approximately 5-10% (18 million) have T1DM.

Carbohydrate metabolism and insulin secretion

T1DM is a disease that targets the endocrine portion of the pancreas. Pancreatic cells pertinent to this autoimmune disease are the β cells within the islet of Langerhans. The islet cells are responsible for secreting insulin in response to carbohydrate ingestion, glucose absorption and glucose uptake into the pancreas via GLUT transporters. Insulin secretion occurs in two separate phases: 1) the triggering pathway and 2) the amplifying pathway. In a positive energy state or post-absorption, glucose is transported into the pancreatic β cells by glucose transporters (GLUT1 in humans, GLUT2 in rodents) via facilitated diffusion. Glucose is committed to various energy pathways, generating adenosine triphosphate (ATP) from glycolysis, Kreb’s cycle and the electron transport chain (ETC).

Glycolytic enzyme glucokinase (GK) and ATP contribute to pancreatic β cell signaling for insulin secretion. Insulin release by the islet cells may be governed by GK, as it participates in
the rate-limiting step in the glycolytic energy pathway.\textsuperscript{13} This enzyme is essential for committing glucose to its phosphorylated form, for its metabolism through glycolysis, the Kreb’s cycle and oxidative phosphorylation by the ETC. For this reason, GK is considered a “pacemaker” for a variety of metabolic reactions, taking substrate glucose and generating products such as pyruvate, lactate, oxaloacetate, citrate, malonyl-CoA, CO$_2$, H$_2$O and ATP.\textsuperscript{13,14}

Insulin secretion is stimulated by a series of reactions following the generation of ATP by these energy pathways. The electron flux produced in the ETC paired with the increased ATP/ADP ratio stimulates a rapid change in mitochondrial membrane potential.\textsuperscript{15} ATP-sensitive potassium (K+) channels are modulated by ATP and close in response to higher levels of this metabolic end product. Depolarization of the pancreatic β cell triggers the opening of voltage-dependent calcium (Ca$^{2+}$) and sodium (Na$^+$) channels, causing a dramatic influx of Ca$^{2+}$ and Na$^+$.\textsuperscript{12}

Increased concentrations of intracellular Ca$^{2+}$ signals soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein complexes to initiate the translocation of insulin-containing secretory vessels to the cellular membrane.\textsuperscript{16} This results in exocytosis of insulin into the bloodstream for glucose homeostasis.\textsuperscript{15} This event is classified as the triggering pathway or the 1$^{st}$ phase of insulin secretion, occurring within the first 10 minutes following glucose uptake and beta cell stimulation.\textsuperscript{16,17} Researchers have proposed that each insulin-secreting phase utilizes a specific set of insulin granules, the readily releasable pool (RRP) and storage-granule pool. Insulin granules docked on the cellular membrane of the β cell are thought to be reserved for immediate release during the 1$^{st}$ insulin-secreting phase following glucose uptake, while majority (>95%) of insulin granules reside in the intracellular storage pool.\textsuperscript{12,15,16,18}

Researchers have proposed that the second insulin-secreting phase is initiated by specific
amplifying pathways that generate products such as GTP, NADPH, Malonyl CoA, LC Acyl-CoA and Glutamate.\textsuperscript{12,17} The insulin storage-pool is stimulated by these pathways, causing the translocation of granules to the cellular membrane. This reserve pool is converted to the RRP and stimulated by the depolarized nature of the β cell. Insulin is then released during the second insulin-secreting phase. Biphasic insulin secretion allows for both a rapid and steady release of insulin into the bloodstream. Hormone insulin binds to insulin receptors throughout circulation, signaling GLUT transporters for glucose absorption. GLUT transporters are recruited from the intracellular pool and undergo translocation to the cellular membrane for glucose uptake. Therefore, insulin is a fundamental part of blood glucose homeostasis.\textsuperscript{12} This mechanism progressively declines with diabetes onset.

![Figure 1.1: Mechanism of glucose absorption and biphasic insulin secretion in beta cells.](image)

Glucose enters the islet cell via facilitated diffusion for catabolism and ATP production. Depolarization of the cellular membrane by ATP and catabolic electron flux leads to the fusion of insulin granules to the membrane for insulin secretion. This event occurs in two separate phases characterized by the location of insulin granules to the cellular membrane.\textsuperscript{12}
Etiology

T1DM manifests from a combination of genetic, epigenetic and environmental factors. Recent studies have investigated >50 genetic polymorphisms related to the development of T1DM. Individuals may possess particular alleles or mutations of the following genes most associated with the development of this chronic disease: human leukocyte antigen (HLA), insulin, protein tyrosine phosphatase 22 (PTPN22), interleukin-2 receptor (IL2Ra) and cytotoxic T-lymphocyte antigen 4 (CTLA4). These susceptibility genes are not sole determinants in disease development. Research suggests the development of T1DM is dependent on a combination of factors including age, family history, genetic susceptibility markers, number of autoantibodies and environmental triggers.

Longstanding research suggests the greatest genetic influence in T1DM development stems from two chromosomal regions: HLA and the insulin gene. A specific locus found in the HLA class II genes on chromosome 6p21 or insulin-dependent diabetes mellitus locus (IDDM1) was confirmed as a primary susceptibility gene related to the onset of various autoimmune diseases including T1DM. Specifically, recent studies have associated 30-50% of T1DM cases with the presence of HLA type II genotypes (MHC Complex). While particular haplotypes of this gene are considered predisposing alleles, others have been found to be protective in nature. HLA-DQB1*0201 and/or HLA DQB1*0302 are associated with T1DM development, whereas DRB1*15011, -DQA1*0102, and -DQB1*0602 haplotypes have the strongest negative association with the disease. Predisposing HLA haplotypes may increase the risk of T1DM development by up to 55% in adolescents. However, the protective alleles are capable of “silencing” the high-risk alleles, even in the presence of islet cell antibodies.
Individuals with first-degree relatives that possess the highest risk haplotype have >20% risk for T1DM development, while those with no known first-degree relatives with T1DM have a 2% risk. Individuals that share high-risk HLA genotypes with a diabetic sibling have an 80% risk for immunity (IA) and a 60% increased risk for T1DM development. Genetically susceptible individuals are particularly vulnerable to environmental triggers that preempt diabetes onset and lead to the production of islet autoantibodies (IAA). Presence of two or more autoantibodies may dramatically increase risk for T1DM, regardless of genetic susceptibility or family history.

Researchers have discovered a modest relationship between the insulin gene and T1DM compared to the HLA locus. Variable number tandem repeat (VNTR) polymorphisms located on the promoter region of the insulin gene on chromosome 11 may be involved in the regulation of insulin expression in the thymus. PTPN22, IL2RA and CTLA-4 susceptibility genes mediate thymus cell (T cell) regulation and other immune related processes. Polymorphisms of these genes can result in alteration of T cell function, affecting a variety of autoimmune diseases such as T1DM, rheumatoid arthritis, celiac disease and Crohn’s disease.

Researchers have recently investigated the severity of risk related to the presence of one or more of the previously mentioned polymorphisms. In a recent study, researchers worked with a combination of different loci including HLA class II genes, insulin gene, PTPN22 and CTLA4. Results revealed that the presence of these particular alleles posed a very high risk for T1DM development. However, the likelihood of an individual possessing all susceptibility genes is very low. Researchers concluded that the presence of high-risk alleles paired with a family history of T1DM leads to an increased hazard ratio (HR) for IA and progression of T1DM.

While these susceptibility genes can increase an individual’s risk for T1DM development, research strongly suggests disease onset involves an environmental trigger or a combination of
several. Detectability of this autoimmune disease begins with the presence of four major autoantibodies including insulin (IAA), glutamic acid decarboxylase (GADA), the tyrosine phosphatases: insulinoma-associated protein 2 (IA-2) and insulinoma-associated 2B (IA2B), and zinc transporter 8 (ZnT8A).\textsuperscript{28} Exposure to specific environmental factors is thought to trigger the diabetic autoimmune response. This leads to the progressive destruction of β cells, infiltration of IAA into the pancreas causing insulinitis and subsequent perturbation of carbohydrate metabolism and hyperglycemia.\textsuperscript{20} Disease manifestation is variable, lasting up to several years before symptoms are clinically diagnosed.

Several viruses have been linked to T1DM onset but there is insufficient evidence to support a causal relationship.\textsuperscript{20} The most common viruses associated with the development of T1DM are enteroviruses or coxsackieviruses. Researchers have proposed that Coxsackievirus B4 (CVB4) target β cells and cause insulinitis in genetically susceptible mice.\textsuperscript{20} Similar evidence was found in children that were previously infected with the virus and later developed T1DM. Other research studies have reinforced this positive correlation, finding the presence of enteroviruses in 44 of 72 (approximately 60\%) recently diagnosed T1DM patients and only 3 of 50 control participants.\textsuperscript{20} Congenital rubella syndrome and T1DM are positively correlated as evidenced by various research studies. Proposed mechanisms suggest that the infection inhibits β cell growth and/or increases susceptibility for DM-associated predisposing HLA haplotypes in children.\textsuperscript{20}

Collective results investigating viral environmental triggers are inconsistent, illustrating the variability associated with autoimmune diseases. Ongoing research is being conducted to investigate factors such as the amount of time between viral infections and disease onset and the presence/absence of insulinitis or autoantibodies prior to viral infection. While a virus may trigger the immediate progression of T1DM, researchers also suggest that viruses play a dormant
role, initially affecting gut mucosa and later translocating to the pancreas to cause the autoimmune event prior to T1DM onset. Further research must be conducted to better characterize these relationships.

Researchers are also investigating the role of gut microbial balance and T1DM onset. The alteration of microbiota via antibiotics and probiotics may change the immunological tolerance of the gut, which may alter the integrity of the immune system and increase an individual's risk for autoimmune diseases including T1DM. Interestingly, researchers are finding that while gut microbiota may play a role in the development of this T1DM, it may also be important in the treatment of this autoimmune disease. Proposed environmental triggers related to T1DM are plentiful, including viruses, bacteria and nutritional components such as cow’s milk, wheat proteins, meat nitrites/nitrates and vitamin D. It is unknown whether these factors work together or alone in stimulating disease progression.

Figure 1.2: Factors leading to β cell failure in type 1 diabetes mellitus onset. Symptoms leading to clinical presentation of the autoimmune disease.
Type 1 diabetes onset

Researchers have proposed many hypotheses regarding the process of T1DM onset and disease development. The most widely accepted is named the linear β-cell decline hypothesis, presented by Eisenbarth in 1986. This model illustrates the presence of genetic susceptibility markers and an environmental trigger, leading to the production of IAA, insulinitis and progressive loss of β cell mass (Figure 1.2). This event eventually leads to clinical onset as identified by uncontrolled hyperglycemia and other characteristic diabetic symptoms. 12

Beta cell mass describes the number and size of these pancreatic cells. In normal individuals, β cell mass progressively increases from birth and throughout adolescence, reaching peak mass in early adulthood and then declining with age. Diabetic individuals experience a more rapid decline in β cell mass in conjunction with IAA production and insulinitis, most often at a younger age. Clinical diagnosis occurs after the loss of 70-80% of β cell mass. The timeline for disease manifestation is largely variable, progressing over a period of months to several years depending on the individual. Clinical onset is determined by the presence of uncontrolled fasting blood glucose and other symptoms of T1DM including polyuria, polydipsia, polyphagia, weight loss, fatigue, and ketoacidosis in severe cases. 12

β cell death occurs following an autoimmune reaction that may be initiated by viral infections or inflammation causing endoplasmic reticulum (ER) stress in the pancreas. Beta cell apoptosis may then cause secretion of β-cell antigens, tumor necrosis factor alpha (TNFα) and interferon (IFN-γ). The release of these antigens from necrotic β cells stimulates antigen-presenting cells (APC’s) to activate naïve T cells in the pancreatic lymph nodes. Active T-cells will then remain in the islet cells of the pancreas, releasing inflammatory cytokines and contributing to pancreatic insulinitis upon reexposure to these β cell antigens. Inflammatory
cytokines stimulate transcription factors nuclear factor kappa beta (NFκβ) and signal transducer and activator of transcription 1 (STAT-1). This process causes ER stress, amplified release of cytokines and disruption of proteins responsible for regulating insulin production and secretion. Research also suggests that islet cells are more vulnerable to oxidative stress in comparison to other bodily tissues. Circulating cytotoxic free radicals and nitric oxide (NO) may contribute to islet cell damage. Collectively, this process seems to be a cycle of reactions leading to the progressive destruction of pancreatic β cells, insulinitis and development of type 1 diabetes.

Figure 1.3: Autoimmune response and β cell apoptosis. Induction and progression of insulitis and loss of beta cell mass by an autoimmune inflammatory cascade.
Clinical symptoms and disease complications

Increased presence of autoantibodies normally precedes the diagnostic symptoms associated with T1DM, with autoantibodies present in 85-90% of patients upon diagnosis. Clinical diagnosis of T1DM is characterized by elevated fasting blood glucose (FBG; ≥126 mg/dL) caused by impaired insulin secretion and abnormal carbohydrate metabolism. Synthetic insulin administration is needed in order to keep blood glucose in homeostatic conditions (70-100 mg/dL) and prevent common symptoms of diabetes seen in newly diagnosed patients. Uncontrolled hyperglycemia can lead to various clinical indicators including polyuria, polydipsia, polyphagia, weight loss and diabetic ketoacidosis in severe circumstances. When blood glucose exceeds the renal “glucose load” of 180 mg/dL, glucose spills over into the urine causing osmotic diuresis. Water and other solutes move from a lower concentration to a higher concentration, following large glucose molecules to be excreted as urine. Polyuria or increased urinary excretion and elevated excretion of solutes lead to dehydration and increased thirst (polydipsia). Research consensus has deemed weight loss, polyuria and polydipsia the most common symptoms associated with T1DM onset.

Aberrant carbohydrate metabolism forces the body to revert to other energy pathways to maintain body functioning, such as gluconeogenesis and lipolysis. The breakdown of triglycerides to free fatty acids leads to rapid weight loss and increased hunger (polyphagia). Prolonged hyperglycemia and dependence on lipolysis metabolism can lead to the accumulation of acetyl-CoA, leading to ketone production and elevated ketone bodies in the blood and urine. Diabetic ketoacidosis (DKA) is characterized by low blood pH, with a pH 7.1-7.35 or plasma bicarbonate 10-21 mmol/L classifying mild to moderate DKA and a pH of <7.10 and plasma
bicarbonate <10 mmol/L classifying severe DKA. Presence of DKA can dramatically increase an individual’s risk for neurological damage, hyperglycemic coma or death.

Synthetic insulin administration is the primary treatment utilized by T1DM patients to keep blood glucose at homeostasis and allow for normalized metabolism. Prolonged uncontrolled hyperglycemia can lead to a multitude of adverse health effects including both microvascular and macrovascular complications. Hemoglobin AIC is a standard biomarker used to assess long-term glycemic control, aiding in diabetes management and prevention of complications. The primary microvascular complications associated with this disease are diabetic retinopathy, nephropathy and neuropathy. These complications are preceded by biomarkers and structural changes such as increased excretion of albumin (microalbuminuria) and declining glomerular filtration rate (GFR), renal hypertrophy, declining autonomic nervous system function and altered retinal microvasculature.

Chronic hyperglycemia can drastically alter the extracellular and intracellular structure and function of many body tissues. Uncontrolled blood glucose leads to the formation of advanced glycation end products (AGEs) when glucose molecules target nucleic acids, lipids and proteins causing glycation and oxidation of these macromolecules. AGEs accumulate in vascular tissues and are therefore indicative in the pathophysiology of microvascular and macrovascular complications associated with diabetes. These compounds are capable of altering intracellular protein function, disturbing gene expression, releasing pro-inflammatory cytokines and free radicals and irreversibly modifying the extracellular matrix.

AGEs form reactive oxygen species (ROS), which bind to cellular receptors to initiate cross-linking at the extracellular matrix (ECM). AGEs may form cross-links with large matrix molecules such as collagen and elastin, causing increased surface area of the matrix and a more
rigid vasculature. Accumulation of AGEs can trigger the receptor for advanced glycation end products (RAGE), which increases endothelial permeability to larger solutes in the vasculature. RAGE also targets smooth muscle cells, macrophages and proteins related to low-density lipoprotein (LDL) oxidation. Collectively, these alterations contribute to the formation of atherosclerotic lesions, rigidity of arterial walls, decreased elasticity of blood vessels, and therefore increased risk for atheromas and myocardial infarction. These structural changes increase the incidence of hypertension (HTN) and together characterize cardiovascular disease (CVD).

Diabetic retinopathy is characterized by structural changes to the retinal basement membrane, vascular occlusion and hyperpermeability of capillaries causing vascular leakage associated with the accumulation of AGEs. Diabetic retinopathy is the leading cause of blindness, while also contributing to cataract formation, microaneurysms and hemorrhages. Diagnostic symptoms of diabetic neuropathy include pain or numbness of extremities and declining nerve and sensory motor function. Myelin is vulnerable to glycation by AGE’s in uncontrolled diabetes, causing demyelination in addition to nerve conduction occlusion.

Diabetic nephropathy is the primary cause of renal failure and is characterized by declining GFR, thickening of the glomerular basement membrane and proteinuria. Accumulation of AGEs in the renal vasculature contributes to the structural and functional changes of the glomerulus by stimulating the release of growth factors that initiate synthesis of collagen and other ECM proteins. The progressive thickening of the basement membrane eventually leads to renal filtration dysfunction, HTN and kidney failure. Structural damage to the nephrons paired with increased vascular permeability leads to hyperfiltration of small molecular weight proteins such as albumin and many other nutrients.
Declining GFR is associated with a multitude of adverse health effects including HTN, anemia, malnutrition, bone disease, neuropathy and CVD. Declining GFR and proteinuria (microalbuminuria/macroalbuminuria) are used to identify the progression of diabetic nephropathy or chronic kidney disease (CKD) to renal failure. These biomarkers are indicative of renal function and widely used in classifying the severity of chronic kidney disease by appropriate stages. Albumin is a large circulating protein, used as a sensitive biomarker for various disease states including malnutrition, diabetes, glomerular disease and HTN. Creatinine is an additional biomarker used widely to assess GFR and CKD appropriately. The ratio of albumin:creatinine is highly predictive in renal disease classification, with a ratio of >30 mg albumin/1 g of creatinine indicating kidney damage as a result of poor glycemic control and disease progression.

Urinary albumin excretion (UAE) is classified as microalbuminuria at 20-200 µg/min and macroalbuminuria at ≥200 µg/min. Albuminuria is present during the early stages of CKD with GFR between 60-90 mL/min/1.73 m². Moderate (stage 3) to severe (stage 4) CKD is classified by a GFR of 30-59 and 15-29 mL/min/1.73 m², with renal failure occurring when GFR falls below 15 mL/min/1.73 m². Normal serum creatinine concentrations are between 0.8-1.4 mg/dL in men and 0.6-1.2 mg/dL in women, with creatinine levels between 1.3-3.0 mg/dL indicating diabetic nephropathy. Collectively, GFR and related proteins are used regularly to determine disease severity in order to develop and implement appropriate pharmaceutical and behavioral treatment methods for diabetic patients.

Diabetes is the leading cause of CKD, with approximately 40% of diabetic individuals progressing from diabetic nephropathy to CKD and end stage renal failure (ESRF). CKD and subsequent HTN are strongly associated with CVD, presenting an increased risk for stroke,
peripheral artery disease, coronary heart disease, atrial fibrillation and heart failure. CVD mortality is inversely related to GFR or severity of renal impairment, accounting for 58% of deaths in CKD patients in a Canadian cohort study and 71% of deaths in a Taiwanese cohort.\textsuperscript{5}

Mechanisms linking CKD and CVD are plentiful and many are independent of HTN, diabetes and other traditional risk factors. Researchers postulate that left ventricular hypertrophy (LVH) associated with HTN, renal anemia, vascular rigidity and underexpression of coronary endothelial regulatory proteins, may be responsible for increased CVD risk. LVH can lead to reduced cardiac reserve and impaired myocardial contractility, which may be attributed to impaired coronary dilation and reduced capillary density.\textsuperscript{44}

Other factors leading to CKD-associated heart disease include dyslipidemia, inflammation, metabolic byproducts and alteration of the renin-angiotensin system.\textsuperscript{44} CKD-induced dyslipidemia results from secondary hyperthyroidism and perturbed gene regulation in lipid metabolism, resulting in downregulation of lipoprotein lipase, upregulation of lipase inhibitor Apolipoprotein C-III and reduced catabolism and clearance of lipoproteins. The disruption of low-density lipoprotein (LDL) receptor and acyl-coenzyme A cholesterol acyltransferase (ACAT) has also been correlated with the impaired lipid metabolism and accumulation of LDL-cholesterol in the serum. Together, these alterations lead to LDL oxidation in blood vessels, leading to the inflammatory cascade that preempts the formation of atheromas.\textsuperscript{45}

Increased oxidative stress is characterized by elevated levels of ROS and is associated with the accumulation of AGEs in diabetes and activation of the renin-angiotensin system in CKD.\textsuperscript{16} Angiotensin II stimulates enzymatic systems such as NADPH oxidase, generating ROS, inflammatory cytokines and chemokines, and other molecules involved in the atherosclerotic inflammatory cascade.\textsuperscript{5} Circulating ROS directly affects endothelial function, resulting in
functionality changes and hyperpermeability of the vascular endothelium. This allows for the passage of LDL molecules into the blood vessels for lipid oxidation, further stimulating inflammatory processes and the formation of atherosclerotic lesions.\textsuperscript{46}

CKD can lead to various health effects aside from CVD, including anemia, bone and mineral disease and malnutrition.\textsuperscript{47} Declining renal function causes hyperfiltration of proteins and other nutrients such as vitamin D. Increased urinary excretion of 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}; calcitriol), the major circulating form of this nutrient, leads to decreased calcium absorption and subsequent hypocalcemia, secondary hyperparathyroidism and renal osteodystrophy.\textsuperscript{46} The role of vitamin D associated with T1DM is extensive, as deficiency of this nutrient may associate with disease onset and/or be affected by the disease itself.

\textbf{Introduction to Vitamin D}

Vitamin D deficiency is associated with many pathologies including CVD, osteoporosis and other adverse bone diseases, cancer, neurological disorders, CKD and autoimmune diseases. A multitude of research has been geared toward understanding the relationship between vitamin D deficiency and these known pathological conditions.\textsuperscript{49} Current literature supports the link between vitamin D deficiency and T1DM. Interestingly, research has investigated two scenarios regarding this relationship. Does the autoimmune disease cause vitamin D deficiency or does the nutrient deficiency exacerbate the autoimmune event that leads to the onset of this disease?

\textbf{Vitamin D and its immunomodulatory role in type 1 diabetes progression}

Vitamin D plays an immunomodulatory role in the prevention of this autoimmune disease as evidenced by the presence of the vitamin D receptor (VDR) in APC’s, active T cells and pancreatic β cells. Studies using a non-obese diabetic (NOD) model of T1DM, found that vitamin D deficient mice were more likely to develop the disease. The autoimmune event was
exacerbated in mice that were vitamin D deficient earlier on, suggesting that nutrient homeostasis may be imperative during childhood, especially in individuals with genetic predisposition to the disease. Additionally, vitamin D supplementation in NOD mice reduced the severity of symptoms characteristic of the diabetic state, preserved β cell function and prevented/attenuated clinical onset. While many studies have shown supplementation to be beneficial, others have proven these positive effects are only achievable if the rodent or individual is initially vitamin D deficient.

The mechanism by which vitamin D deficiency stimulates T1DM progression involves a variety of processes regulated by VDR in the pancreas and 1,25 (OH)₂D₃ throughout the immune system. 1,25 (OH)₂D₃ exhibits protective effects in the pancreatic islet cells, inhibiting the production of inflammatory cytokines and chemokines, increasing regulatory immune cells, decreasing T cell activation and infiltration and downregulating MHC II proteins involved in cytotoxic T cell recruitment. Additionally, 1,25 (OH)₂D₃ prevents beta cell apoptosis through several mechanisms including 1) downregulation of A20 protein gene related to NO production and the subsequent production of inflammatory factors 2) stimulation of dendritic cell turnover, preventing differentiation and maturation into APC’s that preempts the autoimmune event observed T1DM onset and 3) direct inhibition of IL-6 inflammatory cytokines. A 2004 study implemented a vitamin D deficient diet in pregnant NOD mice, acclimated offspring to this diet following birth for 100 days and analyzed the immune system. Vitamin D deficient NOD mice were found to have extreme abnormalities in macrophage cytokine profiles and elevated circulating proinflammatory factors, leading to aggressive disease progression. Given its imperative immunological role, vitamin D may act as an environmental modulator in the prevention or progression of T1DM.
**Overview of vitamin D metabolism**

Vitamin D₃ (cholecalciferol) can be obtained in the diet or be synthesized in the skin via ultraviolet radiation. Ultraviolet B (UVB) photons penetrate the epidermis and dermis layers of the skin to activate pre-vitamin D₃ (7-dehydrocholesterol) to vitamin D₃. Vitamin D₃ is transported through circulation via vitamin D binding protein (DBP) for hydroxylation in the liver, resulting in 25-hydroxyvitamin D₃ (25(OH)D₃; calcidiol), the major circulating form of vitamin D in the body. Clinically, this form of vitamin D is the best reflection of vitamin D status. DBP then transports 25(OH)D₃ to cellular tissues for uptake and utilization.¹³ In the kidney, the DBP-25(OH)D₃ complex is filtered across the glomerulus where it then binds to the cellular membrane of the proximal renal tubule, activating endocytic receptors megalin, cubilin and disabled-2 (dab-2) proteins for reabsorption. There, 25(OH)D₃ is further hydroxylated by 1α-hydroxylase to form biologically active 1,25(OH)₂D₃.⁵⁴

**Vitamin D and type 1 diabetes**

Structural and functional changes in the kidneys associated with diabetic nephropathy can dramatically alter filtration and appropriate reabsorption of nutrients, affecting vitamin D status. The megalin-cubilin complex is responsible for reabsorption of many proteins and nutrients including DBP and 25(OH)D₃. Previous studies using megalin-knockout mice observed increased excretion of DBP, 25(OH)D₃, albumin and other low-molecular weight proteins.⁵⁴ Similar results have been observed in T1DM animal models, with positive correlations between chronic hyperglycemia and DBP excretion.⁴ Streptozotocin (STZ)-induced models of T1DM have confirmed these hypotheses, observing decreased expression of megalin in the proximal tubules and increased urinary excretion of megalin, cubilin and DBP.⁵⁵
Additional studies have observed increased expression of renal 1α-hydroxylase as a compensatory mechanism to maintain vitamin D status in diabetic rodent models. However, the upregulation of this hydroxylase enzyme was typically associated with decreased 1,25(OH)D₃ concentrations due to 25(OH)D₃ deficiency, the substrate for 1,25(OH)D₃ activation. Collectively, the results suggest that the progression of diabetic nephropathy leads to the downregulation and excretion of endocytic receptors, causing the impaired reabsorption of DBP and 25(OH)D₃, resulting in vitamin D deficiency.⁵⁴

**Introduction to Methyl Group Metabolism**

Folate, homocysteine and methyl group metabolism are interrelated pathways that encompass one-carbon metabolism.⁵⁶ Together these pathways regulate methyl group supply for a multitude of cellular pathways and metabolic reactions. One-carbon methyl groups are essential for over 100 transmethylation reactions and play a key role in DNA methylation and regulation of gene expression. The disruption of these pathways is related to a variety of pathologies including birth defects, CVD, osteoporosis, neurological disorders, cancer, metabolic syndrome and vascular diseases.⁵⁷,⁵⁸ Understanding the mechanism by which these pathways are perturbed may provide a sound link between chronic disease and associated complications.

Methionine, folate, betaine and choline act as methyl donors, transferring one-carbon units throughout four regulating pathways within methyl group metabolism. Transmethylation, transsulfuration, folate-independent remethylation and folate-dependent remethylation mediate methyl group supply and homocysteine metabolism. These pathways are regulated by a variety of mechanisms to fuel transmethylation reactions that require an adequate supply of methyl groups. This section of the literature review will summarize each of these pathways and highlight the key metabolic steps affected by the diabetic state.
Figure 1.3: One carbon methyl group metabolism and folate metabolism. Methionine is converted to homocysteine in the transmethylation pathway. Homocysteine can be remethylated to methionine via folate-dependent or folate-independent mechanisms.\textsuperscript{59}

**Transmethylation**

The transmethylation pathway is present in most bodily tissues, beginning with an ATP-dependent reaction catalyzed by methionine adenosyltransferase (MAT). MAT is a tissue-specific enzyme that is expressed by three separate isoforms. MAT I and III are solely expressed in the liver, while MAT II is functional in most tissues.\textsuperscript{60} This enzyme donates an adenosyl group to the amino acid methionine to form S-adenosylmethionine (SAM). Methyltransferases (MTs) mediate methyl group transfer from SAM to a variety of substrates including lipids, proteins, nucleic acids and neurotransmitters.\textsuperscript{61,62} A methylated product and S-adenosylhomocysteine (SAH), a competitive inhibitor of MTs are generated following methyl group removal from SAM. SAH is then hydrolyzed to form homocysteine and adenosine via S-adenosylhomocysteine hydrolase (SAHH).\textsuperscript{62,63}
Methylated substrates formed through the transmethylation pathway are imperative for a variety of cellular processes. Methylated lipids are essential components to the structural integrity of cellular walls, bile formation and cellular signaling, while methylation of proteins stimulates protein activation via post-translational modification. Methylated proteins are functional in cellular signaling, transcription and gene regulation. Nucleic acids comprised of ribonucleic acids (RNA) and deoxyribonucleic acid (DNA) are methylated in this pathway and are vital in regulating gene expression. Epigenetic modifications of methylated substrates have been directly correlated with the development of numerous pathological conditions including cancer, CVD and autoimmune diseases. Therefore, it is crucial to ensure adequate methyl group supply and promote homeostasis of key proteins within one-carbon metabolism.

Transmethylation is dependent on and regulated by a variety of mechanisms, including allosteric regulation via SAM:SAH ratio and the action of specific regulatory proteins in response to this ratio. The ratio of SAM:SAH in the transmethylation pathway influences homocysteine levels, methyl group usage and the pathways utilized in methyl group metabolism. Elevated concentrations of either compound can result in inhibition of processes regulated by it’s counterpart. Elevated SAH concentrations inhibit SAM-dependent reactions, potentiating disturbed methyl group maintenance. Conflicting results have been found regarding the relationship between SAH and hypomethylation, suggesting the multitude of factors involved in regulating these metabolic pathways. Increased concentrations of SAM 1) stimulate the catabolism of homocysteine to cysteine via cystathionine beta synthase (CBS), utilizing the transsulfuration pathway and 2) allosterically inhibit 5-CH₃-THF, preventing folate-dependent remethylation when SAM concentrations are sufficient. These mechanisms promote SAM:SAH homeostasis, homocysteine regulation and conserve methionine for vital methylation reactions.
The transmethylation pathway utilizes three primary methyltransferase proteins to regulate methyl group metabolism and homocysteine concentrations.\(^{62}\) Glycine N-methyltransferase (GNMT) is an enzyme that accepts and catabolizes adenosine to sarcosine, a metabolite with no known metabolic role.\(^{56}\) This mechanism provides a pathway to discard excess methyl groups which can help regulate SAM:SAH ratio and/or lead to hypomethylation.\(^{61}\) Enzymes that assist to yield SAH include phosphatidylethanolamine N-methyl transferase (PEMT) and guanidinoacetate N-methyltransferase (GAMT), forming phosphatidylcholine and creatine, respectively.\(^{62}\) These proteins are the largest known methyl consumers and therefore, vital in regulating homocysteine concentrations.\(^{59}\)

**Folate-dependent remethylation**

Two separate pathways, folate-dependent remethylation and folate-independent remethylation pathways can generate methionine from homocysteine. The folate-dependent pathway utilizes methionine synthase (MS), a B\(_{12}\) dependent protein to catalyze the remethylation of homocysteine with the addition of a methyl group donated by folate derivative, 5-methyltetrahydrofolate (5-CH\(_3\)-THF). Coenzyme FADH\(_2\) assists coenzyme methylenetetrahydrofolate reductase (MTHFR), to reduce 5,10 methylenetetrahydrofolate (5,10-CH\(_3\)-THF) to 5-CH\(_3\)-THF in this rate-limiting irreversible reaction. Remethylation of homocysteine results in the formation of methionine and tetrahydrofolate (THF).\(^{56}\)

Allosteric regulation allows for these interrelated pathways to maximize methyl group supply, while also normalizing homocysteine and other key proteins throughout methyl group metabolism. Elevated methyl group supply and SAM concentrations inhibit the activity of MTHFR, preventing the formation of methyl donor 5-CH\(_3\)-THF and downregulating the folate-
dependent remethylation pathway. Conversely, when methyl group supply is depleted and SAM concentrations are low, MTHFR is upregulated allowing for remethylation of homocysteine.\textsuperscript{58,68}

**Folate-independent remethylation**

Folate-independent remethylation is an alternative pathway used to regenerate methionine from homocysteine. This utilization of this pathway depends on the dietary protein intake that corresponds to methionine concentrations and methyl group supply, SAM:SAH ratio and the availability of vitamins B\textsubscript{12}, B\textsubscript{6} and folate. Choline is an essential nutrient that may be oxidized in the body via choline oxidase/dehydrogenase to form metabolite betaine, the methyl donor in the folate-independent remethylation of homocysteine to methionine.\textsuperscript{63} Betaine-homocysteine S-methyltransferase (BHMT), a protein primarily expressed in mammalian hepatic tissue, accepts and transfers methyl groups from betaine to homocysteine when methyl groups are depleted and SAM concentrations are low.\textsuperscript{60} This pathway is highly favored when B vitamins are not available to fuel folate-dependent remethylation. However, BHMT is allosterically inhibited by SAM when methyl group supply is sufficient and SAM:SAH ratio is near homeostasis.\textsuperscript{69} Collectively, these pathways work in sync to maintain vital methylation reactions and promote optimal body functioning.

**Transsulfuration**

Transsulfuration is a B\textsubscript{6}-dependent irreversible metabolic pathway that is upregulated in correlation with elevated homocysteine levels. This allosteric mechanism allows for the catabolism of homocysteine in the event of elevated methionine levels, folate/B\textsubscript{12} deficiency and/or the physiological need for cysteine, it’s catabolic product. Transsulfuration is active in the liver, kidney, pancreas, intestine and brain.\textsuperscript{60} Altered expression and activity of key transsulfuration enzymes CBS and γ-cystathionase have been observed in tissues specific to
diabetes. These observations may be largely due to the hyperhomocysteinemic state characteristic of T1DM development and severity of CKD.\textsuperscript{6}

**Disruption of methyl group metabolism and pathological implications**

Research has proven the importance of methyl group maintenance in preventing the occurrence of disease. Maintenance of methyl group metabolism is affected by numerous factors including the following 1) dietary protein deficiency, which reflects methionine concentrations and methyl group supply 2) deficiencies of specific nutrients that fuel one-carbon metabolism (i.e. choline, folate, B\textsubscript{6}, B\textsubscript{12})\textsuperscript{70} 3) medications 4) environmental toxins 5) genetic polymorphisms of one-carbon metabolic enzymes and 6) complications associated with metabolic diseases.\textsuperscript{71}

Disturbed methylation directly alters gene expression, which may lead to a multitude of health disparities. Undermethylation of DNA typically leads to overexpression of genes, while elevated methylation causes gene silencing. Altered gene expression due to undermethylated DNA has been suggested to increase the incidence of oncogenes.\textsuperscript{56} Several studies have found a strong correlation between methyl group deficient diets and the increased expression of carcinogens leading to hepatocellular cancer. Wagner et al. identified a 51\% increase in carcinogenic liver cells in rats fed methionine and choline deficient diets for 13 to 24 months.\textsuperscript{68}

The body responds to methyl group deficiency by downregulating GNMT enzyme, preserving SAM-donated methyl groups for essential methylation reactions when methionine and SAM concentrations are low. Research studies have also supplemented homocysteine in conjunction with methyl-deficient diets to determine if remethylation of homocysteine would provide sufficient methionine to promote methyl group maintenance. Consensus of these results is that while homocysteine can be used to regenerate methionine, methyl group deficient diets
decrease growth in rats, suggesting that remethylation has a limited capacity for maintaining vital methylation reactions.\textsuperscript{72,73}

Other studies have investigated the effects of methyl and folate deficient diets in methyl group metabolism. Deficiencies of nutrients essential to one-carbon metabolism are highly associated with perturbed central nervous system functioning and progression of neurological disorders. Additionally, B vitamin deficiencies may lead to depression, birth defects, anemia and CVD related to hyperhomocystemia. Folate and methyl deficient diets in rats have caused accumulation of homocysteine in the brain as a result of perturbed remethylation and transsulfuration pathways in one-carbon metabolism. Elevated homocysteine levels and methyl deficiency induced oxidative damage may be indicative in the etiology of CVD and neurodegenerative disorders including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and amyotropic lateral sclerosis.\textsuperscript{70}

Plasma homocysteine levels between 5-15 $\mu$mol/L are considered normal in humans.\textsuperscript{74} Hyperhomocystenemia is categorized based on severity, with moderate (>10 $\mu$mol/L), intermediate (>30 $\mu$mol/L) and severe (>100 $\mu$mol/L) classifications.\textsuperscript{3} Homocysteine levels are affected by numerous factors including age, sex, smoking, medications, and many disease states, especially those associated with impaired renal function. Homocysteine concentrations are typically higher in men compared to women and progressively increase with age in both sexes.\textsuperscript{75} A parallel relationship between smoking and elevated plasma homocysteine has been replicated in many studies. Researchers propose that this relationship may be attributed to impaired vitamin B$_6$ status and subsequent perturbation of the transsulfuration pathway, causing homocysteine accumulation.\textsuperscript{75}
A variety of drug-nutrient interactions impact homocysteine concentrations due to their affect on vitamin absorption and disturbance of one-carbon metabolism and folate metabolism. Medications used to treat high blood cholesterol and non-insulin dependent diabetes including cholestyramine and metformin, interfere with absorption of B vitamins, essential for maintaining metabolic pathways associated with homocysteine regulation. Other drugs such as methotrexate, fibric acid and nicotinic acid are also associated with elevated homocysteine levels.66 A 2001 study observed a 53-57% increase in plasma homocysteine levels in patients treated with ciprofibrate for hypertriglyceridemia.67 Patients are urged to take caution with any of these prescriptions, as hyperhomocysteinemia can lead to many adverse health affects including CVD.

Various genetic polymorphisms influence methyl group metabolism, altering methylation reactions and homocysteine levels. MTHFR genetic mutations are highly associated with hyperhomocystenemia and considered genetic risk factors for CVD. The primary MTHFR mutation is the C667T variant and is characterized by the accumulation of 5-CH3-THF, inhibiting the folate-dependent remethylation pathway for regeneration of methionine from homocysteine.68 “Methyl trapping” is commonly observed in individuals with this genetic mutation and is exacerbated by B vitamin deficiencies, inhibiting folate-independent remethylation of methionine via MS enzyme and catabolism of homocysteine via transsulfuration.69 Less common one-carbon mutations related to hyperhomocysteinemia include transsulfuration proteins, CBS and γ-cystathionase.70

**Methyl group metabolism and type 1 diabetes**

One of the largest determinants in total plasma homocysteine is renal function as illustrated by the inverse relationship between GFR and homocysteine. This is one of the strongest links between two detrimental chronic diseases, T1DM and CVD. The kidneys play a key role in
reuptake of many nutrients including amino acids and their metabolites. The exact mechanism by which the kidney functions in homocysteine metabolism and clearance has not been confirmed. However, researchers postulate that homocysteine filtration is similar to that of cysteine, lysine, arginine and other amino acids. Therefore, impairment of glomerular filtration associated with renal disease may cause decreased homocysteine clearance and subsequent plasma homocysteine elevation. Some researchers have suggested that in the event of renal impairment, homocysteine-regulating enzymes are hormonally upregulated to maintain normal plasma homocysteine levels.\(^3\)

T1DM is typically associated with hyperhomocysteinemia as a result these mechanisms. However, previous studies have identified hypohomocysteinemia in STZ-induced diabetic rodent models. While counterintuitive, the mechanism for lower circulating plasma homocysteine concentrations may be attributed to compensatory increases in one-carbon metabolic enzymes, CBS and \(\gamma\)-cystathionase. These proteins fuel transsulfuration, an alternative metabolic pathway capable of catabolizing homocysteine when concentrations are elevated.\(^59\) Lower circulating concentrations of homocysteine have been observed in T1DM patients in the early stages of the disease, characterized by the absence of microvascular complications. Hyperhomocysteinemia and downregulation of transmethylation and transsulfuration pathways was assessed in concurrence with CKD progression.\(^81,82\) These results indicate that homocysteine regulation is largely dependent on disease severity and functionality of one-carbon metabolic pathways to keep homocysteine in homeostatic conditions and maintain methylation reactions.

Various proteins throughout one carbon methyl group and folate metabolism mediate plasma homocysteine concentrations and alterations of these proteins have been observed in T1DM.
Perturbed methyl group metabolism has been observed using rodent models of T1DM, indicated by the upregulation of GNMT, CBS and γ-cystathionase and BHMT, enzymes present in transmethylation, transsulfuration and folate-independent pathways. These enzymes play a key regulatory role in homocysteine regulation and methyl group maintenance. Alterations in protein expression may indicate the presence of nutritional and/or hormonal modulation of these enzymes associated with the diabetic state.

Our previous research has demonstrated retinoic acid (RA) and dexamethasone (DEX) as independent signals in GNMT induction in a STZ-treated rat model. Insulin administration alleviated GNMT upregulation in this diabetic model, suggesting its role as a hormonal modulator of regulatory methyl group metabolism proteins. However, we would also like to explore the possibility of glucose as an independent signal for GNMT induction and other associated homocysteine-regulatory proteins. Hormone insulin functions to regulate postprandial blood glucose. Therefore, normalization of GNMT could be attributed to the administration of insulin and/or achieving blood glucose homeostasis.

Taken together, the goal of this experimental study was to investigate the effect of dietary resistant starch (RS) in attenuating hyperglycemia in diabetes, as a means to prevent disease complications and associated perturbations in methyl group metabolism. In addition, this study aims to investigate RS and glucose as potential hormonal and nutritional modulators in methyl group metabolism using a STZ-induced model of T1DM.

**Resistant Starch**

**Starch background**

Starch is a type of polysaccharide and a primary source of carbohydrates. This polysaccharide is made up of monosaccharides linked together by α-D-(1,4) and α-D-(1,6)
glycosidic linkages. Starch is comprised of two primary molecules, amylose and amylopectin. Amylose and amylopectin differ both in structure and digestibility. Amylose is a linear polymer chain of glucose molecules connected via \( \alpha-D-(1,4) \) glycosidic linkages, which typically constitutes 25% of starch. Amylopectin is a highly branched glucose molecule that is linked by both \( \alpha-D-(1,4) \) and \( \alpha-D-(1,6) \) glycosidic linkages making up the remaining 75% of this carbohydrate.$^{83,84}$

Starch is digested by \( \alpha \)-amylases found in the saliva, pancreas and small intestine. The branching of glucose molecules in amylopectin allows for increased enzymatic action and therefore more efficient digestibility than amylose.$^{84}$ Advances in biotechnology have geared toward improving the structure, functionality and yield of different starches. Altering genotypes of native starches has resulted in the amplified production of high quality crops. Biotechnology has allowed for researchers to better understand how functionality and structure affect digestion and overall health.$^{83}$

Digestibility of starch depends on the chemical makeup of the particular molecule, which determines the functional characteristics. Resistant starches are found in nature and can be synthetically developed via gene alteration. RS cannot be digested by the \( \alpha \)-amylases that reside in the saliva and throughout the gastrointestinal tract. Therefore, these starches completely or partially bypass the small intestine for fermentation in the large intestine. Research has shown evidence of prebiotic health benefits associated with consumption of this product. Colonic fermentation seems to produce specific metabolites that have been associated with many health benefits including normalization of macronutrient metabolism, reduction in colonic carcinogenic precursors and hormonal regulation resulting in potential mental and physical health benefits.$^{85}$
Definition and classifications of resistant starch

RS is divided among 5 subcategories referred to as RSI, RSII, RSIII, RSIV and RSV. RS1 is a type of starch that is structurally protected by a strong protein complex and a dense cell wall, inhibiting water absorption and gelatinization and therefore preventing appropriate enzymatic action, digestion and absorption in the small intestine. RSII is crystalline in structure and resistant to enzymatic hydrolysis in an uncooked state. Examples of RSII sources include potato starch, green banana starch, gingko starch and high amylose maize starch. RSIII becomes resistant following retrogradation at refrigeration temperatures (4-5°C). This phenomenon causes amylose and amylopectin to form of double helices, shortening the chain length of these molecules and inhibiting water binding and enzymatic hydrolysis. RSIV is formed via chemical modification, which can implement cross-linking throughout the molecule. Cross-linking can inhibit both water absorption and enzymatic action by amylases, causing a resistant effect during digestion and fermentation. RSV is a thermally stable product that is formed when starch and lipid molecules interact. This interaction causes the formation of a helical amylose-lipid complex, preventing water absorption and swelling and enzymatic hydrolysis.

Resistant starch: applications in health and disease

Amylose constitutes approximately 20-35% of naturally occurring maize, with high amylose starches comprising greater than 40%. Breeding of high amylose starch has been commercialized and widely utilized in research, with amylose contents exceeding 90% of total composition. Resistant starches have confirmed positive physiological effects according to numerous research studies. RS may play an important role in the prevention of colon cancer and gallstone formation, improvement of blood lipid profiles and treatment of gastrointestinal
diseases and diabetes. Other studies have shown that RS may attenuate fat accumulation and increase absorption of key minerals such as calcium and iron.\textsuperscript{84}

**Resistant starch and glycemic control in diabetes**

RS has been investigated as a known hypoglycemic agent that can be implemented for therapeutic uses in the treatment of diabetes. Typically, the digestion of starch begins immediately following consumption of carbohydrate meals. However, RS is digested at a slower rate, taking between 5-7 hours to reach the large intestine. The rate of digestion and absorption of glucose is slower following RS consumption, preventing dramatic increases in postprandial blood glucose, which may possibly reduce adverse health effects associated with chronic hyperglycemia.\textsuperscript{84}

We have demonstrated the ability of RS to prevent hyperglycemia and associated diabetic complications in rodent models of T1DM and type 2 diabetes mellitus (T2DM). Attenuation or prevention of long-term diabetic complications may be a result of the protective effect of RS on kidney function and nutrient homeostasis. Complications associated with diabetes are highly associated with microvascular issues, causing deteriorating kidney function and therefore disrupting nutritional status. Using an RSII based diet as a replacement for cornstarch, we have observed attenuated DM-associated weight loss and hyperglycemia, prevented diabetic nephropathy as evidenced by normalized serum creatinine, urinary volume and urinary albumin excretion, prevented excretion of VBP and 25(OH)D\textsubscript{3} and normalized gene expression of renal endocytic proteins megalin and dab2, respectively.\textsuperscript{55}

**Resistant starch and the gut microbiome**

RS is a notable substrate for fermentation of short chain fatty acids (SCFA’s) including butyrate, acetate and propionate in the colonic gut. Dietary RS has been shown to favor the
production of butyrate among other SCFA’s, however this largely depends on the type, amount and structure of RS, all of which can affect microbial fermentation and relative SCFA proportions. Typically, acetate represents the highest proportion (60%) among colonic SCFA’s and therefore acetate is the most abundant in peripheral circulation. However, peripheral circulation is generally low for these fermentable by-products.\textsuperscript{87}

SCFA’s have been associated with reduced inflammation in monocytes and peripheral blood mononuclear cells (PBMC), mediated by FFAR2 (immune cells) or FFAR3 (adipocytes) and normalization of cytokine/chemokine profiles and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}).\textsuperscript{87} Other studies have observed neutrophil and leukocyte recruitment stimulated by FFAR2 to alleviate bacterial-associated inflammation. Low levels of SCFA’s are typically found in circulation, limiting anti-inflammatory action.

Typically, there are low levels of SCFA’s found in circulation, limiting anti-inflammatory action to the colon. However, pathogenic bacteria are capable of producing SCFA’s throughout circulation and subsequently elevating peripheral concentrations. This hypothesis supports the immunological role of SCFA’s in the colonic gut, peripheral tissues and adipocytes. The action of SCFA’s in these tissues is significant given the inflammatory pathogenesis of T2DM and various gastrointestinal diseases.\textsuperscript{88}
CHAPTER 2: RESISTANT STARCH PROMOTES REGULATION OF METHYL GROUP METABOLISM

Abstract

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by pancreatic β cell dysfunction, insulin deficiency and abnormal carbohydrate metabolism. Chronic hyperglycemia causes structural damage to the kidneys, resulting in ultrafiltration and imbalance of nutrients such as vitamin D and disruption of various proteins associated with methyl group metabolism and homocysteine regulation.\textsuperscript{3-5} Using streptozotocin (STZ) treated rats as a model of T1DM, we have demonstrated that dietary resistant starch (RS) attenuates many complications associated with T1DM including increased urinary excretion of 25 hydroxyvitamin D\textsubscript{3} (25(OH)D\textsubscript{3}) and vitamin D binding protein (DBP).\textsuperscript{55}

The focus of this study was to characterize the impact of RS on methyl group metabolism using a STZ-induced diabetic rat model. Male Sprague Dawley rats (n=38) were randomly assigned to respective groups: control starch (CS, n=12), diabetes-CS (DM-CS, n=12) and diabetes-RS (DM-RS; n=14). CS and DM-CS rats were fed a standard cornstarch diet throughout the 9-wk study, whereas the DM-RS group was acclimated to the CS diet for 2-wk and transitioned to a high amylose RS diet (RS was 37\% resistant to digestion) for the remaining 7-wk. Rats were sacrificed, tissues were extracted and plasma and urine were collected for analysis. DM-RS rats exhibited symptoms characteristic to DM including polyuria, hyperglycemia and decreased urinary creatinine excretion (2.3 fold). In addition, glycine N-methyltransferase (GNMT) activity was elevated in hepatic (1.2 fold) and renal (~2 fold) tissues. However, there was no change in ribonucleic acid (mRNA) abundance of one-carbon proteins GNMT and betaine-homocysteine S-methyltransferase (BHMT) in DM rats fed either diet.
Dietary RS prevented or attenuated DM-complications and normalized hepatic and renal GNMT, an important protein to one-carbon metabolism.

**Introduction**

Recent developments in nutritional sciences have geared researchers toward implementing therapeutic dietary strategies in the prevention and treatment of various pathologies including gastrointestinal conditions, neurological disorders and autoimmune diseases. Resistant starch (RS) is a type of dietary fiber that resists digestion in the small intestine and undergoes fermentation in the colon. Consumption of carbohydrates that are abundant in RS have exhibited many physiological and metabolic health benefits. Subsequently, RS has been widely used as a dietary preventative and treatment method for a variety of diseases including diabetes, cardiovascular disease, obesity and cancer.

Colonic fermentation of RS results in the production of short chain fatty acids (SCFA’s), proven as mediators in these pathological conditions. Gut hormones stimulated by SCFA’s are associated with increased energy expenditure and lipolysis in humans, which provides implications for RS in prevention and treatment of obesity, type 2 diabetes mellitus (T2DM) and metabolic syndrome. SCFA absorption in adipocytes is also linked to improved blood lipid profiles. Furthermore, SCFA have an immunological role by producing anti-inflammatory effects in the colonic gut, peripheral tissues and adipocytes. This mechanism has shown benefits in inflammatory-related diseases such as T2DM, gastrointestinal disorders and cancer.

The protective effect of RS in diabetes is likely related to several mechanisms. Dietary RS bypasses the small intestine, resisting typical carbohydrate digestion and absorption and assisting in glycemic control. Various studies have shown a reduction in post-prandial blood glucose associated with RS consumption. Due to the multitude of complications associated with
chronic hyperglycemia, the use of RS as a therapeutic dietary agent may provide favorable outlooks for diabetic patients, slowing and/or preventing the progression of these complications and inevitable mortality. Diabetic nephropathy is a common vascular disease of the kidneys associated with poor glycemic control in diabetes. Declining renal function results in hyperfiltration and reduced clearance of various proteins and nutrients associated with vitamin D metabolism and one-carbon metabolism.\(^2\)\(^5\) RS has previously shown to attenuate/prevent complications associated with uncontrolled hyperglycemia such as weight loss, increased urinary output and hyperfiltration of proteins and nutrients associated with declining renal function.\(^5\)\(^5\) Most recently, we have shown RS to restore vitamin D status as evidenced by reduced vitamin D binding protein (DBP) and 25 hydroxyvitamin D\(_3\) (25(OH)D\(_3\)) excretion and normalized expression of endocytic vitamin D receptors in the kidneys.\(^5\)\(^5\)

Reduced homocysteine clearance and elevated plasma homocysteine levels are associated with chronic kidney disease (CKD) progression in DM.\(^3\) Subsequent induction of one-carbon regulatory proteins glycine N-methyltransferase (GNMT), betaine-homocysteine S-methyltransferase BHMT, cystathionine beta synthase (CBS) and \(\gamma\)-cystathionase have also been observed and hypothesized as compensatory mechanisms to maintain methyl group supply and normal homocysteine levels.\(^6\) In this study we hypothesized that RS supplementation would alleviate abnormalities in methyl group metabolism by controlling blood glucose and preserving kidney function.

**Materials and Methods**

**Rodents.** Animal studies were approved and conducted under the guidelines provided by the Iowa State University Laboratory Animal Resources. Male Sprague Dawley (N=38; Harlan Teklad, Madison, WI) rats weighing 110-140 g were housed individually in plastic-wired cages
under a 12-hr light:dark cycle. Rodents were housed for 9 wk and had access to food and water *ad libitum*.

**Diets.** The standard control diet composition is listed in Table 2.1. High-amylose maize RS was used as a replacement for cornstarch. Cornstarch (CS) was replaced with RS, 37% resistant to digestion. Starches were cooked prior to mixing with other ingredients.

**Table 2.1 Diet Compositions**

<table>
<thead>
<tr>
<th>Diet Ingredient</th>
<th>Control (g/kg)</th>
<th>RS (g/kg)</th>
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<tbody>
<tr>
<td>Casein, vitamin-free</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Glucose</td>
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<td>150</td>
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<tr>
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**Treatment groups.** Sprague Dawley rats were acclimated to the control diet for 14 d. Rats were then randomly divided into three groups, including control (group 1; n=12), diabetes mellitus (DM; group 2; n=12) and DM+RS (group 3; n=14). Following the acclimation period, group 1 (control) and 2 (DM) were continued on the standard diet and groups 3 (RS) was transitioned to the RS diet. This summarizes the 3 groups and 2 diets that were administered over the 50 d experimental period.

**Streptozotocin injection.** Following the acclimation period, rats transitioned to the diet regimen specified for each group for approximately 21 d. On d 35, diabetic rats (groups 3-6), were given an intraperitoneal injection of streptozotocin (STZ; 60 mg/kg BW) to induce diabetes. Control
rats (groups 1-2) were given a vehicle injection (10 mmol/L citrate buffer, pH 4.5). Rats were killed 3 wk post-STZ injection.

**Data collection.** All rodents were housed in metabolic cages for urine collection and fasted overnight (12h) prior to the kill. A cocktail of ketamine:xylamine (90:10 mg/kg BW) was prepared and used to anesthetize rats via intraperitoneal injection. Whole blood was collected via cardiac puncture and centrifuged for plasma collection. After euthanasia, liver and kidney tissues were extracted and submerged in liquid nitrogen for preservation. Collected tissues, blood and urine were stored at -80°C for later analysis.

**Assessment of renal function.** Urinary creatinine, serum creatinine and serum albumin concentrations were assessed using a commercial colorimetric kit (QuantiChrom Creatinine Assay Kit and QuantiChrom BCG Albumin Assay Kit; Bioassay Systems, Hayward, CA).

**Assessment of blood glucose.** To assess blood glucose in all rats, fasting blood glucose (FBG) was measured via glucometer (Bayer Healthcare) using serum collected via cardiac puncture at the time of euthanasia.

**Assessment of DM symptoms.** To assess weight loss and increased urinary output associated with DM, 12-hr urine samples were collected and measured prior to euthanasia. Body weight was determined daily throughout the study to calculate weight loss/gain following diet implementation and STZ injections in DM rats.

**Assessment of serum 25(OH)D₃ status.** Vitamin D status was assessed by measuring serum 25(OH)D₃ using a commercial enzyme immunoassay kit (Immunodiagnostic Systems, Scottsdale, AZ).

**Enzyme analysis.** GNMT activity was assessed in hepatic and renal tissues using the Cook and Wagner method with slight modifications. Each sample assay was performed in triplicate using
250 µg aliquots and combined with a reaction mix containing 0.2 M Tris buffer (pH 9.0), 0.2 mM S-adenosyl-L-[methyl-³H]methionine (PerkinElmer, Waltham, MA), 2 mM glycine and 5 mM dithiothreitol. A heat-killed control was prepared for triplicate samples and aliquots were incubated for 30 min at 25°C. The reaction was halted by the addition of 10% trichoroacetic acid and activated charcoal was added to aliquots, vortexed and centrifuged (14,000 x g) to remove excess, unreacted SAM. Supernatant layers were isolated and extracted to be used for liquid scintillation counting.

**Real-time PCR.** Hepatic tissue was removed from -80°C storage and ~0.1g samples were cut and dispersed in Trizol Reagant (Invitrogen, Carlsbad, CA). RNA was isolated using SV Total RNA Isolation System (Promega, Madison, WI) and quantified via UV detection (Nanodrop, 280/260). Extracted RNA was used for cDNA synthesis using a High Capacity cDNA synthesis kit with RNase inhibitor (Applied Biosystems, Foster City, CA). Reactions were performed in duplicate from single cDNA stocks created for each liver sample. Hepatic GNMT and BHMT gene expression was analyzed via real-time PCR using iScript SYBR Green Detection reagents (Bio-Rad, Hercules, CA), 4µL cDNA/well and forward and reverse primer sets for BHMT and GNMT using 18S mRNA as the control or housekeeping gene (Table 2.2). Gene expression was assessed via fold-induction relative to non-diabetic control rats.

**Statistical Analysis**

Statistics were calculated using SigmaPlot 9.0 software (Systat, Chicago, IL). Treatment group means were compared using a one-way ANOVA, followed by the Fisher least-significant difference post-test. ANOVA on ranks was performed when normality or equal variance tests failed. Significant differences were noted at P < 0.05.
Results

Serum 25(OH)D$_3$ was moderately elevated in DM.

No statistical differences between groups were yielded in this assay. However, 25(OH)D$_3$ was moderately elevated in DM rats compared to control and RS-treated rats. This may be attributed to increased consumption of food, leading to increased vitamin D status.

Dietary RS attenuated weight loss and hyperglycemia, and prevented polyuria.

Weight loss. No statistical difference in cumulative weight gain was observed between groups prior to STZ-injection (Table 2.2). DM rats fed the control diet lost 27% of total body weight following STZ-injection, while control rats gained 11% of total body weight. DM rats fed the RS diet gained 1% of total body weight following STZ-injection (Figure 3.3). Hyperglycemia.

Elevated blood glucose (527±52.2 mg/dL) was observed in DM rats at 9 wk 140% compared to CS blood glucose (221.6±17.1 mg/dL). Hyperglycemia was not prevented in RS rats, however there was a statistically significant 32% reduction in blood glucose (358.4±46.6 mg/dL) compared to the DM-CS group (p=0.008). Polyuria. Polyuria is evident in DM and is used as a marker for renal dysfunction. Urine was collected over a 12-hour period and measurements illustrated a 53% reduction in urinary volume of RS rats (6.7±1.9 mL) relative to DM rats (12.7±2.2 mL) fed CS diet (p=0.022). There were no statistically significant differences observed between the control and RS group (p=0.533) (Figure 2.5).

Dietary RS normalized urinary creatinine excretion, but had no affect on serum creatinine or serum albumin concentrations. Decreased urinary creatinine levels were observed in DM group, ~60% lower than the control (p=0.006) (Figure 2.2A). RS normalized creatinine concentrations in the urine. No statistical differences were observed between DM-RS and CS
groups (p=0.434). There were no significant differences in serum creatinine concentrations (p=0.417; Figure 2.2B) or serum albumin concentrations (p=0.384) between groups (Figure 2.3).

**Dietary RS significantly decreased renal GNMT activity and attenuated hepatic GNMT activity.** Hepatic GNMT Activity. Similar to other studies in our lab, GNMT activity was elevated in the liver of diabetic rats and significant differences were observed between all groups (p=0.027). This activity was not entirely normalized by RS, however there was a slight reduction (p=0.127). Renal GNMT Activity. Renal GNMT Activity is generally lower compared to hepatic tissue. However, increased GNMT activity in DM groups was significantly reduced (23%) by the RS diet (p=0.039). There were no observable differences between the control and DM-RS groups (p=0.018).

**No differences in GNMT and BHMT mRNA expression in hepatic tissue found between groups.** Induction of hepatic GNMT (~4 fold) and BHMT mRNA (2.1 fold) was illustrated in DM-CS rats relative to control. RS attenuated expression of both proteins (Figure 2.6), however the results were not statistically significant (Table 2.4).

**Discussion**

Resistant starch has been confirmed as an effective dietary strategy for glycemic control and alleviation of complications characteristic of diabetes.\textsuperscript{1,5,51-93} We hypothesized that RS consumption would normalize abnormalities in methyl group metabolism regarding induction of key regulatory proteins GNMT and BHMT by diabetes. As demonstrated in previous studies, GNMT activity was elevated in kidney and liver tissues of diabetic rats. In addition, elevated hepatic mRNA expression of BHMT and GNMT was observed in DM rats.\textsuperscript{5,95,96} Previous research has shown the use of hormones and nutrients to normalize the expression of these
proteins in one-carbon metabolism. Similar results were obtained in this model, suggesting RS to be another mediator for these proteins.

Weight loss associated with prolonged hyperglycemia is a characteristic symptom of diabetes. Interestingly, the therapeutic use of RS has been used in many T2DM and obese models as a dietary method for weight loss. This mechanism is related to the production of GLP-1 (glucagon like peptide-1) by butyrate in the colonic gut and is associated with increased energy expenditure and lipolysis in humans. RS attenuated hyperglycemia and subsequently weight loss in this T1DM model, suggesting that this mechanism did not have a profound impact.

Normal FBG in humans ranges between 70-100 mg/dL. However, glucose concentrations in rodents are much higher under fasting conditions. One study evaluated blood glucose after 5 and 11 hours in Wistar rats, determining an average FBG between 135-190 mg/dL. Other studies have used 200 mg/dL and 250 mg/dL as benchmarks for DM classification. However, we have observed FBG exceeding 300 mg/dL in some control rats. Therefore it is important to evaluate other markers that can assess DM severity including serum creatinine, urinary albumin and polyuria.

Polyuria, decreased serum creatinine and microalbuminuria are indicative of renal function. A model of diabetic nephropathy in mice showed elevated serum creatinine, with 0.08-0.11 mg/dL classifying the normal range, with albumin:creatinine ratio >1000 mg/g. This dramatically exceeds ratios characterizing CKD in humans. There were no significant differences in serum creatinine concentrations found between groups and mean concentrations were below this normal range (0.501-0.519±0.0383 mg/dL). However, urinary creatinine and albumin concentrations are direct markers for impaired renal filtration and typically better standards to
use in CKD assessment. Urinary creatinine concentrations were depressed in DM rats, suggesting the perturbed clearance of this metabolite in the glomerulus. Polyuria and decreased urinary creatinine were evident in our study and both symptoms were alleviated in RS treated rats.

In addition to these biomarkers, disruptions in vitamin D metabolism and methyl group metabolism are observed in diabetes. In our laboratory we have assessed the induction of key regulatory proteins GNMT, BHMT and cystathionine beta synthase (CBS) associated with diabetic nephropathy. Upregulation of these enzymes may be related to impaired homocysteine clearance by the proximal tubules and the subsequent rise in plasma homocysteine. Transmethylation, transsulfuration and remethylation pathways in one-carbon metabolism are governed by homocysteine concentrations and the S-adenosylmethionine: S-adenosylhomocysteine (SAM:SAH) ratio. Hyperhomocystenemia is a consequence of CKD and may be associated with the upregulation of these proteins and pathways. BHMT functions to remethylate methionine from homocysteine. In transmethylation, GNMT accepts and disposes methyl groups in the form of sarcosine. Transsulfuration enzymes CBS and γ-cystathione are also induced, allowing for the catabolism of homocysteine to cysteine. These mechanisms may help regulate methyl group supply and homocysteine in a diabetic state. However, increased catabolism of methyl groups via GNMT may lead to hypomethylation, as GNMT takes methyl groups from vital methyltransferases and impairs vital methylation reactions.

In this study, hepatic and renal GNMT activity were elevated and GNMT and BHMT mRNA abundance was markedly increased in DM rats. In our laboratory, administration of retinoic acid (RA), dexamethasone (DEX) and insulin has been shown to normalize the genomic expression of these proteins in DM rats. Insulin administration alleviated GNMT upregulation in
this diabetic model, suggesting the changes are diabetes-specific.\textsuperscript{6} Hormone insulin functions to bring blood glucose to homeostasis in diabetes.\textsuperscript{12} Therefore, normalization of GNMT could be attributed to the administration of insulin and/or the normalization of blood glucose. While previous studies have shown the prevention of diabetic nephropathy and associated complications using RS, we have observed these results in the presence and absence of hyperglycemia.\textsuperscript{55} Although this does not rule out the possibility of glucose as a hormonal modulator in one-carbon metabolism, it suggests another mechanism may be responsible.

New developments in research have characterized the role of SCFA produced by RS in the colonic gut. SCFA’s are immunological mediators in the colon, peripheral tissues and adipocytes. Acetate, butyrate and propionate exhibit an anti-inflammatory effect in the body in response to bacterial infections.\textsuperscript{88} Of particular interest is the role of butyrate as a mediator for TGF-\(\beta\)-1 (transforming growth factor-\(\beta\)-1), a profibric cytokine expressed during advanced renal disease and associated with renal fibrosis.\textsuperscript{103}

![Figure 2.1: Renal fibrosis.\textsuperscript{104}]

CKD is characterized by structural and functional changes to the renal tubules, ECM and glomerulus.\textsuperscript{5-5} Renal fibrosis occurs in the late stages of CKD and involves an inflammatory cascade that begins with an injury to the kidney. This stimulates the release of pro-inflammatory cytokines, which recruit macrophages and T cells for infiltration at the site of injury. The activation of fibroblasts, mesangial cells and epithelial cells leads to the accumulation of
collagen, fibrinogen and other matrix components. Excessive accumulation in the extracellular matrix (ECM) leads to cross-linking, excessive scarring and loss of renal function.\textsuperscript{104}

Cytokine TGF-\(\beta\)-1 and its associated similar to mothers against decapentaplegic (SMAD) receptors are upregulated in CKD. In healthy kidneys, TGF-\(\beta\)-1 is tightly regulated by SMAD antagonists, which include ski-related novel N (SnoN), Sloan-Kettering Institute (Ski) and TG-interacting factor (TGIF). Recent studies have demonstrated low circulating levels of these antagonists in CKD, leading to amplified expression of this cytokine. Additionally, this cytokine is regulated via induction and post-translational modification.\textsuperscript{104} Potential modulators of TGF-\(\beta\)-1 include elevated glucose, angiotensin II and butyrate. Together this potentiates glucose and RS in the form of butyrate, as hormonal and nutritional modulators in diabetes.

Mechanisms by which glucose mediates this pro-inflammatory cytokine involve a variety of reactions. AGEs associated with chronic hyperglycemia stimulate the production of ROS.\textsuperscript{1} Circulating ROS activates protein kinase C (PKC), the hexosamine pathway, extracellular signal-related kinase (ERK) pathway, p38 mitogen activated protein kinase (MAPK) pathway and TGF-\(\beta\)-1 transcription factors. Specifically PKC and the hexosamine pathway are related to glucose-stimulated TGF-\(\beta\)-1 synthesis.\textsuperscript{104} Interestingly, recent developments have highlighted butyrate in the mitigation of TGF-\(\beta\)-1 stimulated renal fibrosis by inhibiting MAPK and ERK pathways.\textsuperscript{103} Taken together, these results have indicated that both glucose and RS are mediators in DM. Moreover, RS in the form of butyrate exhibits a protective effect in the kidneys of diabetic patients, preventing the progression of CKD and associated complications.

Collectively, our study demonstrated the prevention/attenuation of diabetic complications with the use of dietary RS. New developments in research have suggested butyrate to play an immunological role in in CKD as in inhibits several pathways related to renal fibrosis and TGF-
β-1 synthesis. Because RS supplementation attenuates hyperglycemia in DM rats, the production of butyrate by RS directly and indirectly mediates these inflammatory processes characteristic in DM. The protective effect of RS in the kidneys thus prevents a multitude of complications including weight loss, polyuria, hyperfiltration of proteins and nutrients and altered clearance of metabolites. In this study, we have demonstrated the normalization of key regulatory proteins in methyl group metabolism, speculating RS as an indirect modulator in one-carbon metabolism.
### Table 2.2 Real-time RT-PCR primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
</tr>
</thead>
</table>
| GNMT   | F*: ACA ACA AAG CCC ACA TGG TAA CCC  
       | R*: AGC CGA AAC TTA CTG AAG CCA GGA  |
| BHMT   | F: ATC TGG GCA GAA GGT CAA TGA AGC  
       | R: TGA CTC ACA CCT CCT GCA ACC AAT  |
| 18S    | F: GAA CCA GAG CGA AAG CAT TTG CCA  
       | R: ATG GTC GGA ACT ACG ACG GTA TCT  |

*F represents forward primer, R represents reverse primer
Figure 2.2: Creatinine concentration in urine (A) and serum (B). RS normalized urinary creatinine concentrations in DM rats. No statistical differences were observed between DM-RS and CS groups (p=0.434).
Figure 2.3: RS diet has no effect on serum albumin concentrations in DM rats. There were no significant differences in serum albumin concentrations between groups (p=0.384)
Figure 2.4: RS significantly reduces hyperglycemia associated with DM. Elevated blood glucose was observed in DM rats at 9 wk 140% compared to blood glucose in control rats. Hyperglycemia was not prevented in RS rats, however there was a statistically significant 32% reduction in blood glucose compared to the DM-CS group (p=0.008).
Table 2.2: RS diet significantly reduces weight loss associated with DM after STZ injection

<table>
<thead>
<tr>
<th></th>
<th>Total Weight Gain to STZ (42 d), g</th>
<th>Total Weight Gain after STZ (21 d), g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>202±6.7\textsuperscript{a}</td>
<td>30.0±2.4\textsuperscript{a}</td>
</tr>
<tr>
<td>DM-CS</td>
<td>208±4.1\textsuperscript{a}</td>
<td>-62.4±7.6\textsuperscript{b}</td>
</tr>
<tr>
<td>DM-RS-37</td>
<td>212±5.1\textsuperscript{a}</td>
<td>-1.4±7.1\textsuperscript{c}</td>
</tr>
<tr>
<td>P Value</td>
<td>0.420</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data shown as group mean ±SE; letters denote significant differences between groups (p<0.05)

Figure 2.5: Cumulative weight loss/gain. No statistical difference in cumulative weight gain observed between groups prior to STZ-injection. DM-CS rats lost 27% of total body weight following STZ-injection, while control rats gained 11% of total body weight. DM-RS rats gained 1% of total body weight following STZ-injection. Data shown as group mean ±SE; letters indicate significant differences between groups (p<0.05); dotted lines indicate diet transition and STZ injection at 14 d and 43 d, respectively.
**Figure 2.6: RS prevents polyuria in DM rats.** Following a 12-hr fasting period, there was a 53% reduction in urinary volume of DM-RS rats (6.7±1.9 mL) relative to DM-CS rats (12.7±2.2 mL; p=0.022). No statistically significant differences were observed between the CS and DM-RS group (p=0.533)
Table 2.3: RS diet normalizes renal GNMT activity and attenuates increased hepatic GNMT activity associated with the diabetic condition

<table>
<thead>
<tr>
<th></th>
<th>Renal GNMT Activity, pmol/min*mg</th>
<th>Liver GNMT Activity, pmol/min*mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>5.1±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.6±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM-CS</td>
<td>9.1±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.2±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM-RS-37</td>
<td>7.0±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.4±1.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P Value</td>
<td>0.027*</td>
<td>0.027*</td>
</tr>
</tbody>
</table>

Data are means ±SE (n=x); letters denote significant differences between groups (p<0.05)

Figure 2.7: Hepatic and renal GNMT activity. GNMT activity was elevated DM liver and kidney tissues and significant differences were observed between all groups (p=0.027). GNMT activity was not normalized by RS in the liver. However, Renal GNMT activity in DM groups was significantly reduced (23%) by the RS diet (p=0.039). Data shown as group mean ±SE; letters indicate significant differences between groups (p<0.05)
Table 2.4: Hepatic mRNA abundance of one-carbon metabolism enzymes in diabetic rats fed CS and RS diets relative to control rats.

<table>
<thead>
<tr>
<th></th>
<th>Hepatic GNMT mRNA Relative Expression</th>
<th>Hepatic BHMT mRNA Relative Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>1.0±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM-CS</td>
<td>3.9±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM-RS-37</td>
<td>1.8±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P Value</td>
<td>0.307</td>
<td>0.739</td>
</tr>
</tbody>
</table>

Data are means ±SE (n=x); letters denote significant differences between groups (p<0.05)

Figure 2.8: Hepatic GNMT and BHMT mRNA abundance relative to the control. There were no statistically significant reductions in GNMT and BHMT mRNA expression with RS administration; letters indicate significant differences between groups (p<0.05)
General Conclusion

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that manifests from a combination of genetic, epigenetic and environmental factors. This disease is characterized by insulin deficiency, impaired carbohydrate metabolism and subsequent hyperglycemia. Chronic hyperglycemia associated with uncontrolled DM causes progression of microvascular and macrovascular complications characteristic of the disease. Advanced glycation end products (AGEs) associated with hyperglycemia infiltrate microvascular tissues, ultimately leading to vascular disease of the nervous system, eyes and kidneys.  

Diabetic nephropathy is one of the most common complications associated with DM and the leading cause of chronic kidney disease. Approximately, 40% of diabetic individuals progress from diabetic nephropathy to CKD and end stage renal failure (ESRF) during their lifetime. CKD is associated with numerous pathologies including cardiovascular disease (CVD), vitamin D deficiency and impaired methyl group metabolism. There are several mechanisms that link CKD and CVD including hypertension, endothelial dysfunction and hyperhomocystenemia.  

Hypertension contributes to the progression of CKD and is also a consequence of the disease. Renal dysfunction causes hypertension in diabetic patients by 1) disrupting sodium and volume concentrations that are correlated with blood pressure 2) causing autonomic dysregulation, which leads to increased peripheral resistance, blood volume, cardiac output and subsequently increased blood pressure and 3) activating the renin-angiotensin system (RAS) which may lead to an inappropriate rise in blood pressure. RAS activation also contributes to endothelial dysfunction and the development of CVD by activating Angiotensin II. Angiotensin II and circulating AGEs stimulate the production of inflammatory factors and reactive oxygen species (ROS), which directly contribute to endothelial dysfunction and atherosclerosis.
Structural damage to the nephron paired with endothelial dysfunction leads to increased vascular permeability and impaired filtration of proteins and many other nutrients.\textsuperscript{5} Loss of renal function has been linked to vitamin D deficiency and hyperhomocystenemia.\textsuperscript{2,5,54} Structural damage to the glomerulus causes hyperfiltration of nutrients including vitamin D binding protein (DBP), 25 hydroxyvitamin D\textsubscript{3} (25(OH)D\textsubscript{3}) and vitamin D endocytic receptors megalin, cubilin and dab2.\textsuperscript{2,54} Vitamin D deficiency can lead to anemia, osteoporosis, cancer and autoimmune diseases.\textsuperscript{49}

Impaired glomerular filtration may also cause decreased clearance of nutrients including homocysteine, a key metabolite in methyl group metabolism. Decreased homocysteine clearance can lead to the subsequent rise in plasma homocysteine levels. Hyperhomocystenemia is an independent risk factor for CVD and is highly correlated with declining glomerular filtration rate.
(GFR) in severe CKD. Anomalous upregulation of pathways and proteins in one-carbon metabolism are also associated with severe CKD. Researchers postulate these alterations as compensatory mechanisms for changes in homocysteine concentrations.³ Induction of key proteins glycine-N-methyltransferase (GNMT) and betaine-homocysteine-methyltransferase (BHMT) in CKD can lead to methyl group wastage and subsequent hypomethylation, which is associated with many adverse health conditions including cancer, neurological disorders, birth defects and CVD.⁵⁷,⁵⁸

Diabetes mellitus (DM) affects approximately 180 million individuals nationally, with 5% of individuals living with T1DM.¹¹ Uncontrolled hyperglycemia in DM can prime the development of many other diseases including CKD, vitamin D deficiency, cancer, neurological disorders and CVD. Therefore, prevention and treatment of this disease is imperative for optimal quality of life and longevity in diabetic patients. Many therapeutic dietary strategies have been developed and used in research to determine their affect in DM control including high fiber diets, lower glycemic index diets, prebiotics and probiotics.¹⁰⁷ Resistant starch (RS) is a dietary fiber that resists digestion, bypasses the small intestine and undergoes fermentation in the colonic gut.⁸⁵ RS consumption slows digestion and absorption of glucose, acting as a lower glycemic agent to prevent dramatic increases in post-prandial blood glucose.⁸⁴ Attenuated hyperglycemia associated with RS consumption may possibly reduce the development of comorbidities related to uncontrolled DM.

Previous research in conjunction with this study has demonstrated the ability of RS to attenuate hyperglycemia and therefore prevent diabetic complications and abnormalities associated with vitamin D metabolism and methyl group metabolism.⁵⁵ Proposed mechanisms for this phenomenon include the normalization of blood glucose and/or the production of
butyrate by RS in the colon, which acts as an immunological modulator in CKD.\textsuperscript{6,88} The protective effect of RS in diabetes and other pathological conditions presents many positive implications in health and disease.\textsuperscript{89,90}

In addition to promoting blood glucose homeostasis, RS has been shown to 1) improve satiety due to a slower rate of digestion 2) increase lipid oxidation and energy expenditure 3) improve colonic mucosal integrity and reduce inflammation via immunological effects exerted by butyrate and other SCFA’s produced during RS fermentation and 4) improve insulin sensitivity.\textsuperscript{108} With these health benefits, RS may be used as a treatment for various disease states such as T2DM, colon cancer, CVD, inflammatory gastrointestinal conditions and autoimmune diseases. RS has a dramatic impact on the gut microflora by promoting microbiome activity and diversity and producing SCFA’s, both linked to disease prevention. Researchers are working to better understand how RS and other carbohydrates influence the gut microflora. The mechanism behind these physiological benefits may be directly attributed to specific bacterial populations produced by RS.\textsuperscript{107}

There are many risk factors associated with T1DM including age, family history, genetic susceptibility and environmental factors such as vitamin D deficiency and viruses.\textsuperscript{19} More recently, research has geared toward understanding the role of the immune system in disease development as it relates to the gut microbiome. T1DM is associated with increased intestinal permeability, gut leakiness and altered intestinal immunity.\textsuperscript{109} Prebiotics and probiotics have immunomodulatory properties in disease, promoting optimal microbial diversity and exerting anti-inflammatory effects in the body.\textsuperscript{110}

The gut microbiome hosts a plethora of bacteria, capable of producing both pro- and anti-inflammatory responses. Therefore, the composition of the microflora may be inherently related
to immune system functioning and disease development. In a recent study, researchers performed a gut microbiome analysis in non-obese diabetic rats, finding low abundance of healthy bacteria and decreased diversity of the microbiome. The bacterial strains observed in this study differed significantly compared to the gut composition of control rats. Control rats possessed probiotic bacterial species that have been shown to promote gut integrity and prevent autoimmunity such as *Lactobacillus* and *Bifidobacterium*. Another study investigated the gut microbiome in genetically susceptible children, whom possessed at least two islet autoantibodies. Similar results were obtained in this human model including 1) comprised gut integrity 2) low bacterial diversity 3) gut instability, defined as decreased similarity between gut microflora in autoimmune children and 4) altered ratio of *Firmicutes* to *Bacteroidetes*, the most notable differences between control and autoimmune microbiomes.

The mechanisms that link T1DM with the autoimmune microbiome are variable. Some researchers suggest that these bacteria may be more highly exposed to foreign substances, given the increased gut permeability and leakiness associated with the disease. Interaction between the autoimmune gut microbiome and pathogenic GI bacteria can result in altered T cell regulation and increased production of pro-inflammatory factors, preceding the autoimmune event that characterizes T1DM. These research studies have classified the autoimmune microbiome in T1DM and have identified plausible mechanisms that contribute to disease development.

Several studies have investigated the use of prebiotics and probiotics as a means to normalize the gut microbiome and prevent occurrence of disease. In an NOD model, researchers observed reduced insulinitis, beta cell destruction and decreased incidence of T1DM with probiotic administration. Additionally, prebiotics such as RS have been shown to positively alter the microbial composition of the gut, promoting gut integrity and preventing bacterial
invasion.\textsuperscript{114,115} These results suggest that probiotics and prebiotics may be optimal dietary strategies in the treatment and prevention of various pathologies including diabetes, obesity and gastrointestinal disorders.

Collectively, these research findings suggest the microbiome and immune system to be critical epigenetic factors in the development of T1DM.\textsuperscript{116} This provides positive implications for administration of probiotics and prebiotics as a means to normalize the gut microbiome and prevent the onset of T1DM in susceptible individuals. Overall, research suggests that RS may be implemented as a therapeutic dietary aid for prevention and treatment of T1DM.
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55. Smazal AL, Borcherding NC, Anderegg AS, Schalinske KL, Whitley EM, Rowling MJ. Dietary resistant starch prevents urinary excretion of 25-hydroxycholecalciferol and


