2016

The effect of whole egg on DNA hypomethylation and disruption of one-carbon metabolism in insulin dependent diabetes mellitus

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by

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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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2016

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LIST OF ABBREVIATIONS

5caC: 5-carboxycytosine

5hmC: 5-hydroxymethylcyotsine

5fC: 5-formalcytosine

5mC: 5-methylcytosine

AGEs: advanced glycation end-product(s)

AOPPs: advanced oxidation protein product(s)

BHMT: betaine-homocysteine S-methyltransferase

CAT: catalase

CBS: cystathionine β-synthase

Cp: ceruloplasmin

CVD: cardiovascular disease

DMG: dimethylglycine

DNMT: DNA methyltransferase

GAMT: guanidinoacetate N-methyltransferase

Ghb: glycated hemoglobin

GNMT: glycine N-methyltransferase

GSH: glutathione

GSSG: glutathione disulfide

MAT: methionine adenosyl transferase

MDA: malondialdehyde

MMP-9: matrix metalloproteinase-9

MS: methionine synthase

MTHFR: methylenetetrahydrofolate reductase

NAFLD: non-alcoholic fatty liver disease

NO: nitric oxide

PEMT: phosphatidylethanolamine methyltransferase

PLP: pyridoxal phosphate

Pol II: RNA polymerase II

SAH: S-adenosyl homocysteine

SAM: S-adenosyl methionine

SOD: superoxide dismutase

STZ: streptozotocin

T1DM: type 1 diabetes mellitus

T2DM: type 2 diabetes mellitus

TBARS: thiobarbituric acid reacting substances

TDG: thymine DNA glycosylase

TET: ten-eleven translocase

THF: tetrahydrofolate

TMG: trimethylglycine

VLDL: very low density lipoproteins

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GENERAL INTRODUCTION

Diabetes and its complications are a steadily increasing cause of mortality worldwide and has a significant financial impact on the U.S. to the tune of $245 billion annually as of 2012 [1]. While there is ongoing research into potential cures for Type 1 diabetes mellitus (T1DM), understanding the mechanism behind the increased prevalence of diabetic comorbidities is critical to gaining a better understanding of chronic health conditions as a whole. The intention of this review of previous literature is to elucidate both the basics of epigenetics, which is the alteration of gene expression without changing the physical genetic structure, and the one-carbon metabolism pathway. During this exploration, the basic pathway will be defined and common perturbations to this pathway will be discussed. The relationship between these pathway alterations and chronic disease states will be examined with a specific eye towards their relationship to diabetes. In chapter 2, the use of a dietary intervention and its effect on the changes to one-carbon metabolism will be studied, and the results will be discussed in detail. Dietary interventions play a primary role in the treatment of diabetes, and this research is evaluating the use of diet in prevention of diabetic comorbidities at the level of one-carbon metabolism.

CHAPTER 1: LITERATURE REVIEW

Introduction

DNA methylation involves a set of interdependent reactions which, when disrupted, can lead to serious metabolic consequences. Methyl groups added to DNA via DNA methyltransferase (DNMT) enzymes control gene expression, and are donated almost exclusively by the conversion of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH). Methyl groups are attached to CpG islands primarily at either the 3' or 5' end of the gene. CpG islands are a series of attached Cytosines and Guanines in the nucleic acid sequence, connected by a phosphodiester bond. Genes are transcribed as RNA polymerase II (Pol II) travels from the 5' end of a gene towards the 3' end of a gene matching a new set of nucleotides, thereby forming a new chain of RNA. Pol II has been shown to pause and accumulate just before promoter regions, a method of regulating RNA and controlling gene expression. The signaling for this pause is the methylation of promoter regions of the gene, which blocks Pol II and leads to its eventual release from the gene, preventing gene transcription. DNA methylation at the 3' end of a gene can result in a more stable mRNA strand. The methylation of the 3' end also causes Pol II to pause transcription allowing for a more efficient termination of transcription [2].

Counter to the DNA methylation pathway, there exists an active method of DNA demethylation. In order for a methyl group to be removed from a CpG island, it must first undergo several conformational changes. The ten-eleven-translocase (TET) family of enzymes are responsible for catalyzing these changes. TET1 can oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which can then be oxidized to 5-formalcytosine (5fC) by TET2,

which can be further oxidized by TET3 to 5-carboxycytosine (5caC). Both 5fC and 5caC can be actively converted back to cytosine by thymine DNA glycosylase (TDG) mediated base excision repair [3].

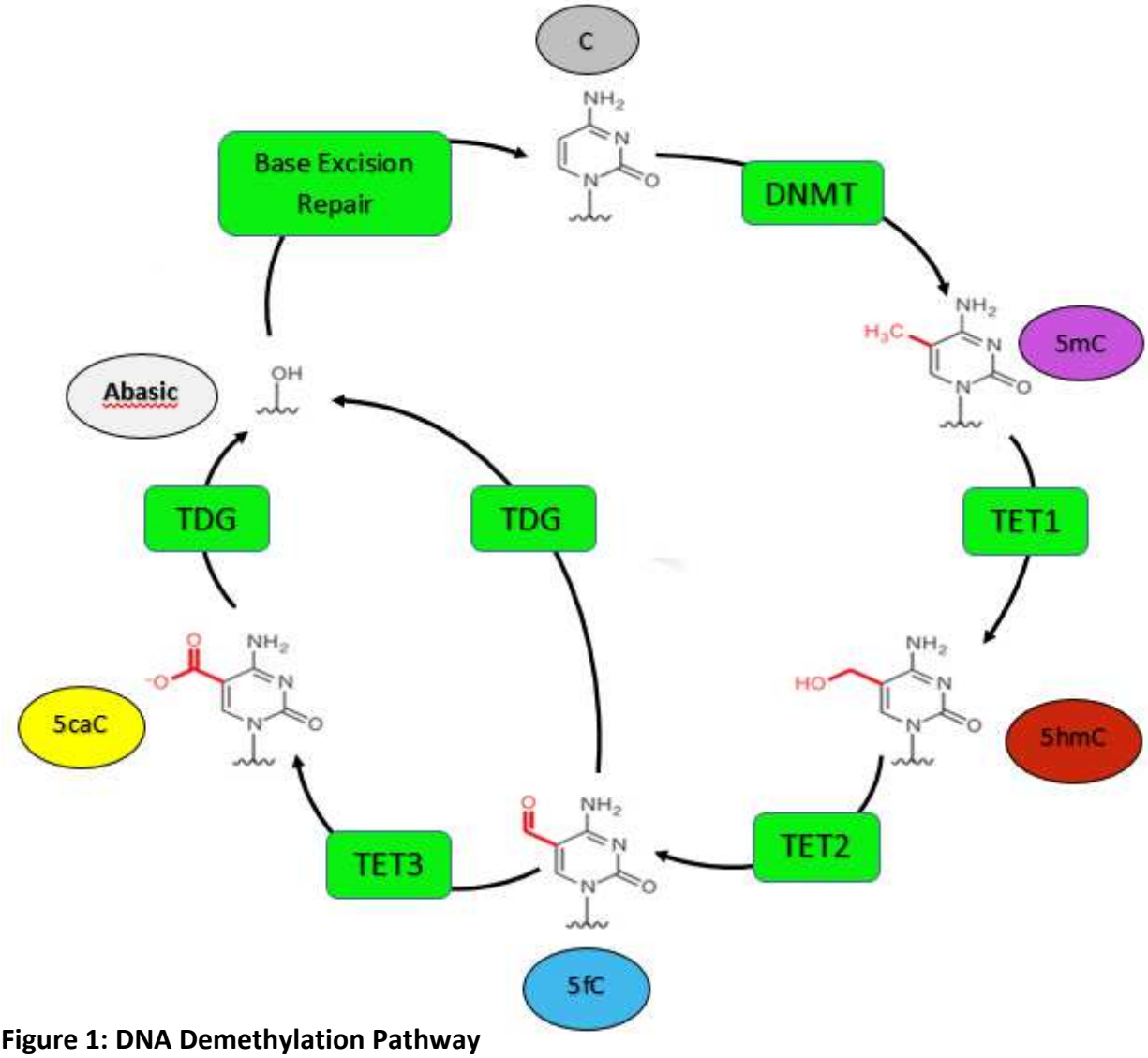

Figure 1: DNA Demethylation Pathway

One-carbon metabolism

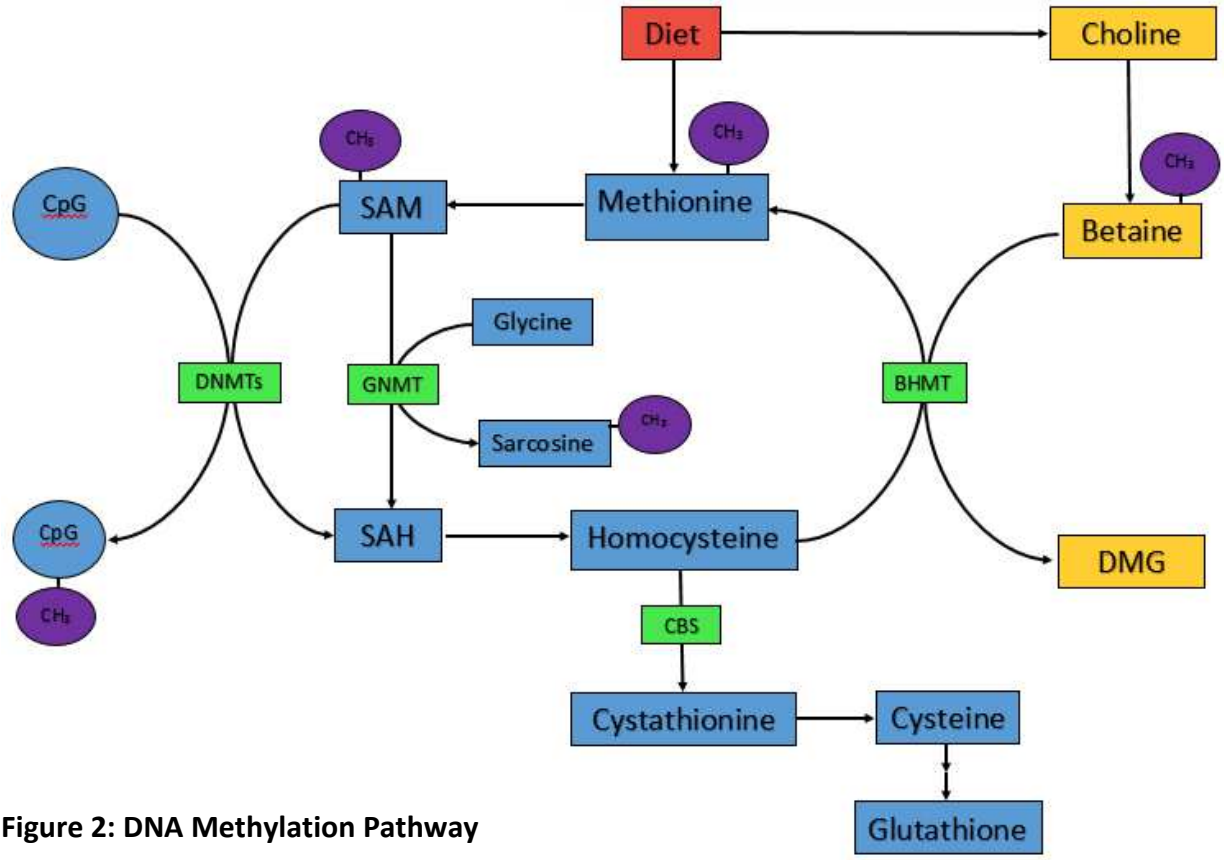

Figure 2: DNA Methylation Pathway

SAM is generated from the adenylation of methionine by methionine adenosyltransferase (MAT). Depending on the tissue, MAT can have several different isoforms. MAT I and MAT III are expressed in the liver and MAT II is not. MAT I and MAT II are allosterically inhibited by SAM. MAT III is not regulated by SAM, which allows for the prevention of methionine build-up when SAM is abundant.

SAM can undergo many different transmethylation reactions. The previously described DNMT reaction combines SAM and an unmethylated CpG island. The result of the transmethylation is SAH and methylated DNA, with the methyl group from SAM being removed

and placed onto the CpG island. Other key methyltransferases include phosphatidylethanolamine N-methyltransferase (PEMT), Glycine-N-methyltransferase (GNMT), and guanidinoacetate N-methyltransferase (GAMT). Combined, PEMT, responsible for creating phosphatidylcholine, and GAMT, responsible for creatinine synthesis, account for 85% of all transmethylation reactions that are SAM dependent [4]. GNMT maintains the SAM:SAH ratio by accepting the methyl group from SAM and attaching it to glycine to form sarcosine [5, 6]. SAH hydrolase cleaves the adenosyl group off SAH, leaving homocysteine which has several important pathways through which it can proceed. Homocysteine can be methylated to regenerate methionine via a folate-dependent pathway or a folate-independent pathway.

The folate-dependent pathway

The folate-dependent pathway occurs in all bodily tissues and involves a B12 dependent transmethylation reaction where methionine synthase (MS) converts homocysteine to methionine. 5,10-methylenetetrahydrofolate is converted to 5-methyltetrahydrofolate by methylenetetrahydrofolate reductase (MTHFR). Subsequently, 5-methyltetrahydrofolate has its methyl group removed by MS and added to homocysteine regenerating methionine. The remaining folate is in the form of tetrahydrofolate (THF). Folate cannot be synthesized in the human body, making this pathway dependent on dietary intake. Folate is absorbed from the diet in the form of THF, which is converted to 5,10-methylenetetrahydrofolate for its use in this pathway. This transmethylation pathway ties back into SAM and SAH regulation because 5-methyltetrahydrofolate allosterically inhibits GNMT, which increases SAM levels when they

become low. High SAM levels can inhibit MTHFR activity, which allows GNMT to return SAM levels to normal [5].

The folate-independent pathway

The folate-independent pathway occurs predominately in the liver and kidneys and regenerates methionine from homocysteine using betaine (trimethylglycine, TMG) as the methyl donor. Choline from the diet is oxidized to TMG. Betaine-homocysteine S-methyltransferase (BHMT) transfers a methyl group from TMG to homocysteine, resulting in the regeneration of methionine and leaving dimethyl glycine (DMG). This pathway also has a regulatory function where elevated SAM levels inhibit BHMT, decreasing homocysteine recycling and lowering SAM levels. When SAM levels are depleted, BHMT activity is not inhibited, increasing homocysteine recycling and returning SAM levels to normal [7].

Glutathione pathway

Homocysteine can also be converted to cysteine via the B6 (PLP) dependent cystathione-β-synthase (CBS). CBS catalyzes a transsulfuration reaction that is allosterically regulated by SAM. Homocysteine is converted to cystathionine through a a transsulfuration reaction with serine. Subsequently γ-cystathionase converts cystathionine to cysteine. Cysteine can then be utilized by the body or γ-glutamylcysteine synthetase can combine glutamate and cysteine to form γ-Glutamylcysteine. Glycine and γ-Glutamylcysteine are then condensed into

glutathione through a reaction catalyzed by Glutathione synthetase [8]. Glutathione acts as a primary antioxidant in the body as glutathione peroxidase catalyzes a reaction between 2 GSH molecules and a hydrogen peroxide radical. The resulting oxidized glutathione (GSSG) can then be reduced by other antioxidants like ascorbic acid or by the conversion of NADPH + H^+ to $NADP^+$ and water.

Diseases and methylation

While adequate dietary intake of folate, B6, B12, choline, and protein is essential for regulating one-carbon metabolism and maintaining optimum health in a healthy person, this is not necessarily sufficient in certain medical conditions. In regards to one-carbon metabolism, folate is required for the recycling of homocysteine through the donation of a methyl group. Folate deficiency is most commonly associated with neural tube defects during fetal growth [9]. 5,10-methylenetetrahydrofolate donates a methyl group to deoxyuridine monophosphate to create thymidine monophosphate, a reaction catalyzed by thymidylate synthetase. Thymidine monophosphate is a nucleic acid needed for DNA synthesis leading folate to play a crucial role in protein regulation, red blood cell formation, and the development of some cancers [10, 11]. In addition to limiting the rate at which MS can regenerate methionine from homocysteine by limiting the substrate THF, inadequate dietary intake of folate can inhibit gene expression by negatively impacting DNA synthesis [10]. B12 is a key cofactor for MS function and inadequate B12 intake can also limit the ability of MS to function correctly.

PLP is a cofactor for CBS which is a key enzyme in the synthesis of glutathione, a major antioxidant. Reactive oxygen species are strongly linked with CVD, cancer, retinopathy, osteoporosis, and any number of other serious medical conditions [12-14]. Choline is also an important dietary factor with regards to one-carbon metabolism. Choline provides the substrate for BHMT to convert homocysteine to methionine. Deficiencies in all of these molecules result in increased levels of homocysteine as it cannot be converted to either methionine or cystathionine.

In addition to dietary factors, genetics can also be a source of perturbations of one-carbon metabolism. A polymorphism to the MTHFR gene can severely inhibit the folate-dependent pathway of methionine regeneration. The most common polymorphism to the MTHFR gene is a single nucleotide, C677T, which has been linked to hyperhomocysteinemia and disruptions to one-carbon metabolism [15, 16].

As of 2012, 29 million U.S. citizens had diabetes with 1.4 million people being diagnosed with diabetes every year [17]. Diabetes and its comorbidities have strong ties to altered DNA methylation. Diabetes itself disturbs one-carbon metabolism through increased secretion of glucagon and cortisol. In previous research, cortisol increased GNMT activity, a perturbation seen in diabetics [18, 19]. This alteration to one-carbon metabolism plays a role in altered gene expression, increasing the prevalence of diabetic comorbidities.

A study of both Type 1 and Type 2 diabetics in Australia found a significantly increased risk for numerous types of cancers [20]. Certain cancers, like liver cancer, are linked to perturbed DNA methylation. For example, rats fed a low methyl diet will form liver tumors [21,

22]. Studies have also shown strong correlations between CVD and altered one-carbon metabolism due to perturbations of homocysteine concentrations. Hyperhomocysteinemia is an independent risk factor for CVD, but hypohomocysteinemia can also be problematic as it limits the synthesis of glutathione, a crucial antioxidant. The peroxidation of lipoproteins is an important step in the development of CVD, a process normally kept under control by glutathione. Since CVD is the most common cause of death among diabetics, it seems there is a relationship between altered one-carbon metabolism, diabetes and its comorbidities [23].

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a potential precursor to liver cancer and affects 50% of diabetic patients [24]. Choline is a key molecule in the synthesis of very low density lipoproteins (VLDL), the primary carrier of triglycerides secreted by the liver. Phosphatidylcholine is formed from the methylation of phosphatidylethanolamine by PEMT in the liver, or from choline from the diet. Without phosphatidylcholine to form VLDL, triglycerides cannot by transported out of the liver and will accumulate leading to NAFLD [25]. A study of DNA methylation patterns in rats fed a low choline diet found gene specific hypermethylation accompanied a global trend of demethylation. Six-week-old male rats were fed a choline-deficient L-amino acid-defined diet, some of which were sacrificed at 4 days, 8 days and 3 weeks. Methylation of genes E-cadherin, Cx26, Rassf1a, c-myc, and c-fos was specifically measured. In the low choline diet, E-cadherin, Cx26, and Rassf1a were all methylated, whereas in normal livers these genes were unmethylated [21, 22]. However, both c-fos and c-myc were

methylated in the livers of control rats, but both genes were hypomethylated in the livers of rats fed low choline diets. This study suggested gene-specific hypermethylation patterns in the development of hepatocarcinogenesis, as both c-fos and c-myc are known oncogenes [21, 22]. The trend of global hypomethylation with gene specific hypermethylation is a trend seen in numerous types of cancer.

Oxidative stress

The exact mechanism(s) between diabetes and its comorbidities is not known. Increased oxidative stress and/or protein glycation are supported by research as possible mechanisms [26]. Diabetes has been shown to increase levels of malondialdehyde (MDA), glycosylated hemoglobin (GHb), nitric oxide (NO), advanced glycation endproducts (AGEs), advanced oxidation protein productions (AOPPs), and thiobarbituric acid reacting substances (TBARS), which are all circulating biomarkers of oxidative stress [26]. At the same time, diabetes has been shown to lower levels of GSH, and decrease superoxide dismutase (SOD) activity, catalase (CAT) activity, and ceruloplasmin (Cp) activity, all key molecules in the body's defense against oxidative stress [26]. Copper ions play a significant role in the defense against oxidative stress. Ceruloplasmin and SODs are copper containing molecules, whose activity is severely inhibited in a hyperglycemic state. The inhibition of SOD activity in diabetics is due to the loss of both Zn^{2+} and Cu^{2+} from the enzyme [27, 28]. In a study of the effect of diabetes on trace element metabolism, Cu^{2+} plasma levels increased significantly while Mg^{2+}, Ca^{2+}, and Zn^{2+} levels all dropped significantly. The increased Cu^{2+} levels are caused by glycation and release of Cu^{2+}

ions from copper-containing enzymes, which is supported by the decreased reactivity of SOD and Cp. In addition to decreased activity of antioxidant enzymes, the increased plasma copper levels accelerate the creation of reactive oxygen species. The transition from O2- to H2O2 is a process normally controlled by SOD. However, glucose autoxidation, which can be catalyzed by Cu2+ ions, increase the production of H2O2, OH- , O2- and ketoaldehydes [27]. Ketoaldehydes are protein reactive molecules that can fragment normal proteins via free radical mechanisms [29]. Increased AOPPs are evidence of increased protein glycation, and specifically relate back to CVD. Diabetes is associated with increased levels of LDL that has been glycated or oxidized [30]. Oxidized LDL promotes atherogenesis through increased levels of TBARS [31]. Heinecke et al. [32] proposed that levels of TBARS are increased due to copper ions catalyzing lipid peroxidation, increasing LDL oxidation in diabetics.

Reduced glutathione levels were also decreased in diabetics. Glutathione is a major antioxidant in the body, and 2 GSH molecules are oxidized to GSSG, which can then be reduced by vitamin C or other dietary antioxidants. Glutathione is produced via the previously described transsulfuration pathway. This pathway is blocked in diabetes; γ-glutamyl-cysteine synthetase activity is inhibited by a lack of Mg2+ ions, which prevents the combination of cysteine and glutamate to form glutathione [33]. This leads to the build-up of cysteine seen in diabetics [33]. Oxidized glutathione is normally reduced back to GSH, keeping GSH concentrations relatively static. However, glutathione reductase, the FAD dependent enzyme catalyzing the reduction of GSSG to GSH, is inhibited by high circulating levels of copper ions [34]. This means that in addition to a lack of new glutathione production, existing glutathione has been removed from circulation due to a lack of active reduction reactions.

Nephropathy

AGEs result from the increased rate of protein glycation due to hyperglycemia seen in diabetes mellitus. Frequent and/or prolonged bouts of hyperglycemia lead to the accumulation of AGEs which have been shown to accumulate in the kidneys at higher rates among diabetics [35]. This accumulation is specific to the mesangial cells, renal tubules, and glomerular basement membranes [36, 37]. This AGE accumulation can increase production of glomerular extracellular matrix α1 (IV) collagen, laminin B1, and transforming growth factor β [38]. AGEs may also be able to crosslink or chemically modify matrix or plasma proteins. All these factors build towards basement membrane thickening, glomerular hypertrophy, and mesangial extracellular matrix expansion, which are linked to glomerulosclerosis progression, proteinuria, and albuminuria [39]. This shows a pathogenic pathway linking hyperglycemia to nephropathy.

Retinopathy

Similar to the mechanism above, AGEs accumulate in the neuroglia and retinal vessels of people with diabetes and there is an apparent cell-type specific pattern of accumulation of AGEs [40-43]. Recent research has shown that if preformed AGE albumin, albumin that has intentionally been glycated, is injected into non-diabetic rats it will accumulate in the microvasculature of the retina, specifically in pericytes and smooth muscle cells. These two cell types are of note since they undergo early cell death during diabetic retinopathy [44, 45].

AGEs and DNA methylation are intertwined in that DNA hypomethylation can prevent the body from being able to remove AGEs from the system. For example, a study on wound healing in diabetic foot ulcers found an abnormally high expression of matrix metalloproteinase-9 (MMP-9), which can suppress wound healing. AGEs were found to increase the expression of MMP-9 in skin primary keratinocytes [45]. It was shown that TET2 upregulation and MMP-9 promoter demethylation were both present in diabetics, providing an explanation for poor wound healing seen in diabetics [45]. As previously mentioned, T1DM is associated with increased activation of the demethylation pathway including TET2, and this study highlights the link between the body's ability to defend against AGEs and DNA methylation [46].

Hypomethylation

There are two known pathways that result in a hypomethylated state. Decreased levels of methyl donors (SAM), as was seen in rats on low methyl diets, results in the decreased ability to add methyl groups to DNA. Increased activity of the pathway responsible for removal of methyl groups from DNA is also a possibility, where the methyl groups removed from DNA are reintroduced to the one-carbon metabolism pathway. The most likely scenario is a combination of the two pathways where both a lack of methyl donors and an increase in DNA demethylation occur simultaneously.

Numerous studies have shown altered levels of substrate components in one-carbon metabolism pathways in various disease states. While looking at the effect of diabetes on

wound healing, Dhliwayo et al. [3] found that Type 1 diabetes induced increased levels of 5hmC and 5fC in zebrafish. This coincided with increased levels of TET family enzymes and increased TDG activity, supporting the hypothesis that T1DM results in increased removal of methyl groups from DNA to supplement depleted SAM levels. Williams et al. [47] studied the effect of uncontrolled diabetes on hepatic DNA methylation over an 8 week period. Williams found increased activity and abundance of GNMT and increased activity of phosphatidylethanolamine N-methyltransferase (PEMT). Increased GNMT activity indicates methyl groups being bled off for purposes other than DNA methylation, and increased PEMT is used in the natural creation of choline in the body from phosphatidylcholine. They found increased BHMT activity and decreased MS activity, which indicated a shift away from the folate-dependent pathway and towards the folate-independent pathway for methionine regeneration. While not studied, the preferential utilization of the folate-independent pathway could be due to the end product of DMG, which has been shown to have some antioxidant properties [48]. The enzymes dimethylglycine dehydrogenase and sarcosine dehydrogenase can convert DMG to glycine in rat livers**.** Glycine is a required substrate for GNMT transmethylation, and is more readily produced by heavy reliance on the folate-independent pathway through the conversion of DMG to glycine. While tied more closely with T2DM, a genome-wide association study showed a significant correlation between low plasma DMG levels and high blood glucose concentrations [49]. Williams et al. also found an increased abundance of CBS indicating that T1DM results in the increased conversion of homocysteine to cystathionine; however the downstream synthesis of glutathione is limited such that hepatic levels of glutathione (GSH) are decreased and decreased levels of GSH is a risk factor for atherosclerosis [50, 51]. This supports the hypothesis

that DMG is preferentially produced for its antioxidant properties, and that the body reacts to diabetic hyperglycemia by increasing antioxidant production to account for the loss of both SOD to glycation and glutathione to inhibition. Williams et al. also found that both SAM and SAH levels, as well as the SAM:SAH ratio did not differ between T1DM rats and control rats. This could be due to dietary replenishment or a possible explanation for the increased removal of DNA methylation. As homocysteine is being removed from circulation at an increased rate, less is being recycled to methionine and some deficit would seem inevitable without significant dietary intervention. If the methyl groups removed from DNA can re-enter the cycle as methionine, this could provide a possible explanation for the lack of change in absolute values of SAM and SAH.

The alteration to homocysteine remethylation could be a key factor in understanding the long term effects of diabetes. In T1DM patients without renal complications, plasma homocysteine levels were decreased compared to a person without diabetes [51]. Insulin treatment returned homocysteine levels to normal in a dose dependent manner [52]. This is important because homocysteine can form reactive oxygen species (ROS) in plasma and decrease glutathione (GSH) peroxidase activity, the enzyme catalyzing the oxidation of glutathione [53]. This would seem to indicate diabetics are at risk for CVD complications during both hyperglycemia through protein glycation and hypoglycemia through insulin induced GSH inhibition.

Another aspect of this reaction is increased glucagon and cortisol secretion. Rats treated with doses of glucagon showed decreased levels of homocysteine, but increased activity of

GNMT, CBS, BHMT, and cystathionine gamma-lyase in the liver. Levels of SAM and SAH were also increased [54, 55]. Similarly, dexamethasone treated rats had increased GNMT activity [18, 19]. In STZ-induced diabetic rats, glucagon secretion was elevated at baseline and in response to arginine. These increases were not inhibited to the same degree by high concentrations of glucose or high doses of insulin compared to control. However, glucagon secretion was inhibited by insulin when endogenous secretion was relatively high. This is an important distinction in that it provides a potential explanation for the hypermethylation seen in T2DM. If the perturbations to one-carbon metabolism are due to excessive glucagon, insulin-independent diabetics would not necessarily experience the same issues because the glucagon secretion is normalized by the endogenous secretion of insulin. This indicates that in STZ-induced diabetes, hyperglucagonemia could be responsible for the changes to one-carbon metabolism and subsequent transmethylation reactions [56].

T1DM was also shown to increase DNMT1 abundance, which initially seems counterintuitive with the global hypomethylation seen with T1DM. Recent research has shown that DNMT1 has a higher affinity for DNA strand breaks, gaps, and uracil than for partially methylated CpG islands. DNA strand breaks, gaps, and uracil are common in the early stages of folate and methyl deficiency [57]. Diets low in methionine, choline, and folic acid were associated with DNA strand breaks and global hypomethylation and increased DNMT activity [58]. Hypomethylation decreased the stability of chromatin structure, making it more vulnerable to DNA-damage [58]. Diabetes has the potential to induce choline deficiency by over-activation of the folate-independent pathway due to the use of choline, converted to betaine, as the primary methyl donor for the regeneration methionine. This would lead to

similar genetic outcomes as a low methyl diet. This coincides with recent research into diabetes showing global hypomethylation and increased DNMT activity.

In human pre-neoplastic cells, DNA strand breaks and gaps are frequently seen, showing a link between diabetic hepatic hypomethylation and liver cancer among diabetics. Williams' results showed hepatic CpG hypomethylation and a trend towards increased genomic DNA hypomethylation in T1DM rats [47], which supports the previous studies findings.

Given the switch of primary remethylation pathways of homocysteine from folate-dependent to folate-independent, the question becomes: Is choline abundance, or lack thereof, a limiting factor in maintaining one-carbon metabolism and subsequent DNA methylation. Eggs provide an excellent opportunity to increase three key substrates into one-carbon metabolism to test this theory. Eggs have around 250mg of choline per 100g of egg [59]. Eggs also have upwards of 144mg cysteine and 242mg of methionine per egg [60]. The choline provides the methyl donor for BHMT to convert homocysteine to methionine, the cysteine has the potential to regulate the glutathione pathway by reducing the need for *de novo* synthesis from homocysteine, and methionine increases the amount of substrate for adenylation to SAM. This makes eggs an ideal dietary vehicle to determine whether one-carbon metabolism is inhibited by the lack of substrate availability in diabetics.

CHAPTER 2: THE EFFECT OF WHOLE EGG ON DNA HYPOMETHYLATION IN MALE STZ-INDUCED DIABETIC RATS

ABSTRACT

Diabetes mellitus is linked with a higher risk of cardiovascular disease and certain types of cancer [61-63]. A study of Type 1 and Type 2 diabetics in Australia showed multi-fold increases in the risk for several types of cancer. Diabetics are also two to four times more likely to have CVD than an adult without diabetes. The common thread among these three medical conditions is oxidative stress. To date, little work has been done on the impact of dietary intervention on the body's ability to handle the increased oxidative stress caused by diabetes. In this study, male Sprague-Dawley rats were fed a high choline diet for 4 weeks (3 weeks acclimation and 1 week experimental). Diabetes was induced using STZ and after 1 week the rats were sacrificed and liver tissue was harvested for study. There was a significant decrease in GNMT abundance between the non-diabetic control and whole egg diet groups, but not between the diabetic control group and whole egg diet group. There was no attenuation of hepatic hypomethylation in the group fed the high choline diet in comparison to the diabetic control group ($P = .121$). No significant difference was seen in serum TG levels between groups ($P = 0.462$).

INTRODUCTION

In 2014, an estimated 9% of adults globally had diabetes mellitus [64]. The World Health Organization predicts that by 2030, diabetes will be the 7th leading cause of death, moving ahead of HIV/AIDS and diarrheal diseases common in the developing world [65]. This is a very misleading statistic though, as diabetes is rarely listed as the cause of death. A 2001 study showed that 50% of people with diabetes die of cardiovascular disease, which is then recorded as the cause of death [66]. Harding et al. recently found a trend towards increased cancer risk in people with diabetes, specifically liver cancer, which had a 3 fold increase in the incidence rate [20]. The cost for treating diabetes has risen from $147 billion in 2007 to $245 billion in 2012 prompting a huge influx of studies in regards to health outcomes for diabetics [1].

However, little work has been conducted in the area of dietary interventions on DNA methylation in diabetes. Williams et al., studied the effects of diabetes on one-carbon metabolism by comparing an STZ-induced hyperglycemic state to a normoglycemic state [47]. Results showed normal levels of SAM, SAH, and SAM to SAH ratio, as well as increased abundance of DNMT1, and increased hepatic GNMT activity. Dhliwayo et al., studied the demethylation mechanism of diabetes in zebrafish and found increased activity of ten-eleven translocase 2 and 3, increased Thymine-DNA glycosylase (TDG), in addition to increased presence of 5-hyrdoxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine [3]. These results combine to suggest that increased rates of demethylation likely contribute to hepatic demethylation in diabetes. Providing further evidence of this, rat studies have shown rats fed a low methyl group diet have consistently developed liver cancer within a year [67].

This showed a strong relationship with disturbed one-carbon metabolism and liver cancer, which coincides with disrupted one-carbon metabolism in the liver triggered by diabetes. The disruption of one-carbon metabolism by diabetes is due to the increased secretion of glucagon and cortisol. Cortisol increases GNMT activity, a key enzyme in the regulation of one-carbon metabolism.

The Dhliwayo and Williams studies each establish a baseline measure of diabetic one-carbon metabolism for a piece of the overall pathway. However, neither answers the question as to whether disruptions to one-carbon metabolism caused by diabetic hyperglycemia can be remedied through dietary intervention. We sought to fill this gap in knowledge by not only looking at GNMT and DNA methylation like the Williams and Dhliwayo studies, but also looking at the impact of a whole egg diet on the changes to DNA methylation seen with diabetes. The combination of these methods will give a more complete picture of diabetes-induced changes to one-carbon metabolism, and the impact of choline on both aspects of methylation.

MATERIALS AND METHODS

All animal studies were approved by the Institutional Animal Care and Use Committee, and Iowa State University Laboratory Animal Resource Guidelines were followed. Male Sprague-Dawley rats (N=18) were individually housed in clear plastic cages on a 12 hour light-dark cycle. Rats were divided into 3 groups: a non-diabetic control group, a diabetic control group, and an intervention group. The two control groups were fed ad libitum on a diet containing 20% protein by weight from casein, the diabetic intervention group was fed a diet

where the 20% protein came entirely from whole egg (Table 1). Following a 24 day acclimation period, the diabetic control group (n=6) and intervention group (n=6) were given an intraperitoneal injection of Streptozotocin (STZ; 60 mg/kg bodyweight) to induce diabetes. After 1 week, the rats were fasted for 24 hours and blood glucose was measured to ensure hyperglycemia in the diabetic groups. All three groups were anesthetized by intraperitoneal injection of ketamine (90:10 mg/kg bodyweight). Whole liver tissue was snap frozen in liquid nitrogen for later study (Figure 1).

Liver concentration of DNA methylation was quantified using a commercially available colorimetric ELISA kit (EpiGentek, New York). DNA was extracted from a lysate by the Autogen Quickgene810 DNA Isolation machine. A lysate was formed following the Autogen QG-810 protocol.

Hepatic GNMT abundance was measured using a commercially available colorimetric assay kit (MyBioSource, California). Liver tissue was homogenized in phosphate buffered saline solution with 100ul of proteinase and 100ul of EDTA at a ratio of 10:1 PBS to liver tissue (0.1g tissue to 1mL PBS). Statistics were calculated using SigmaStat (Systat, Chicago, IL).

Serum levels of triglycerides were also measured using a commercially available colorimetric assay kit (BioAssay Systems, San Francisco). Plasma was diluted 5-fold to allow comparison to standards before being assayed.

Colorimetric assay plates were read on a BioTek Synergy H1 Hybrid Reader. Group means were compared using a one-way ANOVA, and Fisher LSD post-test with a p value of 0.05.

RESULTS

A high choline (whole egg) diet did not significantly change hepatic DNA methylation (P = .121, one-way ANOVA). Total hepatic DNA methylation was 1.56% in the non-diabetic control group, 0.94% in the diabetic control group, and 1.3% in the whole egg diet group. The diabetic control group showed a significant (39.7%) drop in global DNA methylation compared to the non-diabetic control group (P = 0.044), while the whole egg diet group had a 17% reduction in global DNA methylation. This puts the whole egg dietary group in the middle of the two control groups leaving it without statistical significance (Figure 2).

A high choline diet significantly decreased hepatic GNMT abundance. GNMT abundance was not significantly different between the non-diabetic control group and the diabetic control group. The non-diabetic control group had 114.7 $\pm$ 3.95 ng/g of GNMT in liver tissue, while the diabetic control group showed 99 $\pm$ 16 ng/g. The whole egg diet group had 88.4 $\pm$ 6.5 ng/g, a significant reduction in comparison to the non-diabetic control group; however, it was not significantly different from the diabetic control group (Figure 3).

A high choline diet did not significantly change serum triglyceride levels. While a diabetic condition is associated with increased serum triglyceride concentrations, the differences between groups in this study were not significantly different (P = 0.462), and there was no impact by high choline intake (Figure 4).

DISCUSSION

Previous research showed increased GNMT activity in diabetic rats[47]. Our experiment did not show a significant difference in GNMT abundance between groups. This is possibly due to the experimental model where the rats were only diabetic for 1 week. Previous work showing increased GNMT expression had samples taken starting at two weeks from diabetic inducement. It is likely that 1 week is not enough time to see the induced changes in GNMT. However, our results did show a significant decrease in GNMT abundance in the whole egg diet group compared to the non-diabetic control group. This points to a possible ability of a high choline diet to reduce the increased GNMT expression seen in T1DM. As one of the hypotheses regarding DNA hypomethylation is lack of substrate availability due to increased conversion of SAM to SAH by GNMT, the ability of the high choline diet to mitigate or prevent this change in abundance becomes critical to preventing DNA hypomethylation and its subsequent negative outcomes. This is supported by the change seen with total hepatic DNA methylation. The whole egg dietary group cut the rate of hypomethylation in half compared to the diabetic control group. This could be due to increased SAM availability or some as yet unknown mechanism.

Going forward, it will be critical to evaluate the impact of the whole egg diet over a longer period of time. If the rate of hypomethylation is merely slowed, there will come a point where DNA hypomethylation will drop to the same level as the diabetic control group. On the surface, this would seem to limit the usefulness of the high choline diet, but it retains its usefulness when translated to humans. Insulin therapy significantly decreases the rate at which

DNA is de-methylated. However, after an initial diagnosis of T1DM, human beings will undergo a potentially prolonged period of glycemic instability as the complexities and individual characteristics of diabetes are figured out. This time period is fraught with perturbations to one-carbon metabolism and subsequent DNA hypomethylation. This would provide an ideal time for a high choline diet to limit the damage being done over a relatively small time period, aiding in the prevention or slowing down the rate of progression for chronic diseases associated with T1DM.

Serum triglycerides were measured due to the high incidence of hypertriglyceridemia among diabetics. Previous work has shown that hyperglycemia can cause increased plasma triglyceride levels in diabetic rats [68]. This could be due to hyperglycemia causing favoritism of the folate-independent pathway of one-carbon metabolism. This limits the availability of choline, which over time leads to NAFLD. There is a strong, positive correlation between serum triglycerides and NAFLD [69]. By adding large amounts of choline, we hoped to show reduction in serum triglyceride levels as means of showing possible prevention of NAFLD. Serum TG concentrations, and not liver TGs, were measured because dyslipidemia is an early sign of NAFLD. None of the three dietary groups showed significant differences, which is again likely due to the shortened time frame. One week is not enough time to see significant changes to serum triglyceride levels. Similarly, four weeks on the high choline diet also did not show any changes to triglyceride levels in the rats. To our knowledge, this is the first exploration of dietary intervention in the prevention of DNA hypomethylation in diabetics.

Table 1: Nutrient breakdown of control and experimental diets.

	Casein	Whole Egg
Whole Egg/Casein		
Lipid		163g/kg
Protein	200g/kg	200g/kg
Unknown		45g/kg
Corn Oil	163g/kg	N/A
Cornstarch	437g/kg	392g/kg
Glucose (monohydrate)	150g/kg	150g/kg
Mineral Mix	35g/kg	35g/kg
Vitamin Mix	10g/kg	10g/kg
Choline bitartrate	2g/kg	2g/kg
L-Methionine	3g/kg	3g/kg

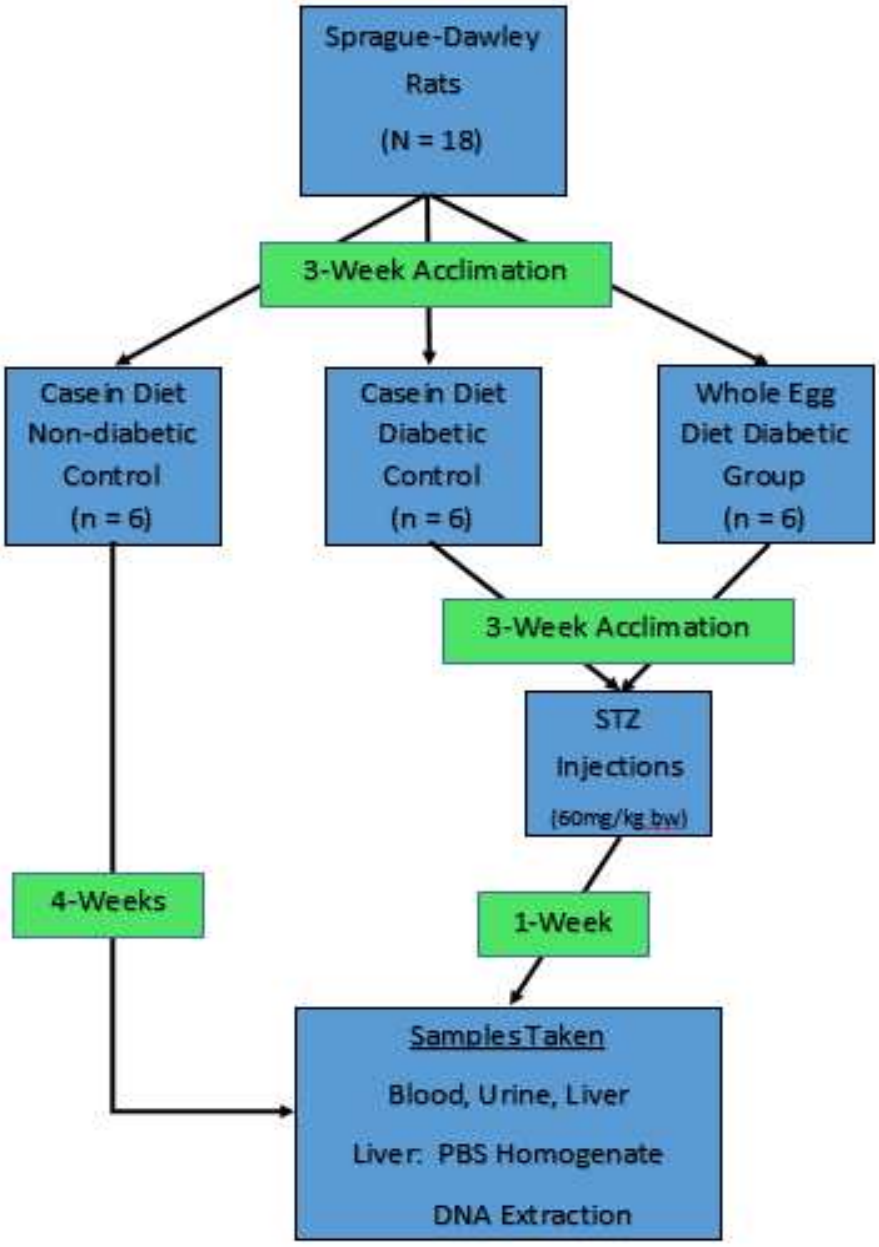

Figure 3: Experimental design.

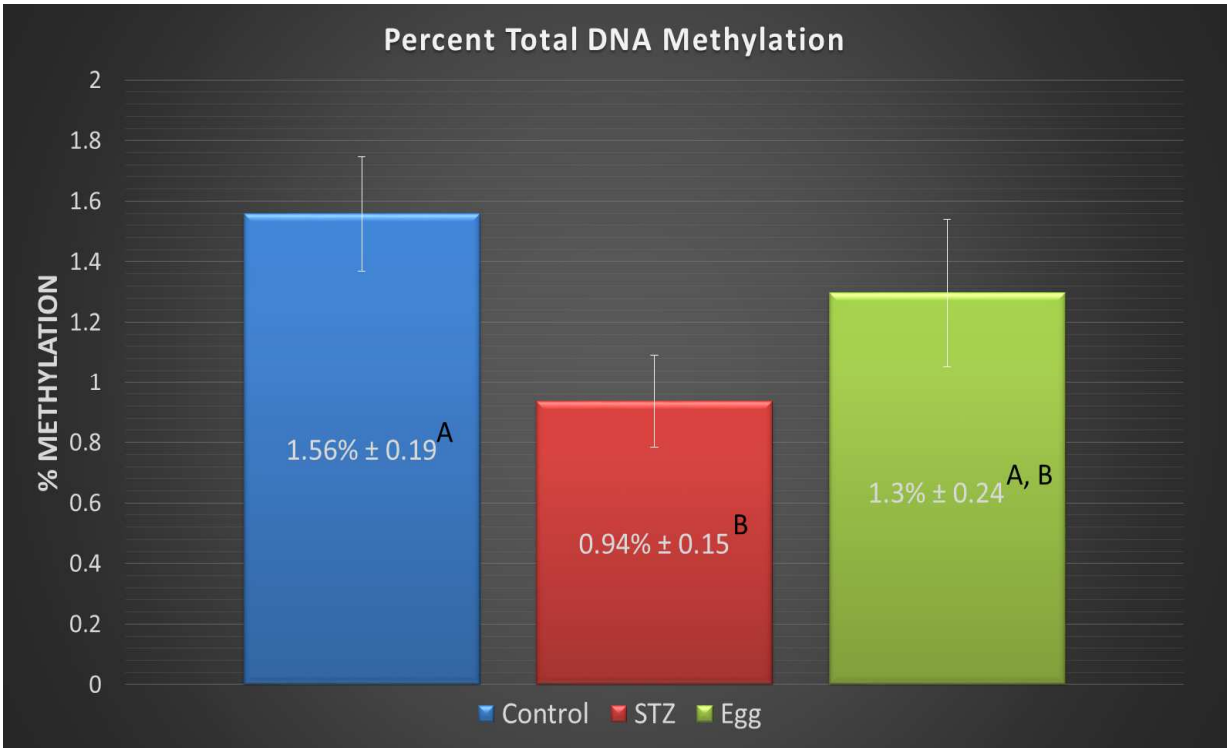

Figure 4: Percent Total Hepatic DNA methylation. Data shown as mean ± SEM. Letters indicate significant differences between groups ($p<0.05$).

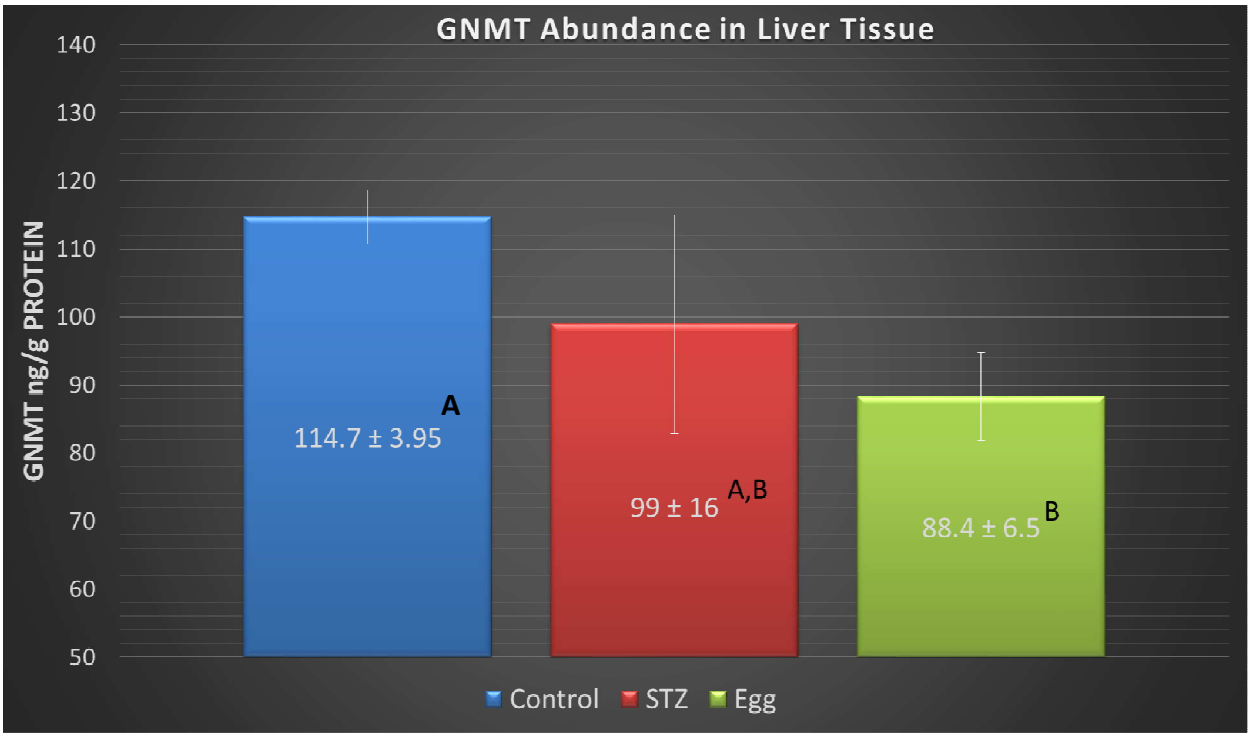

Figure 5: GNMT abundance in liver tissue. Data shown as mean ± SEM. Letters indicate significant differences between groups ($p<0.05$).

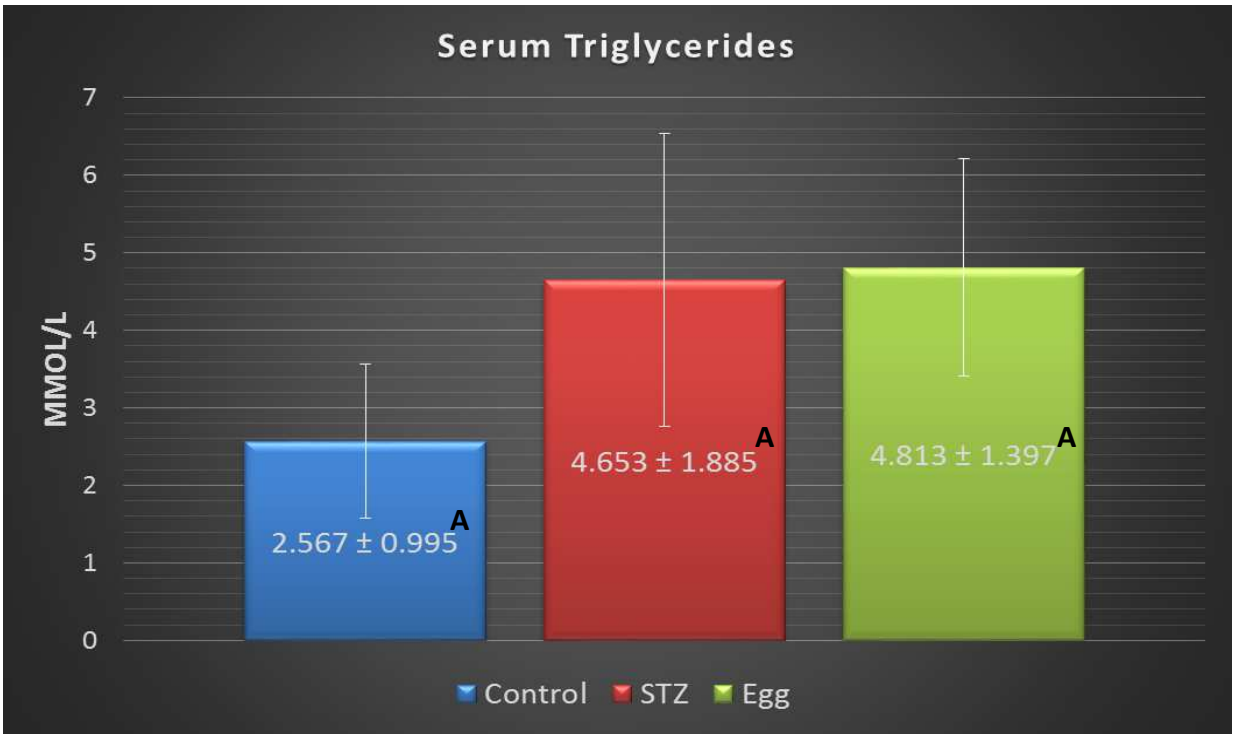

Figure 6: Serum triglyceride levels. Data shown as mean ± SEM. Letters indicate significant differences between groups ($p<0.05$).

REFERENCES

1. American Diabetes, A., *Economic costs of diabetes in the U.S. in 2012.* Diabetes Care, 2013. **36**(4): p. 1033-46.
2. Jonkers, I. and J.T. Lis, *Getting up to speed with transcription elongation by RNA polymerase II.* Nat Rev Mol Cell Biol, 2015. **16**(3): p. 167-77.
3. Dhliwayo, N., et al., *Parp inhibition prevents ten-eleven translocase enzyme activation and hyperglycemia-induced DNA demethylation.* Diabetes, 2014. **63**(9): p. 3069-76.
4. Mudd, S.H. and J.R. Poole, *Labile methyl balances for normal humans on various dietary regimens.* Metabolism, 1975. **24**(6): p. 721-35.
5. Wagner, C., W.T. Briggs, and R.J. Cook, *Inhibition of glycine N-methyltransferase activity by folate derivatives: implications for regulation of methyl group metabolism.* Biochem Biophys Res Commun, 1985. **127**(3): p. 746-52.
6. Yeo, E.J. and C. Wagner, *Tissue distribution of glycine N-methyltransferase, a major folate-binding protein of liver.* Proc Natl Acad Sci U S A, 1994. **91**(1): p. 210-4.
7. Park, E.I., M.S. Renduchintala, and T.A. Garrow, *Diet-Induced Changes in Hepatic Betaine-Homocysteine Methyltransferase Activity Are Mediated By Changes in the Steady-State Level of Its mRNA.* The Journal of Nutritional Biochemistry, 1997. **8**(9): p. 541-545.
8. Njalsson, R. and S. Norgren, *Physiological and pathological aspects of GSH metabolism.* Acta Paediatr, 2005. **94**(2): p. 132-7.
9. Zaganjor, I., et al., *Describing the Prevalence of Neural Tube Defects Worldwide: A Systematic Literature Review.* PLoS One, 2016. **11**(4): p. e0151586.
10. Eto, I. and C.L. Krumdieck, *Role of vitamin B12 and folate deficiencies in carcinogenesis.* Adv Exp Med Biol, 1986. **206**: p. 313-30.
11. Glynn, S.A. and D. Albanes, *Folate and cancer: a review of the literature.* Nutr Cancer, 1994. **22**(2): p. 101-19.
12. Halliwell, B., *Oxidative stress and cancer: have we moved forward?* Biochem J, 2007. **401**(1): p. 1-11.
13. Husain, K., et al., *Inflammation, oxidative stress and renin angiotensin system in atherosclerosis.* World J Biol Chem, 2015. **6**(3): p. 209-17.
14. Basu, S., et al., *Association between oxidative stress and bone mineral density.* Biochem Biophys Res Commun, 2001. **288**(1): p. 275-9.
15. Jacques, P.F., et al., *Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations.* Circulation, 1996. **93**(1): p. 7-9.
16. Remacha, A.F., et al., *Vitamin B12 deficiency, hyperhomocysteinemia and thrombosis: a case and control study.* Int J Hematol, 2011. **93**(4): p. 458-64.
17. Prevention, C.f.D.C.a., *National diabetes statistics report, 2014*. 2014.
18. Rowling, M.J., *Nutritional and hormonal modulation of glycine N-methyltransferase: implications for aberrant methyl group metabolism*. 2004, Digital Repository @ Iowa State University.
19. Rowling, M.J. and K.L. Schalinske, *Retinoic acid and glucocorticoid treatment induce hepatic glycine N-methyltransferase and lower plasma homocysteine concentrations in rats and rat hepatoma cells.* J Nutr, 2003. **133**(11): p. 3392-8.
20. Harding, J.L., et al., *Cancer risk among people with type 1 and type 2 diabetes: disentangling true associations, detection bias, and reverse causation.* Diabetes Care, 2015. **38**(2): p. 264-70.

21. Shimizu, K., et al., *Disturbance of DNA methylation patterns in the early phase of hepatocarcinogenesis induced by a choline-deficient L-amino acid-defined diet in rats.* Cancer Sci, 2007. **98**(9): p. 1318-22.
22. Wainfan, E. and L.A. Poirier, *Methyl groups in carcinogenesis: effects on DNA methylation and gene expression.* Cancer Res, 1992. **52**(7 Suppl): p. 2071s-2077s.
23. Federation, I.D. *Complications of diabetes*. 2015 [cited 2016 3/11/16]; Available from: http://www.idf.org/complications-diabetes.
24. Pagadala, M.R. and A.J. McCullough, *The relevance of liver histology to predicting clinically meaningful outcomes in nonalcoholic steatohepatitis.* Clin Liver Dis, 2012. **16**(3): p. 487-504.
25. Cole, L.K., J.E. Vance, and D.E. Vance, *Phosphatidylcholine biosynthesis and lipoprotein metabolism.* Biochim Biophys Acta, 2012. **1821**(5): p. 754-61.
26. Abou-Seif, M.A. and A.A. Youssef, *Evaluation of some biochemical changes in diabetic patients.* Clin Chim Acta, 2004. **346**(2): p. 161-70.
27. Zbronska, H., et al., *[Activity of superoxide dismutase in erythrocytes and leukocytes and levels of zinc and copper in blood of patients with diabetes. Effect of diabetic treatment on examined parameters].* Pol Arch Med Wewn, 1995. **94**(3): p. 228-34.
28. Lin, J., *[The association between copper ions and peroxidative reaction in diabetic cataract].* Nippon Ganka Gakkai Zasshi, 1996. **100**(9): p. 672-9.
29. Hunt, J.V. and S.P. Wolff, *Oxidative glycation and free radical production: a causal mechanism of diabetic complications.* Free Radic Res Commun, 1991. **12-13 Pt 1**: p. 115-23.
30. Tian, H., et al., *Lipoprotein(a) level and lipids in type 2 diabetic patients and their normoglycemic first-degree relatives in type 2 diabetic pedigrees.* Diabetes Res Clin Pract, 2003. **59**(1): p. 63-9.
31. Maseki, M., et al., *Lipid peroxide levels and lipids content of serum lipoprotein fractions of pregnant subjects with or without pre-eclampsia.* Clin Chim Acta, 1981. **115**(2): p. 155-61.
32. Heinecke, J.W., H. Rosen, and A. Chait, *Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture.* J Clin Invest, 1984. **74**(5): p. 1890-4.
33. Furfaro, A.L., et al., *Impaired synthesis contributes to diabetes-induced decrease in liver glutathione.* Int J Mol Med, 2012. **29**(5): p. 899-905.
34. Rafter, G.W., *Copper inhibition of glutathione reductase and its reversal with gold thiolates, thiol, and disulfide compounds.* Biochem Med, 1982. **27**(3): p. 381-91.
35. Soulis, T., et al., *Effects of aminoguanidine in preventing experimental diabetic nephropathy are related to the duration of treatment.* Kidney Int, 1996. **50**(2): p. 627-34.
36. Miyata, T., et al., *Accumulation of carbonyls accelerates the formation of pentosidine, an advanced glycation end product: carbonyl stress in uremia.* J Am Soc Nephrol, 1998. **9**(12): p. 2349-56.
37. Gugliucci, A. and M. Bendayan, *Reaction of advanced glycation endproducts with renal tissue from normal and streptozotocin-induced diabetic rats: an ultrastructural study using colloidal gold cytochemistry.* J Histochem Cytochem, 1995. **43**(6): p. 591-600.
38. Yang, C.W., et al., *Advanced glycation end products up-regulate gene expression found in diabetic glomerular disease.* Proc Natl Acad Sci U S A, 1994. **91**(20): p. 9436-40.
39. Vlassara, H., et al., *Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats.* Proc Natl Acad Sci U S A, 1994. **91**(24): p. 11704-8.
40. Hammes, H.P., et al., *Differential accumulation of advanced glycation end products in the course of diabetic retinopathy.* Diabetologia, 1999. **42**(6): p. 728-36.
41. Hammes, H.P., et al., *Aminoguanidine inhibits the development of accelerated diabetic retinopathy in the spontaneous hypertensive rat.* Diabetologia, 1994. **37**(1): p. 32-5.

42. Stitt, A.W., et al., *Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats.* Am J Pathol, 1997. **150**(2): p. 523-31.
43. Murata, T., et al., *The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas.* Diabetologia, 1997. **40**(7): p. 764-9.
44. Gardiner, T.A., et al., *Selective loss of vascular smooth muscle cells in the retinal microcirculation of diabetic dogs.* Br J Ophthalmol, 1994. **78**(1): p. 54-60.
45. Kern, T.S. and R.L. Engerman, *Vascular lesions in diabetes are distributed non-uniformly within the retina.* Exp Eye Res, 1995. **60**(5): p. 545-9.
46. Zhang, J., et al., *AGE-induced Keratinocyte MMP-9 Expression is Linked to TET2-mediated CpG Demethylation.* Wound Repair Regen, 2016.
47. Williams, K.T., T.A. Garrow, and K.L. Schalinske, *Type I diabetes leads to tissue-specific DNA hypomethylation in male rats.* J Nutr, 2008. **138**(11): p. 2064-9.
48. Takahashi, T., et al., *N, N-Dimethylglycine decreases oxidative stress and improves in vitro development of bovine embryos.* J Reprod Dev, 2016.
49. Magnusson, M., et al., *Dimethylglycine Deficiency and the Development of Diabetes.* Diabetes, 2015. **64**(8): p. 3010-6.
50. Loven, D., et al., *Effect of insulin and oral glutathione on glutathione levels and superoxide dismutase activities in organs of rats with streptozocin-induced diabetes.* Diabetes, 1986. **35**(5): p. 503-7.
51. Matteucci, E., et al., *Blood levels of total homocysteine in patients with type 1 diabetes (with no complications, diabetic nephropathy and/or retinopathy) and in their non-diabetic relatives.* Nutr Metab Cardiovasc Dis, 2002. **12**(4): p. 184-9.
52. Gursu, M.F., et al., *Insulin increases homocysteine levels in a dose-dependent manner in diabetic rats.* Arch Med Res, 2002. **33**(3): p. 305-7.
53. Chen, N., et al., *Physiologic concentrations of homocysteine inhibit the human plasma GSH peroxidase that reduces organic hydroperoxides.* J Lab Clin Med, 2000. **136**(1): p. 58-65.
54. Jacobs, R.L., et al., *Hyperglucagonemia in rats results in decreased plasma homocysteine and increased flux through the transsulfuration pathway in liver.* J Biol Chem, 2001. **276**(47): p. 43740-7.
55. Karelin, A.A., M.S. Bikmetov, and A. Nikolaev, *[Stimulating effect of glucagon and theophylline on the activity of rat liver betamine-homocysteine-methyltransferase. Role of cyclic adenosine-3',5'-monophosphate].* Vopr Med Khim, 1977. **23**(2): p. 188-93.
56. Weir, G.C., et al., *Glucagon secretion from the perfused pancreas of streptozotocin-treated rats.* Diabetes, 1976. **25**(4): p. 275-82.
57. James, S.J., et al., *Mechanisms of DNA damage, DNA hypomethylation, and tumor progression in the folate/methyl-deficient rat model of hepatocarcinogenesis.* J Nutr, 2003. **133**(11 Suppl 1): p. 3740S-3747S.
58. Pogribny, I.P., et al., *Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl-deficient rats.* Cancer Res, 1995. **55**(9): p. 1894-901.
59. Patterson, K.Y., Bhagwat, Seema A., Williams, Juhi R., Howe, Juliette C., Holden, Joanne M., *USDA Database for the Choline Content of Common Foods Release Two*, USDA, Editor. 2008, USDA.
60. Csonka, F.A., *Nitrogen, methionine and cystine content of hen's eggs. Their distribution in the egg white and yolk.* J Nutr, 1950. **42**(3): p. 443-51.

61. Herrmann, M., et al., *The effect of B-vitamins on biochemical bone turnover markers and bone mineral density in osteoporotic patients: a 1-year double blind placebo controlled trial.* Clin Chem Lab Med, 2007. **45**(12): p. 1785-92.
62. Refsum, H., et al., *Homocysteine and cardiovascular disease.* Annu Rev Med, 1998. **49**: p. 31-62.
63. Miller, A.L., *The methylation, neurotransmitter, and antioxidant connections between folate and depression.* Altern Med Rev, 2008. **13**(3): p. 216-26.
64. Organization, W.H. *Global status report on non-communicable diseases 2014*. Geneva 2014; Available from: http://www.who.int/nmh/publications/ncd-status-report-2014/en/.
65. Mathers, C.D. and D. Loncar, *Projections of global mortality and burden of disease from 2002 to 2030.* PLoS Med, 2006. **3**(11): p. e442.
66. Morrish, N.J., et al., *Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes.* Diabetologia, 2001. **44 Suppl 2**: p. S14-21.
67. Mikol, Y.B., et al., *Hepatocarcinogenesis in rats fed methyl-deficient, amino acid-defined diets.* Carcinogenesis, 1983. **4**(12): p. 1619-29.
68. Hirano, T., et al., *Effect of acute hyperglycemia on plasma triglyceride concentration and triglyceride secretion rate in non-fasted rats.* Diabetes Res Clin Pract, 1990. **9**(3): p. 231-8.
69. Zhang, J., et al., *Association between serum free fatty acid levels and nonalcoholic fatty liver disease: a cross-sectional study.* Sci Rep, 2014. **4**: p. 5832.