MS Thesis Proposal

Discovering Causal Relationships Between The metabolome and DNA methylation in type 1 Diabetes using Bayesian Networks

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Table of Contents

[Abstract 2](#_Toc51258836)

[Introduction 3](#_Toc51258837)

[DNA Methylation 3](#_Toc51258838)

[Figure 1: DNA Methylation Patterns 3](#_Toc51258839)

[Metabolomics 3](#_Toc51258840)

[Figure 2: Micronutrients in One-Carbon Metabolism and DNA Methylation 5](#_Toc51258841)

[Type 1 Diabetes 5](#_Toc51258842)

[Specific Aims 6](#_Toc51258843)

[Primary Aim 1 6](#_Toc51258844)

[Aim 1a 7](#_Toc51258845)

[Aim 1b 7](#_Toc51258846)

[Primary Aim 2 7](#_Toc51258847)

[Aim 2a 7](#_Toc51258848)

[Primary Aim 3 7](#_Toc51258849)

[Aim 3a 7](#_Toc51258850)

[Aim 3b 7](#_Toc51258851)

[Aim 3c 8](#_Toc51258852)

[In addition to gene-based functional analysis of methylation sites, use regulatory annotation from the ENCODE and Roadmap Epigenomic projects to provide additional biological interpretation of methylation site regulatory functions. 8](#_Toc51258853)

[Aim 3d 8](#_Toc51258854)

[Methods 8](#_Toc51258855)

[Cohort 8](#_Toc51258856)

[Methylation 8](#_Toc51258857)

[Metabolomics 8](#_Toc51258858)

[Feature Reduction 9](#_Toc51258859)

[Figure 3: Metabolite, Methylation, and T1D Status Triad with Nominal P Value Cutoffs 9](#_Toc51258860)

[Figure 4: The Causal Inference Testing (CIT) Model 10](#_Toc51258861)

[Bayesian Network Analysis 10](#_Toc51258862)

[Figure 5: Graph Structures 11](#_Toc51258863)

[Figure 6: Graphical separation, conditional independence, and probability decomposition. 11](#_Toc51258864)

[Figure 7: The 24 DAG Structures for a Metabolite, Methylation, and T1D Status Triad 13](#_Toc51258865)

[Preliminary Results 14](#_Toc51258866)

[Table 1: Top 10 Candidates from the CIT Package 14](#_Toc51258867)

[Table 2: Frequency of Network Structure Selection 14](#_Toc51258868)

[Figure 8: Distribution of DIC for Each Network Structure 16](#_Toc51258869)

[References 17](#_Toc51258870)

# Abstract

Type 1 diabetes (T1D) is an auto-immune disease characterized by the destruction of insulin-producing pancreatic β cells, leading to hyperglycemia, nephropathy, and in some cases early death. The disease currently affects over 30 million individuals worldwide and the incidence is increasing by 3-4% per year on average for reasons that are not entirely clear.1 While genetic predisposition accounts for much of the disease etiology, recent studies also suggest roles for DNA methylation and the metabolome in development and progression of T1D.2–8 There is also mounting evidence that DNA methylation affects the metabolome and vice versa. 9–11 Although DNA methylation and the metabolome have been studied separately in T1D and together in other disease states,12–19 the combined nutri-epigenetics of T1D are still unexplored. Here we propose to integrate DNA methylation and metabolomic data using Bayesian network analysis (BNA), to uncover causal relationships between methylation sites, metabolites, and disease state in T1D.

# Introduction

## DNA Methylation

Although all cells in an organism share the same DNA, not all genes are active at the same time in every cell. DNA and histone modifications that impact gene expression without altering the base pair sequence are collectively referred to as “epigenetics,” and are often inherited along with the raw genetic information. These modifications, which can occur as the results of environmental exposure, are “fundamental to the regulation of many cellular processes” and “may be necessary for generating the large range of different phenotypes that arise from the same genotype.”20

Epigeneticists have long recognized the role of DNA cytosine methylation in repression of gene transcription and X chromosome inactivation. A cytosine followed immediately by a guanine nucleotide is called a CpG dinucleotide, and gene silencing is associated with regions of high CpG density. Certain transcription factors have been shown to be sensitive to DNA methylation (in one study, 22% of the 542 transcription factors examined demonstrated decreased binding to methylated motifs). Although mammalian genomes have relatively few CpG sites (~1%), about two thirds of mammalian promoters contain regions, called CpG islands (CGIs), of 200 base pairs (bp) or more in which 50% of the nucleotides (or more) are guanine or cytosine.21 CpG islands are generally unmethylated in normal cells, and in humans about 60% of gene promoters are near a CpG island.20 So, DNA methylation can still have an outsized effect on expression despite low CpG density overall (Figure 1).

Methylation can also change heterochromatin structure through recruitment of DNA-methyltransferase (DNMT) proteins and chromatin remodelers, which in turn alters transcription by making it less likely that transcription factors will bind to DNA.22 Due to DNA methylation’s role in gene expression, abnormal methylation has been linked to many diseases including various cancers.20

### Figure 1: DNA Methylation Patterns

A screenshot of a video game

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From Portela & Esteller (2010)20: Unmethylated CGIs (a) and CpG island shores (b) allow transcription factor binding which in turn leads to gene expression. Methylated sites prevent gene expression.

## Metabolomics

Metabolites are small molecule products of metabolism and are involved in many vital cellular processes including energy storage, cellular signaling and apoptosis, post-translational protein modification and transport, and maintenance of homeostasis in the cellular milieu. For example, ATP, ATP, acetyl-CoA, NAD+, and S-adenosyl methionine (SAM) can regulate post-translational alterations that affect protein activity by acting as co-substrates, and ﻿fatty acids and hormones facilitate transport of plasma proteins through the bloodstream. Some metabolite-protein interactions also have a role in the initiation of signaling cascades.23 As a result of this far-reaching impact at multiple levels of human development, analysis of the metabolome can therefore quantify the integrated response to endogenous and exogenous disease factors or other physiological changes.24

In addition to these well-studied effects of the metabolome, there is mounting evidence that nutrition and metabolism also directly affect DNA methylation25 and histone acetylation10, and that the study of nutri-epigenetics may elucidate the role of diet in many diseases. S-adenosylmethionine (SAM) is the primary methyl group donor for enzymes that methylate nucleic acids and histones. Methylation of a substrate using this methyl group results in S-adenosylhomocysteine (SAH), another intermediate metabolite in the one-carbon pathway (Figure 2). Animal studies have shown that concentrations of SAM and SAH directly influence histone methylation in a way consistent with a signal transduction mechanism, meaning that “concentrations of SAM and SAH are on the order of the kinetic parameters that determine enzyme activity.”11 Also, human studies have confirmed that diet, specifically consumption of folate, choline, betaine, B vitamins and methionine, globally modifies DNA methylation.25

### Figure 2: Micronutrients in One-Carbon Metabolism and DNA Methylation

A close up of a map

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From Anderson et al. (2012)25: Substrates obtained via diet are highlighted in yellow.

## Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease characterized by the production of antibodies which target pancreatic -cells. This leads to both micro and macrovascular complications, and individuals with T1D are at an increased risk of experiencing a major cardiac event (e.g. stroke, angina, and myocardial infarction) and are less likely to have satisfactory outcomes after a coronary event.26 The disease currently affects over 30 million people worldwide,27 and is increasing by 3-4% per year on average.28 However, the global burden of disease is difficult to estimate due to geographic variation in incidence.26,28

Genetic predisposition accounts for some of the etiology of T1D (sibling relative risk has been estimated at 15)29 and explains some geographic variation in incidence.28 Human leukocyte antigen (HLA) genes were the first to be linked to T1D and account for much of the genetic predisposition to the disease, but genome-wide association studies (GWAS) have also identified more than 40 other loci associated with increased relative risk of T1D. However, it is still unclear how these multiple loci interact with one another and the environment to eventually produce a T1D diagnosis. In addition to the complex genetics of T1D, low monozygotic (MZ) twin concordance (approximately 50%) and studies of migrant populations support the theory that non-genetic factors play an important role in T1D development.27

Epigenetic differences may be important contributors to non-genetic T1D etiology. Changes in methylation have been associated with other autoimmune conditions, and monozygotic twins can be epigenetically heterogenous despite sharing identical genetic code.27 Rakyan et al. and Stefan et al. performed epigenome-wide association studies (EWAS) in discordant and concordant twin pairs and found that methylation profiles were more similar among participants with T1D than to unaffected twins. Epigenetics profiles were also combined with GWAS data and differentially expressed methylation sites were mapped to 6 well known T1D susceptibility genes, including two major histocompatibility complex (MHC) genes and several HLA loci.6,27 Finally, the Norris group has found that T1D cases had different longitudinal methylation patterns compared to controls prior to diagnosis.30

Environmental factors including viruses, diet, and the metabolome have also been linked with islet autoimmunity31 and T1D etiology.26 Previous studies have found associations between T1D and differentially expressed phospholipids and sphingolipids, excretion of modified amino acids, and vitamin D (and related compounds on its metabolic pathway).24,26 Branched chain amino acids (BCAAs) have also been associated with insulin resistance.5

Although the effects of the metabolome and DNA methylation have been studied separately in T1D, the combined nutri-epigenetics of T1D remain unclear. Early in-vitro studies confirmed metabolome-dependent alterations in DNA methylation associated with various cancers,13,15 and even identified oncometabolites associated with the development of glioma.14 More recent human studies also support the connection between the metabolome and methylation in breast cancer,12 colorectal cancer,18 and smoking-related diseases.16 A 2018 study by Zaghlool et al. used linear models to link metabolomics and DNA methylation with type 2 diabetes (T2D) and obesity, and Mendelian randomization (MR) analyses suggest “a causal effect of metabolite levels on methylation of obesity-associated CpG sites.”19

Integration of epigenetic and metabolomics data requires statistical methods capable of evaluating associations between many different variables in complex multilevel networks. Bayesian networks are becoming increasingly popular for analysis of large-scale systems biology (“omics”) data, including protein-protein interactions and gene regulatory networks.32 This approach can be used to generate intuitive graphical models, which represent probabilistic dependence between multiple variables32,33 and avoid many of the pitfalls of traditional mediation analyses.34 Also, probabilistic dependence relationships can be interpreted as causal pathways, which clarifies the biological interpretation of results. We hope to derive a graphical model of the relationships between metabolites and methylation sites, to illustrate and make inferences on the epigenetic and environmental pathways that lead to T1D.

# Specific Aims

## Primary Aim 1

Due to the high dimensionality of these data, we will first perform feature selection to identify metabolite and methylation probe candidates for further analysis.

Hypothesis: We will find metabolite-methylation-T1D status triads in which the metabolite and methylation probe are significantly associated.

### Aim 1a

Identify metabolite-methylation-T1D status triads (associated at a nominal p < 0.001 level, and either (or both) of the metabolite or methylation probe is significantly associated with T1D status at the p < 0.05 level) using linear models adjusted for methylation platform. See methods for additional details.

### Aim 1b

Further reduce the triads from Aim 1a using causal inference testing at the p < 0.05 level

## Primary Aim 2

Construct a directed Bayesian network integrating T1D status with multiple metabolites and methylation sites. Previous research has indicated roles for both DNA methylation and the metabolome in T1D, but thus far the combined nutri-epigenetics of T1D remain unclear. Integration of these two omics datasets into Bayesian networks will elucidate the causal effects of methylation sites, metabolites, and T1D status on one another.

Hypothesis: Development of a Bayesian network will reveal previously discovered and novel causal relationships between methylation sites, metabolites, and T1D status.

### Aim 2a

Learn the optimal network structure for each of the triads selected in primary aim 1. If necessary, further reduce the number of triads based on specific network structures of interest, strength of relationships between nodes, or prior knowledge.

## Primary Aim 3

Explore biological interpretations of the final network. The Bayesian network will likely reveal novel associations between these omics datasets, and it is essential to put these relationships into a broader biological context. Doing so will lead to functional insights that could drive future research or even suggest potential T1D biomarkers.

### Aim 3a

Compare methylation sites and metabolites selected for the final networks to previously studied probes and metabolites (in particular the differentially methylated positions and regions previously identified by our group30), in order to contextualize our findings.

### Aim 3b

Map methylation probes to nearby genes and use functional enrichment analysis tools such as DAVID or GSEA35 to provide biologically meaningful insight regarding nearby gene function. Because enrichment generally requires a larger number of probes than we plan to include in the final network, this step will likely need to be performed on the triads, prior to constructing the final network.

### Aim 3c

### In addition to gene-based functional analysis of methylation sites, use regulatory annotation from the ENCODE and Roadmap Epigenomic projects to provide additional biological interpretation of methylation site regulatory functions.

### Aim 3d

If available, use metabolite annotations from the Kyoto Encyclopedia of Genes and Genomes (KEGG) to aid in biological interpretation.

# Methods

## Cohort

The Diabetes Autoimmunity Study in the Young (DAISY) cohort follows 2547 high-risk children in Colorado for the development of IA and T1D. A total of 174 participants (87 T1D cases and 87 frequency-matched controls) were chosen for measurement of DNA methylation.30 Participants with both methylation and metabolomic measures at seroconversion were selected for these analyses. Seroconversion is defined as the second consecutive visit at which a confirmed auto-antibody to insulin, GAD65, IA-2, or ZnT8 was detected.3

## Methylation

DNA methylation was measured in peripheral whole blood, and study participants were split into two groups. One group of 84 participants was measured ﻿using the Infinium HumanMethylation450K Beadchip (“450 K”) and the remaining 90 participants were measured using ﻿the Infinium MethylationEPIC Beadchip (“EPIC”).30 Measurement platform will be included as a covariate, along with age and sex, in all linear models, in order to adjust for potential platform differences. Measurements were collected at up to five time points prior to diagnosis, but we will examine only a cross-sectional cohort at the seroconversion visit. A total of 199,243 probes covering 68.6% of autosomal genes passed rigorous pre-processing procedures, which included normalization, quality control, and filtering using the SeSAMe pipeline.30,36 Batch effects were adjusted for using ComBat.30,37

## Metabolomics

Metabolites and complex lipids at seroconversion were quantified using gas chromatography/time-of-flight mass spectrometry (GC-TOF MS), charged surface hybrid mass spectrometry (CSH-QTOF MS), and at the NIH West Coast Metabolomics Center at the University of California, Davis. Data collection and processing are described in further detail in Johnson et al. (2020), and a total 2,541 metabolites were available for testing.3

## Feature Reduction

First, we will select methylation-metabolite pairs by using linear models to correlate all combinations of probe and metabolite. We will also use logistic regression to find probes and metabolites that are significantly associated with the T1D phenotype. Candidate pairs that are strongly associated (nominal p-value < 0.001) and contain either a probe or metabolite (or both) that is significantly associated with the T1D phenotype (nominal p-value < 0.05) will continue on to the next step (Figure 3). We will not adjust these p-values for multiple comparisons because doing is complicated with many correlated variables, and there are no agreed upon best practices.38 However, careful network development with strict thresholds in later steps should prevent false positives in the final results, as described in Rudra et al.

### Figure 3: Metabolite, Methylation, and T1D Status Triad with Nominal P Value Cutoffs

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Metabolite-methylation pairs fitting the above criteria will be further pared down using causal inference testing. The R package “cit” performs multiple statistical tests associated with causal mediation, and generates an omnibus p-value for the component tests.39 For example, let L represent a methylation probe, G represent a metabolite, and T represent the outcome of T1D (Figure 4). To test for causal relationships, the package fits three linear regression models (with i indexing subject):

And tests four null hypotheses:

The omnibus p-value is simply the highest p-value from the four tests above,40 and if all four null hypotheses are rejected (i.e. an omnibus p-value < 0.05) the methylation-metabolite pair will move on to the Bayesian network analysis.

### Figure 4: The Causal Inference Testing (CIT) Model

A picture containing clock

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From Millstein et al. (2009)40

## Bayesian Network Analysis

A graph consists of a non-empty set of vertices (or nodes) and a finite set of pairs of vertices called arcs, links, or edges (unlike , this set can be empty). If arc is an ordered pair, the arc is said to be directed from to and is usually represented by an arrow of the form . Graphs themselves can also be described as directed if all arcs are directed.41 Sequences of arcs form a path from one vertex to another, and imply that the first node is a cause the and the other an effect.42 Bayesian networks are a specific case of graphical models, where each arc represents a probabilistic dependency between two variables , which are represented as nodes . The graphs must be directed and acyclic (i.e. no path of the form exists) (Figure 5).41

### Figure 5: Graph Structures

A close up of a watch

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From Nagarajan et al. (2013)43: An undirected graph (left), a directed graph (center), and a partially directed graph (right). The directed graph is also acyclic, as there are no paths (e.g. C -> D -> B) from one back to itself, and the nodes can be interpreted as “D depends on C, B depends on A, etc.”

Graphical separation , or the absence of an arc between two given nodes, can be linked to probabilistic separation in three ways. If each node corresponds to random variable and *P* represents the probabilistic dependence structure of , then graph G is an independence map (I-map) if:41

For all disjoint subsets A, B, and C of . Graph G is a dependence map (D-map) of P if:

G is called a perfect map if it is both an I-map and D-Map:

### Figure 6: Graphical separation, conditional independence, and probability decomposition.

A screenshot of a cell phone

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From Nagarajan et al. (2013).

The formal definition of a Bayesian network is a directed acyclic graph (DAG) denoted if and only if G is a minimal I-map of P such that not a single arc can be removed without making G no longer an I-map. From this definition follow two important properties. First, the global distribution P(**X**) can be decomposed into:

Where is the set of parent nodes of . From this decomposition follows the local Markov property of BNs, which states that “each node is conditionally independent of its non-descendants (e.g. nodes for which there is no path from to ) given its parents.”44 Essentially this all means that the probability distribution of the whole network (the global distribution) can be broken down into the product of simpler distributions for each node (see Figure 5).

The first step of fitting a Bayesian model is often called structure learning, and it involves trying to find the best (preferably the minimal I-map) graph structure. There are several popular methods for learning network structures, and these can be grouped into three categories: constraint-based, score-based, and hybrid structure learning algorithms. We will use score-based learning because the rjags package45 allows for simple calculation of the deviance information criterion (DIC), which is the measure of Bayesian model fit recommended by Spiegelhalter et al..46 Other packages such as bnlearn47 implement constraint-based and hybrid structure learning, but these packages do not accept network structures in which a categorical variable depends on a continuous one.

For each pair selected by the causal inference testing, we will use a DIC-based algorithm to find the best network structure using the probe-metabolite-phenotype triad. With no additional restrictions on edge number or direction (aside from the assumptions of Bayesian networks), there are 24 possible network structures for each triple. We will compare all possible network structures using DIC, and the structure with the best (lowest) score will continue to the next selection step. DIC can be considered a Bayesian equivalent to Akaike’s information criterion, a measure commonly used for model comparison in frequentist statistics. It is calculated as the posterior mean deviance (a measure of fit) plus double the effective number of parameters in the model (a measure of model complexity):44

Where

### Figure 7: The 24 DAG Structures for a Metabolite, Methylation, and T1D Status Triad

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# Preliminary Results

From the original 199,243 methylation probes and 2,541 metabolites, 7,157 unique methylation and metabolite pairs (out of a total of 506,276,463 possible combinations) were chosen to move on to the causal inference testing phase of feature reduction (i.e. the pairs were associated with one another at the p < 0.001 level and at least one was associated with T1D status at the p < 0.05 level). These pairs were then evaluated using the cit R package.39 Because there is little prior knowledge regarding the direction of the effect between methylation site and metabolite (i.e. which variable should be considered “L” or “G” in the CIT framework), both configurations were tested. This step is simply for feature reduction, so the direction of the effect is not important. A total of 139 unique pairs with an omnibus p value < 0.05 continued to the next step.

Next, each metabolite and methylation probe pair from the causal inference step was considered as a triad with T1D status. For each triad we fit a Bayesian graphical model for each network structure (Figure 7) using the package rjags45 with uninformative priors. We compared the DIC for each model to determine the best possible network structure for each triad. However, although picking the “best” model is relatively simple, it’s less clear whether the model is a good one or simply the “least bad.” This method appears to favor certain model structures, and although DIC theoretically accounts for the number of nodes in a graph, this method warrants further investigation. Permutation testing is ongoing to assess the distribution of DIC under the null hypothesis of no relationships between the three nodes.

### Table 1: Top 10 Candidates from the CIT Package

|  |  |  |
| --- | --- | --- |
| Methylation Probe | Metabolite | Omnibus P Value |
| cg05045817 | lipid\_79 | 0.0028337 |
| cg12757310 | hilic\_12 | 0.0049853 |
| cg21041956 | hilic\_12 | 0.0090782 |
| cg05045817 | lipid\_647 | 0.0113507 |
| cg18807011 | lipid\_117 | 0.0120120 |
| cg21524155 | lipid\_117 | 0.0134683 |
| cg25104186 | bc\_oxylipin45 | 0.0143077 |
| cg11226183 | lipid\_647 | 0.0154822 |
| cg26074100 | lipid\_117 | 0.0165700 |

### Table 2: Frequency of Network Structure Selection

|  |  |
| --- | --- |
| Structure Number | Frequency |
| 6 | 1 |
| 10 | 27 |
| 14 | 19 |
| 19 | 2 |
| 23 | 60 |
| 24 | 30 |

### Figure 8: Distribution of DIC for Each Network Structure

A close up of a mans face

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