Exploring Epigenetic Links: Stress and Albuminuria in Youth with Type 2 Diabetes

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Table of Contents

Table of Tables

Table of Figures

Abstract

Background:

Youth with Type 2 Diabetes (T2D) often develop early-onset kidney disease, with albuminuria as the initial biomarker. Psychological factors, including perceived stress, are associated with the progression of kidney disease, although the mechanisms remain unclear. This study investigated DNA methylation changes in youth with T2D and albuminuria compared to those without albuminuria and examined whether changes in DNA methylation existed in genes known to be associated with stress functions.

Methods:

This cross-sectional study analyzed data from 213 youth with T2D enrolled in the national iCARE cohort study. Kidney injury was assessed by non-orthostatic albuminuria, and perceived stress was measured using the PSS-14 questionnaire. Whole blood DNA methylation patterns were analyzed using an epigenome-wide association study (EWAS) to identify differentially methylated sites. Associations with albuminuria were tested with multiple linear regression models. A differentially methylated region (DMR) analysis explored broader DNA methylation differences across the genome in areas related to kidney injury. A candidate gene analysis compared CpG sites from our study to the EWAS Atlas, with significance assessed using t-tests.

Results:

Based on the EWAS, no significant sites were associated with albuminuria. Six significant DMRs were identified, corresponding to the genes: TNXB, TSPAN32, ZNF486, ZNF562, ATP5E, and TNFRSF6B. These genes are linked to energy metabolism, immune regulation, and extracellular matrix maintenance. In the candidate gene analysis, we identified 56 CpG sites with significant differences at a p-value < 0.05 and 18 sites at a p-value < 0.01.

Conclusion/Importance:

Although no significant site-level differences were found, the six significant DMRs suggest potential regions of epigenetic variation that could be associated with stress and kidney injury in youth with T2D. Given the exploratory nature of these findings and the limitations of blood-based DNA methylation studies, further research is needed to clarify the role of DNA methylation changes. These findings may help guide future investigations into the role of epigenetics in kidney injury and stress in youth-onset T2D.

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Acronyms

Acronym	Full Term
ACR	Albumin to Creatinine Ratio
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
CKD	Chronic Kidney Disease
CpG	Cytosine-Phosphate-Guanine
CRH	Corticotropin-Releasing Hormone
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DcR3	Decoy Receptor 3
DKD	Diabetic Kidney Disease
DN	Diabetic Nephropathy
DMR	Differentially Methylated Region
DNMT	DNA Methyltransferase
DPN	Diabetic Peripheral Neuropathy
ECM	Extracellular Matrix
eGFR	Estimated Glomerular Filtration Rate
ESKD	End-Stage Kidney Disease
EWAS	Epigenome-Wide Association Study
FARHA	Four Arrows Regional Health Authority
FDR	False Discovery Rate
GDM	Gestational Diabetes
HbA1c	Hemoglobin A1c
HIF	Hypoxia-Induced Factor
HPA	Hypothalamic-Pituitary-Adrenal
iCARE	Improving Renal Complications in Adolescents with Type 2 Diabetes through Research
KDIGO	Kidney Disease Improving Global Outcomes
mQTL	Methylation Quantitative Trait Locus
OCAP	Ownership, Control, Access, And Possession
PAG	Participant Advisory Group
PCA	Principal Component Analysis
PSS-14	Perceived Stress Scale-14 Item Questionnaire
SBP	Systolic Blood Pressure
SNP	Single Nucleotide Polymorphism
SNS	Sympathetic Nervous System
SV	Surrogate Variable
SVA	Surrogate Variable Analysis
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TCPS2	Tri-Council Policy Statement
TODAY	Treatment Options for type 2 Diabetes in Adolescents and Youth
ZNF	Zinc Finger protein

Chapter 1: Introduction

Among youth, type 2 diabetes (T2D) is one of the fastest-growing chronic diseases, which is associated with serious complications such as early-onset kidney disease. Although T2D affects individuals across different ages and ethnicities, First Nations and Métis youth are disproportionately impacted, experiencing higher rates of complications at a younger age.[1] For instance, the Improving Renal Complications in Adolescents with Type 2 Diabetes through Research (iCARE) cohort, the largest prospective cohort of youth with T2D in Canada, found that one-third of youth had prevalent albuminuria within the first two to three years following diagnosis.[1] Albuminuria, defined as an albumin to creatinine ratio (ACR) greater than 3.0mg/mmol on overnight urine collection or first-morning sample, is an early sign of kidney disease.[2,3]

Youth with T2D experience many of the same health complications as adults; however, these complications manifest earlier in the disease process and tend to progress more rapidly.[4] A Canadian population surveillance study has shown that over one-third of youth with T2D present with at least one diabetes-related comorbidity at the time of diagnosis, underscoring the urgent need for research focused on the unique disease progression in this population.[5]

The development of T2D and related complications are affected by multiple external sources, including psychological factors and environmental exposures.[6] Perceived stress, a psychological factor resulting from harmful external influences such as environmental exposures, is reported higher among youth with T2D, who have higher rates of kidney complications such as albuminuria compared to those without.[1] The mechanisms through which psychological and environmental factors confer increased risk for complications in youth with T2D are not well understood, representing a critical gap in the current literature. One possible

mechanism could involve epigenetic modifications, particularly DNA methylation, the most commonly studied mechanism of epigenetic inheritance.[7] DNA methylation, which consists of adding a methyl group to cytosine-phosphate-guanine (CpG) sites on the DNA strand, plays a role in gene expression and may mediate the effects of psychological and environmental exposures on disease outcomes.[8,9]

Considering these gaps, our study explored epigenetic alterations as a potential mechanism underlying the relationship between perceived stress and kidney outcomes in youth with T2D. Specifically, we used data from the iCARE cohort to analyze differences in DNA methylation patterns between youth with T2D who have albuminuria to those without albuminuria. By identifying areas within the genome that exhibit differential DNA methylation and the associated genes involved in metabolic pathways, our study identified potential biological processes that may be associated with kidney complications in this population. We hoped our findings would represent an important step in describing the mechanisms linking psychological stress to kidney complications in this population.

Ultimately, our research contributes much-needed evidence to the field of T2D research in Canada, particularly regarding the health of First Nations and Métis youth, who are disproportionately affected by the disease.[1] The findings from our study will help clarify the role of epigenetic changes in kidney disease progression among youth with T2D and may guide future research aimed at mitigating these severe health outcomes.

Chapter 2: Literature Review

2.1 Diabetes

Diabetes encompasses a spectrum of metabolic disorders characterized by elevated blood glucose levels that result from defects in insulin secretion, action, or both. Various forms of diabetes exist, such as Type 1 diabetes (T1D), gestational diabetes (GDM), and type 2 diabetes (T2D). Each type has a unique pathophysiology and set of clinical manifestations.

T1D is an autoimmune disease characterized by the destruction of pancreatic beta cells, which leads to insulin deficiency.[10] It typically manifests in childhood or adolescence but can occur at any age. Individuals with T1D rely on external sources of insulin for survival.

GDM emerges during pregnancy and primarily arises due to insulin resistance induced by placental hormones at or after the 20th week.[10] It poses risks to both the pregnant person's health and fetal health. GDM requires strict glycemic management to prevent adverse outcomes.

T2D is characterized by a combination of insulin resistance and inadequate insulin secretion.[10–12] It often develops in adulthood but is increasingly diagnosed in children and adolescents.[13]

Diabetes can be associated with specific medical conditions, such as pancreatitis, where inflammation of the pancreas disrupts insulin production, and cystic fibrosis, a genetic disorder affecting multiple organ systems, including the pancreas, which leads to pancreatic insufficiency and diabetes.[10] Other types of diabetes include medication-induced diabetes,[14] neonatal diabetes, and diabetes resulting from genetic abnormalities in insulin secretion and/or action.[10]

Despite their diverse causes, ages of onset, and clinical presentations, all forms of diabetes share the common characteristic of elevated blood glucose levels, which necessitate

management to prevent complications. Effective management strategies often include lifestyle modifications and pharmacotherapy, which may consist of insulin therapy.

While acknowledging the breadth of diabetes subtypes, this project focuses specifically on T2D, given its increasing prevalence, particularly among youth, and its significant impact on health outcomes.

2.2 Type 2 Diabetes

T2D is a complex metabolic disorder characterized by insulin resistance and impaired insulin secretion.[10–12] While T1D was historically classified as a childhood-onset disease and T2D as adult-onset, this distinction has become less clear over time. T1D is characterized by the autoimmune destruction of pancreatic beta cells, whereas T2D is associated with a combination of insulin resistance and beta cell failure secondary to a wide range of genetic, environmental, and lifestyle influences.

In T2D, insulin resistance is a condition that occurs when the body's cells have decreased sensitivity and/or responsiveness to insulin, which is a hormone that helps to regulate blood sugar levels.[15] This results from disruptions in various cellular pathways, leading to decreased responsiveness of peripheral tissues, particularly muscle, liver, and adipose tissue, to insulin.[12] While beta-cell dysfunction and impaired insulin secretion contribute to T2D progression, insulin resistance is the primary driver of hyperglycemia in the early stages of the disease.[15] Unlike T1D, where insulin deficiency is the hallmark, T2D involves a progressive decline in beta-cell function over time, exacerbating hyperglycemia.[11]

A complex interplay of genetic, environmental, and lifestyle factors influences T2D. These factors include obesity, in-utero exposure to diabetes in pregnancy, sedentary behaviours, a dietary intake high in simple carbohydrates and processed foods, food insecurity, poverty, a

family history of diabetes, and ethnic background. [10,12] Unlike T1D, which is primarily driven by genetic susceptibility and autoimmune mechanisms, T2D risk factors encompass a broader range of environmental and lifestyle factors, reflecting the complex nature of the disease.

The incidence of T2D is on the rise worldwide, driven by factors such as rapid urbanization, sedentary lifestyles, and dietary changes.[10] Today, over 400 million people live with T2D, which is expected to reach 600 million by 2035.[16] Unlike T1D and GDM, which constitute a smaller proportion of diabetes cases, T2D accounts for the majority of diabetes cases globally, approximately 90-95% of all cases. It poses a significant public health challenge due to its associated complications and economic burden.[10]

T2D management involves lifestyle modifications, such as improved diet and increased physical activity, often alongside medications to improve insulin sensitivity, enhance insulin section, and reduce blood glucose levels.[12] Unlike T1D, which requires lifelong insulin therapy for survival, many individuals with T2D can initially manage their condition through lifestyle interventions, and sometimes oral anti-hyperglycemia medications. However, insulin therapy may be required in advanced stages of the disease.

2.3 Type 2 Diabetes in Canada

Examining the epidemiology of T2D in specific regions, such as Canada, provides valuable insights into unique demographic and geographic patterns. In Canada, the prevalence of T2D is significantly higher among First Nations populations compared to non-First Nations populations, with rates three to five times greater.[17,18] This disparity underscores the urgent need for targeted public health interventions aimed at primary and secondary prevention, given the projected rise in T2D prevalence among Manitoba's First Nations communities.[17] Addressing lifestyle changes, socioeconomic inequities, and genetic predispositions is imperative for

mitigating the growing burden of T2D in Manitoba and similar regions. Traditional ways of living, such as land-based practices and diets lower in carbohydrates and higher in protein compared to Western-based diets, provide insights into T2D prevention. For example, hunting and gathering traditionally involved high physical activity and low-carbohydrate diets. Incorporating these practices into contemporary health interventions could offer holistic solutions for Indigenous peoples.[19] Bruyere and Garro's work interviewing First Nations individuals in Manitoba highlights the feelings among individuals that the emergence of T2D is attributed to recent changes such as those resulting from environmental pollution and destruction caused by constructing developments.[20] These changes have disrupted traditional ways of life, including the availability of traditional foods, leading to a shift towards adopting dietary practices of non-Indigenous populations.[20] Participants of this study expressed a sense of loss of their cultural practices and identities resulting from a loss of power and control felt in daily life.[20]

Among Indigenous peoples in Canada, T2D must be considered within the broader context of colonialism, institutionalized racism, the historic and ongoing impacts of Residential Schools and the lasting effects of intergenerational trauma.[1] As a result, Indigenous peoples in Canada have had their ways of life and health removed from them, impacting their susceptibility to diseases such as T2D.[1] Within this context, the proposed factors contributing to the observed increased prevalence of T2D among Indigenous peoples in Canada include increased stress, increased risk due to family history of the disease, and socioeconomic disadvantages such as limited resources, which are recognized as resulting from historic and ongoing anti-Indigenous racism and colonialism.[21] For example, having a family history of T2D and the social deprivation that families may experience is directly related to colonialism and the impact it continues to have to this day. A sample of youth from the iCARE cohort study participating in a focus group

discussed that bearing witness to T2D within their communities, as well as the blame, shame, and stigma of T2D, has impacted their mental health and increased their daily stress.[22] The rapid increase in the prevalence of T2D in Indigenous populations over the past several decades is hypothesized to be in part due to increased social stressors and lifestyle factors.[23] Bartlett suggests that colonization and the associated cultural, social, economic, and political impacts place Indigenous peoples in situations of extreme stress.[24] Iwasaki and colleagues aimed to examine stress among Indigenous peoples in terms of their experiences and the meaning of stress.[23] Common themes from the focus groups were that individuals' lives are 'filled with stress' as it is related to health, marginal economic conditions, and trauma and violence.[23]

With regards to T2D, the observed health disparity between Indigenous peoples and the general population in Canada is even more striking when examining the impact on youth. The overall incidence of T2D in children under 18 years of age in 2017-19 was 2.47 per 100,000 people per year in Canada, and in Manitoba, the rate was 31.1.[25,26] The high rate of T2D in Manitoba can be understood in part as a result of the higher Indigenous population (as a percentage of the total population) compared to other provinces. Understanding why Indigenous peoples experience inequitable health outcomes compared to non-Indigenous populations is complex. However, knowledge can be gained by analyzing Indigenous experiences within the context of the legacy of colonialism. Adverse effects of colonization continue to be experienced through socioeconomic disadvantages, increased stress[27], shame and stigma associated with having diabetes, loss of land, and need for self-determination.[21] The high rate of T2D among Indigenous youth is particularly concerning because of the early onset of complications, such as kidney failure, which is associated with significant morbidity and mortality as well as impacts on quality of life.[28–30]

2.4 Youth-Onset Type 2 Diabetes

There is an increasing prevalence of T2D among youth worldwide. This rise parallels the rates of childhood obesity, reflecting the intricate interplay of genetic predisposition, lifestyle factors, and environmental influences. While T2D traditionally manifests in adulthood, its growing incidence in younger populations signals a concerning trend in metabolic health. This situation underscores the need for targeted interventions to address the increasing burden on youth. T2D is more prevalent among youth from ethnic and racial minority groups. Its occurrence often intertwines with complex psychosocial and cultural contexts, posing challenges to maintaining healthy lifestyle habits and self-care practices.

2.4.1 Incidence and Prevalence

Epidemiological studies have documented a notable increase in the incidence and prevalence of youth-onset T2D across diverse geographical regions.[31] In Canada, between 2002 and 2013, the incidence of youth-onset T2D increased from 3.45 to 5.16 per 100,000 individuals, with a projected prevalence of 0.046% by 2030.[32] Notably, females exhibit higher rates of T2D compared to males, reflecting potential sex disparities in disease risk and progression.[32] In Manitoban children, T2D prevalence increased drastically between 2009-10 and 2017-18.[26] Among all children in Manitoba, there was an increase of 87% (66.4 to 124.2/100,000/year), and specifically among First Nation children, the prevalence increased by 83% (from 282.8 to 517.9/100,000/year).[26] Both the incidence and prevalence of youth-onset T2D in Manitoba are significantly greater than rates reported elsewhere in the world.[26]

2.4.2 Differences in Pathophysiology from Adults

Youth-onset T2D presents distinct pathophysiological features and clinical challenges compared to adult-onset T2D. In children and adolescents, T2D is characterized by insulin

resistance, inadequate insulin secretion, increased hepatic glucose production, inflammation, and other metabolic defects.[31] Beta cell dysfunction plays a crucial role in the insufficient insulin secretion observed in youth-onset T2D, contributing to the rapid onset of clinical symptoms and complications in this population.[33] Unlike adults, who may develop complications later in life, youth with T2D experience a rapid onset of clinical symptoms and complications.[31] Adolescents with T2D often face suboptimal responses to treatment and a rapid deterioration in glycemic control, highlighting the need for tailored management strategies.[34]

2.4.3 Management and Psychosocial Challenges

Effective management of youth-onset T2D necessitates a multifaceted approach encompassing lifestyle modifications, pharmacological interventions, and psychosocial support. Lifestyle interventions focusing on weight management, optimal nutrition, and physical activity play a central role in preventing and managing T2D in children and adolescents.[35] However, current treatment options for T2D in youth are less effective than in adults, necessitating further research into novel therapeutic approaches.[35] Addressing socioeconomic disparities, cultural factors, and access to healthcare services is crucial for optimizing outcomes in youth with T2D.[36] Despite these efforts, the psychosocial burden of T2D in youth often undermines treatment adherence and overall health outcomes. Many youth face significant challenges sustaining lifestyle changes due to environmental stressors and emotional strain. This highlights the need for more integrated treatment approaches prioritizing metabolic control and mental health support to improve adherence and quality of life.

2.4.4 Public Health and Mental Health Implications

Youth-onset T2D poses significant public health challenges, with rising incidence and prevalence rates globally. Addressing the complex interplay of genetic, environmental, and

socioeconomic factors driving the high and increasing rates of youth-onset T2D requires comprehensive prevention and management strategies tailored to the unique needs of children and youth. Conventional medication approaches often fall short, highlighting the pressing need for alternative strategies.[37]

Environmental factors further compound these issues, limiting the efficacy of lifestyle interventions.[38] The psychosocial impact of a T2D diagnosis on youth is profound, as the stigma and demands associated with living with the disease can lead to feelings of depression, anxiety, and hopelessness.[39]

Mental health emerges as a critical yet often overlooked aspect of diabetes management, which may result in earlier deterioration of health.[40] However, the accessibility of mental health resources remains inconsistent across communities, leading to worse outcomes for those who are disadvantaged.[41] Efforts to integrate mental health screening and interventions into routine diabetes care, alongside addressing health inequities, could substantially improve outcomes in youth with T2D.

2.5 Stress and Type 2 Diabetes

Managing T2D involves more than just managing physical health. Disease management is complex because it also involves recognizing and supporting the disease's emotional and social aspects and impacts. The interplay between stress and T2D is increasingly recognized as a crucial factor influencing the ability to manage the disease and prevent poor health outcomes effectively.[42]

Stress is commonly described as a mediating presence in one's life.[27] Cohen and colleagues explain that stress is "a process that entails a stimulus, appraisals of it, and a response."[43] This means that when a stimulus or stressor is present, it is seen as a threat and

elicits a psychological response, which is perceived as the feeling of stress. The biological and behavioural responses are aimed at re-establishing homeostasis. Such responses, also known as allostasis,[44] are carried out by activating the autonomic nervous system, the hypothalamic-pituitary-adrenal (HPA) axis, and cardiovascular, metabolic, and immune systems.[44] While these responses are advantageous in re-instating balance on an acute basis, chronic activation of these systems can have negative long-term health implications.[45]

Previous work into the relationship between stress and diabetes has shown that the two main systems involved are the HPA axis, which releases cortisol, and the sympathetic nervous system (SNS), which releases catecholamines such as norepinephrine.[46] Both stress hormones have hyperglycemic effects,[46] and cortisol is proposed to mediate other stress-related factors, such as the regulation of fat via lipid metabolism and storage.[47] Research in this area that focuses on Indigenous peoples living in Canada has looked at the impact of stressful environments and stress modifiers on individuals' health.[27] Stress is prevalent among individuals with T2D, with studies consistently reporting elevated levels compared to that of the general population.[48] The experience of living with a chronic condition like T2D, coupled with the demands of self-management, can lead to chronic stress, exacerbating the physiological dysregulation characteristic of diabetes.[49] Psychological stressors associated with T2D encompass a wide range of factors, including the emotional burden of treatment regimens, fear of complications, and adjustment to lifestyle modifications.[50]

The consequences of stress in T2D extend beyond psychological distress and impact glycemic control and overall health outcomes. Chronic stress triggers biological responses involved in T2D pathogenesis, such as dysregulation of glucose metabolism, inflammation, and neuroendocrine dysfunction.[48] Moreover, stress can undermine health behaviours crucial for

diabetes management, including dietary choices, physical activity, and medication adherence.[48] Consequently, individuals with T2D experiencing high levels of stress are at increased risk of poor glycemic control, diabetes-related complications, and cardiovascular disease.[50]

Several factors contribute to stress in individuals with T2D, including demographic characteristics, disease-related variables, and psychosocial factors. Gender differences exist, with females exhibiting a higher prevalence of stress compared to males.[51] Disease-related complications, such as neuropathy, nephropathy, and retinopathy, are associated with elevated stress levels, creating a bidirectional relationship between stress and diabetic complications.[51] The kidney complications common for youth with T2D identified as albuminuria are more common in those with higher perceived stress scores within the iCARE cohort.[1] Additionally, socioeconomic status, social support networks, and cultural factors influence the experience of stress in individuals with T2D.[52]

In a study by Iwasaki and colleagues, among Indigenous adults with T2D living in Winnipeg, Manitoba, focus group interviews found that individuals living with T2D felt that their perceived stress was a consistent presence in their lives.[27] Individuals from this study made it apparent that their stress was directly linked to living with diabetes. Stress was not only a result of having to manage one's disease but also of their fear of the future, financial aspects of living with diabetes, and potential diabetes-related complications.[27] Coping with stress proved extremely difficult for the adults in Iwasaki's focus group.[27] We expect that managing stress will be even more difficult for adolescents. Mental health professionals, researchers, and Indigenous individuals believe the disproportionate rates of psychological stress are a result of colonialism.[53] The feeling of collective post-traumatic stress and harsh psychosocial impacts resulting from a legacy of colonialism are shared amongst many.[53]

Addressing stress in managing T2D requires multifaceted interventions encompassing individual and systemic approaches. Under cognitive-behavioural principles, stress management training has shown promise in reducing perceived stress levels and improving glycemic control among adults with T2D.[49] These interventions focus on enhancing coping mechanisms, fostering social support networks, and promoting adaptive responses to stressors.[49]

Integrating psychosocial care into routine T2D management is vital to address the holistic needs of individuals with T2D.[52] Comprehensive diabetes care should include regular assessments of stress levels, provision of mental health support services, and incorporation of stress management techniques into diabetes education programs.[52] Collaborative care models involving interdisciplinary teams comprising healthcare professionals and peer support groups can facilitate the delivery of integrated psychosocial care to individuals with T2D[54]

Stress represents a significant modifiable risk factor in the management of T2D. It harms glycemic control, psychological well-being, and overall health outcomes.[55,56] Recognizing the prevalent and ongoing nature of stress in T2D and implementing tailored interventions are essential steps toward optimizing diabetes care and improving patient outcomes.

2.6 Type 2 Diabetes Co-Morbidities and Complications

T2D is often accompanied by various co-morbidities and complications that significantly impact the health and well-being of affected individuals. This section delves into some of the key co-morbidities associated with T2D, including retinopathy, neuropathy, cardiovascular disease, depression, and mental health disorders, which illustrate the systemic and multifaceted nature of the disease. While these conditions are not the primary focus of our study, they provide a critical context for understanding the broader implications of living with T2D and its interconnected health challenges. Given the significance to this project, further discussion on diabetes associated

nephropathy will be provided in section 2.7. T2D-associated co-morbidities and complications exacerbate the challenges of managing T2D and underscore the importance of early detection, intervention, and comprehensive management strategies tailored to youth-onset T2D.

2.6.1 Retinopathy

Retinopathy, a complication of diabetes characterized by damage to the blood vessels in the retina, poses a significant threat to vision among individuals with T2D. Although commonly seen in adult-onset T2D, this condition is increasingly recognized as a concern among youth with T2D.[57] Research indicates that retinopathy can progress rapidly in youth populations, underscoring the critical importance of early detection and intervention.

Studies examining retinopathy in youth with T2D reveal alarming trends. Sellers and colleagues reported that even early in the disease course, 4% of youth had background retinopathy, while another 4% had sight-threatening retinopathy, highlighting the need for vigilant monitoring and management.[58] The severity of retinopathy is further illustrated when reviewing the TODAY study cohort. At the initial assessment, 13.7% of participants had mild retinopathy, while at the 7-year follow-up visit, 51% had some form of eye disease.[4] While findings from the Pima Indian population suggest a lower rate of retinopathy in those diagnosed with T2D during childhood compared to adulthood,[58] cases of blindness before 30 years of age have been documented among Canadian First Nations young adults with youth-onset T2D, indicating the severity of the condition even in younger age groups.[58]

Recent research, such as the meta-analysis conducted by Jensen and colleagues, discusses the increasing prevalence of retinopathy worldwide and identifies major risk factors, such as the duration of diabetes, blood glucose levels, and blood pressure.[59] Retinopathy in youth-onset T2D

is highly prevalent, with 56% of individuals with T2D in Jensen and colleagues' study showing retinal changes at follow-up.[59]

Mayer-Davis and colleagues conducted a pilot study to assess the prevalence of diabetic retinopathy in youth with T1D and T2D. Their research revealed a substantial prevalence of retinopathy in youth, particularly among minority populations and those with T2D (42%).[60]

Rajalakshmi and colleagues aimed to assess the prevalence and risk factors for diabetic retinopathy in young individuals with T1D and T2D.[61] Their findings indicated a high prevalence of retinopathy in both groups within a relatively short duration of diabetes. Over half of those with youth-onset diabetes, regardless of type, have retinopathy within 10–12 years following diabetes diagnosis.[61]

Overall, the evidence suggests that retinopathy is a significant concern for youth with T2D, necessitating early detection, regular screening, and intensive management to prevent vision impairment and blindness.

2.6.2 Neuropathy

In young people with T2D, neuropathy presents a significant health concern, manifesting as tingling, numbness, or pain in the extremities, thereby impairing mobility and affecting their overall quality of life. According to a study conducted by the Treatment Options for Type 2 Diabetes in Adolescents and Youth (TODAY) study group, the prevalence of diabetic peripheral neuropathy (DPN) was notably higher in youth with T2D compared to those with T1D, with rates reaching 22% in the former group. The research identified several risk factors associated with DPN in youth with T2D, including older age, male sex, longer diabetes duration, smoking, and lower HDL cholesterol levels. Moreover, the study highlighted that DPN was apparent early in the course of youth-onset T2D and was closely linked to glycemic control, suggesting the need

for improved glycemic management to mitigate the risk of neuropathy-related morbidity. These findings indicate the necessity to further investigate intervention strategies for addressing neuropathy in youth with T2D, particularly given the association between neuropathy and glycemic control, as well as the high prevalence of risk factors identified in the population.

2.6.3 Cardiovascular Disease

Cardiovascular disease (CVD) presents a significant concern for youth with T2D, substantially increasing morbidity and mortality rates among this population. Hu and colleagues conducted a large-scale cohort study in China, revealing a robust association between early-onset T2D and heightened CVD risk, particularly among individuals with obesity.[62] Moreover, each 5-year decrease in age at T2D diagnosis is linked with a 14% increase in CVD risk, emphasizing the urgency of early intervention and management approaches.[62] The study underscores the critical role of preventive strategies and weight management in mitigating cardiovascular complications in youth with T2D.

In concurrence, Shah and colleagues investigated the progression of cardiovascular risk factors in young adults with youth-onset T2D over a decade.[63] Their findings indicate alarmingly high rates of hypertension, dyslipidemia, and smoking among these individuals, with the prevalence of risk factors escalating over time. Factors such as male sex, non-Hispanic white ethnicity, obesity, and poor glycemic control were associated with a heightened cardiovascular risk profile in youth with T2D, highlighting the multifaceted nature of cardiovascular complications in this population.[63]

The American Diabetes Association (2019) provides comprehensive recommendations for managing CVD risk in individuals with T1D and T2D, advocating for aggressive risk assessment and intervention strategies.[64] The guidelines underscore the importance of blood

pressure control, lipid management, and antiplatelet therapy in reducing CVD risk among patients with diabetes. However, these findings are limited as they do not specifically discuss applicability to youth with T2D. High-intensity statin therapy, which is defined as lowering LDL cholesterol by >50%, is a lifestyle therapy recommended for individuals of all ages who have diabetes and a 10-year CVD risk exceeding 20%.[64]

These studies collectively emphasize the urgency of addressing cardiovascular risk factors in youth with T2D through multifaceted intervention strategies encompassing lifestyle modifications, pharmacological therapy, and comprehensive risk assessment. By targeting modifiable risk factors such as obesity, hypertension, and dyslipidemia early in the disease course, healthcare providers can mitigate the burden of CVD and improve long-term outcomes for youth with T2D.

2.6.4 Depression and Mental Health Disorders

Depression and Mental Health Disorders are prevalent among youth with T2D, with studies indicating a higher risk compared to those who do not have diabetes.[65] A systematic review of 109 studies from 2000-2020 on youth with T1D and T2D showed that youth with T2D have a prevalence of depression of 22.7%.[66] While this review doesn't make comparisons to individuals without T2D or the general population, a study from 2019 in the United States found that among the general population of US children, 3.2% had depression.[67] Diabetes distress is another area of concern, with 23% of a sample of 64 adolescents and young adults living with T2D reporting high diabetes distress.[68] This statistic pales in comparison to Khuwaja and colleagues' study, where out of 889 adults with T2D, 57.9% of participants had anxiety, and 43.5% had depression, according to the Hospital Anxiety and Depression Scale.[69] This may

indicate worsening mental health as people with T2D age, showing just how important it is to manage and improve mental health in youth with T2D.

In a study by Sellers and colleagues, a cohort of children with T2D was retrospectively identified using the clinical database of the Diabetes Education Resource for Children and Adolescents in Manitoba.[70] These children, who predominantly self-identified as First Nations, were compared to a cohort of children with T1D from the same registry and to a matched cohort of diabetes-free peers. Children with T2D were found to have an increased risk of mood or anxiety disorders both before and after diagnosis compared to their diabetes-free peers, with relative risks of 2.38 and 1.70, respectively.[70] Similarly, their risk of suicide attempts or completion was significantly higher both pre-and post-diagnosis, emphasizing the urgent need for mental health interventions tailored to this population.[70] These findings highlight the intersection of T2D, mental health, and social determinants of health, particularly in youth from socioeconomically disadvantaged communities. Additionally, the TODAY study, comprised of young adults with youth-onset T2D, showed that high diabetes distress was reported by 23.6% of participants.[71] In multivariate analyses, diabetes distress was also associated with female sex, HbA1c levels, lack of healthcare coverage, and anxiety.[71]

The differences in the prevalence of youth with and without T2D indicate a substantial impact of T2D on mental health.[66] This burden extends to both genders, with girls (29.7%) exhibiting a higher prevalence of depression compared to boys (19.7%).[66] Additionally, socioeconomic factors play a role, as depression rates are higher in youth living in lower-middle-income countries.[72]

The complex interplay between diabetes management, psychosocial stressors, and hormonal changes during adolescence contributes to the development and exacerbation of

depressive symptoms.[65] Despite this high prevalence, routine screening for depressive symptoms is not universally implemented in clinical practices, leading to a low identification rate of participants with depressive symptoms in medical records.[65] This underscores the need for standardized and time-efficient screening methods to detect depressive symptoms early on and facilitate appropriate referral for treatment.[65] Effective chronic disease management is crucial, particularly in specialist care settings, where individuals with diabetes are at a higher risk of depressive symptoms.[72]

The complexity of T2D adds layers to the psychological burden experienced by affected youth. Factors such as blood glucose level, racial differences, obesity, and insurance status, impact the prevalence of depression.[73] Multidisciplinary treatment approaches are warranted, recognizing that addressing mental health issues in youth with T2D requires more than just pharmacological interventions.[73] The study by Farooqi and colleagues emphasizes the importance of managing depression alongside diabetes to improve overall health outcomes and quality of life in affected youth.[72]

A review of the literature discussing the role of positive characteristics in diabetes outcomes showed that positive traits such as self-esteem, self-efficacy, and adaptive coping were each associated with improved diabetes management and glycemic control in adults with T1D and T2D.[74] Parental monitoring and support were also predictors of positive outcomes for adolescents.[74]

This review reveals that depression, anxiety, stress, and other mental health disorders present significant challenges for youth with T2D. Given the high prevalence of mental health issues and the complex relationship between mental health and T2D, it is vital to further explore options for comprehensive screening, early intervention, and multidisciplinary treatment

approaches to mitigate the effects of mental health. Addressing these concerns alongside diabetes management is essential for enhancing health outcomes and improving the overall well-being of affected individuals.

2.7 Diabetes Associated Nephropathy

2.7.1 Epidemiology and Risk Factors of Diabetes Associated Nephropathy in Indigenous Populations

Kidney disease is the most common comorbidity of T2D, and in Canada, the leading cause of kidney disease is T2D.[75] Indigenous peoples living in Canada have a higher risk of developing T2D and T2D-related complications. Among Indigenous youth with T2D, kidney complications often present in adolescence as albuminuria and culminate in kidney failure by the time the individuals have reached early adulthood.[76] Notably, T2D-related kidney complications can manifest quickly, with albuminuria often present within two to three years following a T2D diagnosis.[77] In a Canadian study, Indigenous youth were found to have alarming statistics where by age 30, 50% of individuals diagnosed with T2D in adolescence will have developed kidney failure. [76] A similar observation was made in Australia, where kidney failure was observed in 49% of individuals who were diagnosed with T2D before age 40, compared to individuals diagnosed with T2D at age 60 or later, whose kidney failure rate was reported as 7%.[78]

With regards to kidney disease, commonly known risk factors, including hypertension and hyperglycemia, do not fully explain the high level of morbidity experienced by Indigenous youth with T2D. Psychological factors such as mental health may play an important albeit indirect role in the development of renal complications.[1] The youth from the iCARE cohort describe their mental health as playing an important role.[1] Mental health challenges, including depression and anxiety, may indirectly affect kidney health by reducing an individual's ability to adhere to

diabetes self-management behaviours such as regular blood glucose monitoring, medication adherence, and dietary control. Additionally, chronic stress associated with mental health conditions may contribute to heightened inflammation and activation of the HPA axis, which are pathways known to accelerate kidney damage.[79,80]

2.7.2 Pathophysiology of Diabetic Kidney Disease

The development of DKD in individuals with T2D is driven by a combination of metabolic, hemodynamic, and inflammatory mechanisms that result in cumulative kidney damage. While the general mechanisms of DKD are well-characterized, the rapid progression observed in youth with T2D highlights unique pathophysiological considerations.

Metabolic factors, particularly chronic hyperglycemia, drive oxidative stress, inflammation, and extracellular matrix (ECM) accumulation.[81,82] Advanced glycation end-products, a hallmark of DKD, thicken the basement membrane and contribute to glomerular dysfunction. Hyperglycemia also induces podocyte injury through detachment, hypertrophy, and apoptosis, leading to irreversible proteinuria and an accelerating disease progression.[83]

Hemodynamic changes, such as glomerular hyperfiltration and increased intraglomerular pressure, amplify mechanical stress on the glomerulus.[84,85] These pressures activate the renin-angiotensin-aldosterone system, further damaging the filtration barrier and contributing to podocyte loss and mesangial expansion.[86,87]

Inflammation worsens DKD progression through cytokines such as interleukin-6 and tumour necrosis factor-alpha, which stimulate fibrosis and glomerulosclerosis. Vascular injury, marked by endothelial dysfunction and ischemia, further promotes tubulointerstitial fibrosis by depriving kidney tissues of oxygen.[OBJ] The temporal progression of DKD, which often begins with glomerular hyperfiltration and podocyte loss, advances to mesangial ECM expansion and

culminates in tubulointerstitial fibrosis and renal failure.[88] Metabolic and hemodynamic pathways interact to create a harmful cycle of oxidative stress, inflammation, and fibrosis.[89,90]

2.7.3 Kidney Pathology in Youth with Type 2 Diabetes

Youth with T2D demonstrate a markedly faster progression of DKD compared to adults. This is characterized by an earlier onset of albuminuria, more rapid development of glomerulosclerosis, and a quicker decline in renal function, often leading to end-stage kidney disease (ESKD) within a shorter timeframe.[76,91,92] Factors such as worse glycemic control, the cumulative metabolic burden over a longer lifetime, and more aggressive disease phenotypes in youth contribute to this accelerated trajectory.[91–93]

Another key distinction between youth and adults with T2D is the nature of the kidney pathology. While the classic diabetic nephropathy characterized by glomerulosclerosis and tubulointerstitial fibrosis is well-documented in adults, recent evidence suggests that youth with T2D frequently present with non-classic kidney diseases.

Sellers and their co-authors offer valuable insight into this area of research, demonstrating that nondiabetic kidney diseases, such as immune complex glomerulopathies, are prevalent in youth with T2D.[94] In their cohort of Canadian First Nations youth with T2D and macroalbuminuria, the majority of renal biopsies revealed immune complex disease or glomerulosclerosis rather than typical diabetic nephropathy.[94] This finding underscores the complexity of renal disease in youth, indicating that clinical and laboratory assessments alone are insufficient for accurately diagnosing the specific type of renal pathology present.[94]

Additionally, glomerular hyperfiltration and intraglomerular hypertension are more pronounced in youth with T2D, potentially due to the combination of early hyperglycemia and renal hemodynamic alterations.[95,96] This hyperfiltration places significant mechanical stress on

the glomeruli, accelerating podocyte loss and basement membrane damage.[97,98] Furthermore, microvascular damage is more severe and occurs earlier in youth.[4] These vascular changes lead to reduced oxygen delivery to renal tissue, contributing to ischemia, hypoxia, and subsequent tubulointerstitial fibrosis.[99]

The accelerated progression to ESKD underscores the urgent need for early diagnostic precision, which often necessitates renal biopsy and individualized therapeutic strategies. These findings reinforce the importance of understanding the unique renal pathophysiology in youth with T2D to mitigate long-term complications and improve outcomes.

2.7.4 Clinical Assessment and Monitoring of Diabetes Associated Nephropathy Progression

Diabetic kidney disease (DKD) progression is closely monitored through assessments of albuminuria and estimated glomerular filtration rate (eGFR).[100] Albuminuria, in particular, serves as an essential biomarker for assessing kidney health, given its characteristic increase in the early stages of the disease. It was the focus of this research project. Albuminuria refers to the presence of albumin, a protein, in the urine, which typically indicates kidney damage.[40] Random urine collection is the most common choice to test the ACR when assessing albuminuria in adults, given the ease of collection.[40] While 24-hour urine collection is the gold standard, it is rarely chosen given the inconvenience to the patient and the level of difficulty to perform correctly.[40] In youth, it is also essential to account for orthostatic albuminuria, a condition where youth excrete more protein in the urine after standing or walking. Therefore, the first morning or overnight urine collection is recommended for accurate assessment in pediatric populations.[40]

Persistent elevation in urinary albumin levels over three months, as evidenced by at least two out of three urine samples over three to six months with an ACR ≥3.0mg/mmol, is considered abnormal.[2,40,101] Such persistence suggests glomerular damage. Notably, albuminuria

in adolescents with T2D predicts adverse outcomes, highlighting its significance as a marker of kidney health.[92] Consequences of persistent albuminuria include increased risk of kidney disease progression and the potential need for dialysis. Given its significance as an indicator of kidney health, urinary albumin testing is the recommended screening test for detecting kidney complications in youth with T2D, as outlined by the Diabetes Canada guidelines.[40]

The Diabetes Canada Clinical Practice Guidelines for the Prevention and Management of Diabetes in Canada (2018) recommends establishing glycemic control as soon as possible after a T2D diagnosis to mitigate the risk of developing kidney disease and slow the progression of overall renal damage.[40] There is controversy over the ideal target for hemoglobin A1c (HbA1c) due to the need to balance tight control with the risk of hypoglycemic events. This results in studies suggesting targets of 7%, <6.5%, and <6%.[40] Diabetes Canada recommends an HbA1c target of ≤7.0% to reduce microvascular complications, with ≤6.5% considered for individuals with a shorter diabetes duration and lower hypoglycemia risk.[40] Maintaining optimal blood pressure is also critical in preventing and managing kidney disease in those with diabetes.[102] Targeting a systolic blood pressure (SBP) <130 mmHg and diastolic blood pressure (DBP) <80 mmHg is recommended for most individuals with diabetes thirteen years old and older to achieve renal protection.[40,101] For those with diabetes who are under the age of thirteen, the target SBP and DBP are below the 95th percentile for sex, age, and height.[101,103] Individuals with blood pressure higher than the aforementioned targets on at least three occasions meet the cut-off for hypertension.[103] By identifying albuminuria early on, individuals can begin working towards maintaining HbA1c and blood pressure targets to mitigate risk.

2.7.5 Role of Psychological Factors in the Development and Progression of Diabetic Kidney Disease

Psychological health can impact the progression and health outcomes associated with DKD. Psychological factors such as depression and anxiety significantly impact Chinese adult T2D patients with DKD, as highlighted by Shen and colleagues.[104] Their study revealed alarming rates of depression (41.3%) and anxiety (45.0%) among DKD patients, with nearly one-third (31.6%) experiencing both conditions simultaneously. Notably, the study emphasized the detrimental effects of depression and anxiety on kidney function, albuminuria, and overall quality of life in DKD patients.[104] Depression and anxiety were independent risk factors for DKD patients with stage three or four CKD and a poor quality of life.[104] Depression and anxiety scores were also negatively correlated with participants' eGFR and quality of life scores, while urinary ACR was positively associated with depression scores.[104] Furthermore, Shen and colleagues hypothesized that these psychological disorders may exacerbate DKD progression through mechanisms involving HPA axis dysregulation and heightened systemic inflammation.[104] However, these pathways remain speculative and require further investigation.

Research from the SEARCH for Diabetes in Youth study provides complementary evidence on the relationship between psychological factors and metabolic/inflammatory markers in individuals with diabetes. In youth with diabetes, higher depression levels were associated with worse metabolic outcomes, such as elevated apolipoprotein B, systemic inflammation, and other indicators of poor metabolic functioning. These findings support a potential biological link between psychological health and disease progression, as inflammation and metabolic dysregulation are known contributors to renal decline. This highlights the importance of

investigating how systemic inflammation and metabolic disturbances driven by psychological factors may influence DKD progression in T2D.

The role of psychological factors in DKD highlights the intricate connection between mental health, systemic inflammation, and metabolic dysfunction. Evidence linking depression and anxiety with worse kidney function and metabolic outcomes emphasizes the importance of addressing psychological health as a critical component of DKD management. These findings suggest that mental health conditions may not only impact quality of life but also contribute to disease progression through potential biological pathways. Therefore, a comprehensive, multidisciplinary approach is essential to effectively address the complex interplay between psychological and physiological factors in DKD, improving clinical outcomes and patient well-being.

2.7.6 Impact of Diabetic Kidney Disease on Health Outcomes and Quality of Life

The impact of kidney disease extends beyond kidney health, affecting various aspects of health-related quality of life, functional status, and overall well-being. Patients with nephropathy have high rates of cardiovascular morbidity and mortality.[57] The link between diabetes-associated nephropathy and cardiovascular complications highlights the significance of our work in gaining a deeper understanding of the potential shared mechanisms contributing to both kidney disease and other cardiovascular complications. While ongoing and future trials aim to fill these knowledge gaps, early recognition and timely interventions are crucial for improving outcomes and enhancing the quality of life for individuals with kidney disease.[105]

2.8 Epigenetics

Epigenetics is the study of changes in gene activity or function without an associated change in the DNA sequence.[8,9] These epigenetic modifications, which include DNA

methylation, histone modifications, and non-coding RNAs, play pivotal roles in orchestrating cellular responses to environmental cues, developmental signals, and disease states.[8,106] Among the diverse array of epigenetic modifications, DNA methylation is the most studied mechanism, given its stability, theorized biological significance, and the tools available to examine it.[107,108] DNA methylation involves the addition of a methyl group to the cytosine nucleotide within CpG sites.[8,9,109] While CpG islands, often found in promoter regions, are frequently studied concerning gene regulation, DNA methylation can occur across various genomic contexts, including gene bodies and intergenic regions.[9,109] Adding methyl groups to a CpG site profoundly affects chromatin structure and accessibility, thereby influencing the binding of transcription factors and RNA polymerase complexes.[110]

The role of DNA methylation extends beyond gene silencing; it serves as a dynamic regulator of transcriptional activity, orchestrating intricate gene expression programs essential for cellular homeostasis and development. DNA methylation patterns are dynamically regulated during development, characterized by global DNA demethylation before implantation and targeted de novo methylation thereafter.[111]

The regulatory functions of DNA methylation extend to encompass diverse biological processes, including genomic imprinting, X-chromosome inactivation, and transposable element repression. These processes are controlled by DNA methyltransferases (DNMTs), enzymes responsible for adding methyl groups to cytosine residues, and demethylases, which remove methyl groups, highlighting the plasticity of the epigenome.[110] This plasticity allows cells to adapt and respond to changing environmental cues and physiological demands.[111]

A review by Jones[110] provides a comprehensive overview of the multifaceted functions of DNA methylation in gene regulation, emphasizing that the effects of DNA methylation are

context-dependent. The review highlights that while DNA methylation near transcription start sites often suppresses gene expression, DNA methylation within gene bodies may promote transcription elongation and influence splicing. Jones underscores that recent genome-wide studies, such as those utilizing bisulfite sequencing, have revealed a more nuanced relationship between DNA methylation and gene regulation than was previously understood. This work serves as a foundation for interpreting how DNA methylation changes contribute to diseases.

In addition, Hernando-Herraez and colleagues offer insights into the evolutionary implications of DNA methylation, emphasizing its role in driving species-specific traits by influencing gene regulation rather than protein sequence differences.[112] They argue that differences in gene regulation, mediated by CpG methylation, are a driving force behind phenotypic diversity and adaptive responses to environmental pressures. The review highlights the relationship between DNA methylation and underlying genome sequences, suggesting that comparative studies of the methylome across humans and nonhuman primates could shed light on adaptive responses to environmental pressures.[112] They underscore the need for integrative studies to explain the complex interplay between genetics, epigenetics, and environmental factors in shaping DNA methylation patterns.

The dysregulation of DNA methylation is intricately linked to the pathogenesis of various diseases, as evidenced by studies which implicate specific genes and regulatory pathways in disease susceptibility and progression.[113–115] The identification of disease-specific DNA methylation signatures holds immense promise for developing targeted therapeutics and personalized treatment strategies for complex diseases.

Epigenetic studies have illuminated the potential role of maternal obesity in predisposing offspring to CKD through epigenetic modifications.[9] However, this phenomenon is best

understood within the framework of intergenerational transmission, where direct exposure or physiological carryover explains the observed effects.[116] These findings suggest that addressing maternal health during pregnancy could be a key factor in reducing disease risk in the next generation.

DNA methylation and other epigenetic mechanisms impart cells with remarkable plasticity, allowing them to respond dynamically to environmental cues, developmental signals, and disease states. For example, DNA methylation patterns have been implicated in diabetes, where hypermethylation of insulin-related genes can impair insulin secretion and sensitivity.[117] Similarly, stress has been shown to influence DNA methylation at stress-response genes, altering HPA axis function.[118] In kidney disease, epigenetic changes, including DNA hypermethylation at inflammation-associated genes, exacerbate disease progression.[119] Understanding these specific pathways underscores the critical role of epigenetics in disease pathogenesis and highlights opportunities for therapeutic interventions targeting epigenetic dysregulation.

2.9 Blood Memory

Blood memory holds significant importance within Indigenous knowledge systems. The concept reflects the intricate relationship between ancestral legacies and genetic and cultural heritage. Cora Weber-Pillwax explains that Indigenous lifelong education is grounded in lived experiences and ancestral wisdom, underscoring the strong bond between Indigenous peoples and their ancestral lands.[120] Blood memory is central to Indigenous learning, as it serves as a way for knowledge to be transmitted and built upon from generation to generation.[120]

As one aims to understand the teachings of blood memory better, it is crucial to recognize blood memory's associated past of colonial erasure and cultural appropriation and the more recent reclamation of its significance by Indigenous scholars.[120] The concept, which was initially

introduced to academic audiences by N. Scott Momaday in his book 'House Made of Dawn' published in 1968, has been further researched and discussed by several other scholars, including Amy Bombay, an Indigenous health practitioner and scientist who explores blood memory and sacred knowledge as central components of Indigenous health knowledge in her work.[120,121]

Notably, Weber-Pillwax reflects on her upbringing within the Métis community, recalling the phrase "it's in the blood," having been commonly used by many to describe the multifaceted nature of the transmission of human traits and behaviours.[120] As such, blood memory serves to pass on ancestral knowledge and cultural practices while explaining individuals' unique characteristics.[120] The Indigenous understanding of blood memory has been affirmed by recent scientific discoveries in epigenetics, showing the role of environmental experiences in shaping gene expression. While epigenetic modifications, such as changes in DNA methylation, may influence offspring through intergenerational transmission, it is essential to note that true multigenerational inheritance, where changes persist unaltered across several generations, has not been proven and widely accepted. Instead, observed effects in descendants are better understood as intergenerational, where environmental and social experiences shape the health and resilience of subsequent generations.

Recent work supports the Indigenous understanding of blood memory. Rogers-LaVanne and colleagues demonstrated that experiences of historical trauma are associated with alterations in DNA methylation patterns at specific CpG sites in Alaska Native peoples, providing biological evidence of trauma embodiment.[122] When they conducted an Epigenome Wide Association Study (EWAS), they identified specific CpG sites at the following genes: DENND1A, ATP2B4, C8orf38, U6, and PCBP3, which showed altered DNA methylation patterns associated with historical trauma responses.[122] Their findings show links between

historical loss symptoms such as sadness, anger, and feeling that the trauma is happening again, and DNA methylation patterns in genes implicated in signal transduction, metabolism, and immune pathways, suggesting that these epigenetic changes may contribute to health disparities in Indigenous communities.[122] Additionally, the study highlights the protective role of cultural identification in promoting general well-being, underscoring the importance of cultural connection as a buffer against the effects of historical trauma.[122] The impact of the experiences of our ancestors on our epigenome cannot be understated. These findings deepen our understanding of how colonial violence can shape health outcomes at the molecular level, offering a framework for addressing health inequities rooted in systemic oppression.

Indigenous elders often emphasize that memory exists not only in shared narratives but also in blood and bone.[123] Echoing this sentiment, Amy Bombay explains that experiences and environments profoundly impact gene expression, shaping future generations' health and well-being.[123] Recognizing the implications of blood memory can help capture the gravity and lasting effects of intergenerational trauma and the strength and resilience promoted by positive cultural affiliations and spiritual practices.[123,124]

Blood memory has been shown to be a testament to the enduring resilience and wisdom of Indigenous peoples, where traditional knowledge is intertwined with contemporary scientific understandings. As Indigenous and non-Indigenous scholars continue to explore the complexities associated with the transmission of who we are and how we act, blood memory may be an important concept that bridges the gap between the past, present, and future while enriching our understanding of human existence and resilience.

2.10 Epigenetics of Youth-Onset T2D

Epigenetics provides a framework for understanding how environmental factors such as diet, physical activity, and exposure to stressors can influence gene expression, shaping susceptibility to and progression of youth-onset T2D. This is particularly relevant as it helps identify molecular mechanisms, such as altered DNA methylation patterns, that could help explain why individuals with similar genetic risks develop the disease at varying rates and severities. By exploring this mechanism, our study aimed to investigate differences in DNA methylation patterns between youth with and without albuminuria, providing insight into the potential epigenetic mechanisms that may be associated with kidney complications in youth-onset T2D. If epigenetic alterations are identified, this research could help lay the groundwork for future studies to explore biomarkers or therapeutic targets to improve disease management.

In exploring the epigenetics of youth-onset T2D, DNA methylation emerges as a factor potentially influencing disease susceptibility. Toperoff and colleagues conducted an EWAS comparing DNA methylation in the peripheral blood of 710 subjects with T2D to 459 controls without T2D.[125] The study revealed a significant association between lower levels of DNA methylation in the first intron of the *FTO* gene in peripheral blood and an increased prevalence of T2D. The *FTO* gene has been reported to be associated with obesity in humans.[126,127] Notably, every 1% decrease in DNA methylation corresponded to a 6% increase in the odds of having T2D.[125] Beyond its potential as a biomarker for T2D, this study showed that DNA hypomethylation in this region may have functional consequences. Specifically, lower DNA methylation levels co-localize with enhancer elements and binding sites for DNA methylation-sensitive transcriptional regulators, suggesting a mechanism through which epigenetic changes may alter transcriptional activity and influence metabolic pathways linked to T2D.[125]

Furthermore, lower DNA methylation at this site has been observed in young individuals prior to T2D onset, underscoring its potential as an early marker of disease susceptibility.[125] This finding highlights the utility of DNA methylation patterns not only as biomarkers but also as contributors to the pathophysiology of T2D, warranting further investigation into their mechanistic role in youth-onset disease progression.

Investigations into specific genetic loci implicated in metabolic regulation show the intricate relationship between DNA methylation and T2D susceptibility. Dayeh and colleagues conducted their study on a cohort of 258 adult participants from Finland, half of whom had T2D and the other half were controls without T2D.[128] They identified DNA methylation differences in blood at loci within *ABCG1* and *PHOSPHO1* as potential biomarkers for predicting future T2D risk, with alterations in DNA methylation levels correlating with metabolic markers and disease onset.[128] Chambers and colleagues demonstrated differential DNA methylation patterns at specific loci, including *ABCG1*, *PHOSPHO1*, *SOCS3*, *SREBF1*, and *TXNIP*, which were associated with T2D susceptibility in their case-control EWAS of 25,372 Indian Asians and Europeans.[129] Moreover, Indian Asians exhibited higher levels of DNA methylation at these loci than Europeans, highlighting the presence of ethnic-specific epigenetic signatures that may contribute to their higher T2D risk. These findings suggest that DNA methylation differences at these loci might influence metabolic pathways involved in T2D pathogenesis, offering potential biomarkers for risk stratification and early intervention.

Epigenetic modifications within key tissues involved in insulin signalling, such as pancreatic islets, display insights into the molecular mechanisms contributing to T2D. Ling and colleagues conducted a study examining pancreatic islets from 60 multi-organ donors, 48 of whom were non-diabetic, and 12 had T2D. They observed a twofold increase in DNA

methylation of pancreatic islets in the *PPARGC1A* gene promoter among the T2D donors.[130] Among those without diabetes, 4.7% of the DNA in the *PPARGC1A* promotor region was methylated, whereas, in individuals with T2D, this DNA methylation was higher, at 10.5%, indicating a higher degree of epigenetic modification in diabetic islets.[130] This difference was linked to a 90% reduction in *PPARGC1A* expression, impairing mitochondrial function and insulin secretion in islets.[130] Experimental downregulation of *PPARGC1A* reduced insulin secretion by 41%, highlighting its role in mitochondrial health. Dysregulated insulin secretion contributes to hyperglycemia and reduced muscle glucose uptake, which impairs oxidative phosphorylation in muscle cells.[130] This underscores how epigenetic modifications, as seen in Ling and colleagues' work, can influence metabolic homeostasis and suggest potential therapeutic targets for mitigating disease progression.

Notably, the epigenetic landscape extends beyond DNA methylation to encompass histone modifications and long non-coding RNAs, which actively shape chromatin structure and regulate gene expression. Histone modifications are key regulators of chromatin states that influence the transcriptional activity of metabolic genes implicated in T2D.[131] For example, trimethylation of *H3K4* marks active transcription start sites, while *H3K27* acetylation identifies active enhancers and promotes transcription.[131] Conversely, *H3K27* histone trimethylation silences key genes, contributing to impaired metabolic pathways.[131]

Similarly, long non-coding RNAs emerge as critical players in gene regulation. By targeting chromatin-modifying enzymes to specific genomic loci, long non-coding RNAs act as modular scaffolds, directing chromatin-modifying complexes like histone acetyltransferases or deacetylases to their targets, thereby influencing metabolic homeostasis.[132] These findings

highlight the dynamic interaction of epigenetic factors, chromatin structure, and gene regulation in the pathogenesis of T2D.[132]

Incorporating environmental factors such as diet, stress, and exposure to pollutants into the discussion provides a comprehensive understanding of the multifaceted etiology of youth-onset T2D. Volkov's methylation quantitative trait locus (mQTL) analysis in human adipose tissue identified genome-wide interactions between genetic variants and DNA methylation patterns. This revealed how genetic effects on metabolic traits, such as obesity and diabetes, may be mediated through changes in DNA methylation, which offers insights into the molecular mechanisms underlying metabolic dysfunction.[133]

Dick and colleagues' investigation into the association between DNA methylation and body mass index (BMI) elucidates the bidirectional relationship between adiposity and epigenetic regulation, with significant DNA methylation changes observed at the *HIF3A* gene, which is associated with increased BMI.[134] While the causal nature of this association remains to be fully explained, the findings at the *HIF3A* gene suggest that increased BMI is linked to increased DNA methylation. Conversely, these DNA methylation changes might affect gene expression through the hypoxia-induced factor (HIF) pathways, potentially influencing metabolic processes that further contribute to weight gain and T2D pathogenesis. While the causal nature of this association remains to be fully explained, the findings at the *HIF3A* gene offer a compelling example of how BMI and epigenetic modifications can influence one another. By considering these environmental factors, we gain clearer insight into how environmental exposures and epigenetic changes interact to influence individual susceptibility to T2D.

The epigenetics of youth-onset T2D represent a complex relationship between inherited genetic factors, environmental influences, and epigenetic modifications. DNA methylation is

emerging as a key mechanism in disease susceptibility and progression. By uncovering the regulatory networks underlying T2D pathophysiology, epigenetic studies provide a foundation for developing novel diagnostic biomarkers and targeted therapies to reduce the disease burden and improve clinical outcomes.

2.11 Epigenetics of Stress

The ability of the stress response system to cohesively respond to external and internal stressors and quickly reinstate homeostasis is crucial for the health of every individual. Stress, particularly when experienced early in life, can have harmful effects on mental and physical health.[135] Epigenetic mechanisms play a crucial role in regulating gene expression in response to various environmental factors, including stress.[136–138] These mechanisms involve modifications to DNA and histone proteins, ultimately influencing chromatin structure and gene transcription.

Central to the body's response to stress is the HPA axis, with glucocorticoids playing a prominent role in regulating its duration and magnitude.[139] Epigenetic modifications to HPA-axis-related genes, such as *FKBP5*, have emerged as an area of interest due to the implications for stress regulation and downstream health effects. For instance, demethylation of the *FKBP5* gene in peripheral blood has been shown to increase gene transcription, exacerbating stress dysregulation.[140,141] Higher levels of DNA methylation of *FKBP5* in blood may also be associated with an increased risk of T2D through significant associations with increased HbA1c and LDL cholesterol.[142] Other genes that are key to the regulation of the HPA axis, such as *CRH* and *CRHBP*, which regulate corticotropin-releasing hormone (CRH) activity, have been shown to undergo stress-related changes in DNA methylation in blood, further underscoring the connection between chronic stress and HPA-axis.[140]

While the *NR3C1* gene, which encodes the glucocorticoid receptor, has also been studied in relation to stress-induced DNA methylation, the reliability of the findings has been questioned due to a lack of replication.[135,143–145]

Epigenetic changes driven by stress can have profound implications for the next generation. While the term "transgenerational" is often misused, what is more accurately observed is the intergenerational transmission of stress-induced epigenetic modifications directly passed from mother to offspring without crossing multiple generations. Maternal stress during pregnancy has been linked to changes in DNA methylation at stress-regulatory loci, such as *FKBP5* and *IGF2*, which may predispose offspring to altered stress responses and heightened risk of psychiatric or metabolic disorders.[141,146] These epigenetic changes may serve as a biological mechanism by which early-life stress becomes embedded, influencing the offspring's stress physiology and long-term health outcomes.

Recent research has strengthened these findings, providing further evidence of how maternal stress shapes offspring biology. Sharma and colleagues identified stress-related DNA methylation markers in newborn saliva, revealing altered CpG sites associated with genes involved in neurodevelopment, neuronal signalling, and psychiatric disorders such as PTSD and schizophrenia.[147] These findings suggest that maternal stress during pregnancy leads to direct epigenetic changes in offspring, with potential lifelong impacts on health and behaviour. Similarly, Grégoire and colleagues found that prenatal maternal stress in mice amplifies offspring sensitivity to chronic pain following nerve injury.[148] This heightened pain sensitivity was linked to sex- and region-specific disruptions in the expression of stress- and epigenetic-regulating genes in the hippocampus and frontal cortex, key brain regions involved in pain processing and stress regulation.[148] These findings underscore the significant and lasting effects

of prenatal stress on the central nervous system, further emphasizing the importance of reducing maternal stress during pregnancy to mitigate these risks.

Collectively, these findings highlight the significant role of prenatal stress in shaping offspring development through epigenetic mechanisms. The observed intergenerational effects reinforce the importance of interventions to reduce maternal stress during pregnancy, both to mitigate immediate effects on maternal and child health and to break potential cycles of health disparities propagated through biological vulnerability.

It is well-established that social and environmental factors interact with genetic and epigenetic mechanisms to influence stress responses and disease susceptibility.[149] Stressors such as low socioeconomic status, early-life adversity, and chronic exposure to environmental stress can exacerbate epigenetic modifications, thereby contributing to health disparities and an increased risk of disease among vulnerable populations. For instance, Naumova and colleagues demonstrated DNA hypermethylation of 28 genes involved in brain development and function, including those regulating the arginine vasopressin 1A receptor, GABA A receptor, and glutamate receptor, in children aged seven to 10 years following institutionalization.[150] These DNA methylation changes may impair affected individuals' neural development and stress regulation. Labonte and colleagues further demonstrated that early adversity in adulthood is associated with hypermethylation of 248 gene promoters, leading to decreased mRNA expression in the hippocampi of adult men who completed suicide.[151] These findings highlight the long-term epigenetic consequences of adverse early environments on stress-regulatory pathways. Moreover, Jawahar and colleagues discussed that early-life adversity is associated with significant global DNA methylation differences, particularly in genes involved in the nervous and immune systems.[152] Together, these studies provide compelling evidence that social

determinants of health, such as early-life adversity, can shape epigenetic regulation, influencing stress resilience and disease risk across the lifespan.

Further extending the role of social factors, Raffington and colleagues demonstrated how epigenetic programming, such as DNA methylation associated with BMI, reflects the long-term impact of early-life socioeconomic stressors on health outcomes. They found that children from lower socioeconomic backgrounds had altered DNA methylation associated with BMI-related genes, correlating with increased disease susceptibility later in life.[153] Notably, their findings suggested a bidirectional relationship between BMI and DNA methylation. Early-life BMI appeared to influence changes in DNA methylation over time, and these epigenetic changes, in turn, shaped future BMI.[153] This relationship highlights how adverse early-life social determinants can establish biological vulnerabilities that persist into adulthood, reinforcing health disparities across generations.

Bridging the gap between basic research and clinical practices in the field of stress and epigenetics will be crucial for identifying and offering new therapeutic approaches for stress-related disorders as well as disorders in which high stress is a common comorbidity.[154] This is because epigenetics connects environmental stressors to molecular changes that alter gene expression without modifying the underlying DNA sequence. Understanding these mechanisms enables the discovery of biomarkers for stress susceptibility, the development of tailored interventions through epigenetic profiling, and the design of innovative gene therapies aimed at reversing harmful modifications. This focus is particularly important because stress has been shown to be associated with epigenetic regulation, which, in turn, affects health outcomes.[136–138] Understanding this relationship may provide insights into the mechanistic underpinnings of complications such as diabetes-related kidney disease. As this field advances, opportunities to

improve personalized interventions that target specific pathways to promote resilience and mitigate the adverse effects of stress on health will be possible.[154]

2.12 Epigenetics of Kidney Disease

2.12.1 Identification of Epigenetic Biomarkers for Kidney Disease

Kidney disease is a significant complication of T1D and T2D.[155] Recent research highlights the involvement of epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNAs, in the pathogenesis of diabetes-related kidney disease.

Several studies link epigenetic modifications and kidney complications in individuals with T2D.[156–158] In 2014, Wing and colleagues published a study with 3939 adult participants showing that within blood, there are specific DNA methylation patterns associated with rapidly progressing CKD.[159] Given the finding, DNA methylation may be used as a tool to predict the CKD progression rate. Maghbooli and colleagues found that the presence of albuminuria among adults with T2D was associated with increases in global DNA methylation in peripheral blood, further supporting the role of epigenetics in diabetic kidney complications.[160]

DNA methylation of saliva also provides valuable epigenetic insights, as demonstrated by Sapienza and colleagues, who identified 39 genes that were involved in kidney development or DN in adult patients with either T1D or T2D and who have ESKD when compared to those who do not have any kidney damage.[158] While saliva is less commonly used for epigenetic studies than blood, it offers a distinct advantage as a non-invasive, easily accessible tissue. However, a key limitation is the lack of direct evidence linking saliva DNA methylation to kidney-specific epigenetic changes. Current evidence broadly compares saliva to blood or brain DNA methylation patterns.

Braun and colleagues demonstrated that saliva DNA methylation correlates with brain methylation patterns with a correlation level comparable to blood-brain DNA methylation comparison.[161] However, blood exhibits a higher proportion of CpG sites significantly correlated with brain tissue, underscoring its reliability for capturing DNA methylation changes relevant to systemic diseases. Smith and colleagues found that DNA methylation patterns in saliva were more similar to those in the brain compared to blood methylation patterns. However, they observed greater variability in CpG sites from saliva than from blood, likely reflecting the heterogeneous nature of saliva samples. The researchers also noted that correlated CpG sites between saliva and blood were more likely to occur in regions of low CpG density, such as CpG shores and open seas.[162] While these studies highlight saliva's potential for capturing certain epigenetic patterns, particularly in systemic contexts, they also emphasize its limitations due to variability and a lack of direct evidence for certain tissue-specific diseases.

The benefit of examining saliva DNA methylation is its non-invasive accessibility and potential to reflect systemic processes. However, its limitations, such as variability in cellular composition and limited evidence for certain organ-specific conditions like kidney disease, must be carefully weighed against research objectives. Among individuals with T2D, there are several links between kidney disease and DNA methylation across multiple genes.

2.12.2 Epigenetic Regulation of Inflammation and Fibrosis in Diabetic Nephropathy

Epigenetic modifications, including DNA methylation, histone modification, and non-coding RNA regulation, are key regulators of gene expression without altering the DNA sequence and play a critical role in the inflammatory processes underlying DN.[163] Studies led by VanderJagt. and Park highlight that genome-wide DNA methylation analyses show associations between DNA methylation changes and kidney injury, inflammation, and kidney dysfunction in

individuals with DN, suggesting a critical role of epigenetic regulation in disease progression.[164,165] Specifically, high glucose conditions show changes to DNA methylation patterns in kidney cells, with notable alterations observed in genes related to renal fibrosis, such as *TGF-β1*, *Col4a2*, *Tceal3*, *Ret*, and *Agt*.[166,167]

Epigenetic mechanisms modify inflammatory pathways in DN, contributing to persistent inflammation, exacerbating renal fibrosis, and ultimately leading to CKD and ESKD.[163] For example, DNA methylation dynamically influences transcriptional regulation in high-glucose environments. Chen and colleagues demonstrated that high-glucose conditions promote aberrant DNA methylation patterns through upregulating DNMTs, such as DNMT1, suppressing the transcription of anti-inflammatory genes like *Nrf2*.[168]

Consequently, anti-inflammatory strategies have emerged as promising avenues for managing DN, with epigenetic modifications serving as potential targets for therapeutic intervention.[163] Notably, histone modification, particularly the inhibition of histone acetyltransferases and histone deacetylases, represents novel therapeutic approaches for improving DN-associated inflammation and fibrosis.[163]

Research exposing the role of specific epigenetic regulators in DN pathogenesis has shown promising results regarding the potential molecular mechanisms underlying kidney damage in diabetes. The *Sirt1* gene, a key epigenetic regulator, protects against kidney damage in diabetes by modulating processes such as histone deacetylation and transcriptional silencing, which regulate genes involved in inflammation and fibrosis. Its downregulation in proximal tubules is linked to the upregulation of Claudin-1 in glomeruli, which was correlated with albuminuria in diabetic mice.[169] Conversely, *Sirt1* overexpression in proximal tubules prevents albuminuria by maintaining nicotinamide mononucleotide concentrations around glomeruli,

which protects podocyte function.[169] Importantly, clinical correlations between *Sirt1* and Claudin-1 expression levels and proteinuria in individuals with diabetes highlight the translational relevance of these findings, suggesting potential therapeutic strategies for mitigating albuminuria in those with diabetes.[169] Understanding the interplay between epigenetic modifications, such as *Sirt1*-mediated histone deacetylation, and renal pathology in diabetes will be crucial for identifying molecular pathways that contribute to kidney dysfunction, ultimately enabling the development of targeted interventions for reducing DN.[163]

2.12.3 Environmental Influences on Epigenetic Changes in Kidney Disease

Environmental factors, including diet, obesity, and toxin exposure, have been shown to influence epigenetic modifications associated with kidney disease.[170] Research indicates a strong correlation between obesity, insulin resistance, and various metabolic abnormalities, which can lead to chronic diseases such as T2D, hypertension, cardiovascular disease, non-alcoholic fatty liver disease, and CKD.[170]

Epigenetic mechanisms, including DNA methylation, histone modifications, and non-coding RNA regulation, are increasingly recognized as key contributors to the development of CKD.[170–172] EWAS have shown substantial changes to DNA methylation patterns in CKD patients.[170] Evidence from EWAS has shown that changes to DNA methylation patterns in CKD patients often occur in promoter and enhancer regions.[171] These regions are linked to regulating genes that promote fibrosis, a critical pathological process driving kidney damage.[159] Such DNA methylation changes are hypothesized to contribute to CKD development by modulating the expression of fibrotic genes, creating a pro-fibrotic environment within the kidney. However, further functional studies are needed to establish causality and identify the precise mechanisms involved.

Among CKD patients, in situations of accelerated renal function decline there is a correlation between DNA methylation changes affecting genes involved in inflammation and fibrosis.[170] Although these changes may initially appear as biomarkers of disease progression, emerging evidence suggests that DNA methylation modifications can actively regulate the transcriptional activity of inflammatory and fibrotic genes, contributing to the pathogenesis of CKD. For example, Smyth and colleagues identified significant DNA methylation changes in genes such as *ELMO1* and *PRKAG2*, with functional analyses supporting their role in CKD development through differential gene expression.[173] Similarly, Ko and colleagues demonstrated that differentially methylated regions in CKD patients are predominantly located in enhancer regions associated with renal transcription factors, highlighting their role in regulating fibrosis-related gene expression, such as collagen-encoding genes.[174] These findings suggest that DNA methylation changes may serve as markers of disease and play an active role in driving pathological processes. However, despite this evidence, further studies are required to definitively establish causality and explore the potential of epigenetic therapies targeting these DNA methylation patterns.

Future studies should explore the relationship between obesity, epigenetic alterations, and the development of CKD to reveal further the mechanisms underlying the transmission of metabolic diseases. Understanding how environmental factors shape epigenetic profiles associated with CKD could pave the way for targeted interventions aimed at mitigating disease risk and progression on a personalized level.

2.13 Evidence Gap in Current Literature

There is strong existing knowledge on the impact of the high rates of T2D and associated kidney complications in Manitoba, particularly among Indigenous peoples. While the epigenetic

modifications to stress-related genes have been researched, there is little known about the role of stress in determining kidney outcomes among Indigenous peoples with T2D in Manitoba. Recent research has presented the increased stress experienced by individuals who have been diagnosed with T2D, the connection between stress and kidney disease, and the connection between kidney disease and T2D, separately.[1,46,175–177] However, there has yet to be an analysis of the association between stress, T2D, and kidney disease examining epigenetic modifications such as DNA methylation as a potential biological mediator of the relationships.

There are limitations to the current literature. First, there is limited research focused on Indigenous youth with T2D who live in Canada. Given the unique disease experience of Indigenous youth, which includes a high prevalence and high morbidity associated with T2D, it is essential to have a body of research tailored to the specific pathophysiology of early renal disease in this population. Second, there is a gap in understanding how stress is transferred to the kidneys, resulting in kidney damage, and if epigenetics is one mechanism by which stress impacts kidney health. While stress-related pathways, such as HPA axis activation, inflammation, and SNS-driven changes, offer theoretical explanations for this connection, studying these mechanisms in human populations is challenging due to ethical and practical constraints. As a potential mediator, epigenetics could be investigated through peripheral biomarker studies or animal models, though confirmation of kidney-specific changes would require direct tissue analysis. Addressing these evidence gaps will be critical for improving tailored interventions and health outcomes for youth with T2D.

Chapter 3: Research Objectives and Questions

This study aims to examine the relationship between stress levels and kidney outcomes in youth with T2D and examine epigenetics as one potential component of the pathway by which this occurs. This was accomplished using data from the iCARE cohort, Canada's largest prospective cohort of youth with T2D. To achieve the research aim, I examined the following research questions:

1. (a) Do DNA methylation patterns differ between youth living with T2D who have albuminuria and youth without albuminuria?

   (b) Do youth living with T2D with albuminuria and without albuminuria have DNA methylation differences in areas of the genome that correspond to stress response?
2. Is there a synergistic effect on DNA methylation between the presence of T2D and varying stress levels as determined by the Perceived Stress Scale-14 (PSS-14), and are those DNA methylation markers associated with albuminuria?

We hypothesized that among youth with T2D, the presence of albuminuria (i.e. ACR greater than 3.0mg/mmol on overnight urine collection or first-morning sample) would be associated with altered DNA methylation both generally across the epigenome and in areas related to stress compared to youth without albuminuria. Additionally, we hypothesized that youth with T2D who experience high stress levels and have albuminuria would exhibit a unique or additive pattern of DNA methylation markers, distinguishing them from those with T2D alone or T2D with only one of stress or albuminuria.

Chapter 4: Methods

4.1 Ethics and Data Access

As a subset project of the iCARE study [Ethics #: HS13255 (B2011:024)], my research project required review from the Biomedical Research Ethics Board at the University of Manitoba Bannatyne Campus and was approved on March 27, 2023 [Ethics #: HS25891 (B2023:029)]. This project used de-identified data, and individual informed consent was obtained prior to the commencement of my project.

My research methods were guided by the Tri-Council Policy Statement (TCPS2) and Ownership, Control, Access, and Possession (OCAP) principles. TCPS2 outlines key aspects that help to ensure a positive relationship and outcome for the researcher and community through ensuring free, informed, and ongoing consent; consideration for participants' needs and feelings; and an acknowledgement of potential power imbalances and how to mitigate them.[178] OCAP aims to ensure that Indigenous communities have control over data collection processes, ownership, access to information, and authority over how their data is used in research.[179]

To uphold these ethical standards, my research actively involved the iCARE Participant Advisory Group (PAG). The PAG comprises adolescents and youth living with T2D and their caregivers, contributing to the governance of the study and providing valuable insights from their lived experiences. The PAG oversees iCARE study priorities, processes, and publications and plays a key role in advising on all aspects of the research.

The iCARE team has fostered an ongoing partnership with the PAG, integrating their input into outputs such as academic publications, media, and presentations at local and national conferences. In addition, community-based gatherings have been hosted to share research

progress with broader community members. Meetings have been held with leadership figures, including Chiefs in Council, to ensure the research aligns with community priorities.

Since 2023, the iCARE investigators have been in partnership with the Anishininew Okimawin to support a research position within the Anishininew Nation Health Organization, which is operated under the Four Arrows Regional Health Authority (FARHA). This partnership has built research capacity and strengthened governance within FARHA, enhancing the community's control over research activities. There is ongoing work between iCARE and FARHA to develop a legal research partnership and data management agreements that adhere to OCAP standards, reinforcing the ethical principles of ownership, control, access, and possession.

In my research project, I based my work on the fundamentals of the TCPS2 and OCAP in several ways. iCARE PAG members were informed about my work throughout the research process. I presented to the iCARE Steering Circle to ensure the suitability of my research methods (December 2023). I presented to the PAG to discuss my research plans, answer questions, receive feedback, and find interested parties to join my investigator group (January 2024). iCARE PAG members were informed about my work throughout the research process, providing opportunities to share findings, gather feedback, and ensure that the research reflected the lived experiences of those directly impacted by T2D (February 2024-January 2025).

To further support community engagement and transparency, reports were created to inform the broader community of research findings and progress. These reports were presented to the FARHA Board of Directors, ensuring the research aligned with the community's interests.

4.2 Overall Study Design

This cross-sectional study utilized de-identified data from the iCARE cohort, a prospective observational longitudinal study. The iCARE project investigates the risk factors for

poor kidney outcomes in adolescents with T2D by examining biological, psychological, and social exposures. The primary outcome measure is non-orthostatic albuminuria, determined by an overnight or first-morning urine sample (further defined below).

4.3 Study Population

Data for this analysis were extracted from the iCARE cohort, collected between July 2011 and August 2023. This cohort includes individuals who enrolled between the ages of 10 and 18 years and are being treated for T2D at one of nine Canadian tertiary care settings. These sites include: The Children's Hospital of Winnipeg (Winnipeg, Manitoba); British Columbia Children's Hospital (Vancouver); Stollery Hospital (Edmonton, Alberta); Alberta Children's Hospital (Calgary, Alberta); McMaster Children's Hospital (Hamilton, Ontario); Children's Hospital of Eastern Ontario (Ottawa, Ontario); Hospital for Sick Children (Toronto, Ontario); McGill University Health Centre (Montreal, Quebec); and the IWK Health Centre (Halifax, Nova Scotia).

The overall iCARE cohort continues to have ongoing enrollment and currently includes 402 youth with T2D. Interested individuals are enrolled if they have a T2D diagnosis according to Canadian Diabetes Association criteria while not meeting any of the following exclusion criteria: 1) diabetes secondary to medication use or surgery; 2) antibodies suggestive of Type 1 Diabetes; 3) treatment with high-dose steroids or immunosuppressive agents that could interfere with cortisol assessment and inflammatory markers; 4) cancer; 5) evidence of alcohol or drug abuse; and 6) inability or unwillingness of the patient or caregiver to provide voluntary informed assent/consent.[180]

The iCARE study began in 2011 with data collected for phenotypic assessments and expanded in 2014 to include psychological evaluations, one of which was the PSS-14

questionnaire.[180] Follow-ups are conducted annually to monitor risk factors and kidney outcomes, such as the progression of albuminuria.[180] The iCARE study is the largest, most diverse, and comprehensive study of youth with T2D in Canada, providing valuable insights into the factors related to the development of kidney disease in this population.

My inclusion criteria for this thesis project required participants to be part of the iCARE cohort, diagnosed with T2D, have an albuminuria result, complete the PSS-14 questionnaire, and complete epigenetic data collection. Thus, 213 participants were included.

4.4 Data Collection

Data collection was completed before the start of my study. The primary exposures, outcome variable, and mediating factor were stress, T2D, albuminuria, and epigenetics, respectively. The methods of data collection for each variable are detailed below.

4.4.1 Exposure 1: Stress

Stress as an exposure was measured through the PSS-14, a validated and widely used measure of perceived stress.[181] Among the iCARE cohort, the PSS-14 was completed with 96.6% of participants at their baseline assessments.[181] The questionnaire was administered by the research associate and completed by participants. The 14-item questionnaire uses a 5-point Likert scale ranging from 0 (‘Never’) to 4 (‘Very often’).[182] Total scores range from 0-56, with higher scores indicating greater perceived stress.[182] The 14 items question an individual’s perception of their life as being unpredictable, uncontrollable, and overloaded over the past month.[183] The PSS-14 has been shown to have high internal consistency, reliability, and test-retest reliability.[184] The tool has been used in various populations ranging from youth to older adults.[184–186] The PSS-14 is not a diagnostic tool, so there are no cut-off scores. Our study used a

stress score of 27 or greater, as used in previous iCARE work and other research papers, to indicate stress.[187]

4.4.2 Exposure 2: Type 2 Diabetes

T2D diagnosis was defined by the clinical criteria used by the Canadian Diabetes Association. Exclusion criteria aligned with the iCARE cohort's inclusion/exclusion criteria as previously described (see 4.3 Study Population).[180]

4.4.3 Outcome: Albuminuria

Urine samples were collected from participants to test for non-orthostatic albuminuria. Preferred samples for analysis were overnight or first-morning urine collections, with random samples used when logistical constraints prevented these.[188] These preferred samples minimize the effects of orthostatic albuminuria, a phenomenon common among adolescents.[189] During adolescence, standing or walking throughout the day may cause small amounts of protein to spill into the urine due to the gravitational impact on kidney function.[189] However, when an individual is lying flat overnight, this gravitational effect is eliminated, allowing for a more accurate measurement of kidney function. Participants provided their urine samples in a sterile urine container. The clinical biochemistry lab at Shared Health Diagnostic Services processed and analyzed urine samples.

Albuminuria in the iCARE cohort was defined as an ACR greater than 3.0mg/mmol on overnight urine collection or first-morning sample, in line with the Kidney Disease Improving Global Outcomes (KDIGO) 2021 Clinical Practice Guideline for the Management of Glomerular Diseases, [190] Confirmation of albuminuria is required through a timed overnight urine or first-morning urine collection to avoid misdiagnosis of orthostatic proteinuria, which occurs in up to 20% of youth.[180] However, due to logistical constraints, not all participants provided first-

morning or overnight samples, and random urine samples were sometimes used instead. While preferred sample types help minimize the risk of false positives caused by orthostatic proteinuria, the study followed the Canadian Diabetes Association guidelines, which recommend confirming persistent albuminuria with two samples taken at least one month apart within six months.[1] However, in practice, persistent albuminuria was confirmed through a review of clinical charts. This approach ensured that albuminuria status was accurately assessed despite variations in sample type.

This research focused on whether an individual's albuminuria status (i.e., whether they meet the cut-off for diagnosis) was associated with differences in DNA methylation patterns across the epigenome and in verified stress-related genes.

4.4.4 Modifying Factor: Epigenetics

Buffy coats from blood samples were collected from participants and assessed in this study to examine DNA methylation. Blood samples were obtained during participants' baseline visits and subsequently every two years; however, only the baseline samples were analyzed for this study.

The buffy coat, comprising less than 1% of a blood sample, contains white blood cells and platelets and is used to examine DNA methylation patterns.[191] Using the buffy coat rather than whole blood allows for a more cell-specific analysis of DNA methylation by focusing on white blood cells, which are immune cells and highly responsive to environmental exposures.[192] Whole blood contains other components, such as red blood cells, that lack nuclei and, therefore, do not contribute DNA for methylation analysis.[192] The buffy coat provides a practical and widely accepted source of DNA for epigenetic studies.

Epigenetic data collection began after the start of the iCARE study data collection, resulting in baseline data for most but not all participants. Approximately 50.5% of the overall iCARE cohort completed epigenetic data collection at baseline. DNA methylation data from blood were already processed and normalized for use in adjacent iCARE projects.

Qualified personnel performed the blood draws, filled the tubes, and inverted them gently between four and 10 times. Within 20 minutes of the blood draw, the plasma and serum tubes were centrifuged at 3000 rpm for 20 minutes at 4°C. The buffy coat, the white layer between the plasma and the red blood cells, was collected using a pipette and aliquoted into one 2 mL cryovial. Immediately after aliquoting, the buffy coat samples were stored in a −80°C freezer by sample and visit type.

DNA was extracted from the buffy coats using the QIAamp DNA blood minikit and bisulfite conversion was carried out using the Zymo EZ DNA methylation kit. DNA methylation data were processed and analyzed using established protocols involving the Illumina Infinium Human Methylation EPIC BeadChip platform, as described in sections 4.5–4.6, which outline the methods for quality control, normalization, and cell type deconvolution.

4.4.5 Covariates:

The a priori covariates I adjusted for included age, sex, ethnicity, duration of diabetes, adiposity (BMI z-scores), glycemic control (HbA1c), hypertension, medication use, smoking, genetic ancestry, batch, and cell-type proportions. I slightly modified the variables included in my model to increase model fit, as described in section 4.8. Detailed information on the inclusion and measurement of the covariates included in my final model is provided in Appendix 2.

4.5 Quality Control and Normalization

The data were pre-processed previously. This was accomplished through several steps using R software. Full details of the previously completed quality control and normalization can be found in Salama and colleagues' publication.[193] The raw intensity values from the Illumina Infinium Human Methylation EPIC BeadChip microarray were analyzed from IDAT files into R by using the *minfi* package. Detection p-values were calculated using a cut-off p-value <0.05. Probe intensity levels were normalized using *preprocessNoob* from *minfi* and probes were excluded if they had a low detection p-value (<0.01 in any sample). Additional exclusion criteria for probes included bead counts less than three in at least three samples, CpG loci in the sex chromosomes, CpG loci commonly affected by a known single nucleotide polymorphism (SNP), and CpG loci with cross-reactive probes. The remaining 705,247 CpG loci were used for analysis. *BMIQ* from the *wateRmelon* package was used to correct for probe type variance and I had 213 samples and 705,247 CpG loci remaining for analysis. Beta and M values were calculated as described by Salama and colleagues.[193]

4.6 Cell Type Deconvolution

I estimated cell type proportions of blood using the same methodology as Salama and colleagues.[193] I employed a flow-sorted blood tissue reference set from *ExperimentHub* and *estimateCellCounts2* function from the *FlowSorted.BloodEPIC* package. The R package *compositions* was used to transform cell type proportion information with isometric log-ratio using the *clr()* function. Then *prcomp()* function was used to extract robust principal components, which were then utilized in linear regression analyses to adjust for differences in DNA methylation resulting from varying cell-type proportions.

4.7 Batch Correction

Batch correction was completed using the *ComBat* method to address the potential issues related to variations in experimental conditions, such as different batches of chips, array rows, and plate runs. The *sva* and *wateRmelon* packages were used for *ComBat* and data conversion, respectively. Principal Component Analysis (PCA) was conducted on the uncorrected data to visualize existing batch effects. This helps identify the sources of variance prior to batch correction. Sequentially, I performed three batch corrections for plate runs, array rows, and slide, with PCA conducted after each step to assess the impact of the correction. Following the three corrections, M-values were converted back to beta values.

4.8 Assessing Model Fit

The a priori covariates included in the model were slightly modified to achieve the best model fit while allowing biological rationale to guide the inclusion/exclusion criteria. My final model adjusted for the following variables: albuminuria status, age at baseline visit, sex, BMI, smoking status, glycemic control (HbA1c levels), genetic ancestry (GeneticPC1-3), and cell-type proportions (CellPC1-4). I excluded the originally proposed covariates of batch, ethnicity, duration of diabetes, hypertension, and medication use, due to potential confounders and data quality issues. Batch was not included in the model because batch effects were already corrected using the *ComBat* method during data preprocessing, making further adjustment redundant. Ethnicity was not included because genetic ancestry was already added to the model (Genetic PC1-3), and this is the preferred method as it controls for relatedness and helps to remove some of the genetic confounding data. Genetic PC1-3 were chosen (leaving out Genetic PC4-5) because the most variance is accounted for in Genetic PC1, followed by Genetic PC2 with the second most variance and so on. I expected that the small amount of variance accounted for in

GeneticPC4-5 was not necessary to include given that their inclusion did not further improve my model fitness and would only contribute to reducing power by including two additional variables in the model. Duration of diabetes was excluded as it was strongly correlated with age at baseline and would reduce model fit to include both variables. Hypertension was excluded due to its association with albuminuria and its failure to improve model fitness. Medication use was excluded to avoid confounding effects and because incomplete data would compromise the model's integrity. Medication use may be confounded with glycemic control, for which I am already controlling. Additionally, medication use had many missing values denoted as 'N/A.' In the R script, variables must be coded as a binary Yes/No, and there cannot be any missing values, as any participants with missing values will be removed from the analysis. The extent of missing data would compromise the model's integrity, and we would not have been able to continue. Details on the inclusion and measurement of covariates are included in Table 1.

Surrogate Variables (SV) help identify underlying sources of variability in complex datasets. Surrogate Variable Analysis (SVA) is an approach used to identify these hidden factors. It is often applied when the data does not fit the expected model or when unexpected variability is observed.

SVA creates surrogate variables by identifying patterns of variation in the dataset that are not explained by the existing model. The process involved removing variables with minimal impact and focusing on the key elements to understand the hidden sources of variability better. The model used the *sva* package to determine the number of SVs that should be included.

After identifying SVs, they were incorporated into the model to correct for what was previously hidden. This correction usually helps to reduce unexplained variability and improves overall model fit. However, when I completed SVA and included one or more SVs in my model,

the model only became more deflated and the overall fit was decreased (Figure 1). As a result, no SVs were included in my final model. The final model that was used for my analysis was slightly inflated with a lambda equal to 1.44 (Figure 1, Figure 2).

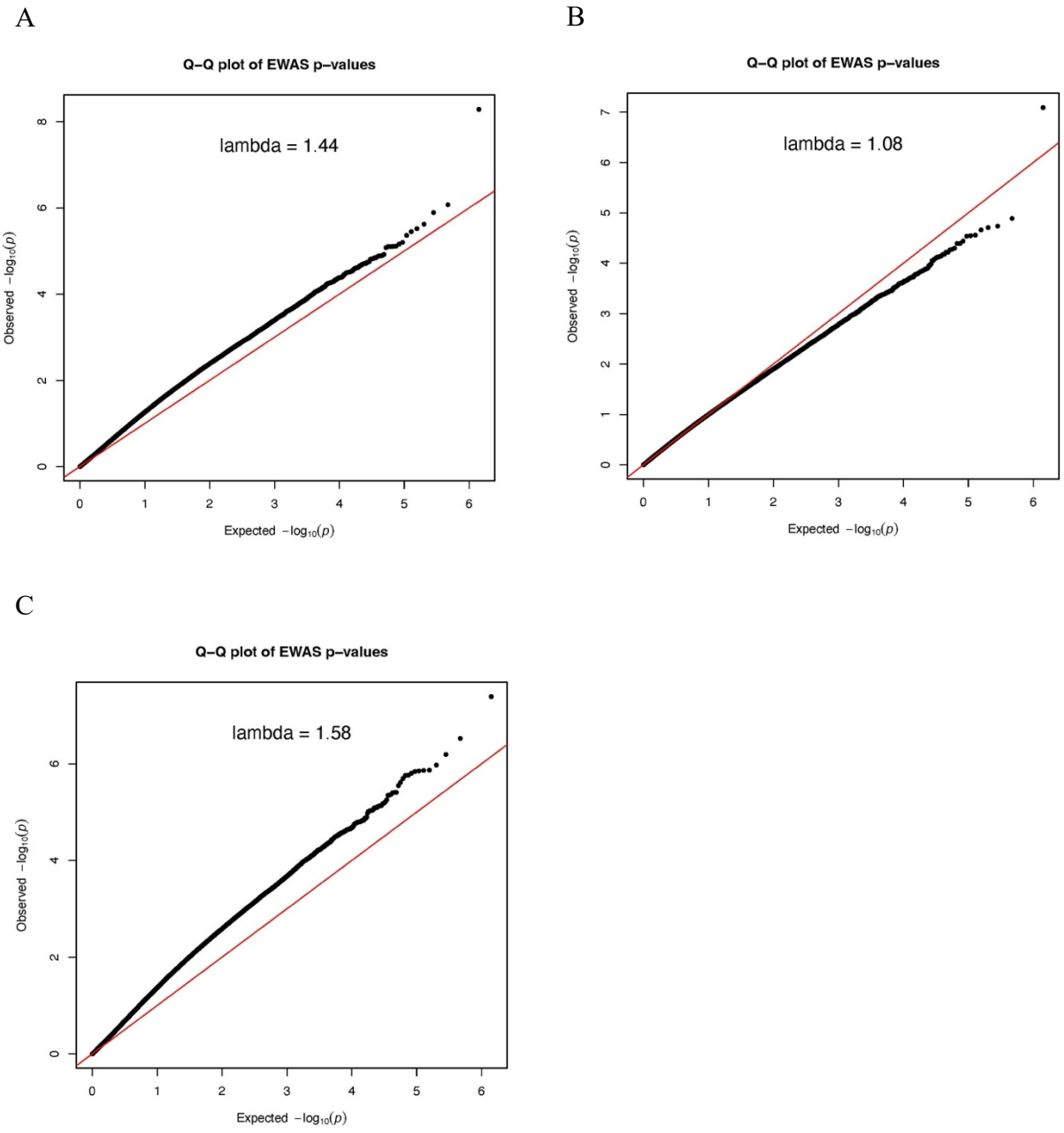


Figure 1. Evidence of systematic inflation in EWAS p-values across models.
The observed -log10(p-values) deviate from the expected distribution under the null hypothesis, indicating potential inflation or model artifacts. The Q-Q plots compare observed -log10(p-

values) (y-axis) to expected -log10(p-values) (x-axis), with the diagonal line representing the null hypothesis distribution. The genomic inflation factor, lambda (λ), shown for each plot, quantifies deviations from the expected distribution. **A.** Model used in the analysis, which included the following variables: albuminuria, age at baseline visit, sex, smoking, glycemic control, genetic ancestry (GeneticPC1-3), cell-type proportions (CellPC1-4), and adiposity. λ=1.44, indicating moderate inflation. **B.** Model with one surrogate variable added. Not used in the analysis. Variables included: albuminuria, age at baseline visit, sex, smoking, exposed to second hand smoke, glycemic control, genetic ancestry, cell-type proportions, adiposity, and surrogate variable 1. λ=1.08, indicating slight deflation, possibly due to over-adjustment. **C.** Model after applying the *bacon* method designed to correct for systematic biases in EWAS. Not used in the analysis. Variables included: albuminuria, sex, smoking, exposure to second-hand smoke, genetic ancestry, cell-type proportions, and adiposity. λ=1.58, indicating moderate inflation.

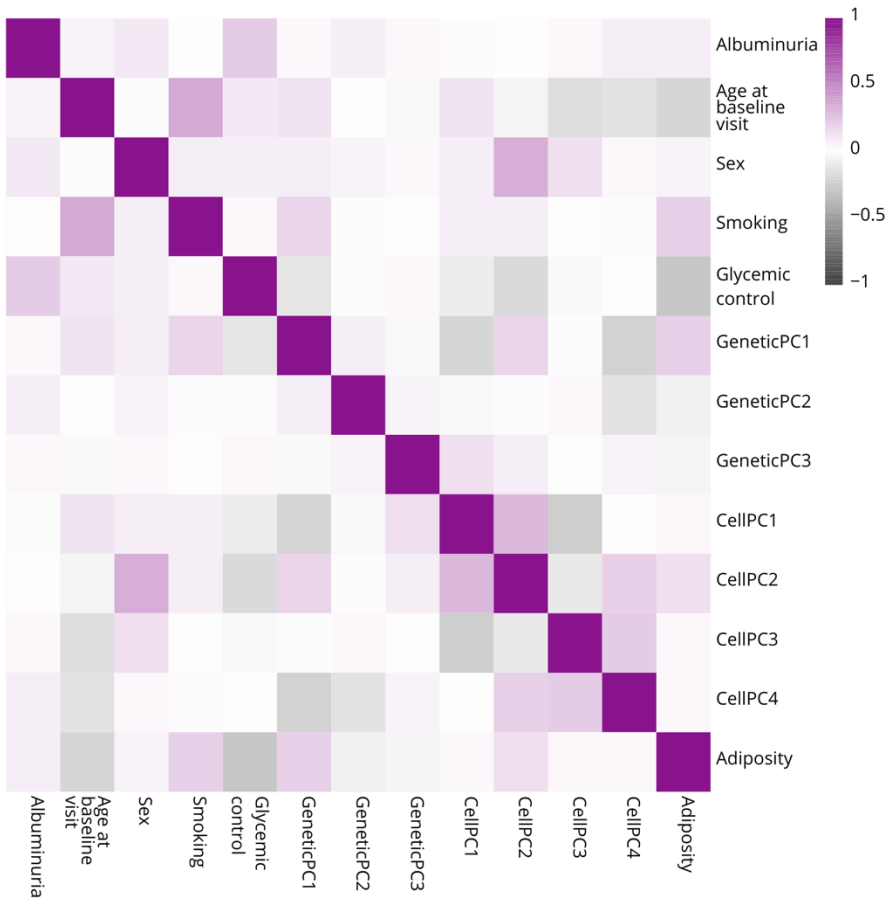


Figure 2. Slight associations identified between several covariates in the metadata.
The heatmap shows the strength and direction of pairwise correlations between covariates, with values ranging from -1 (strong negative correlation) to 1 (strong positive correlation). Continuous covariates are analyzed using Pearson's correlation coefficient, while correlations between continuous and categorical covariates are based on the square root of the R-squared value from a linear model. Associations between categorical covariates are measured using Cramer's V. Cells are colour-coded to reflect correlation strength, with darker colours indicating stronger associations. Rows and columns are ordered according to the covariates in the metadata matrix.

Table 1. Covariate Measurement and Reason for Inclusion.

Covariate	Measurement and Reason for Inclusion
Albuminuria status[180,190]	Categorical variable Yes/No status based on albumin to creatinine ratio (ACR) greater than 3.0mg/mmol on overnight urine collection or first morning sample. With confirmation of persistent albuminuria determined by an ACR of 3.0 mg/mmol on two samples taken at least one month apart within a six-month period. Included as it directly represents the outcome of interest, providing a clear measure of kidney damage and allowing for the investigation of factors associated with its development and progression.
Age at baseline[194]	Categorical variable Self-reported Included to account for known association with albuminuria, reflecting the aging process and impact on kidney function over time.
Sex[194]	Categorical variable Self-reported Included because there are hundreds of sex-associated differentially methylated CpG sites, the majority of which are hypermethylated in females. Sex will be controlled for because of its association with changes in global DNA methylation levels.
Adiposity[195,196]	Continuous variable BMI z-score Included because it is a crucial risk factor for albuminuria.
Glycemic Control[197,198]	Continuous variable Quantified using hemoglobin A1c (HbA1c) analyzed on a Roche Cobas Integra 800 CTS Included because it is a critical determinant of kidney function and has a direct impact on albuminuria status.
Smoking Status[199]	Continuous variable Self-reported based on number of cigarettes smoked per day. Included because of alterations smoking may cause on DNA methylation patterns.
Genetic Ancestry[200–202]	Categorical variable Measures ancestry informative marker SNPs

	Included Genetic PC1-3 Included to control for population structure as this marker is used to infer ancestry.
Cell-Type Proportions2025-03-20 11:26:00 AM	Categorical variable DNA methylation reference matrix Included CellPC1-4 Included to account for variability in cellular composition, which can influence DNA methylation patterns.

4.9 Question 1(a) Methods

4.9.1 Hypothesis

The planned analysis for research question 1(a) involved an EWAS. I hypothesized that the presence of albuminuria would be associated with altered DNA methylation.

4.9.2 Statistical Methods

Epigenome Wide Association Study Analysis

This research project utilized a case-control EWAS grouping unrelated participants by the phenotype of interest, albuminuria status, and compared the CpG methylation levels of those with albuminuria to the controls without albuminuria. The aim was to identify specific sites of DNA methylation associated with the presence of albuminuria. The EWAS analyzed hundreds of thousands of CpG sites using high-throughput technologies such as the Illumina Infinium Human Methylation EPIC BeadChip.

This analysis involved performing a linear regression for each of the 705,247 CpG sites using the *limma* package. Benjamini-Hochberg procedure was used to correct for multiple tests with the level of significance set at a false discovery rate (FDR) <0.05, and two effect size cut-offs: 5% and 1%.

To avoid stratification by ethnicity, a sub-analysis was conducted on participants in the iCARE cohort who self-identified as First Nation, comprising 176 of the total 213 participants.

Identification was based on a self-declaration of up to three ethnicities during the baseline iCARE visit. All participants who self-identified as First Nation or Métis were included in this subset. The same linear regression model applied to the whole cohort on 705,247 CpG sites was also used for the First Nation subset of 176 participants.

Power calculations are rarely utilized in conducting EWAS due to the vast number of tests (705,247 CpG sites) and the variability of specific parameters, such as variance and effect size, across these tests, making their estimation uncommon.

While the EWAS effectively detects differences in DNA methylation at individual CpG sites across the genome, it does not consider broader genomic patterns or regions where multiple CpG sites may collectively show significant changes. This led me to the following method.

Differentially Methylated Regions Analysis

After completing the EWAS, I identified differentially methylated regions (DMRs). DMR analysis provides insight into DNA methylation patterns across contiguous DNA regions, revealing larger epigenetic changes that may be more biologically relevant than changes at individual CpG sites alone.[204] DMR analysis improves statistical power by aggregating signals from multiple CpG sites, which is particularly important when individual sites may not reach significance due to variability. The *Enmix* package facilitated this analysis. The process examined the beta values, M values, probe annotation data, and differential DNA methylation data. The *combp()* function was utilized to identify DMRs and plot the results, mapping them to the appropriate chromosome and position ranges.

By combining both EWAS and DMR analyses, the study was able to examine both individual CpG sites associated with albuminuria and broader genomic regions where consistent

DNA methylation differences were present. This complementary approach helps provide a more comprehensive view of the epigenetic modifications associated with albuminuria.

Following the EWAS and DMR analyses, it was possible to examine further the cellular functions of specific sites exhibiting differential DNA methylation when comparing individuals with albuminuria to those without. To accomplish this, I performed a targeted literature search on the identified genes to explore their known roles in biological processes and disease pathways, focusing on evidence linking them to stress and kidney function.

Candidate Gene Analysis

Given that other studies of DNA methylation have identified associations with kidney disease, I utilized the National Genomics Data Center EWAS Atlas to identify other EWAS studies that had detected DNA methylation differences associated with kidney disease.

Due to limited results, I expanded my search to the general term ‘kidney disease’ rather than the more specific ‘albuminuria,’ for which there were no search results. The studies identified under the search term ‘kidney disease’ in the EWAS Atlas were then loaded into R and pre-processed. My analysis integrated my study data and publicly available EWAS Atlas datasets to explore CpG sites of interest that were common to both sources. These common CpG sites were identified, and the datasets were merged into a single data frame.

To visualize the data, I created Chicago plots displaying the change in DNA methylation in sites across the genome. Additionally, I generated a Manhattan plot to highlight CpG sites with significant DNA methylation changes (≥1%) between groups. Following this, I performed t-tests on each site to determine whether there were significant differences in DNA methylation between those in our study with and without albuminuria. Benjamini-Hochberg procedure was used to correct for multiple tests with the level of significance set at a FDR <0.05, and two effect

size cut-offs: 5% and 1%. Further, I created Gviz plots of each of the six regions identified in the DMR analysis to show where the CpG sites are located relative to the gene.

Figure 3 summarizes the complete workflow, from sample collection and DNA methylation analysis to the statistical methods used in the study.

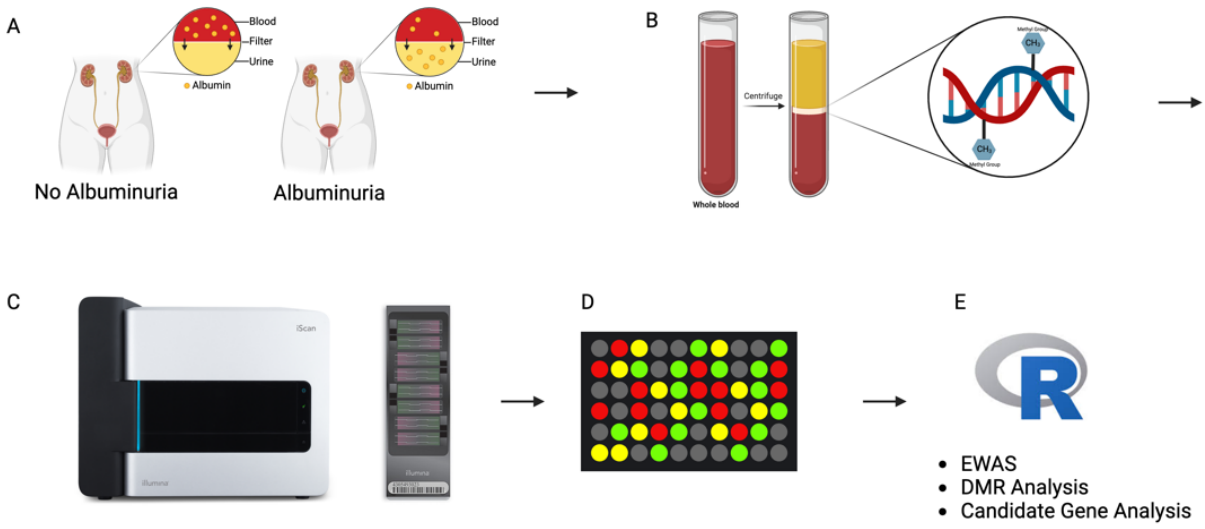


Figure 3. Overview of methods.

Overview of our methods for examining the relationship between levels of stress and kidney outcomes in youth with T2D and examining epigenetics as one potential component of the pathway by which this occurs. A comprehensive workflow was used to investigate the epigenetic modifications associated with albuminuria in youth with Type 2 Diabetes (T2D). **A.** Schematic representation of kidney function, illustrating how albumin passes through the kidney's filtration system from the blood into the urine in cases of albuminuria. **B.** Buffy coat extracted from whole blood was used for DNA methylation analysis, focusing on white blood cells to obtain DNA for epigenetic analysis. **C.** Bisulfite sequencing was performed using the Illumina Infinium Human Methylation EPIC BeadChip platform, with samples scanned on the iScan machine to quantify DNA methylation levels across the genome. **D.** DNA microarray data were analyzed to assess genome-wide DNA methylation, comparing participants with and without albuminuria. **E.** Statistical analyses, including epigenome-wide association study, differentially methylated region analysis, and candidate gene analysis, were conducted in R to identify DNA methylation changes associated with albuminuria.

4.10 Question 1(b) Methods

4.10.1 Hypothesis

Based on Question 1(b), I hypothesized that the presence of albuminuria would be associated with altered DNA methylation in areas of the genome that correspond to the stress response.

4.10.2 Methods

No formal statistical analysis was performed for research question 1(b). The original planned methodology was to conduct gene ontology, which would provide insights into the biological processes, cellular components, and molecular functions associated with differentially methylated genes. Given the small number of sites identified in the DMR analysis, I pivoted by researching the gene functions of the genes identified in my DMR analysis, focusing on potential relationships with stress pathways and other variables of interest.

4.11 Question 2 Methods

4.11.1 Hypothesis

I hypothesized that the combination of T2D and increased stress would have an additive effect on changes to DNA methylation patterns associated with the presence of albuminuria.

4.11.2 Methods

The planned analysis for research question 2 was not pursued because the conditions necessary to assess the additive effects of T2D and stress were not met within our data set.

Chapter 5: Results

5.1 Demographics

Of the 213 youth in the cohort for my thesis analysis, 68% were female, and 32% were male. On average, participants were 16 (±2.9) years old and had T2D for 3.3 (±2.7) years at entry into the study. Fifty-five participants (25.8%) had albuminuria at their baseline visit with an average ACR of 16(±23) (Figure 4). Fifty-five percent of participants (118) were stressed according to the PSS-14 questionnaire, with rates between those with and without albuminuria very similar (Figure 5). Ethnicity was self-identified by participants. Our cohort comprised 83% First Nation, 3% Métis, 7% Caucasian, and 7% from other ethnic backgrounds, reflecting the heightened risk of T2D and related complications among First Nations and Métis populations and other marginalized groups in Canada. The majority of our cohort lived in Manitoba (73%), with the remaining participants coming from Ontario, Alberta, Nova Scotia, and Saskatchewan. Participants without albuminuria had a mean BMI (z-score) of 2.4 (±0.95), while the albuminuria group had a slightly higher BMI of 2.6 (±0.97). HbA1c levels differed between those with and without albuminuria. Those without albuminuria had a mean level of 9.0 (±2.4), and the albuminuria group had a mean level of 10 (±2.6). Hypertension was more common in youth with albuminuria (86.5%) compared to those without albuminuria (64.3%). Higher rates of hypertension among those with albuminuria was consistent for daytime and nocturnal hypertension (Figure 6). While 23% of participants reported smoking regularly or occasionally, 58% of participants reported being exposed to second-hand smoke. For a complete list of demographic characteristics, see Table 2.

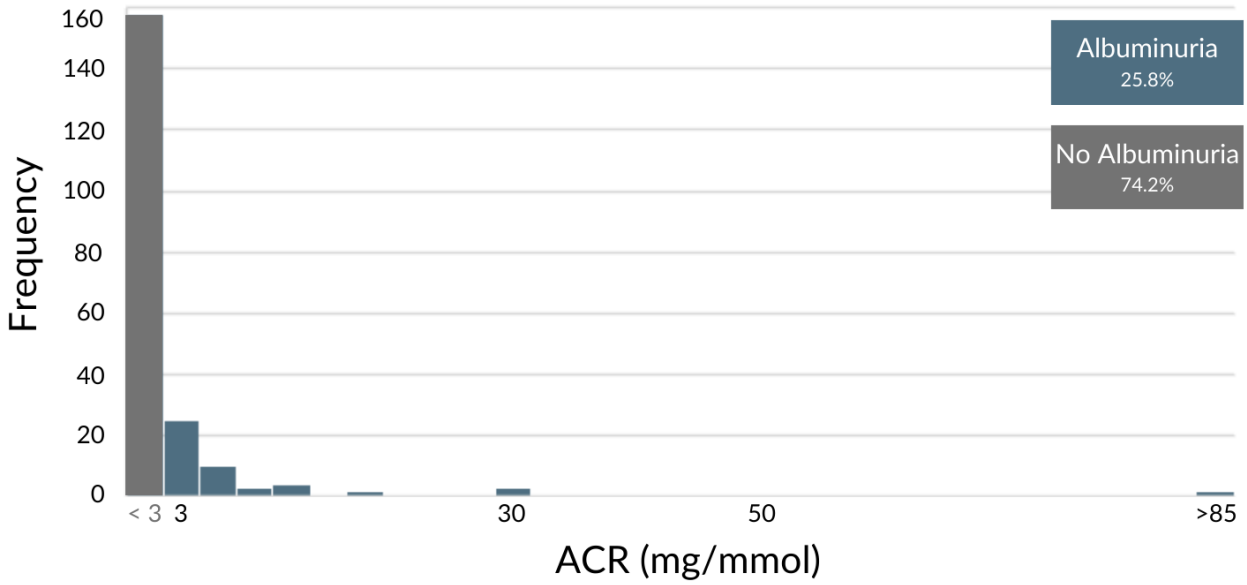


Figure 4. The majority of participants have low ACR values, with a few displaying significantly higher values.

The distribution of Albumin-to-Creatinine Ratio (ACR) values is shown for all participants. The x-axis represents the best available measure of the ACR for each participant, prioritized from an overnight, then first morning, and lastly a random urine sample. The y-axis indicates the number of participants. The right-skewed distribution highlights that most participants have low ACR values, with a few outliers having higher values. Fifty-five participants have albuminuria (ACR ≥ 3.0 mg/mmol). The mean score among participants meeting the cut-off is 15.62mg/mmol.

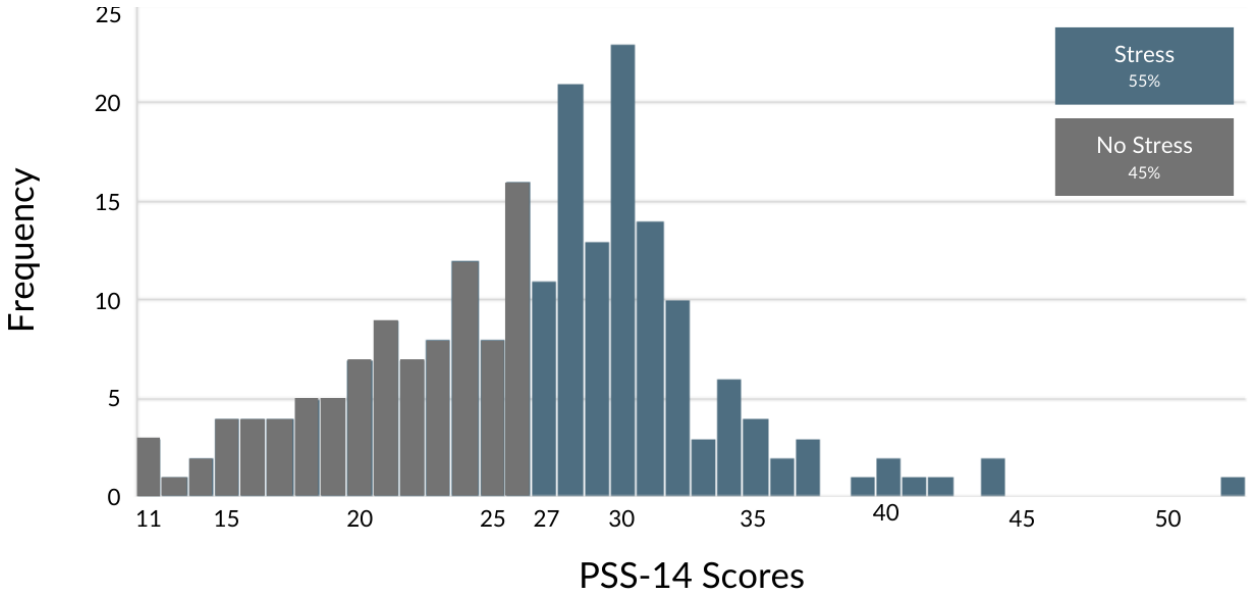


Figure 5. Most participants reporting stress have PSS-14 scores just above the threshold, with a few showing higher levels of perceived stress.

The distribution of total Perceived Stress Scale (PSS-14) scores is presented for all participants. The x-axis represents total PSS-14 scores, and the y-axis shows the number of participants in each score range. The distribution indicates that most participants who are stressed cluster just above the stress threshold (≥27), with a small number of outliers reporting significantly higher stress levels. The mean score among participants meeting the cut-off is 31.14.

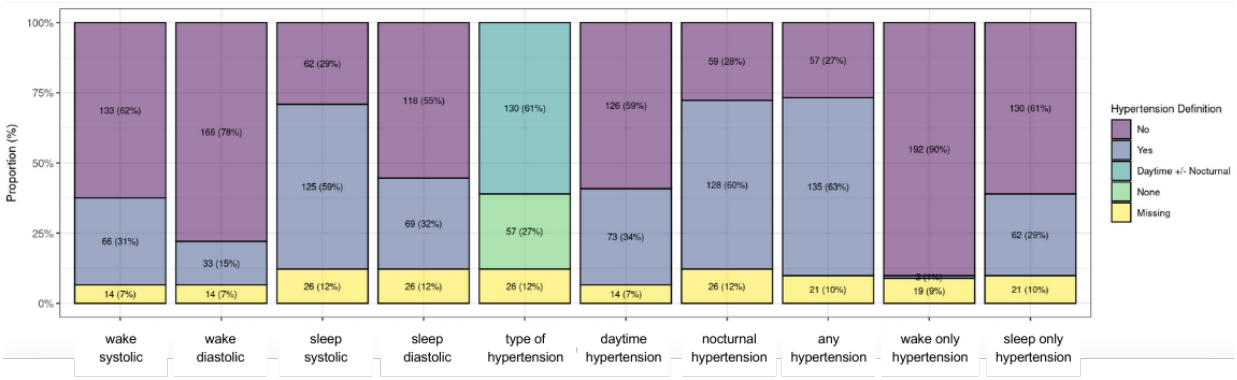


Figure 6. Most participants have daytime and/or nocturnal hypertension, with only 27% showing no hypertension.

The bar plot shows the proportion of participants classified under different hypertension definitions based on age, height, and blood pressure readings. 27% have no hypertension, and 61% have daytime +/- nocturnal hypertension.

Table 2. Characteristics of iCARE Cohort Participants.

	Total Participants (N=213)	**No Albuminuria (N=158)**	**Yes Albuminuria (N=55)**	**P value**
Sex				
Female	145 (68 %)	103 (65 %)	42 (76 %)	0.172872**
Male	68 (32 %)	55 (35 %)	13 (24 %)	
Age (years)				
Mean (SD)	16 (± 2.9)	16 (± 2.8)	16 (± 2.9)	0.3517821*
Ethnicity				
First Nation	176 (83 %)	127 (80 %)	49 (89 %)	0.6605164**
Caucasian	15 (7 %)	11 (7 %)	4 (7 %)	
Metis	6 (3 %)	6 (4 %)	0 (0 %)	
South East Asian	5 (2 %)	4 (3 %)	1 (2 %)	
African American/Black	4 (2 %)	4 (3 %)	0 (0 %)	
South Asian	4 (2 %)	3 (2 %)	1 (2 %)	
Mexican/South and Central American	2 (1 %)	2 (1 %)	0 (0 %)	
West Indian/Caribbean	1 (0 %)	1 (1 %)	0 (0 %)	
Diabetes Duration (years)				
Mean (SD)	3.3 (± 2.7)	3.2 (± 2.7)	3.4 (± 2.8)	0.587567*
Albumin-to-Creatinine Ratio (ACR ≥3.0mg/mmol)				
Mean (SD)	4.6 (± 13)	0.74 (± 0.75)	16 (± 23)	1.593811e-05*
PSS-14 Score				
Mean (SD)	27 (± 6.4)	27 (± 6.1)	27 (± 7.1)	0.6411074*
Stressed (PSS-14 ≥ 27)				
No	95 (45 %)	70 (44 %)	25 (45 %)	1.0**
Yes	118 (55 %)	88 (56 %)	30 (55 %)	
WHO BMI (Z-score)				
Mean (SD)	2.4 (± 0.95)	2.4 (± 0.95)	2.6 (± 0.97)	0.2553827*
Smoking Status				
No	164 (77 %)	122 (77 %)	42 (76 %)	1.0**
Yes	49 (23 %)	36 (23 %)	13 (24 %)	
Exposed to second hand smoke?				
No	90 (42 %)	68 (43 %)	22 (40 %)	0.8147042**
Yes	123 (58 %)	90 (57 %)	33 (60 %)	
HbA1c (%)				
Mean (SD)	9.3 (± 2.5)	9.0 (± 2.4)	10 (± 2.6)	0.002070703*
Hypertension				
Daytime +/- Nocturnal	130 (61 %)	88 (56 %)	42 (76 %)	0.007226077**
None	57 (27 %)	50 (32 %)	7 (13 %)	
Missing	26 (12.2%)	20 (12.7%)	6 (10.9%)	
Province				
AB	3 (1 %)	2 (1 %)	1 (2 %)	0.6771447**
SK	1 (0 %)	1 (1 %)	0 (0 %)	
MB	155 (73 %)	116 (73 %)	39 (71 %)	
NS	6 (3 %)	3 (2 %)	3 (5 %)	
ON	48 (23 %)	36 (23 %)	12 (22 %)	

*t-test used

**chi-square test used

5.2 Findings

5.2.1 Research Question 1a

The EWAS showed no significant DNA methylation differences at individual sites between participants with albuminuria and those without (Figure 7). DMR analysis identified six regions that are significant when correcting for multiple testing using a FDR <0.05. The number of DMRs is reduced to four when using Sidak, the more conservative method for correcting for multiple testing.[205] The six regions from the DMR analysis were located on chromosomes six (associated with *TNXB*), 11 (*TSPAN32*), 19 (*ZNF486, ZNF562*), and 20 (*ATP5E, TNFRSF6B*) (Figure 8).

In addition to the DMR analysis, a candidate gene analysis was undertaken to identify significant sites of DNA methylation differences between groups. Individual t-tests were conducted on CpG sites that were common between our study and those identified in studies listed on the EWAS atlas. The individual t-tests identified several significant CpG sites. There were 56 CpG sites found to be significant with a p-value of <0.05 and 18 CpG sites significant at the <0.01 level (Figure 9). However, none of the sites remain significant after correction for multiple testing.

5.2.2 Research Question 1b

After researching the six significant regions identified by the DMR analysis, *TNXB, TSPAN32*, *ATP5E*, and *TNFRSF6b* were found to have known or hypothesized associations with kidney complications and/or stress (further discussed in section 6.2.2).

5.2.3 Research Question 2

There are no findings to report for research question 2 as no analysis was completed, given the lack of significant findings from the EWAS.

A B

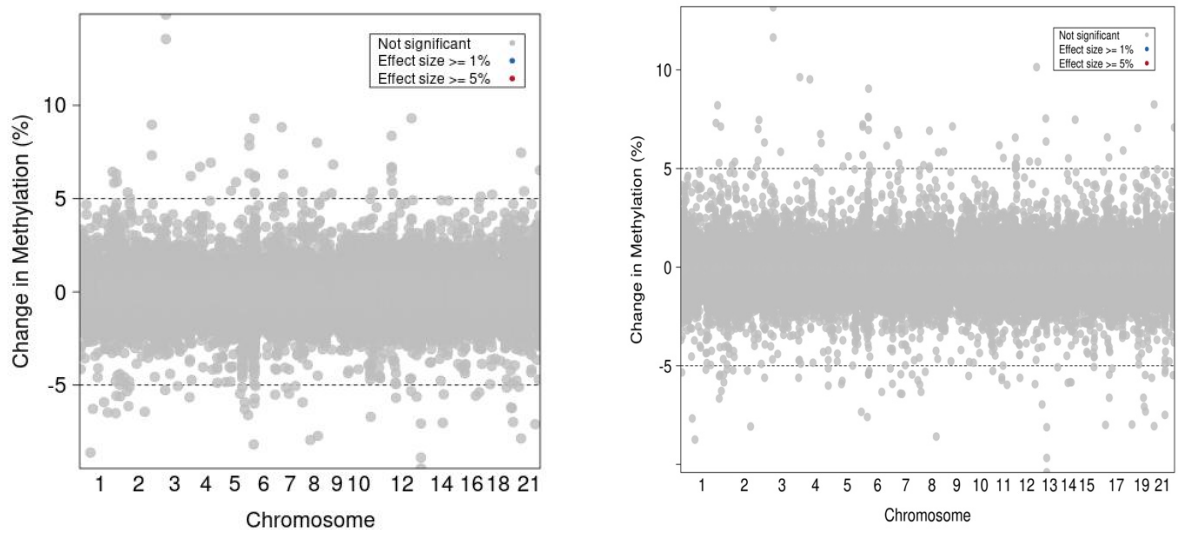


Figure 7. No differentially methylated CpG sites were detected in the cohort or the First Nations subset of the EWAS analysis.

The Chicago plot displays the change in DNA methylation (%) (y-axis) against the genomic location (x-axis) in 705,247 sites across the human genome in youth with T2D and albuminuria compared to controls without albuminuria. Each dot represents a CpG site, with non-significant sites shown in grey. Associations were tested using multiple linear regression models from *limma*, adjusting for albuminuria, age at baseline visit, sex, smoking, glycemic control, genetic ancestry, cell-type proportions, and adiposity. **A.** No differentially methylated CpG sites were identified in blood samples of the entire cohort (N=213). **B.** No differentially methylated CpG sites were identified in blood samples of the First Nations subset (N=176).

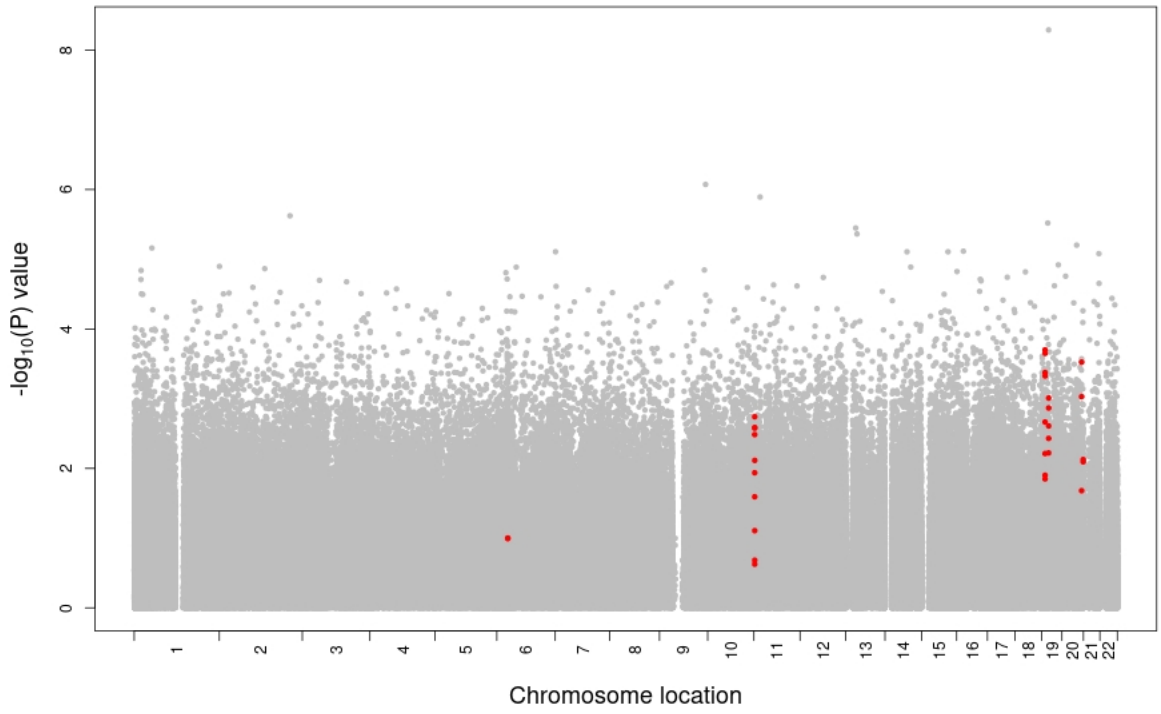
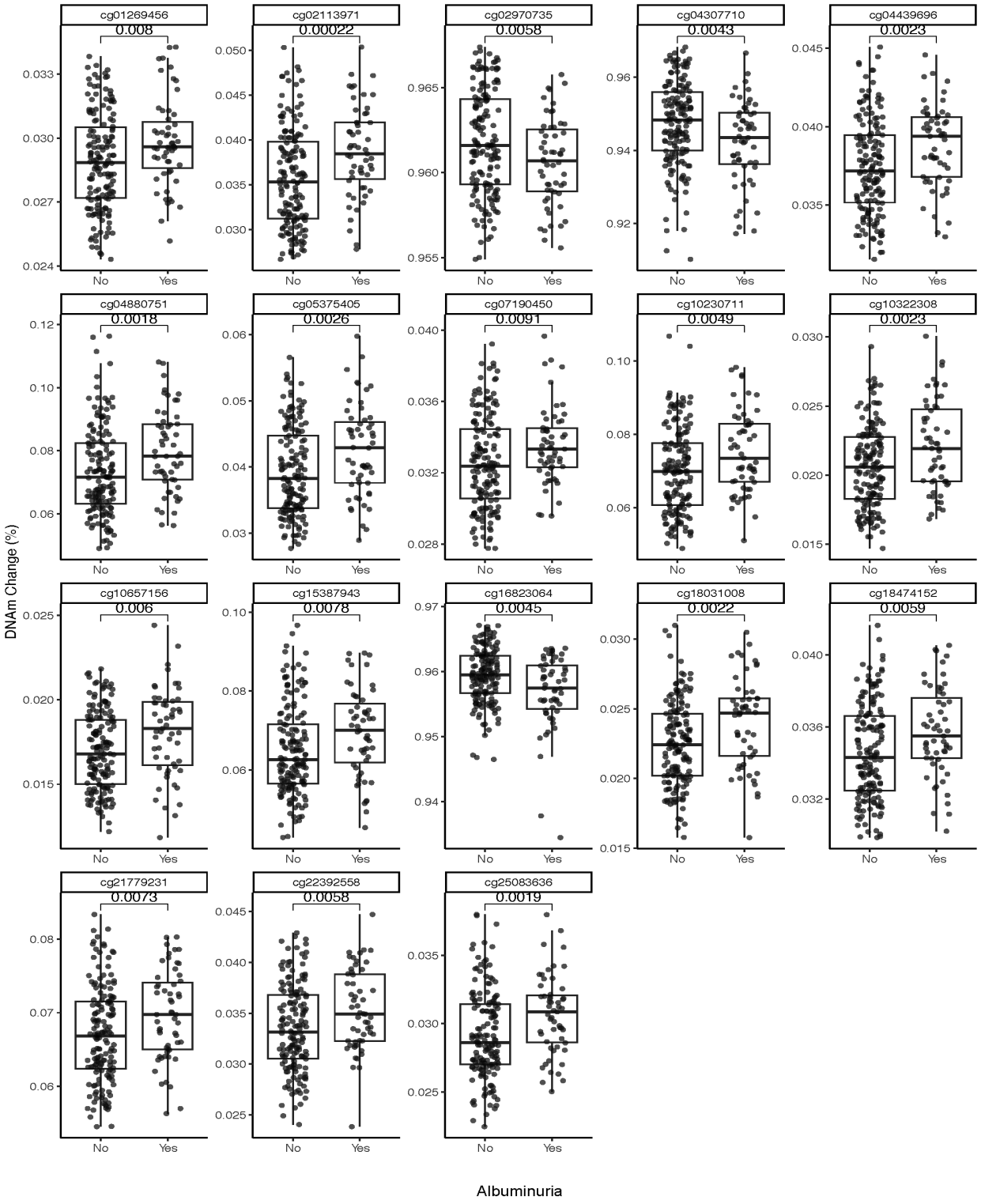


Figure 8. Six significant DMRs identified (FDR < 0.05), with four regions remaining significant under the Sidak correction.

The plot illustrates the results of the Differentially Methylated Region (DMR) analysis, with each dot representing a site tested for differential DNA methylation. The y-axis shows -log10(p) values, while the x-axis indicates genomic location. Significant DMRs are highlighted in red, while non-significant regions are shown in grey. Six DMRs were identified as significant using FDR < 0.05, located on chromosomes six, 11, 19, and 20. When applying the more conservative Sidak correction for multiple testing, four DMRs remained statistically significant.

A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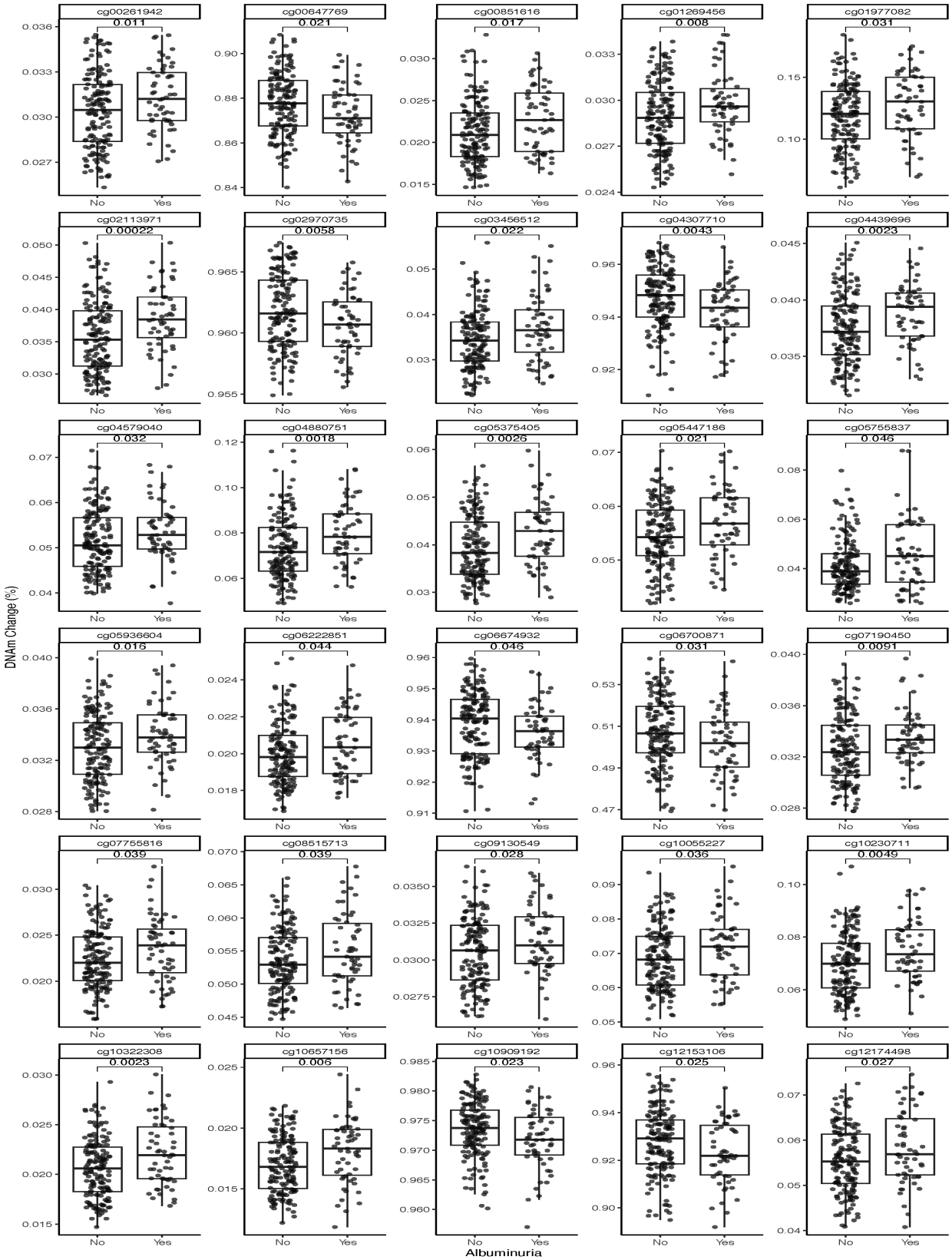
0.0019
No
Yes
DNAm Change (%)
Albuminuria

B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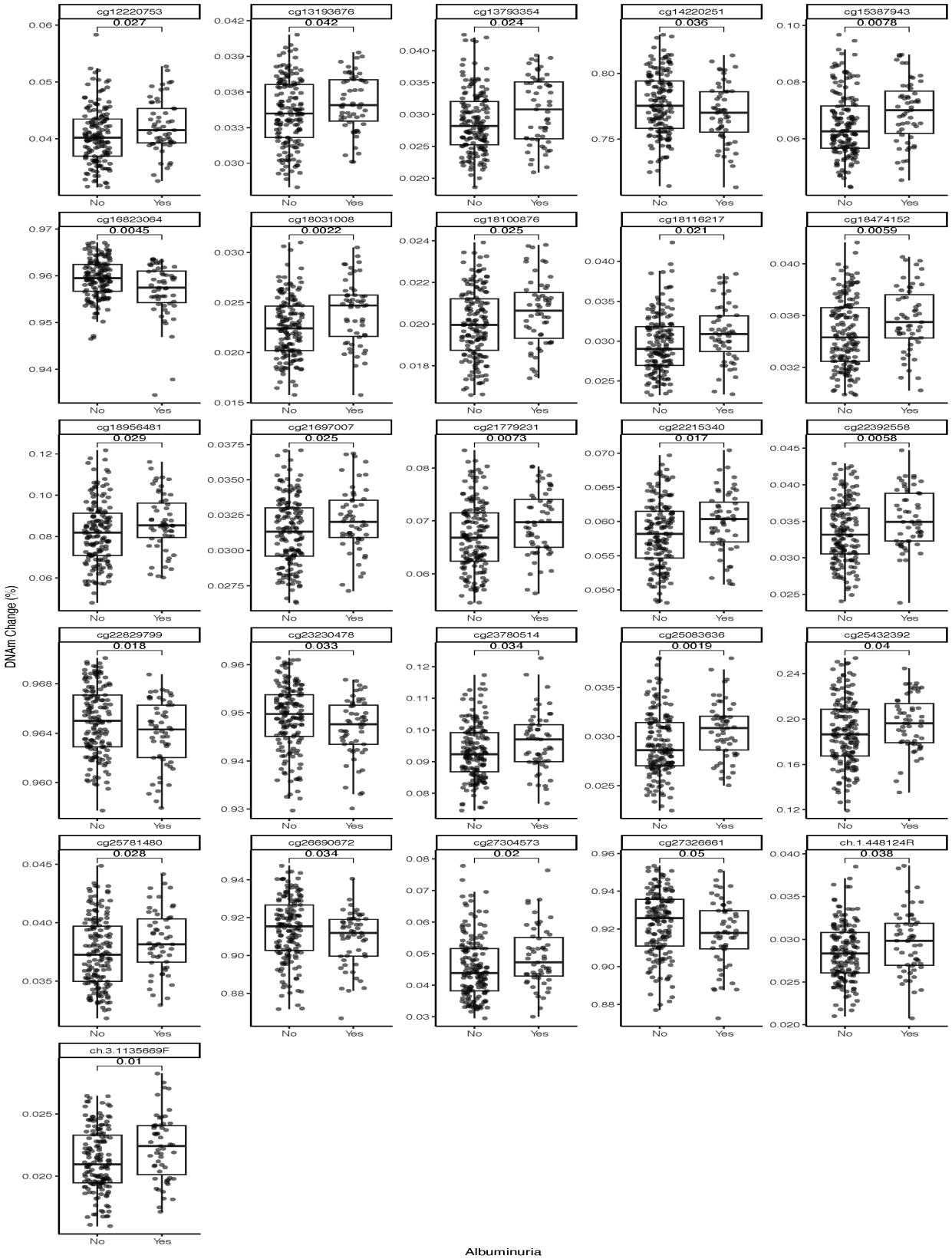
No
Yes
DNAm Change (%)
Albuminuria

B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0.02
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0.05
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0.038
ch.3.1135669F
0.01
No
Yes
DNAm Change (%)
Albuminuria

Figure 9. Significant but small differences in DNA methylation were observed at CpG sites within candidate genes associated with kidney disease in other studies.

The figure shows DNA methylation levels (%) at candidate CpG sites, comparing participants with and without albuminuria. The y-axis represents DNA methylation levels, and the x-axis indicates albuminuria status (No vs. Yes). Each dot represents an individual sample, with the median DNA methylation value for each group shown by the horizontal line within the box. Statistical comparisons were performed using two-sided t-tests. **A.** 18 CpG sites showed significant, albeit small, differences at $p < 0.01$. **B.** 56 CpG sites showed significant, albeit small, differences at $p < 0.05$.

Chapter 6: Discussion

6.1 Summary of Main Findings

Our study aimed to investigate the relationship between stress levels and kidney outcomes in youth with T2D, specifically focusing on the potential role of epigenetics in this process. High rates of T2D in Canada, particularly among Indigenous populations, have highlighted the need to explore factors contributing to disease progression, such as stress. The study aimed to understand whether DNA methylation patterns, detectable in blood and potentially influenced by stress, could be linked to kidney complications such as albuminuria. While kidney tissue would provide more direct insights into kidney-specific DNA methylation changes, using blood as a surrogate tissue is based on the general understanding that systemic processes, such as inflammation, oxidative stress, and immune response, can influence stress response and kidney disease progression.[206] These processes are relevant to stress and kidney disease progression, though studies linking these mechanisms to the genes identified in this study, particularly in blood-based DNA methylation, remain limited. Despite these limitations, blood-based DNA methylation patterns may offer a feasible and non-invasive proxy for investigating systemic processes that reflect kidney outcomes, including stress-mediated pathways relevant to disease progression.[206,207]

The data was from the iCARE cohort, the largest prospective group of youth with T2D in Canada, aimed at uncovering actionable insights that could inform future care pathways and interventions for managing kidney health in youth with T2D.

Findings from this study highlight the complexity of the relationship between stress, epigenetics, and kidney outcomes in youth with T2D. Firstly, there were no significant DNA methylation differences at individual CpG sites. Contrary to our initial hypothesis, our study did

not identify significant differences in DNA methylation patterns between youth with T2D who had albuminuria and those who did not. This suggests that epigenetic changes related to kidney function may not be pronounced at early stages of the disease, especially in younger populations. Additionally, the reliance on blood samples, as opposed to kidney tissue, may have limited the detection of relevant DNA methylation patterns.

Secondly, there may be broader epigenetic changes at regional levels. Despite the lack of site-specific findings, the study identified DNA methylation differences in broader genomic regions. Six significant DMRs were found (FDR <0.05), four of which remained significant following the more conservative Sidak correction for multiple testing.[205] These regions are linked to genes involved in cellular processes such as energy metabolism, immune regulation, and extracellular matrix maintenance (e.g., *ATP5E, TSPAN32, TNFRSF6B*). These findings hint at complex potential pathways through which stress may be associated with kidney outcomes. However, further research is needed to confirm their relevance to kidney-specific functions and disease mechanisms.

Thirdly, a candidate gene analysis provided further context for the findings but lacked statistical robustness. This analysis identified 56 CpG sites previously associated with kidney disease in other studies that reached significance at $p < 0.05$, with 18 sites reaching $p < 0.01$. However, none of these associations remained significant after correcting for multiple testing. While these findings are consistent with existing research linking DNA methylation to kidney disease, the lack of statistical robustness underscores the need for validation in larger studies with greater analytical power.

These findings collectively support the study's objective of investigating the relationship between stress and kidney outcomes in youth with T2D. The identification of DMRs and their

links to genes involved in stress and immune pathways provides preliminary evidence for potential stress-related epigenetic mechanisms underlying kidney outcomes. Importantly, this study contributes to the growing understanding of the complex and multifactorial pathways that may connect stress, epigenetic regulation, and kidney disease progression. These results highlight the value of exploring regional rather than site-specific epigenetic changes and suggest that systemic processes may play a more significant role than previously understood.

While this study used blood-based methylation patterns to explore stress-related epigenetic mechanisms, further research is needed to directly investigate epigenetic changes in kidney tissue. Such efforts would provide a more precise understanding of the biological pathways associated with kidney complications in youth with T2D. Ultimately, this study highlights the potential of epigenetic research to identify biomarkers of renal risk, which could guide the development of targeted prevention and intervention strategies in this population.

6.2 Interpretation of Findings

6.2.1 Main Finding 1

We completed an EWAS of DNA methylation patterns among youth with T2D, comparing individuals with and without albuminuria. Contrary to our initial hypothesis, we did not identify any significant individual sites in peripheral blood with >1% difference in DNA methylation between the two groups.

Several factors may influence the absence of significant CpG methylation differences. First, the younger age of our participants likely reflects an earlier stage of disease progression, where epigenetic modifications may not yet be fully evident. Studies involving adults with T2D have identified DNA methylation changes associated with diabetic kidney disease,[208] but these alterations likely emerge over time as hyperglycemia, inflammation, and other metabolic

stressors accumulate. In contrast, the youth in our cohort may not have experienced the prolonged exposure necessary to see measurable epigenetic changes. However, other completed research with the iCARE cohort has identified DNA methylation changes in youth associated with T2D.[193] Another potential is that the absence of significant individual CpG site methylation differences reflects the unique pathophysiological trajectory of DKD in youth with T2D. Unlike adults, youth demonstrate a faster progression from microalbuminuria to ESKD, with earlier glomerular hyperfiltration, podocyte injury, and severe vascular changes.

My analysis focused on DNA methylation observed in blood, which may not accurately represent epigenetic changes in the kidneys. The tissue-specific nature of DNA methylation means that key epigenetic changes in kidney cells may not be detectable in blood cells, potentially weakening any association between DNA methylation patterns and albuminuria in this study.[209]

Previous research on DNA methylation and kidney disease has primarily focused on adult populations. The lack of findings in our study may suggest that epigenetic modifications are more likely to emerge after the onset of significant kidney damage or that in youth with T2D who are part of the iCARE cohort, albuminuria is not predominantly associated with epigenetic mechanisms. The absence of significant DNA methylation changes suggests that other biological processes, potentially transcriptional regulation or metabolic dysregulation, may play a more central role in the development of albuminuria among youth with T2D.[210,211] This underscores the complexity of albuminuria and the need to consider non-epigenetic pathways in future research.

Although we did not identify statistically significant differences at individual CpG sites, this does not disqualify the possibility of broader epigenetic alterations associated with

albuminuria. DNA methylation often functions in a coordinated manner across multiple nearby sites, where changes within a region, rather than at single CpG sites, may play a more critical biological role.[212,213] For this reason, we pivoted to a different analytical approach by conducting a DMR analysis. DMRs can provide insights into more extensive, functionally relevant epigenetic modifications that may be missed when only examining individual sites.

6.2.2 Main Finding 2

We used a spatially adjusted p-value <0.05 and two probes as the minimum number thereof in our DMR analysis. These were chosen because spatially adjusted p-values account for the correlation between nearby CpG sites, recognizing that DNA methylation changes often occur in clusters rather than in isolation. By aggregating significant CpG sites into regions and adjusting for spatial correlation, this approach reduces false positives, reduces the number of tests, and retains a conventional threshold ($p < 0.05$). Using a minimum of two probes, the DMR analysis identifies regions, reflecting coordinated DNA methylation changes across multiple CpG sites rather than noise at a single site. Without this requirement, the analysis would essentially be the same as the site-level EWAS performed earlier. This combination of statistical rigour and biological plausibility enhances the reliability of the results. Using the above-noted p-value and a minimum of two probes, we identified six significant DMRs when correcting for multiple testing using a FDR <0.05. The number of DMRs is reduced to four when using the more conservative Sidak method.[205]

The findings presented in this section are based on DNA methylation patterns observed in blood samples, as this was the only sample type analyzed in our study. While these patterns may provide insights into systemic processes, it remains speculative to extend these findings to other tissues, such as the kidneys, without further validation. Any suggested connections to kidney-

specific processes should be interpreted with caution and considered hypotheses requiring additional research.

The six regions from the DMR analysis were located on chromosomes six (associated with *TNXB*), 11 (*TSPAN32*), 19 (*ZNF486*, *ZNF562*), and 20 (*ATP5E*, *TNFRSF6B*) (Figure 8, Figure 10). *TNXB* and *TNFSF6B* were the two genes that did not reach significance when correcting for multiple testing using the Sidak method (Table 3). These regional patterns of DNA methylation may still be associated with albuminuria in youth with T2D, even when individual CpG site differences are not detected. These findings reinforce the importance of considering site-specific and regional DNA methylation changes when studying complex diseases such as albuminuria.

TNXB is a gene located on chromosome six. *TNXB* encodes the protein tenascin-X, a member of the tenascin family of extracellular matrix glycoproteins.[214] The differentially methylated CpG sites are situated in the gene body within a CpG island, often associated with active transcription, but can serve other regulatory roles depending on the context. These intragenic CpG islands may also influence transcription by regulating alternative promoters or affecting transcription elongation, highlighting their potential to affect *TNXB*'s expression in response to environmental and physiological cues.[215,216]

The active transcription of *TNXB* is essential for maintaining the structural integrity of connective tissues. Tenascin-X plays a critical role in the structural integrity and elasticity of connective tissues by interacting with other extracellular matrix components, such as collagen and fibronectin.[217,218] This gene is also involved in regulating cell adhesion, migration, and tissue repair, making it essential for maintaining the normal function of various organs.[217,218] Its role in tissue repair processes suggests that *TNXB* may be particularly important under conditions of

chronic inflammation or injury, such as those driven by prolonged metabolic stress in T2D or DKD.

Stress, which is a known risk factor in T2D progression, can influence extracellular matrix dynamics, which could include *TNXB* activity.[219–221] Chronic stress, characterized by prolonged elevations in cortisol, has been shown to alter the extracellular matrix by modulating the expression and function of key extracellular matrix proteins, which could implicate *TNXB* in stress-related tissue damage and kidney complications.[222] This aligns with the broader understanding of stress-induced disruptions in tissue homeostasis and may provide a potential mechanism linking *TNXB* DNA methylation changes to DKD pathology.

While *TNXB*'s role in maintaining the extracellular matrix is particularly important in organs such as the kidneys, the direct connection between *TNXB* DNA methylation changes in blood and kidney-specific processes remains hypothetical. The kidney filtration system relies on a stable extracellular matrix to support the glomeruli and other structures involved in filtration.[223] Disruption in *TNXB* expression can lead to alterations in the extracellular matrix,[224] potentially contributing to conditions like DKD, where the balance between tissue repair and fibrosis is disturbed. Wunnenburger and colleagues examined SNPs associated with CKD using data from over 5000 CKD patients.[225] They discovered that a risk variant at the *TNXB* gene was associated with a higher risk for CKD among individuals with T1D.[225] Sapienza and colleagues identified *TNXB* as one of 187 candidate genes where two or more CpG sites were differentially methylated when comparing patients with end-stage renal disease who were being treated with hemodialysis and diabetes patients without nephropathy.[158] Another association between *TNXB* and kidney function was found in a cohort of patients with impaired kidney function, where a mutation to the *TNXB* gene was significantly associated with an increased risk of progression to

ESKD.[226] These findings highlight the role of *TNXB* mutations in the early onset and progression of kidney complications.[226]

TSPAN32, located on chromosome 11, belongs to the tetraspanin family, a group of proteins characterized by four transmembrane domains.[227] Tetraspanins are involved in organizing membrane microdomains, which influence various cellular processes, including cell adhesion, migration, and signal transduction.[228] The CpG sites are located in the TSS1500 region, further upstream of the transcription start site, and are in the Open Sea region far from CpG islands, which may have a more subtle effect on gene regulation.[229] However, the observed increase in DNA methylation among youth with albuminuria in this area may still influence long-range regulatory mechanisms, potentially affecting *TSPAN32* expression and possibly impacting its role in immune cell regulation.[230]

Given its role in fundamental cellular processes, alterations in *TSPAN32* expression or function could affect various physiological systems, including those relevant to kidney function and T2D. However, this extension is speculative, and the role of *TSPAN32* in kidney function is not well-established. The kidney is highly dependent on well-coordinated cellular processes for its proper function, which can be influenced by tetraspanins such as *TSPAN32*.[230] Discussion of the involvement of *TSPAN32* in kidney-related processes, such as maintaining the integrity of the glomerular filtration barrier, which relies on the proper functioning of various cellular structures and signalling pathways to maintain kidney filtration and prevent albuminuria, is purely speculative. Similarly, while *TPSAN32*'s role in immune cell regulation could suggest a potential influence on inflammatory responses in the kidney, there is no direct evidence linking this gene to kidney disease, and such connections cannot be inferred from this study's findings.

Stress is a known factor in the progression of many diseases, including T2D and its associated complications.[220] *TSPAN32* might be implicated in the body's response to stress through its role in immune regulation and apoptosis, a process of programmed cell death that helps eliminate damaged or unnecessary cells.[230] Stress-induced changes in immune function can exacerbate inflammation and immune responses,[231] potentially leading to tissue damage in various organs. Moreover, stress is associated with epigenetic modifications, such as DNA methylation, which could alter the expression of genes like *TSPAN32*.[136–138] If stress plays a role in *TSPAN32* expression, this could potentially impact immune regulation and apoptosis pathways, further contributing to kidney damage in individuals with T2D.

In the context of T2D, the immune system plays a significant role in the development of insulin resistance and the chronic inflammation of the disease.[232] *TSPAN32*, through its role in immune cell regulation, could be involved in these processes. Dysregulation of *TSPAN32* might exacerbate inflammatory responses, contributing to the metabolic disturbances seen in T2D. However, since our study's data is limited to blood-based DNA methylation patterns, any potential effects of *TSPAN32* on kidney-related complications, such as albuminuria, should be considered speculative and require further investigation in other tissues to confirm.

ZNF486 and ***ZNF562*** are members of the zinc finger protein (ZNF) family, characterized by the presence of zinc finger domains and believed to enable binding to DNA, RNA, or proteins.[233] Both *ZNF486* and *ZNF562* are located on chromosome 19. *ZNF486* is situated in the Open Sea region, far from CpG islands, where higher DNA methylation is less likely to directly affect transcription but may influence regulatory elements.[229] *ZNF562*, located in both the CpG island and south shore regions, shows higher DNA methylation in regions near the transcription start site, which are critical for regulating transcription.[107,110] Shores refer to

regions of DNA that are 0–2 kb away from CpG islands and are known to play a role in gene regulation by being sensitive to changes in DNA methylation.[234] Increased DNA methylation in the CpG island may silence *ZNF562*, while DNA methylation in the South Shore could play a more subtle regulatory role.[110,235] These proteins are primarily involved in transcriptional regulation, influencing gene expression by binding to specific DNA sequences.[233]

While the precise functions of *ZNF486* and *ZNF562* remain largely unexplored, they are presumed to play roles in gene expression regulation and may participate in various cellular processes such as development, differentiation, and response to external stimuli.[233] Recent research has implicated *ZNF562* in diabetic foot ulcers, suggesting that *ZNF562*, along with other genes, may play a role in tissue repair.[236] Further research is needed to explain their exact roles and to determine whether they could serve as potential targets for therapeutic intervention for individuals with T2D-related kidney complications or other related conditions.

ATP5E is a gene located in a CpG Island of chromosome 20 that encodes the epsilon subunit of adenosine triphosphate (ATP) synthase, a crucial enzyme involved in the synthesis of ATP during oxidative phosphorylation in mitochondria.[237] ATP is the primary energy source for cells, fueling numerous physiological processes, including those essential for maintaining cellular homeostasis.[237] The differentially methylated CpG sites for *ATP5E* are located in the TSS200 region, indicating they are close to the transcription start site. The observed increase in DNA methylation in this area could inhibit transcription factor binding, thereby reducing *ATP5E* expression and affecting ATP production in blood cells.

The kidneys are highly metabolic organs that rely on a substantial amount of ATP to perform functions such as filtration, reabsorption, and secretion.[238] Dysregulation in *ATP5E* synthesis could, hypothetically, have broad implications, particularly for energy-intensive organs

such as the kidneys. Although mitochondrial dysfunction has been associated with kidney disease in existing literature,[239,240] our study does not provide direct evidence of such an association in kidney tissue.

Notably, a proteomic study displayed significant changes in mitochondrial proteins, including *ATP5E*, in autoimmune-related kidney diseases.[241] These proteins, which are essential in oxidative phosphorylation pathways, were altered, particularly in patients with IgA nephropathy and lupus nephritis.[241] This highlights a potential link between mitochondrial dysfunction and kidney pathology, suggesting that *ATP5E*-related mitochondrial proteins may play a role in maintaining kidney tissue health.

While our study examined stress cross-sectionally, chronic stress has been shown to induce mitochondrial dysfunction, affecting ATP synthesis.[242] The *ATP5E* gene, a critical component of the ATP synthase complex, could be influenced by stress through epigenetic modifications, affecting mitochondrial function. If such epigenetic changes were to occur in other tissues, like the kidneys, they could exacerbate metabolic challenges in individuals with T2D.

In the context of T2D, energy metabolism is often disrupted due to insulin resistance and hyperglycemia.[243] The *ATP5E* gene could play a role in this metabolic imbalance. Reduced efficiency in ATP production might worsen insulin resistance as cells struggle to maintain energy homeostasis, further complicating the management of blood glucose levels.[244] However, since this study's data are limited to blood samples, any extrapolation to kidney function or glucose metabolism in other tissues remains speculative. Further research is required to determine whether *ATP5E* DNA methylation in other organs, particularly the kidneys, plays a role in these processes.

TNFRSF6B is located on chromosome 20. It encodes a protein known as Decoy Receptor 3 (DcR3), which belongs to the tumour necrosis factor receptor superfamily.[245,246] The gene shows higher DNA methylation across multiple regions, including the TSS200 region and is in the North Shore, which may affect gene expression by influencing nearby CpG islands.[107,235] DcR3 functions by binding to certain ligands and preventing them from interacting with their respective receptors.[247] This action effectively inhibits apoptosis and modulates immune responses, making *TNFRSF6B* an important player in immune regulation and cell survival.[247,248]

Recent studies have identified *TNFRSF6B* in kidney tissue as a significant biomarker for predicting the progression of CKD.[248,249] In predictive models that include traditional risk factors such as proteinuria and eGFR, the addition of *TNFRSF6B* improved accuracy.[249] *TNFRSF6B* expression has been linked to renal fibrosis, suggesting a role in CKD progression.[249] These findings highlight the importance of *TNFRSF6B* not only in immune modulation but also in kidney health, although further research is needed to establish its expression in kidney tissues of CKD patients.

The specific role of *TNFRSF6B* in kidney health remains speculative. However, one potential hypothesis is that DNA methylation changes in *TNFRSF6B* observed in the blood may reflect systemic inflammatory processes common to both blood and kidney tissues. Inflammation is a key driver of CKD progression, and changes to DNA methylation of *TNFRSF6B* in blood could indicate dysregulated immune signalling, paralleling, or even influencing inflammatory pathways in the kidney. This potential systemic link between blood DNA methylation patterns and kidney health could provide indirect insights into CKD progression, even without direct kidney tissue analysis.

Although *TNFRSF6B* may influence the inflammatory processes that contribute to the progression of DKD, this hypothesis requires further investigation in kidney tissue.[248] While DcR3 can reduce apoptosis in renal cells, potentially protecting against kidney damage, any implications for kidney protection or damage are speculative.[248]

It has been shown that *TNFRSF6B* plays a role in immune modulation, potentially influencing the progression of kidney diseases through its effects on immune responses.[248] Studies in both human and animal models suggest that *TNFRSF6B* may be involved in regulating immune pathways that affect kidney disease outcomes.[248]

Stress can dysregulate immune function and apoptosis,[231] processes in which *TNFRSF6B* is involved. Stress exacerbates inflammatory responses, and *TNFRSF6B* might modulate these effects by influencing the balance between pro- and anti-apoptotic signals. Epigenetic modifications associated with stress, such as changes in DNA methylation patterns,[136–138] could alter the expression of *TNFRSF6B*, thereby impacting its role in immune regulation and apoptosis,[246,248] but the specific impact of stress-induced changes in *TNFRSF6B* on kidney health cannot be concluded from our study. These alterations could have implications for immune regulation and apoptosis in various tissues, including the kidneys, but further research is needed to explore these potential connections in different organs.

Chronic inflammation and immune dysregulation are key factors contributing to disease progression and complications in individuals with T2D.[250] *TNFRSF6B*, by modulating the immune response and apoptosis, may play a role in these processes. Altered *TNFRSF6B* expression could impact immune dysregulation or kidney-related complications in T2D. Reduced apoptosis of immune cells could exacerbate chronic inflammation observed in individuals with T2D and remains a potential area of future research. *TNFRSF6B* represents a

gene of interest for understanding broader immune responses and, potentially, therapeutic interventions aimed at immune regulation.

Identifying these six significant DMRs (FDR <0.05), four of which are significant using the Sidak method to correct for multiple testing, offers a compelling starting point for understanding the potential epigenetic mechanisms that may link stress with albuminuria among youth with T2D. Notably, these six genes are involved in various cellular functions, from energy metabolism and immune regulation to extracellular matrix maintenance and gene expression. These genes provide a possible link between stress and cellular function relevant to kidney health, but it is crucial to acknowledge that our study's findings are based on blood DNA methylation data, and further work is required to determine their relevance to kidney-specific processes.

The DMRs identified in this study, particularly those linked to *TNFRSF6B* and *ATP5E*, align with pathways central to DKD pathophysiology. *TNFRSF6B* encodes a decoy receptor involved in immune regulation and apoptosis, processes known to drive inflammation and glomerulosclerosis in DKD. *ATP5E*, a subunit of ATP synthase, plays a role in mitochondrial energy production. Mitochondrial function and energy metabolism are known to be disrupted in hyperglycemia-induced oxidative stress.[251] The association of these regions with kidney complications highlights the potential for stress to exacerbate these molecular pathways through epigenetic modifications, contributing to early kidney damage. This aligns with the known rapid disease trajectory in youth with T2D and underscores the need to explore further stress-related epigenetic changes in influencing renal hemodynamics and inflammation in this population.

The key takeaway from this finding is that the relationship between stress and albuminuria is complex and multifactorial. The identified DMRs were located in genes

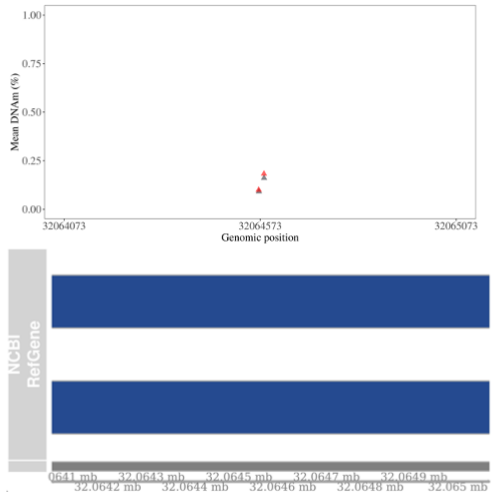
previously implicated in pathways relevant to stress, inflammation, and kidney health. These findings suggest that epigenetic modifications may play a meaningful role in the observed associations. However, while the statistical significance strengthens the robustness of these results, it is crucial to recognize the potential for subtle, systemic patterns rather than direct, isolated effects of stress on epigenetic changes. This complexity may reflect indirect pathways through which stress influences albuminuria, such as systemic inflammation, oxidative stress, or HPA-axis dysregulation.

These findings highlight the need for further research to better understand the interplay between epigenetics and other biological mechanisms. Additionally, these connections may vary between individuals due to external factors, such as genetic background, environmental exposures, and disease duration.

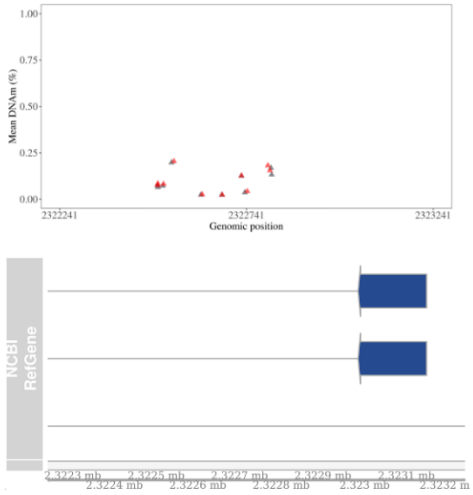
Table 3. Six Differentially Methylated Regions (DMRs) associated with albuminuria in youth-onset type 2 diabetes participants in peripheral blood (n=213).

DMR	chr	start	end	p	fdr	sidak	# of probes	probes
1	19	9785511	9786078	5.72E-12	3.43E-11	7.11E-09	8	cg08464036; cg10755961; cg14073063; cg17704570; cg20154743; cg21610904; cg22391421; cg24874111
2	19	20278013	20278145	2.55E-08	7.64E-08	0.00013607	5	cg02427230; cg03570035; cg07227744; cg15748470; cg24749688
3	11	2322500	2322809	3.59E-07	7.18E-07	0.000819343	10	cg00041575; cg00502099; cg02537342; cg05509777; cg10782575; cg13592872; cg15924868; cg19766471; cg21027517; cg22210337
4	20	57607569	57607693	2.32E-06	3.48E-06	0.013114334	3	cg01503881; cg11595749; cg11919138
5	20	62327968	62328015	0.000506817	0.00060818	0.999502964	2	cg14697761; cg23773946
6	6	32064573	32064579	0.041392124	0.041392124	1	2	cg13400512; cg15196197

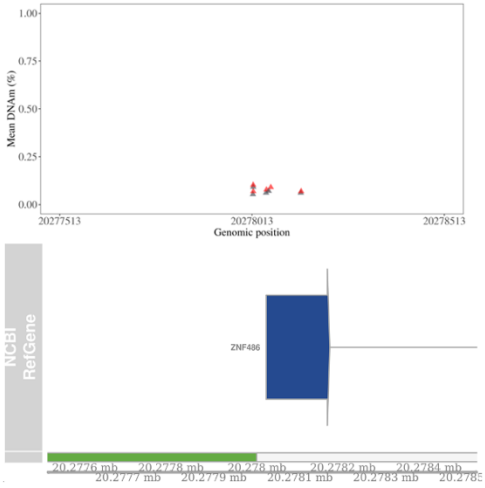
A. TNXB



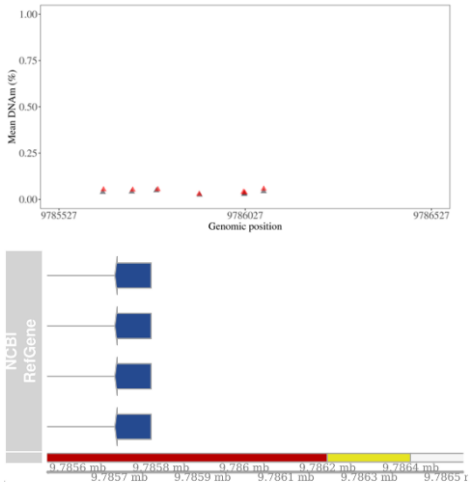
B. TSPAN32*



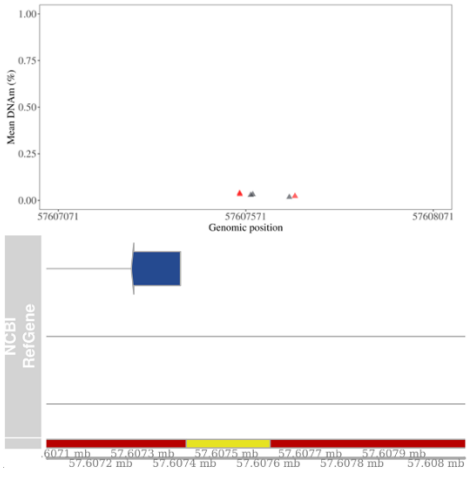
C. ZNF486*



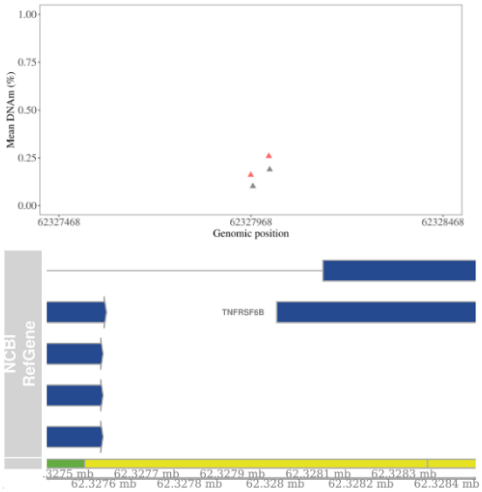
D. ZNF562*



E. ATP5E*



F. TNFRSF6B



Significant using Sidak

Figure 10. The DMR analysis identified six significant DMRs (FDR <0.05) in participants with and without albuminuria, four of which remained significant when using the more conservative Sidak method for multiple testing correction.

Each region has distinct DNA methylation patterns, gene structures, and chromatin state annotations. Red triangles represent DNA methylation levels in participants with albuminuria, and grey triangles represent those without albuminuria. The x-axis indicates genomic coordinates, and the y-axis shows DNA methylation levels as percentages. NCBI RefGene annotations detail the structure of relevant genes, including exons, which are coding regions (blue boxes), and introns, which are non-coding regions (connecting lines). Multiple isoforms of the gene are shown, reflecting alternative splicing events. The coloured bar below the NCBI RefGene represents ChromHMM annotations, which classify functional regions, such as promoters, enhancers, and transcriptional states, using a defined colour scheme. The colours observed in this figure and the associated meaning are red representing an active promotor region, yellow representing a weak/poised enhancer region, green representing transcriptional transition/elongation, and light grey representing repetitive/copy number variation.

6.2.3 Main Finding 3

After conducting an EWAS that yielded no significant findings, we turned to a candidate gene approach. This decision was motivated by recognizing that individual CpG sites linked to kidney function or disease may have been previously identified in other studies. Still, these findings might not have shown in the broader EWAS due to differences in study design, sample size, or population characteristics. The EWAS Atlas provides a comprehensive database of epigenome-wide studies, serving as a valuable resource for comparing findings across different populations. Given that many studies in the Atlas have focused on adult populations or those

with more advanced kidney disease, we hypothesized that some of the DNA methylation sites associated with kidney function in other studies might still be relevant in our cohort, even if they were not detected in the initial EWAS.

The EWAS Atlas was used to identify CpG sites previously associated with kidney function or disease in other populations. This repository aggregates findings from numerous studies and allows researchers to cross-check the significance of specific DNA methylation sites across different cohorts. We specifically targeted CpG sites that had shown associations with kidney disease in past research, as these traits are directly relevant to our study on youth with T2D. The candidate gene approach involved conducting t-tests to compare DNA methylation levels at these pre-identified CpG sites between individuals with and without albuminuria in our cohort.

From the candidate gene analysis, we found 56 significant sites at a p-value of <0.05 and 18 sites at <0.01. However, it is important to note that after correcting for multiple testing, none of the identified sites remained statistically significant, highlighting the increased likelihood of false positives in the initial analysis. As a result, the apparent significance of these sites may not be reliable, particularly in the context of an epigenome-wide study where thousands of comparisons are made simultaneously. Despite this limitation, the fact that these sites had been identified in prior research adds weight to the possibility that they may still be associated with albuminuria in youth with T2D, even if our sample lacked the power to detect them after correction for multiple testing. It is also possible that DNA methylation changes relevant to albuminuria are more pronounced in kidney tissue than in blood, which could explain the lack of significant genome-wide findings in our analysis.

While the candidate gene analysis revealed significant sites common between our study and those in the EWAS Atlas, the differences in findings between our study and those of others likely reflect several factors, including the age of our participants and the stage of kidney disease. In adult populations who have had a longer duration of T2D and more advanced kidney outcomes, epigenetic changes may be more pronounced, and the effect sizes larger, leading to stronger associations. In contrast, our cohort of youth with T2D may be at an earlier stage in disease progression, where epigenetic changes among those with albuminuria show less pronounced deviations in DNA methylation than those without albuminuria. While our study lacked the statistical power to detect significant genome-wide changes, the possibility that subtle, context-dependent effects contribute to these differences should not be discounted and warrant further exploration. The lack of significant findings after correcting for multiple testing further implies that characteristics of our cohort may differ from those of the populations included in the EWAS Atlas studies we compared to, potentially due to differences in age, disease progression, demographic factors, or a fundamental difference in the factors related to kidney disease development in our population.

Despite these challenges, the overlap between our findings and those from other studies suggests that the identified DNA methylation sites warrant further investigation. Future studies with larger sample sizes and greater statistical power may help determine whether these sites are genuinely associated with albuminuria in youth with T2D. Furthermore, longitudinal studies could help clarify whether these epigenetic changes emerge as disease progression accelerates over time**.**

Our findings both align with and diverge from the existing literature. Identifying significant CpG sites consistent with the EWAS Atlas supports prior research linking these sites

to kidney function in adults.[252,253] However, the lack of statistical significance after correcting for multiple testing highlights differences from other studies. This discrepancy may be due to the age and stage of disease progression in our cohort, where epigenetic changes are likely more subtle than in adults with advanced kidney disease.[254] Additionally, other studies on kidney disease have shown that DNA methylation differences are often more pronounced in the later stages of the disease.[255] Our findings suggest that while DNA methylation differences may exist, their detection in youth may require larger samples or more sensitive statistical approaches.

The key takeaway from this finding is that while the candidate gene analysis initially identified several CpG sites previously associated with kidney disease, none remained statistically significant after correction for multiple testing, which weakens the strength of these findings. Despite this, the overlap between our initial results and prior research suggests that these sites could still be biologically relevant. Future studies with larger sample sizes and more stringent statistical approaches are needed to confirm these associations.

6.3 Study Framework

This study aimed to explore the complex relationship between stress and kidney function through potential epigenetic mechanisms in youth with T2D. Given the high rates of youth onset T2D in Manitoba, particularly among First Nations and Métis populations, understanding these associations is crucial for improving early identification, prevention, and interventions to improve care and outcomes for affected youth. Our framework seeks to provide a comprehensive understanding of whether DNA methylation patterns differ between youth with and without albuminuria and whether these differences correspond to areas related to stress response. Epigenetics offered a lens through which we could examine how psychological factors, such as stress, may be associated with biological complications such as early kidney disease through

changes in gene expression without altering the DNA sequence itself.[16] In youth with T2D, higher stress levels are common and are thought to exacerbate the disease's progression and complications.[2]

My research integrates multiple dimensions, the biological mechanisms of epigenetics, the environmental influence of stress, and the clinical outcomes in albuminuria and T2D to form a holistic understanding of disease progression. The findings aim to bridge the gap between environmental exposures and inherited biological susceptibilities, offering insights that could lead to targeted interventions for preventing and managing T2D-related complications in youth.

The study framework emphasizes the multifaceted relationship between albuminuria, stress, and epigenetic changes (Figure 11). By focusing on albuminuria as a key outcome, this study aims to highlight the broader implications of high rates of albuminuria and stress among youth with T2D by assessing whether DNA methylation is the mechanism by which this occurred. This integrated approach advances our understanding of the pathophysiological processes involved and paves the way for developing holistic intervention strategies to address both environmental and genetic factors in managing T2D in youth. Such strategies could include targeted efforts to reduce stress and improve overall health through lifestyle interventions, which may, in turn, positively influence the biological mechanisms contributing to disease progression, ultimately improving health outcomes for affected populations.

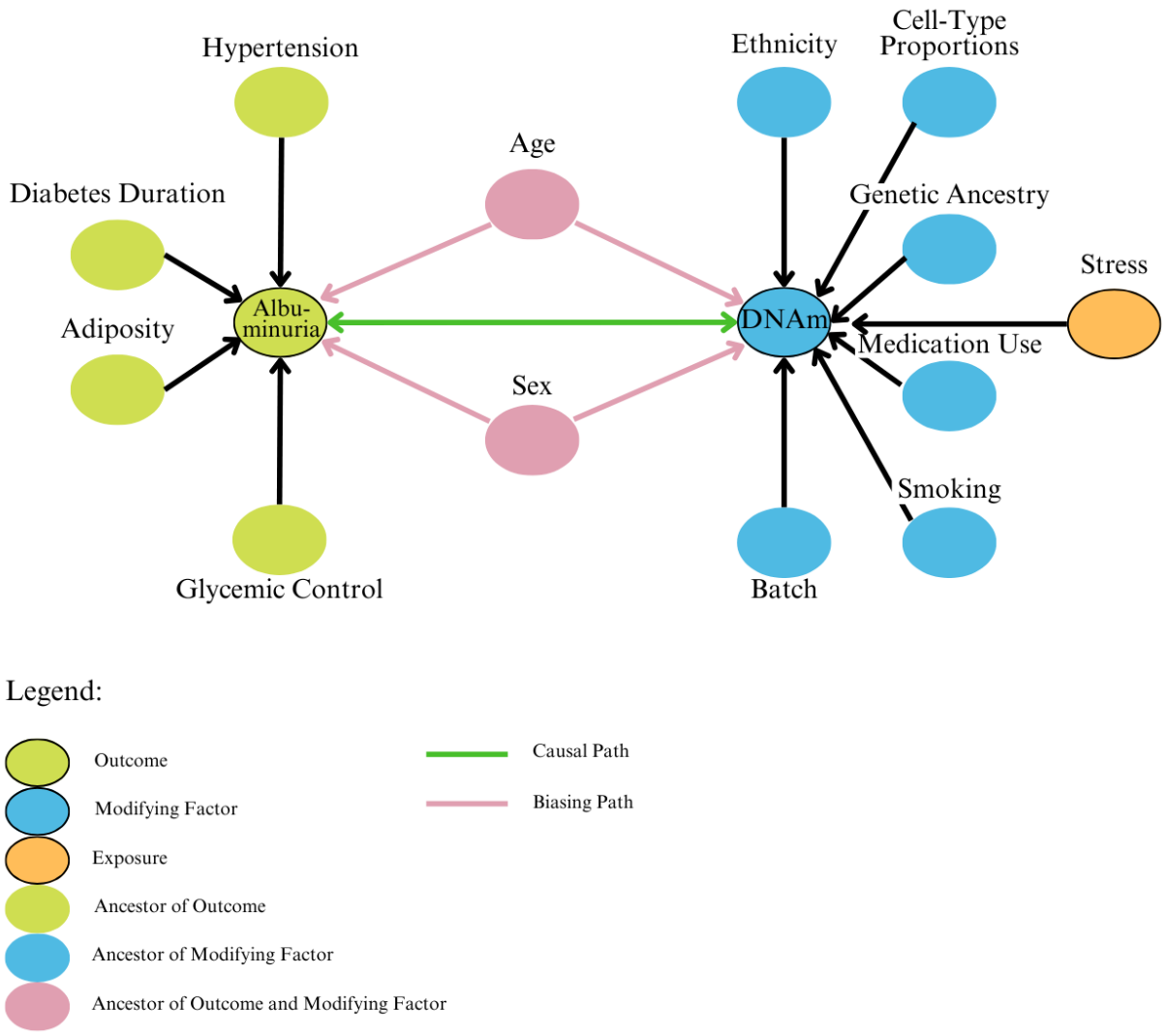


Figure 11. Study framework a priori hypothesized relationship between albuminuria and DNA methylation (DNAm), and potential confounders for research question 1(a).

The green arrow represents the direct relationship being investigated between albuminuria and DNAm, while the pink arrows depict hypothesized indirect relationships and confounding pathways.

6.4 Knowledge Gap Filled

To better understand the determinants of kidney outcomes among youth with T2D, this research aims to contribute to the literature regarding the association between stress and albuminuria among youth with T2D in Manitoba. This study's addition of knowledge is necessary given that T2D incidence is increasing globally among youth.[16] Indigenous peoples, who are disproportionately affected by T2D, are experiencing worse health outcomes related to T2D.[2] The need for the knowledge that this project adds is apparent. There is a gap in research on the association between stress and kidney outcomes among youth with T2D in Manitoba. The idea that epigenetics is a possible link has not been analyzed before this study.

Our research explores DNA methylation patterns in youth with T2D and albuminuria. No significant differences in individual CpG sites were found between youth with and without albuminuria. Our study found six significant DMRs (FDR < 0.05), four of which remained significant using the more conservative Sidak method.[205] I further investigated the six regions, some of which include genes involved in energy metabolism, immune regulation, and extracellular matrix maintenance (*TNXB, TSPAN32, ATP5E, TNFRSF6B*). These findings suggest that stress may be associated with kidney health through broader genomic regions rather than individual DNA methylation sites and that the relationship is complex and likely multifactorial.

It is important to acknowledge that the absence of significant site-specific DNA methylation changes may, in part, reflect a sample size limitation rather than a definitive biological finding. Although individual CpG sites did not reach significance, identifying six significant DMRs suggests that biologically meaningful changes may still occur at a regional level. With a larger sample size providing greater statistical power, some individual CpG sites

within these DMRs could also achieve statistical significance. While this study cannot definitively distinguish between these explanations, the significant DMR findings highlight the potential for regional-level mechanisms to play a role in stress-related epigenetic changes, and they suggest that further research with larger cohorts is necessary to confirm these findings and identify site-specific effects. Tissue-specific DNA methylation analyses should also be considered to explore these patterns fully.

By focusing on the relationship between stress and epigenetics in youth with T2D, this study addresses a critical gap in understanding how systemic and psychological factors influence the rapid progression of DKD in younger populations. Previous research has established the hemodynamic and inflammatory mechanisms driving DKD, including podocyte injury, oxidative stress, and glomerular hyperfiltration.[257–259] However, these studies primarily focus on adults, leaving the accelerated progression in youth underexplored. This research highlights the potential for stress-related epigenetic changes to intersect with kidney complications, underscoring the importance of future studies examining the temporal dynamics of DNA methylation changes in kidney-specific tissues.

Additionally, this research brings attention to First Nations and Métis youth with T2D, a group affected by both the disease and heightened stress levels, whose experiences are often underrepresented in epigenetic studies due to a history of exploitation and the associated ethical concerns surrounding genetic research on Indigenous communities.[260] By acknowledging the challenges posed by sample size while identifying broader genomic alterations, this study opens the door for future investigations into stress-related epigenetic pathways that may be associated with kidney complications. Ultimately, these findings help bridge the knowledge gap and can

potentially inform more targeted interventions to improve healthcare outcomes for youth with T2D in Manitoba, helping address the complex disease burden experienced by this population.

6.5 Study Strengths

One of the primary strengths of this study is the use of a large and well-established cohort of youth with T2D. Although the cohort size may be considered undersized for an EWAS, it is the largest cohort in North America focused on youth with T2D. This unique aspect ensures that the findings are robust and applicable to a specific, high-risk population historically underrepresented in epigenetic research. The inclusion of First Nations and Métis youth, who are disproportionately affected by T2D and its complications, adds immense value to this work by highlighting the vulnerability of this population. This increases the potential for the research to inform targeted public health interventions to address health inequities.

Another strength of the study lies in its advanced use of epigenetic techniques, including EWAS, DMR analysis, and candidate gene analysis. These sophisticated methods allow for the exploration of epigenetic changes across the genome while simultaneously focusing on specific regions of interest. By employing such techniques, the study provides a comprehensive and nuanced understanding of how stress may be associated with the development of kidney complications in youth with T2D. My chosen methods enhance the study's ability to detect subtle epigenetic changes that could explain the underlying aspects of stress-related health outcomes despite the relatively small cohort size for an EWAS.

Our study's multidimensional approach is a valued strength. This research goes beyond a simple association between stress and kidney outcomes by assessing environmental, biological, and psychological factors. It considers the complex relationship between perceived stress, epigenetic modifications, and disease outcomes. This holistic perspective enriches the study's

findings and provides a more comprehensive understanding of the multifactorial nature of T2D-related complications in youth. This integrative analysis is critical in populations that face both social and biological vulnerabilities, such as Indigenous youth, offering insights that could lead to more effective and culturally appropriate interventions.

6.6 General Study Design Limitations

6.6.1 Causality Issues

A key consideration when reviewing this study's findings is the design limitation, given that we could not interrogate causation. While variations in DNA methylation can potentially cause disease, they may also result from the disease. Consequently, this study focused solely on examining associations between variables rather than attempting to establish causality. The study's design did not allow us to determine whether the observed DNA methylation changes were a cause or effect of the disease.

However, it is essential to note that non-causal associations can still provide valuable insights. Even if not causal, DNA methylation patterns can serve as important biomarkers for disease. Biomarkers can help in early detection, risk stratification, and monitoring of disease progression or response to treatment.[261,262]

Non-causal findings can also play a crucial role in monitoring disease progression. Changes in DNA methylation patterns over time can indicate worsening or improving disease states, guiding treatment adjustments and clinical decisions. These findings contribute to understanding disease heterogeneity by helping to uncover molecular differences among individuals with the same disease, shedding light on varying disease subtypes and informing more personalized treatment strategies.

Additionally, non-causal findings can guide hypothesis generation for future studies, helping to identify pathways or biological mechanisms that warrant further investigation. Even without establishing causality, these findings can highlight molecular changes that occur in conjunction with disease processes, offering new targets for therapeutic intervention or preventive strategies.

Finally, biomarkers can help circumvent the tissue-specificity problem often encountered in epigenetic research. Since direct tissue sampling is invasive and impractical in large-scale human studies, peripheral biomarkers such as blood-derived DNA methylation can provide an accessible and less invasive means of monitoring disease-related changes across tissues. This expands the potential for clinical application without the need for direct tissue sampling.

Building on these insights, it is also valuable to consider how early DNA methylation changes might precede disease onset, potentially indicating long-term susceptibility. This perspective aligns with findings from Toperoff and colleagues, who observed DNA methylation changes in young adults before disease onset, suggesting that some DNA methylation patterns may be established early in development and persist, thereby increasing susceptibility to diseases such as T2D later in life.[125]

In contrast, our study population may reflect a different trajectory, where significant DNA methylation changes could occur later in development, after the critical windows in which early developmental changes are typically established. This delayed onset of DNA methylation differences might explain why such changes were not detected in our cohort. To better understand these dynamics, future projects utilizing iCARE data could address this limitation by collecting and examining data for individuals who did not have albuminuria at baseline but later developed it. By analyzing follow-up epigenetic data after the onset of albuminuria, it would be

possible to compare DNA methylation profiles across individuals with varying stages of albuminuria, thereby providing insights into potential causal relationships.

6.6.2 Sample Size

Sample size is a potential limitation, as the 213 participants may have been insufficient to detect significant epigenetic differences between youth with (n=55) and without (n=158) albuminuria. A smaller sample size reduces the study's statistical power, making it more challenging to identify subtle effects. While a sample size of 200 when conducting an EWAS is not completely rare, much larger sample sizes (>1000 participants) are becoming increasingly common. Altmüller and colleagues present an analysis of over 100 association studies, where they discuss the difficulty of finding significant associations and argue for the need for larger sample sizes.[263] With a larger cohort, the study might have uncovered stronger associations. For example, Wing and colleagues conducted a study with 3,939 participants. They identified a unique set of epigenetic patterns in participants with a rapid decline in kidney function compared to those with stable or improving kidney function. This might be attributed to their larger sample size, which provides greater statistical power to detect subtle epigenetic changes.[159] In comparison, our study's relatively smaller cohort may have lacked the power to detect such subtle differences.

However, studies like those led by Maghbooli (n=123), Qiu (n=181), and Sapienza (n=48) worked with sample sizes closer to ours and still reported significant DNA methylation differences.[156,158,160] It is important to note that these studies were not conducted very recently and were conducted at a time when smaller cohorts were more common in epigenetic research. While this suggests that smaller sample sizes can yield significant findings, it also highlights how research practices have evolved because it is now more common to have larger cohorts.

Additionally, the potential effect size of the relationship between stress and albuminuria may have been small, making detecting subtle epigenetic changes even more difficult with a limited sample size.

While our smaller sample size is a concern, it may not entirely explain our study's absence of significant findings. Even if we had a larger sample size, the relationship between stress and albuminuria might remain weak if other factors, such as high baseline stress or alternative pathways, are more influential. While the sample size is a reasonable concern, the lack of significant findings could also reflect a genuine lack of relationship between DNA methylation changes and albuminuria among the iCARE cohort.

6.6.3 Confounding Variables

Confounding variables are another potential limitation that could have influenced the results. Factors such as socioeconomic status, access to healthcare, or comorbid conditions might confound the relationship between stress and albuminuria. These variables could exaggerate or mask the true association, leading to inaccurate conclusions. For instance, if a confounding variable positively correlates with stress and albuminuria (e.g., poor access to healthcare), it might exaggerate the observed relationship between those factors, making the association appear stronger than it truly is. Conversely, if a confounder negatively correlates with one variable while being positively associated with the other (e.g., higher socioeconomic status leading to better health outcomes despite higher stress), it could mask the actual relationship, weakening the apparent association. Although efforts were made to control for known confounders, it is possible that residual confounding or unmeasured variables still affected the results. The potential for confounding remains a concern, but it is not definitive that it wholly accounts for the observed findings.

6.7 Specific Methodological and Contextual Limitations

6.7.1 Impact of Stress and Alternative Pathways

Our study population had a high baseline stress level with little variation between those who were stressed (mean PSS-14 score 27 (± 6.4)). This could mask the relationship between stress and albuminuria. If high stress is prevalent across the population, with little variability in stress levels, the ability to detect a significant association between stress and epigenetic changes related to albuminuria may be lessened because the lack of variability means that there is no proper control group. This could explain the lack of significant sites found in this study. Future research might benefit from studying populations with more varied stress levels or using more sensitive measures of stress to capture its potential impact on albuminuria better.

Additionally, it is crucial to consider that stress may impact kidney outcomes through mechanisms beyond DNA methylation, such as hormonal changes over time. If stress primarily affects kidney function via alternative physiological pathways, such as cortisol levels or other stress-related hormones, then epigenetic changes might not be the primary mechanism linking stress with albuminuria. This could mean that the study's focus on DNA methylation did not capture the full spectrum of stress-related physiological changes, thereby limiting the observed association between stress and albuminuria outcomes. Exploring alternative pathways in future studies could provide a more comprehensive understanding of the multifaceted ways in which stress affects kidney health.

6.7.2 Heterogeneity and Duration of Kidney Disease

The heterogeneity in the population may also be a significant limitation, particularly in the development of albuminuria among youth with T2D. The heterogeneity in kidney disease development in youth with T2D may be influenced by non-classic kidney pathologies, which are

more prevalent in this population compared to adults. This variation could mean stress-related epigenetic changes are more relevant to specific kidney disease subtypes. For example, DMRs linked to immune regulation (e.g., *TNFRSF6B*) may reflect pathways specific to non-classic immune-mediated kidney damage, which may explain some of the variability in epigenetic findings within the cohort. Recognizing this variability is crucial for interpreting the study's findings and highlights the need for personalized approaches to understand kidney disease etiology.

The length of time participants have albuminuria may also be a limitation. Epigenetic changes and their potential impact on disease outcomes may accumulate or manifest differently depending on how long the disease has been present. For example, a study by Sapienza and colleagues discussed significant findings when comparing patients with diabetes and ESKD to those without any kidney damage.[158] The differences between ESKD and no kidney damage may be more pronounced than our comparison of differences between individuals with and without albuminuria, as the transition from a healthy kidney to albuminuria represents a shorter duration of kidney disease and less extensive damage. This discrepancy could explain why Sapienza found significant DNA methylation changes while our study did not. Participants with a longer history of albuminuria might show more pronounced epigenetic alterations, while those with a more recent diagnosis might not yet exhibit such changes. This may lead to an underestimation of the relationship between stress and albuminuria. Conversely, if no strong association is found regardless of disease duration, this could suggest that stress-related epigenetic changes are not a key factor in the association with albuminuria. The duration of albuminuria is a critical factor to consider, but its role in this study’s findings is uncertain without further analysis.

6.8 Epigenetic-Specific Limitations

6.8.1 Tissue Specificity

The biological relevance of epigenetic research, particularly when examining DNA methylation in blood, is an important consideration. There is no assurance that the DNA methylation patterns observed in blood mirror those in kidney tissue. Research by Lowe et al. demonstrated that DNA methylation in whole blood exhibits roughly double the amount of tissue-specific differentially methylated positions compared to other somatic tissues, including the kidney.[264] While correlations between DNA methylation patterns across different tissues have been documented, as noted by Husby, this remains difficult to study in humans due to the accessibility of blood samples compared to other tissues.[265] This limitation complicates the direct application of blood-based DNA methylation findings to kidney tissue, though the study still offers valuable insights into the relationships between stress and albuminuria.

Of note, Dayeh and colleagues found significant blood-based DNA methylation differences linked to T2D susceptibility.[128] T2D-related genes may be more easily detectable in blood due to the systemic nature of T2D, which affects multiple organs and is reflected in the circulatory system. Additionally, blood plays a central role in glucose transport and insulin regulation, making it a more relevant tissue for detecting epigenetic changes tied to T2D pathophysiology. In contrast, kidney- or albuminuria-related changes might be less prominent in blood, which could partly explain why our results were mostly non-significant. Qiu and colleagues published a study with a cohort size comparable to ours and identified significant blood-based DNA methylation changes; however, after adjusting for multiple comparisons, none reached genome-wide significance.[156] This finding underscores the challenges of detecting epigenetic changes in blood when the true biological effects may be more pronounced in kidney

tissue. Our study's lack of significant results in the EWAS and candidate gene analysis after multiple testing correction could reflect the complexities of tissue specificity and the inherent limitations of relying on blood samples for assessing kidney-related epigenetic alterations.

6.8.2 Bias and False Positives

A limitation inherent to EWAS is its susceptibility to bias and inflation, which can lead to the overestimation of statistical significance and an increased likelihood of false positives. Although the original plan included using a Bayesian estimation of empirical null distribution, as implemented in *R* with the *bacon* package, to control for false positives while maintaining statistical power, this approach was ultimately unsuccessful. When applied to the data, *bacon* further inflated my model rather than mitigating the bias, leading to its exclusion from the final analysis (Figure 1).

A potential cause of this may be the specific characteristics of the data itself, such as population heterogeneity or unaccounted confounding factors. These factors may have influenced the effectiveness of the *bacon* adjustment, as they distort the empirical null distribution, which *bacon* relies on to recalibrate test statistics. For instance, population heterogeneity introduces variability that reflects true biological or environmental differences rather than technical artifacts. The *bacon* package assumes that most associations are null, and this additional variability can lead to misestimation of the baseline variability, resulting in over- or under-shrinkage of the test statistics. Similarly, unaccounted confounders can create systematic biases in the data that mimic actual signals, making it more challenging for *bacon* to differentiate between genuine effects and artifacts, which can inflate the model. These limitations underscore the need for further refinement of tools like *bacon* or alternative methods to address data-specific challenges in EWAS.

6.9 Future Research Directions

The findings of this study have highlighted several promising areas for future research, particularly in uncovering the mechanisms linking stress, epigenetic modifications, and kidney outcomes in youth with T2D. An important outcome was the detection of six significant regions from the DMR analysis. Future research should focus on exploring these regions further using larger, more diverse samples. These areas may hold the potential for revealing critical epigenetic pathways related to stress and albuminuria. In addition, employing objective, longitudinal measures of stress, such as hair cortisol or 24-hour cortisol profiles, could provide a more precise and comprehensive understanding of stress exposure and its cumulative impact on kidney health over time. This would offer a more robust picture of experienced stress beyond the current self-reported, cross-sectional data.

As a prospective cohort study, this research also presents the opportunity to collect epigenetic data at multiple points in time, not just at baseline. Future studies could leverage this design to perform longitudinal epigenetic analyses, enabling a more comprehensive understanding of how exposures over time influence epigenetic modifications and contribute to adverse health outcomes. This could include kidney-specific epigenetic analyses, which would offer insights into how stress-related DNA methylation changes affect pathways such as podocyte injury, oxidative stress, and glomerular hyperfiltration, all of which are central to DKD pathophysiology. This type of temporal analysis would allow researchers to trace the changes in stress markers and their potential role in the development of albuminuria and other complications in youth with T2D. This would clarify how early exposures and chronic stress impact long-term health through epigenetic mechanisms.

Another option for future research is shifting the narrative toward resilience by focusing on the factors contributing to kidney protection rather than solely examining disease mechanisms. Instead of focusing exclusively on those at higher risk, future studies could explore how youth with lower stress levels and more favourable health outcomes maintain advantageous epigenetic patterns. Research could examine whether supportive environments, positive coping strategies, and cultural practices have measurable impacts on epigenetic markers and confer protection against T2D-related kidney damage. This perspective shift from deficit to resilience could uncover critical pathways that foster kidney protection, providing valuable insights for prevention and intervention strategies. Insights from an iCARE patient partner highlight the importance of researching both positive and negative coping mechanisms, including potentially harmful ones like substance use, to identify protective factors that foster resilience and reduce stress-related health risks in this population.

In addition to the above, future research may wish to consider how socio-economic factors and access to healthcare play a role in modifying stress and health outcomes in these youth. Studies could investigate the impact of interventions that improve access to culturally sensitive healthcare, mental health resources, and stress-reduction programs. This would address immediate health concerns and contribute to long-term strategies for reducing the burden of T2D-related complications, such as kidney disease. An iCARE patient partner's observation about the absence of critical diabetes management resources in many Indigenous communities, such as diabetes educators and mental health supports, underscores the importance of investigating how improved access to care influences outcomes. Future studies should also account for caregiving dynamics, such as grandparents raising children, as these roles may add unique stressors that influence health outcomes.

Considering the unique stressors faced by Indigenous youth and their relationship with health outcomes, future studies could explore community-led interventions designed to reduce stress while respecting cultural values and autonomy. As emphasized by iCARE patient partners, engaging elders and knowledge keepers in the research process can ensure that findings are communicated in ways that resonate with community experiences and cultural frameworks.

6.10 Policy Implications

While our findings showed six significant DMRs, further research is needed to determine whether DNA methylation is a mechanism linking stress and kidney outcomes. If future studies confirm this connection, it will emphasize the need for public health policies aimed at reducing stress among youth with T2D, particularly those presenting with early signs of kidney complications, such as albuminuria. Chronic stress has been shown to contribute to adverse health outcomes,[266] and if further research confirms that DNA methylation is proven to play a role, addressing stress will become an urgent public health priority. Stress should be recognized not only as a psychological burden but also as a substantial biological risk factor that can contribute to long-term kidney disease through epigenetic changes. This aligns with existing evidence surrounding the burden of intergenerational trauma experienced by Indigenous peoples,[267] much of which stems from the lasting impacts of colonialism. Policies to reduce stress in youth with T2D could help modulate the pathophysiological pathways driving DKD progression. By implementing culturally sensitive stress-reduction programs, such as those emphasizing traditional practices and Indigenous languages, it may be possible to mitigate these biological pathways. This holistic approach could alleviate the inflammatory and oxidative stress burden contributing to DKD in Indigenous youth, ultimately improving long-term kidney

outcomes. Reducing stress in Indigenous youth, who represent a significant portion of youth-onset T2D cases in Canada, could play a vital role in mitigating these adverse health outcomes.

Public health policies should prioritize the development of culturally appropriate interventions aimed at lowering stress among Indigenous youth. For example, fostering environments that emphasize cultural resilience, such as promoting Indigenous language use and integrating traditional practices into healthcare, may improve mental health and reduce the negative epigenetic changes related to stress.[268] This culturally sensitive approach has been shown to improve health outcomes in Indigenous communities, as those where cultural practices are maintained tend to experience better overall health.[268] Additionally, iCARE patient partners emphasized the importance of consulting elders and knowledge keepers to design culturally grounded interventions that resonate with community values. Engaging elders in mental health initiatives could strengthen intergenerational ties and foster a sense of cultural continuity, which may contribute to greater resilience and reduced stress.

Health policies, especially in northern and remote regions, should seek to integrate Indigenous knowledge systems into current Western medical practices, such as building partnerships between Western healthcare providers and Indigenous healers. This could alleviate the long-standing stress burden on youth with T2D, ultimately leading to improved kidney health outcomes. Public health strategies should shift from a purely biomedical model of care to a more holistic approach that considers environmental, cultural, spiritual, and community factors. By expanding healthcare services that are responsive to the cultural needs of Indigenous youth, such as the integration of traditional and Western medicine in healthcare settings, the stress levels in this population may be reduced. Chandler and Lalonde showed that aspects of cultural continuity, such as the use of traditional languages, are associated with lower suicide rates

among British Columbia's Indigenous communities,[269] which promotes the idea that public health policies may benefit from focusing on enhancing resilience by developing mental health and stress-reduction programs tailored to the unique cultural and social contexts of Indigenous youth. iCARE patient partners also highlighted that stress is rarely recognized as a standalone factor but is deeply connected with grief, loss, and trauma stemming from colonial histories. Policies must move beyond measuring stress in isolation and instead address these interconnected factors through programs that prioritize healing from intergenerational trauma. This includes fostering environments where children feel secure, supported, and engaged in meaningful cultural practices that help mitigate stress-related health disparities.

Knowledge translation will also play a crucial role in ensuring that the findings from this research are effectively communicated to stakeholders, including policymakers, healthcare providers, and Indigenous communities. Ethical considerations, such as those outlined in the TCPS2 and the OCAP principles, will guide the dissemination of knowledge to ensure the equitable involvement of Indigenous communities in the research process. Ongoing collaboration with the iCARE PAG will ensure that the findings are scientifically rigorous, culturally relevant, and beneficial to the communities involved. Reports, presentations, and consultations will be used to share the research outcomes with the Four Arrows Regional Health Authority Board of Directors and other relevant bodies, ensuring that the study contributes meaningfully to public health policy and Indigenous health sovereignty.

The results of our study highlight the urgent need to reduce stress among youth with T2D, not only as a potential means of preventing long-term kidney damage but also as a way to promote overall well-being in this vulnerable population. Culturally appropriate, community-led,

and resilience-focused interventions should be prioritized to address both the immediate and long-term health challenges faced by Indigenous youth with T2D.

Chapter 7: References

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Chapter 8: Appendix

Appendix 1. Definition of Variables

Variable	Definition	Criteria
Type 2 Diabetes[270]	A chronic condition characterized by elevated blood glucose levels caused by the body's ineffective use of insulin.	Diagnosis based on clinical criteria: presence of obesity, other evidence of insulin resistance, family history of type 2 diabetes, intrauterine exposure to hyperglycemia, and family heritage from a high-risk ethnic group and the absence of insulin and glutamic acid decarboxylase antibodies.
Albuminuria[2]	The presence of an abnormal amount of albumin, a type of protein, in the urine, which can indicate kidney damage or dysfunction.	Defined by albumin-to-creatinine ratio greater than 3.0mg/mmol on overnight urine collection or first morning sample, confirmed with at least two abnormal results..
Stress[183]	A physiological and psychological process involving a stimulus, individual appraisal, and a response.	Measured using the 14-item Perceived Stress Scale (PSS); a score ≥27 indicates significant perceived stress.
DNA Methylation[8,9]	The addition of a methyl group to the cytosine-phosphate-guanine (CpG) site on a DNA strand, which can influence gene expression without altering the DNA sequence.	Measured through epigenetic analysis, focusing on DNA methylation levels at CpG sites associated with specific genes or regions of interest.

Appendix 2. Description of Tool

Scale	Description	Validity	Additional comments
Perceived Stress Scale - 14[183]	-14 item assessment using 5- point Likert scale - The respondent rates frequency of feelings and thoughts related to events and situations in the last month - Total score of 27 or greater used to indicate stress in our study	-High validity, internal consistency -Chronbach's alpha 0.82 -Highly correlated with stress (coefficient r = 0.644), depression (r = 0.606), and anxiety (r = 0.542) subscales from Depression Anxiety and Stress scale	- Focuses on the person's perception of the degree to which they are experiencing symptoms of stress related to life demands and their ability to cope with life's demands. - Shown in extensive previous research to be related to immune system functioning.