

Elucidating Genetic and Epigenetic Mechanisms of Type 2 Diabetes Mellitus Pathogenesis



Thesis for Doctoral Degree (Ph.D.)

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Submitted by

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Declaration

I hereby declare that this thesis, entitled “**Elucidating Genetic and Epigenetic Mechanisms of Type 2 Diabetes Mellitus Pathogenesis**,” is the result of my own original research work. All sources of information, whether published or unpublished, have been duly acknowledged and appropriately referenced throughout the thesis.

To the best of my knowledge and belief, this thesis has not been submitted, either in whole or in part, to any other university or institution for the award of any degree, diploma, or other academic qualification. Furthermore, no part of this work has been used to satisfy the requirements of any other examination or academic award.

I take full responsibility for the authenticity and integrity of the work presented in this thesis.

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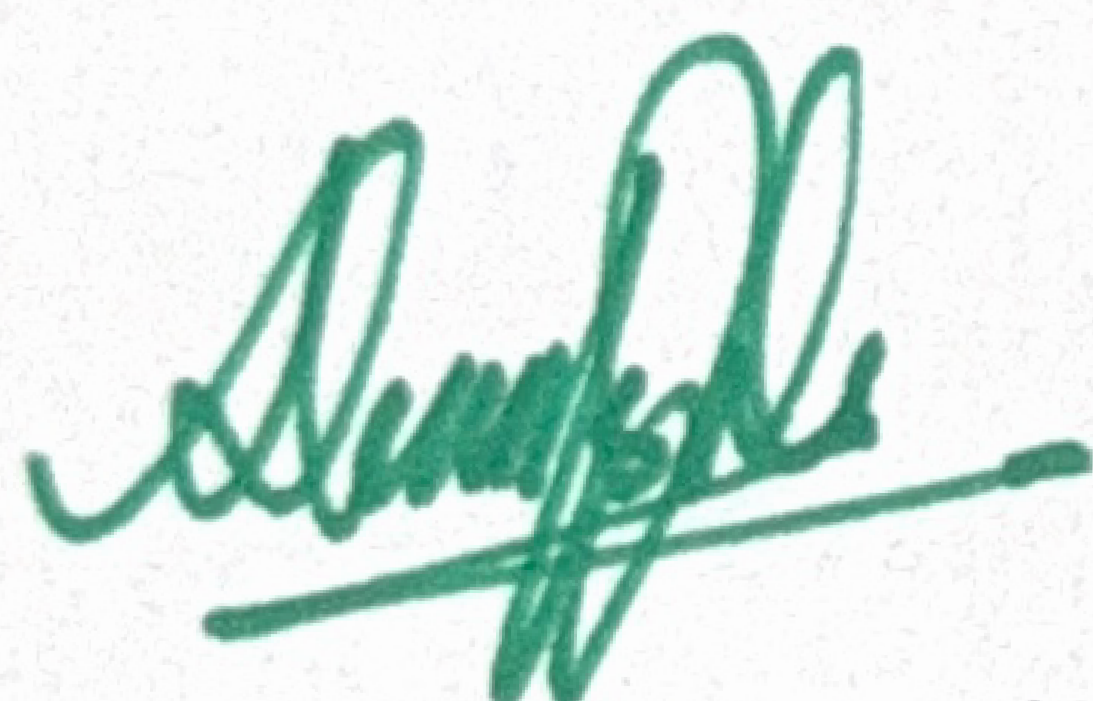
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This is to certify that Nilima Barman (Registration No. PhD-02/2021-2022; Session: 2021-2022) has carried out the research work embodied in this thesis entitled "Elucidating Genetic and Epigenetic Mechanisms of Type 2 Diabetes Mellitus Pathogenesis" under our direct supervision.

We further certify that this research is original and has been conducted by the candidate in accordance with the requirements of the degree. To the best of our knowledge, the work presented in this thesis has not been submitted elsewhere for the award of any degree or diploma.

In our opinion, the thesis is of a satisfactory standard and is worthy of submission for the award of the degree of Doctor of Philosophy (PhD) in Genetic Engineering and Biotechnology of the University of Dhaka.



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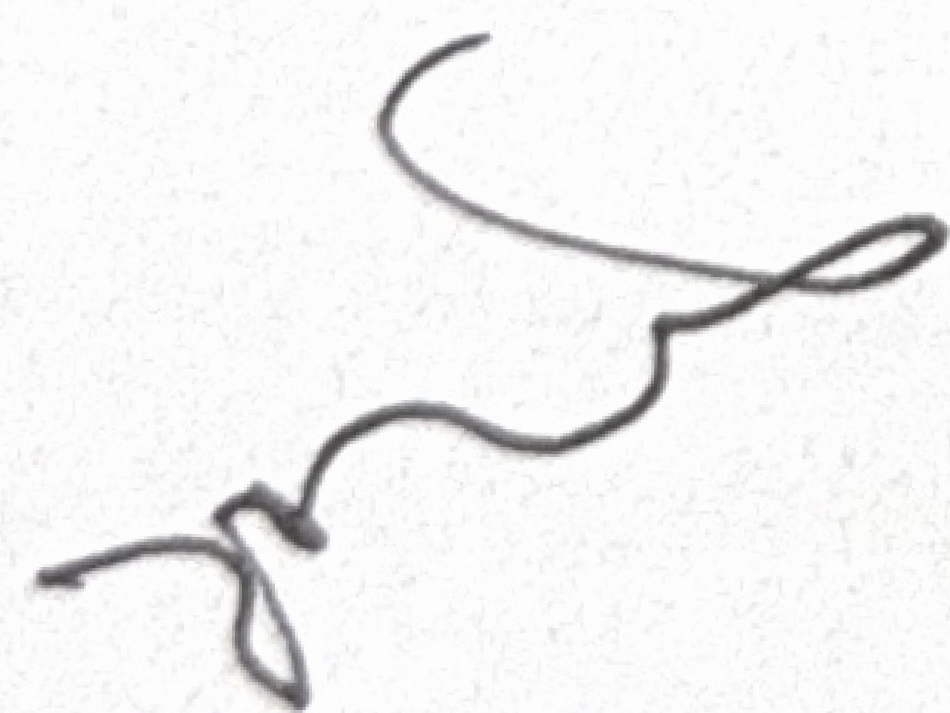
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*Dedicated
To
My Beloved Family*

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List of Abbreviations

Abbreviation	Full Form
ACE	Adverse Childhood Experience
ACEs	Adverse Childhood Experiences
ACME	Average Causal Mediation Effect
ACTH	Adrenocorticotrophic Hormone
ADA	American Diabetes Association
AgRP	Agouti-Related Peptide
AgRP/NPY	Agouti-Related Peptide/Neuropeptide Y
ARC	Arcuate Nucleus
ATP	Adenosine Triphosphate
BER	Base Excision Repair
BMI	Body Mass Index
BP	Blood Pressure
cAMP	Cyclic Adenosine Monophosphate
CCK	Cholecystokinin
CpG	Cytosine-Phosphate-Guanine
CRH	Corticotropin-Releasing Hormone
CTD	C-Terminal Domain
DAG	Diacylglycerol
DBP	Diastolic Blood Pressure
DMC	Differentially Methylated Cytosine
DMH	Dorsomedial Hypothalamus
DMR	Differentially Methylated Region
DMV	Dorsal Motor Nucleus of the Vagus
DNA	Deoxyribonucleic Acid
EGP	Endogenous Glucose Production
ERC	Ethical Review Committee
EST	Expressed Sequence Tag
EWAS	Epigenome-Wide Association Study

Abbreviation	Full Form
FBS	Fasting Blood Sugar
FDR	False Discovery Rate
FFA	Free Fatty Acid
G6Pase	Glucose-6-Phosphatase
GADAs	Glutamic Acid Decarboxylase Autoantibodies
GDM	Gestational Diabetes Mellitus
GIP	Glucose-Dependent Insulinotropic Peptide
GLP-1	Glucagon-Like Peptide-1
GLUT2	Glucose Transporter 2
GWAS	Genome-Wide Association Study
HbA1c	Glycated Hemoglobin
HDL	High-Density Lipoprotein
HDMAX2	High-Dimensional Mediation Analysis 2
HMG	High-Mobility Group
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HPA	Hypothalamic-Pituitary-Adrenal
HWE	Hardy-Weinberg Equilibrium
ICA	Islet Cell Autoantibody
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IGT	Impaired Glucose Tolerance
IHME	Institute for Health Metrics and Evaluation
IL	Interleukin
IML	Intermediolateral Nucleus of the Spinal Cord
INSR	Insulin Receptor
IPA	Ingenuity Pathway Analysis
IQR	Interquartile Range
IRS-1	Insulin Receptor Substrate-1
KATP	ATP-Sensitive Potassium Channel

Abbreviation	Full Form
LADA	Latent Autoimmune Diabetes in Adults
LDL	Low-Density Lipoprotein
LH	Lateral Hypothalamus
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MBD	Methyl-CpG-Binding Domain
MD	Molecular Dynamics
MODY	Maturity-Onset Diabetes of the Young
NCD	Non-Communicable Disease
NCDs	Non-Communicable Diseases
NF- κ B	Nuclear Factor Kappa B
NR3C1	Glucocorticoid Receptor Gene
NTS	Nucleus Tractus Solitarius
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
PC	Principal Component
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PEPCK	Phosphoenolpyruvate Carboxykinase
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PME	Particle Mesh Ewald
POMC	Proopiomelanocortin
PPAR γ	Peroxisome Proliferator-Activated Receptor Gamma
PSS-4	Perceived Stress Scale-4
PVN	Paraventricular Nucleus
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation

Abbreviation	Full Form
ROS	Reactive Oxygen Species
RVLM	Rostral Ventrolateral Medulla
SAH	S-Adenosyl-L-Homocysteine
SAM	S-Adenosylmethionine
SASA	Solvent-Accessible Surface Area
SBP	Systolic Blood Pressure
SCFA	Short-Chain Fatty Acid
SCFAs	Short-Chain Fatty Acids
SD	Standard Deviation
SEA	South-East Asia
SGLT2	Sodium-Glucose Cotransporter 2
SLC30A8	Solute Carrier Family 30 Member 8
SNARE	Soluble NSF Attachment Protein Receptor
SNP	Single Nucleotide Polymorphism
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TCF/LEF	T-Cell Factor/Lymphoid Enhancer Factor
TCF7L2	Transcription Factor 7-Like 2
TDG	Thymine DNA Glycosylase
TLR4	Toll-Like Receptor 4
TMD	Transmembrane Domain
TNF- α	Tumor Necrosis Factor Alpha
TRD	Transcriptional Repression Domain
VMH	Ventromedial Hypothalamus
WHO	World Health Organization
ZnT8	Zinc Transporter 8

Abstract

Type 2 diabetes mellitus (T2DM) is a rapidly escalating public health crisis in Bangladesh, which now ranks seventh globally, with approximately 13.9 million adults living with the disease. T2DM is a multifactorial disorder arising from complex interactions among genetic susceptibility, epigenetic dysregulation, and environmental exposures. Despite the growing burden, molecular-level evidence from Bangladeshi populations remains scarce. This study aimed to investigate the genetic, environmental, and epigenetic factors associated with T2DM in the Bangladeshi population by examining genetic variations in the *TCF7L2* rs12255372 and *SLC30A8* rs13266634 genes, exploring the association between adverse childhood experiences (ACEs) and adult T2DM risk, characterizing genome-wide DNA methylation alterations in T2DM, and assessing the potential mediating role of DNA methylation in the relationship between ACE exposure and T2DM susceptibility.

A case-control study was conducted among Bangladeshi T2DM patients and healthy controls. Clinical, anthropometric, biochemical, and psychosocial data were collected, and ACE exposure was assessed using the standardized ACE questionnaire. Candidate gene polymorphisms were analyzed by PCR amplification and Sanger sequencing. The structural consequences of the *SLC30A8* rs13266634 substitution were investigated via molecular dynamics simulation. Genome-wide DNA methylation profiling was performed using whole-genome enzymatic methyl sequencing on peripheral blood-derived genomic DNA, followed by bioinformatic analysis using the nf-core/methylseq pipeline and differential methylation analyses with edgeR and DMRcate. Pathway enrichment analysis was conducted using Ingenuity Pathway Analysis. High-dimensional mediation analysis (HDMAX2) was applied to explore the potential mediating role of DNA methylation in the association between ACE and T2DM.

The study demonstrated that both genetic and environmental factors contribute significantly to T2DM susceptibility in the Bangladeshi population. The *TCF7L2* rs12255372 T allele was significantly associated with increased T2DM risk, with individuals carrying either GT or GT-TT genotypes showing higher odds of disease. Similarly, the *SLC30A8* rs13266634 wild-type CC genotype and major C allele were associated with increased T2DM susceptibility, whereas the variant TT genotype

appeared to confer a protective effect. Molecular dynamics simulations further suggested that the ZnT8 p.R325W substitution increased structural rigidity and reduced conformational flexibility, potentially affecting zinc transport and insulin granule physiology.

In addition to genetic susceptibility, ACEs were strongly associated with T2DM, as individuals exposed to four or more ACEs had more than two-fold higher risk of developing the disease. Genome-wide DNA methylation profiling identified 608 differentially methylated CpG sites, the majority of which were hypermethylated. Pathway enrichment analyses revealed epigenetic dysregulation in neurotransmitter signaling, fatty acid metabolism, adiponectin signaling, insulin resistance pathways, and mitochondrial regulation. Furthermore, high-dimensional mediation analysis identified multiple methylated loci potentially involved in the association between ACE exposure and T2DM, while pooled mediation analysis indicated that the indirect effect through DNA methylation accounted for approximately 41.1% of the total ACE–T2DM association. These findings provide statistical evidence consistent with a potential epigenetic pathway through which early-life adversity may influence adult metabolic disease risk, rather than establishing a definitive causal relationship.

In conclusion, this study provides integrated evidence that genetic susceptibility, adverse childhood experiences, and epigenetic dysregulation collectively contribute to T2DM in the Bangladeshi population. The findings enhance understanding of the molecular mechanisms underlying T2DM and may contribute to future strategies for early risk prediction, prevention, and personalized management of diabetes in Bangladesh.

1. Introduction

1.1 Background

Diabetes mellitus has become one of the most important global public health challenges of the twenty-first century. According to the International Diabetes Federation (IDF) 2025 report, an estimated 589 million adults aged 20-79 years were living with diabetes in 2024, representing 11.1% of the global adult population, and this number is projected to increase to 852.5 million by 2050 (IDF, 2025). Diabetes was responsible for more than 3.4 million deaths in 2024, and diabetes-related healthcare expenditure exceeded one trillion US dollars for the first time, reflecting its enormous clinical and economic burden. The burden is particularly severe in the South-East Asia region, where rapid urbanization, lifestyle transition, and population growth are driving a steep rise in prevalence. Reflecting this regional trend, Bangladesh now ranks seventh worldwide in the number of adults with diabetes. Among adults, type 2 diabetes mellitus (T2DM) accounts for more than 90% of all diabetes cases (IDF, 2025; IHME, 2024).

T2DM is a complex and multifactorial disease caused by the interaction of genetic, epigenetic, environmental, behavioral, and metabolic factors. Classical non-genetic risk factors include ageing, excess energy intake, physical inactivity, obesity, rapid urbanization, and obesogenic environments (Hu et al., 2019; Inolopú et al., 2019; Temelkova-Kurktschiev & Stefanov, 2012). In addition to these adult-life exposures, early-life stressors are increasingly recognized as important contributors to later metabolic disease. Adverse childhood experiences (ACEs), also referred to as childhood maltreatment, include potentially traumatic exposures occurring before 18 years of age, such as abuse, neglect, and household dysfunction (Finkelhor et al., 2015). These events occur during sensitive periods of neurodevelopment and may produce long-lasting biological consequences (Bick & Nelson, 2016). Several studies suggest that childhood adversity is associated with increased risk of T2DM in later life, indicating that early environmental exposures may become biologically embedded and influence adult metabolic health (Felitti et al., 1998; Huang et al., 2015).

Besides non-genetic factors, genetic susceptibility plays a central role in T2DM pathogenesis. Twin and family studies have estimated the heritability of T2DM to range

between 50% and 90%, supporting a strong inherited component in disease development (Mezuk et al., 2015; Sung et al., 2015; Willemsen et al., 2015). Genome-wide association studies have identified numerous loci associated with T2DM risk. Earlier studies reported more than 120 susceptibility variants (Prasad & Groop, 2015), while later large-scale analyses identified 139 common genetic variants associated with T2DM (Xue et al., 2018). Among the genetic loci associated with T2DM, *TCF7L2* and *SLC30A8* are particularly relevant because of their functional roles in pancreatic β -cell biology and insulin secretion. Transcription factor 7-like 2 (*TCF7L2*) is one of the most consistently replicated susceptibility genes for T2DM across different populations (Del Bosque-Plata et al., 2021; Grant et al., 2006; Jouvett & Estall, 2017; Tong et al., 2009). Variants such as rs7903146 and rs12255372 have repeatedly been associated with increased disease risk and are believed to influence β -cell proliferation, insulin biosynthesis, and incretin-mediated insulin secretion (Del Bosque-Plata et al., 2021; Peng et al., 2013). However, differences in allele frequency and effect size across populations indicate the importance of population-specific investigations (Guinan, 2012).

Similarly, *SLC30A8*, which encodes the zinc transporter ZnT8, has emerged as a biologically significant candidate gene for T2DM because of its β -cell-specific expression and essential role in insulin granule function. ZnT8 regulates zinc transport into insulin secretory vesicles, facilitating insulin crystallization, storage, and secretion. A notable non-synonymous variant in *SLC30A8*, rs13266634 (C>T), results in an arginine-to-tryptophan substitution at position 325 of the ZnT8 protein within its C-terminal domain (Parsons et al., 2018; Rutter & Chimienti, 2015). Because both *TCF7L2* and *SLC30A8* are directly involved in insulin production and pancreatic β -cell function, variants in these genes are more likely to reflect primary defects in insulin secretion rather than secondary metabolic consequences. Therefore, investigation of these candidate genes in the Bangladeshi population may provide valuable insight into the genetic basis of insulin deficiency related T2DM susceptibility.

Although genetic studies have identified important T2DM risk loci, genetic variants alone do not fully explain disease development. These known variants explain only a limited proportion of disease heritability, with estimates suggesting that common T2DM-associated SNPs account for only about 10–20% of the inherited risk (Barrès &

Zierath, 2016; Xue et al., 2018). This “missing heritability” indicates that additional molecular mechanisms, including gene–environment interactions and epigenetic regulation, may be important in explaining T2DM pathogenesis. This gap has encouraged researchers to explore epigenetic mechanisms as possible links between environmental exposures, genetic susceptibility, and metabolic disease. Epigenetics refers to functionally relevant modifications that regulate gene activity without altering the underlying DNA sequence. Major epigenetic mechanisms include DNA methylation, histone modification, non-coding RNA signaling, and chromatin remodeling (Klengel et al., 2014). These modifications may occur during early development and persist across the life course, thereby influencing long-term disease susceptibility (Cunliffe, 2015). Because epigenetic marks are responsive to both intrinsic and environmental factors, they provide a plausible biological mechanism through which lifestyle, nutrition, stress, and early-life adversity may influence T2DM risk.

DNA methylation is one of the most widely studied epigenetic modifications because of its relative stability, biological relevance, and feasibility of measurement. It involves the covalent addition of a methyl group to the fifth carbon of cytosine, forming 5-methylcytosine, most commonly at cytosine-phosphate-guanine dinucleotide sites, known as CpG sites (Ziller et al., 2011). CpG sites may cluster in CpG islands, which are frequently located in gene promoter regions. Methylation in promoter regions is generally associated with transcriptional repression or gene silencing, although the functional effect of methylation may vary depending on genomic context (Eccleston et al., 2007; Jones, 2012). Because DNA methylation can influence gene expression and may be altered by environmental exposures, it is increasingly being investigated as a molecular mediator of chronic metabolic diseases. Evidence from both locus-specific and genome-wide studies supports a relationship between altered DNA methylation and T2DM. Previous studies have reported DNA methylation differences in individuals with T2DM and in tissues relevant to glucose metabolism, including pancreatic islets, adipose tissue, and skeletal muscle (Dayeh et al., 2016; Nilsson et al., 2014; Walaszczyk et al., 2018). DNA methylation has also been associated with insulin sensitivity, including HOMA-IR, suggesting that epigenetic variation may influence early metabolic dysfunction before overt diabetes develops (Arner et al., 2016; Mannar et al., 2023). Importantly, DNA methylation may not only be associated with T2DM but may

also provide a molecular interface through which environmental exposures influence metabolic disease susceptibility.

Epigenetic regulation is highly sensitive to environmental exposures, particularly during early developmental periods. In addition to classical metabolic risk factors, early-life adversity may influence T2DM risk through epigenetic modification of stress-response pathways, especially those related to hypothalamic-pituitary-adrenal (HPA) axis regulation. Epigenome wide studies investigating ACEs have identified methylation changes in several genes implicated in obesity, fatty acid metabolism, neuroendocrine regulation, and metabolic dysfunction. Genes such as *FKBP5*, *NR3C1*, *POMC*, *BDNF*, and *SLC6A4* have been associated with childhood adversity and may also contribute to T2DM susceptibility and related metabolic disturbances (Hao et al., 2018). For example, altered methylation of *FKBP5* has been associated with increased HbA1c, LDL cholesterol, body mass index, and waist circumference in T2DM patients (Ortiz et al., 2018). Similarly, methylation changes in the *NR3C1* promoter region have been linked with subclinical atherosclerosis (Zhao et al., 2015), while altered methylation of *POMC* and *BDNF* has been associated with dysregulated insulin metabolism, obesity, and diabetes-related traits (Liu et al., 2016; Yoo et al., 2014). These findings support the concept that environmental stress during early life may become biologically embedded through epigenetic mechanisms and contribute to long-term metabolic disease risk.

Despite substantial global progress in understanding the genetic and epigenetic basis of T2DM, corresponding evidence from Bangladeshi populations remains limited. Most studies conducted in Bangladesh have primarily focused on clinical, biochemical, and lifestyle-related risk factors, whereas comparatively few investigations have explored molecular mechanisms underlying disease susceptibility. Given the high and rapidly increasing prevalence of T2DM in Bangladesh, population-specific molecular studies are urgently needed. Genetic susceptibility may vary across ethnic populations because of differences in allele frequencies, linkage disequilibrium structures, and environmental exposures. Likewise, epigenetic profiles may differ according to nutritional, psychosocial, environmental, and lifestyle-related influences unique to a population. Therefore, integrated investigation of both genetic variation and DNA

methylation patterns may provide a more comprehensive understanding of T2DM pathogenesis in Bangladeshi individuals.

T2DM is a chronic progressive disease characterized by a prolonged asymptomatic phase during which metabolic dysfunction and vascular damage may silently develop. By the time clinical diagnosis is established, many individuals may already have developed microvascular and macrovascular complications. Identification of molecular markers associated with early disease susceptibility could therefore facilitate risk prediction, early detection, preventive intervention, and improved disease management. In this context, the present study aims to explore both inherited and environmentally responsive molecular mechanisms contributing to T2DM pathogenesis. By focusing on biologically relevant candidate genes such as *TCF7L2* and *SLC30A8*, together with DNA methylation-based epigenetic regulation, this study seeks to bridge the gap between genetic susceptibility, environmental exposure, and metabolic disease. Such an integrated approach is particularly relevant for Bangladesh, where the burden of T2DM is increasing rapidly and molecular evidence remains scarce. Improved understanding of these mechanisms may contribute to future strategies for early detection, prevention, and personalized management of T2DM in this population.

1.2 Literature Review

1.2.1 Global and regional scenario of diabetes

Diabetes mellitus has become one of the most important global public health challenges of the twenty-first century. According to the IDF Diabetes Atlas 2025, an estimated 589 million adults aged 20–79 years were living with diabetes in 2024, representing 11.1% of the global population in this age group, and this number is projected to increase to 852.5 million by 2050. In addition, 635 million adults were estimated to have impaired glucose tolerance and 488 million to have impaired fasting glucose, indicating a very large population at high risk of future diabetes. Diabetes was responsible for more than 3.4 million deaths in 2024, while diabetes-related health expenditure exceeded one trillion US dollars for the first time. Another major concern is that about 42.8% of adults with diabetes remain undiagnosed, delaying timely intervention and increasing the risk of complications. Type 2 diabetes mellitus (T2DM) accounts for more than 90% of all diabetes cases worldwide and is therefore the principal driver of this growing burden. It is currently the eighth leading cause of global disease burden and is projected to become the second leading cause by 2050. Moreover, T2DM is associated with a substantially increased risk of cardiovascular disease, dementia, retinopathy, and vision-threatening complications, further amplifying its clinical and societal impact (IDF, 2025; IHME, 2024).

The burden is particularly pronounced in the South-East Asia (SEA) region, where rapid urbanization, lifestyle transitions, and population growth are driving a steep rise in prevalence. The International Diabetes Federation projects that the number of people with diabetes in the SEA region will increase by approximately 73%, reaching around 185 million by 2050, with prevalence rising to 13.2%. The region also has one of the highest proportions of undiagnosed diabetes (approximately 42.7%) and the highest proportion of pregnancies affected by hyperglycemia (27.8%). Furthermore, the SEA region has the highest age-standardized prevalence of impaired glucose tolerance, indicating a large population at risk of developing diabetes. Despite accounting for nearly one-fifth of the global diabetes population, the region contributes only about 1% of total global diabetes-related healthcare expenditure, reflecting a substantial mismatch between disease burden and resource allocation (IDF, 2025).

In Bangladesh, the scenario reflects this regional and global trend, with a rapidly increasing number of affected individuals. According to the IDF Diabetes Atlas 2025, Bangladesh ranks seventh globally in the number of adults with diabetes, with approximately 13.9 million people affected in 2024 and about 5.4 million remaining undiagnosed. The age-standardized prevalence in adults aged 20–79 years is estimated at 13.2%, and the total number of people living with diabetes is projected to rise to 23.1 million by 2050 (IDF, 2025). These findings indicate that diabetes, particularly type 2 diabetes, is an increasingly serious public health challenge in Bangladesh and will continue to place substantial pressure on the healthcare system in the coming decades.

1.2.2 Diagnostic criteria for diabetes

Most international guidelines follow the diagnostic criteria for diabetes proposed by the World Health Organization (WHO) and the International Diabetes Federation (IDF). These criteria are primarily based on plasma glucose measurements. In addition, the American Diabetes Association (ADA) includes glycated haemoglobin (HbA1c) as a diagnostic tool for both diabetes and prediabetes. WHO also endorses the use of HbA1c $\geq 6.5\%$ for the diagnosis of diabetes; however, it does not recommend HbA1c for identifying intermediate hyperglycaemia, largely due to concerns regarding the availability and standardization of HbA1c testing across different regions (WHO, 2006).

Currently, WHO and IDF recommend the use of the 75-gram oral glucose tolerance test (OGTT), with measurement of both fasting plasma glucose and 2-hour post-load glucose, for the detection of impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and diabetes. This approach remains an important standard method for diagnosing abnormalities in glucose metabolism. Nevertheless, emerging evidence suggests that measurement of plasma glucose at 1 hour during the OGTT may offer greater sensitivity in detecting early dysglycaemia and identifying individuals at risk of developing diabetes (Bergman et al., 2024).

In clinical practice, the diagnosis of type 2 diabetes can also be established based on a single random plasma glucose value ≥ 11.1 mmol/L in the presence of classic symptoms of hyperglycaemia, such as polyuria, polydipsia, and unexplained weight loss. In such

cases, confirmatory testing is not required, as the combination of symptoms and markedly elevated glucose levels is considered diagnostic (IDF, 2025).

1.2.3 Regulation of glucose homeostasis

1.2.3.1 Pancreatic regulation of glucose homeostasis

The pancreas plays a central role in maintaining glucose homeostasis through the coordinated secretion of several hormones from the islets of Langerhans. These endocrine islets constitute only 1–2% of the pancreatic tissue and contain different hormone-producing cells, including insulin-secreting β -cells, glucagon-producing α -cells, somatostatin-producing δ -cells, pancreatic polypeptide-producing γ -cells, and ghrelin-producing ϵ -cells. Among these hormones, insulin and glucagon are the principal regulators of blood glucose balance. Insulin lowers blood glucose levels by promoting glucose uptake and storage, whereas glucagon increases blood glucose levels through hepatic glycogenolysis and gluconeogenesis. The balanced and opposing actions of these hormones maintain blood glucose within a narrow physiological range of approximately 4–6 mM (Brissova et al., 2005; Katsuura et al., 2002).

During fasting or in between meals, reduced blood glucose stimulates α -cells to release glucagon, which enhances hepatic glycogen breakdown and glucose production to maintain energy supply. Conversely, after food intake, elevated blood glucose stimulates β -cells to secrete insulin. Insulin facilitates glucose uptake into insulin-sensitive tissues such as skeletal muscle and adipose tissue and promotes glycogenesis, lipogenesis, and protein synthesis, thereby exerting anabolic effects on metabolism (Göke, 2008).

Insulin secretion from pancreatic β -cells is regulated through a highly coordinated glucose-sensing mechanism. Following a meal, circulating glucose enters β -cells via the glucose transporters. Intracellular glucose metabolism through glycolysis increases the ATP/ADP ratio, leading to closure of ATP-sensitive potassium (KATP) channels. The resulting membrane depolarization opens voltage-dependent calcium channels, causing an influx of calcium ions that triggers exocytosis of insulin-containing granules. The secretory process occurs in two phases: an initial rapid phase involving release of pre-docked insulin granules, followed by a slower sustained phase dependent

on recruitment of additional granules. Vesicle fusion and insulin exocytosis are mediated by SNARE proteins, including SNAP-25, syntaxin-1, and synaptobrevin, together with calcium-sensing synaptotagmin proteins (Henquin, 2000; Komatsu et al., 2013).

In addition to glucose, several external modulators influence pancreatic hormone secretion. Glucose metabolism activates adenylate cyclase-mediated cAMP signaling pathways that amplify insulin secretion through protein kinase A (PKA) and Epac2 activation. Gut-derived incretin hormones, particularly glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP), further potentiate insulin release following nutrient ingestion. These hormones bind to their respective G-protein-coupled receptors on β -cells, stimulating cAMP production and enhancing intracellular calcium signaling, thereby augmenting glucose-stimulated insulin secretion (Röder et al., 2016).

1.2.3.2 Neuroendocrine regulation of glucose homeostasis

The maintenance of glucose homeostasis depends not only on the intrinsic glucose-sensing ability of pancreatic islet cells but also on complex neuroendocrine networks involving the central nervous system and autonomic pathways. The hypothalamus and brainstem function as major neuroendocrine centers that integrate hormonal, nutritional, and neural signals to regulate feeding behavior, autonomic tone, and endocrine responses (Gallardo et al., 2025). Specialized hypothalamic glucose-sensing neurons detect fluctuations in circulating glucose levels through mechanisms analogous to pancreatic β -cells and initiate adaptive autonomic and behavioral responses to preserve metabolic balance. Among the hypothalamic neuronal populations, proopiomelanocortin (POMC) neurons are glucose-excited and promote insulin sensitivity and peripheral glucose uptake during nutrient abundance, whereas agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons are activated during hypoglycemia and stimulate hepatic glucose production to restore normoglycemia (Berglund et al., 2012; Krashes et al., 2011; Parton et al., 2007; Routh et al., 2014).

The paraventricular nucleus (PVN) of the hypothalamus acts as a major autonomic effector by modulating sympathetic and parasympathetic outflow to regulate hepatic glucose production, glycogen storage, and systemic glucose availability (Zsombok et

al., 2024). Hypothalamic nuclei, including the arcuate nucleus (ARC), lateral hypothalamus (LH), and PVN, communicate extensively with brainstem autonomic centers such as the nucleus tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMV), and intermediolateral nucleus of the spinal cord (IML). Through these interconnected pathways, the hypothalamus coordinates pancreatic endocrine secretion, hepatic glucose metabolism, adipose tissue function, and thermogenesis, thereby forming a central-peripheral regulatory loop for glucose homeostasis.

The autonomic nervous system further regulates pancreatic hormone secretion through both sympathetic and parasympathetic innervation. Sympathetic stimulation enhances glucagon release from pancreatic α -cells while suppressing insulin secretion from β -cells, particularly during stress or hypoglycemia. In contrast, parasympathetic vagal stimulation promotes insulin secretion, especially during the cephalic phase of feeding, when sensory stimuli such as smell, taste, and sight prepare the body for nutrient intake (Roh et al., 2016; Rosario et al., 2016). Additionally, gut-derived hormones including glucagon-like peptide-1 (GLP-1), glucose-dependent insulinitropic peptide (GIP), cholecystikinin (CCK), and serotonin interact with vagal pathways to modulate pancreatic endocrine function and glucose metabolism (Owyang & Heldsinger, 2011). Thus, neuroendocrine regulation of glucose homeostasis represents an integrated network linking the hypothalamus, brainstem, autonomic nervous system, gut, liver, and pancreatic islets to maintain systemic metabolic stability.

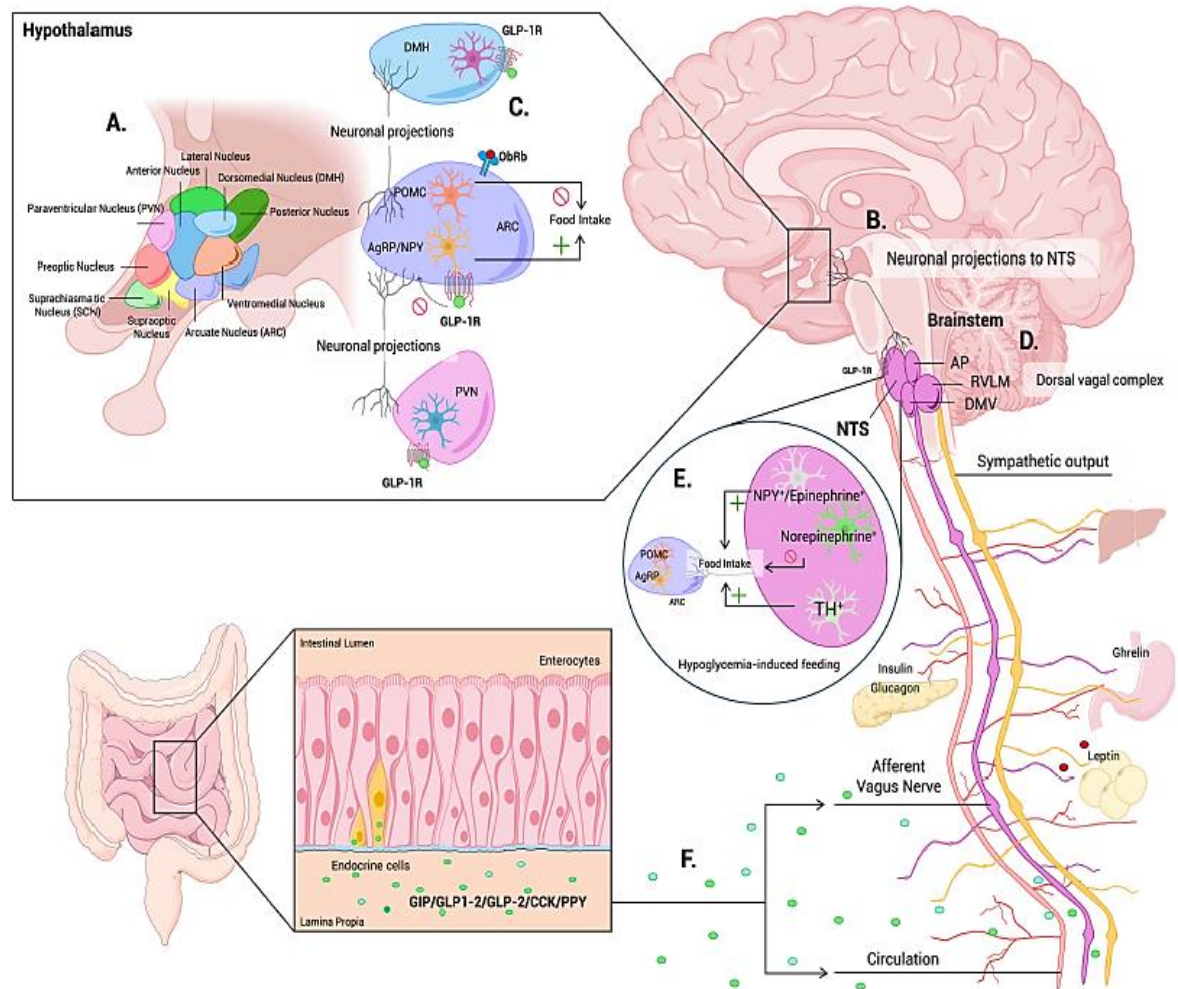


Figure 1.1: Neuroendocrine networks integrating hypothalamic and brainstem mechanisms of glucose homeostasis. Modified from Gallardo et al. (2025)

Figure 1.1 summarizes the central neuroendocrine pathways involved in glucose homeostasis. **(A)** illustrates the major hypothalamic nuclei, including the arcuate nucleus (ARC), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), lateral hypothalamus (LH), and paraventricular nucleus (PVN), which integrate hormonal and nutrient-derived signals. **(B)** shows neuronal projections linking hypothalamic centers with the brainstem, particularly the nucleus tractus solitarius (NTS). **(C)** depicts glucose-sensitive neuronal populations within the hypothalamus, including AgRP/NPY and POMC neurons, which regulate food intake and energy balance in response to metabolic signals. **(D)** illustrates the dorsal vagal complex of the brainstem, comprising the NTS, dorsal motor nucleus of the vagus (DMV), area postrema (AP), and rostral ventrolateral medulla (RVLM), which coordinate autonomic regulation of glucose metabolism. **(E)** highlights neuronal circuits within the NTS

involved in hypoglycemia-induced feeding responses and glucose counter-regulation. **(F)** represents the gut–brain axis, where enteroendocrine hormones including GLP-1, GIP, CCK, and PYY communicate nutrient-derived signals to the central nervous system through vagal afferents and the circulation. Together, these interconnected hypothalamic, brainstem, and gut-derived pathways regulate hepatic glucose production, glycogen metabolism, pancreatic hormone secretion, appetite, systemic energy balance, and overall glucose homeostasis.

1.2.4 Pathogenesis of type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a progressive metabolic disorder characterized by chronic hyperglycemia arising from a complex interplay of insulin resistance, pancreatic β -cell dysfunction, and multiple organ-level defects. Its pathogenesis is multifactorial, involving peripheral tissues, the liver, adipose tissue, the gut, and the central nervous system.

Insulin Resistance: Muscle and Liver

Insulin resistance in skeletal muscle and the liver represents the central pathophysiological defect in T2DM. Under normal conditions, skeletal muscle accounts for approximately 80% of insulin-mediated whole-body glucose uptake; however, in T2DM, this capacity is markedly impaired due to defects in glucose transport, glucose phosphorylation, and glycogen synthesis. At the cellular level, these defects are linked to impaired insulin receptor tyrosine kinase activity, reduced insulin receptor substrate-1 (*IRS-1*) tyrosine phosphorylation, and diminished activation of PI3K/Akt signaling, which together disrupt GLUT4 translocation to the cell membrane. Concurrently, the liver exhibits resistance to insulin's suppressive effect on endogenous glucose production (EGP), resulting in excessive hepatic glucose output, the primary determinant of elevated fasting plasma glucose in T2DM. This is driven largely by accelerated gluconeogenesis, fueled by increased delivery of gluconeogenic precursors such as lactate, alanine, and glycerol, along with elevated free fatty acid (FFA) oxidation and hyperglucagonemia. Dysregulation of key transcription factors including PGC-1 α , HNF-4 α , and FoxO further amplify gluconeogenic gene expression, upregulating rate-limiting enzymes such as PEPCK and G6Pase (Guo et al., 2023; Solis-Herrera et al., 2021; Yaribeygi et al., 2019).

Inflammation

A central feature of T2DM is insulin resistance, in which target tissues - particularly the liver, skeletal muscle, and adipose tissue - exhibit reduced sensitivity to insulin. Chronic low-grade inflammation plays a pivotal role in its development. Elevated pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 impair insulin signaling by promoting serine phosphorylation of IRS-1, thereby disrupting downstream signaling cascades. Activation of NF- κ B and MAPK pathways by high glucose further amplifies this inflammatory state, contributing to pancreatic β -cell dysfunction and peripheral insulin resistance (Donath & Shoelson, 2011; Guo et al., 2023; Panahi et al., 2018).

β -Cell Dysfunction and the Progressive Nature of T2DM

While insulin resistance is an early and persistent feature of T2DM, overt hyperglycemia emerges only when pancreatic β -cells can no longer compensate adequately. In the natural history of T2DM, β -cell secretory capacity progressively declines, with evidence suggesting that up to 80% of β -cell function may already be lost by the time of clinical diagnosis. This deterioration is accelerated by "glucotoxicity," "lipotoxicity," and the recently expanded concept of "nutri-toxicity," wherein chronic overexposure of β -cells to glucose, lipids, and amino acids induces oxidative stress, endoplasmic reticulum dysfunction, mitochondrial impairment, and ultimately β -cell de-differentiation and apoptosis. Autophagy also plays a protective role in this context; impaired autophagic clearance of damaged organelles and toxic protein aggregates within β -cells contributes to cellular dysfunction and reduced insulin secretion (Guo et al., 2023; Jouvett & Estall, 2017; Röder et al., 2016; Solis-Herrera et al., 2021).

The Role of Free Fatty Acids and Adipose Tissue

Adipose tissue dysfunction is a critical contributor to T2DM pathogenesis, particularly in obese individuals who constitute more than 80% of the diabetic population. Insulin resistance in adipocytes leads to unrestrained lipolysis and chronically elevated plasma FFA concentrations. Elevated FFAs impair insulin signaling in muscle and liver through activation of protein kinase C (PKC), serine phosphorylation of IRS-1, and inhibition of PI3K activity. In the liver, FFAs stimulate gluconeogenesis by activating pyruvate

carboxylase and PEPCK, while simultaneously inducing hepatic insulin resistance. Additionally, visceral adipose tissue releases pro-inflammatory adipokines including TNF- α , IL-6, resistin, and leptin, that further promote systemic insulin resistance and chronic low-grade inflammation. Elevated TNF- α inhibits peroxisome-proliferator-activated receptor γ (PPAR γ) function and activates JNK signaling, compounding the disruption of the insulin signaling cascade (Guo et al., 2023; Han et al., 2016; Saltiel, 2015; Solis-Herrera et al., 2021).

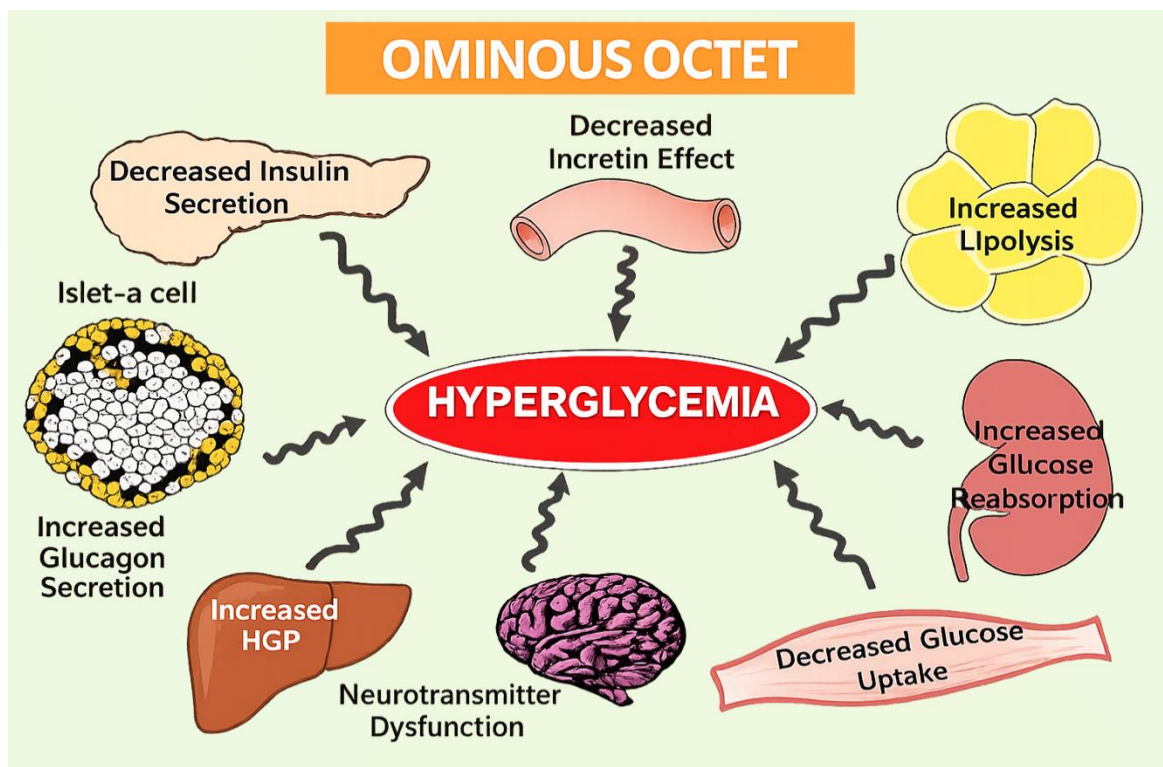


Figure 1.2: Ominous Octet

Overview of the eight key mechanisms responsible for the development of hyperglycemia in individuals with type 2 diabetes. Adapted from Solis-Herrera et al. (2021)

The "Ominous Octet": A Multiorgan Framework

The complexity of T2DM pathogenesis is best encapsulated by the "Ominous Octet" framework (Figure 1.2), which identifies eight principal organ-level mechanisms contributing to hyperglycemia: decreased insulin secretion, increased glucagon secretion, reduced incretin effect, augmented hepatic glucose production, increased lipolysis, impaired renal glucose excretion, decreased peripheral glucose uptake, and neurotransmitter dysfunction. Particularly noteworthy is the "incretin defect," defined

as inadequate postprandial secretion of or response to glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP), which contributes to both deficient insulin and excessive glucagon secretion. Furthermore, hypothalamic insulin resistance impairs the central nervous system's ability to suppress EGP, adding another dimension to the dysregulation of glucose homeostasis. The kidney also contributes pathologically: enhanced tubular glucose reabsorption via upregulated SGLT2 activity prevents appropriate glycosuria despite hyperglycemia, perpetuating the diabetic state (DeFronzo, 2009; Nauck et al., 1986; Solis-Herrera et al., 2021).

Gut Microbiota and Inflammation

Emerging evidence implicates gut dysbiosis in the pathogenesis of T2DM. Reductions in butyrate-producing bacteria such as *Faecalibacterium prausnitzii* and *Roseburia*, combined with increases in opportunistic pathogens, disrupt intestinal barrier integrity and allow lipopolysaccharides (LPS) to enter systemic circulation, triggering chronic low-grade inflammation via TLR4 activation and promoting insulin resistance. Disrupted microbial metabolites including short-chain fatty acids (SCFAs), bile acids, and branched-chain amino acids, further impair glucose homeostasis and β -cell function. Importantly, commonly used antidiabetic agents such as metformin exert part of their therapeutic effect by favorably reshaping the gut microbiome (Guo et al., 2023; Solis-Herrera et al., 2021; J. Wang et al., 2012).

Mitochondrial Dysfunction

Mitochondrial dysfunction represents an additional and increasingly recognized dimension of T2DM pathogenesis. In both skeletal muscle and pancreatic β -cells, impaired oxidative phosphorylation, reduced mitochondrial biogenesis (linked to decreased PGC-1 α expression), and abnormal mitochondrial fusion-fission dynamics lead to excessive reactive oxygen species (ROS) production, oxidative stress, and impaired energy metabolism. These mitochondrial defects further compromise insulin secretion and peripheral glucose utilization, reinforcing the vicious cycle of hyperglycemia and insulin resistance (Solis-Herrera et al., 2021; Wada & Nakatsuka, 2016).

1.2.5 Risk factors of T2DM

Diabetes mellitus is influenced by a wide range of risk factors that vary across its different types. Understanding these risk factors is essential for early identification, prevention, and targeted management of the disease (Banday et al., 2020; Guo et al., 2023; Solis-Herrera et al., 2021).

Genetic Predisposition and Family History

A positive family history of diabetes is one of the most consistent risk factors across all diabetes types. In T2DM, individuals with a first-degree relative with the condition carry a significantly elevated risk. The frequent clustering of T2DM within certain racial and ethnic groups including Native Americans, African Americans, Asian Americans, and Hispanic/Latino populations, further highlights the role of hereditary factors (Banday et al., 2020).

Obesity and Overweight

Obesity is one of the strongest and most well-established modifiable risk factors for T2DM. More than 80% of individuals with T2DM are overweight or obese. Not only the total amount but also the distribution of body fat determines risk; visceral or abdominal obesity is more strongly associated with T2DM than peripheral or subcutaneous fat accumulation. Obesity is similarly a major risk factor for GDM, and its prevalence among pregnant women has contributed to rising GDM rates globally.

Age

Advancing age is a well-recognized risk factor for T2DM. The risk increases progressively with age, with older adults being disproportionately affected. This is attributed to age-related changes in body composition, reduced physical activity, and declining metabolic reserve (Banday et al., 2020).

Physical Inactivity and Sedentary Lifestyle

A sedentary lifestyle and physical inactivity are established risk factors for T2DM. Lack of regular physical activity is closely associated with weight gain, impaired metabolic

function, and reduced insulin sensitivity in peripheral tissues, all of which contribute to an elevated risk of developing T2DM (Banday et al., 2020).

Impaired Glucose Tolerance and Impaired Fasting Glucose

Individuals with impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), collectively referred to as prediabetes, are at significantly elevated risk of progressing to overt T2DM (Banday et al., 2020).

Dietary Habits

Unhealthy dietary patterns, including excessive caloric intake, high consumption of refined carbohydrates, saturated fats, and fructose-rich foods, are recognized contributors to T2DM risk. Such dietary habits are strongly associated with weight gain, dyslipidemia, and metabolic dysregulation, all of which increase susceptibility to the disease.

Gestational History and Hormonal Factors

Women with a previous history of GDM carry a substantially increased lifetime risk of developing T2DM and require regular long-term screening for glucose intolerance (Banday et al., 2020). Polycystic ovary syndrome (PCOS) is another significant hormonal risk factor, commonly associated with insulin resistance and abnormal glucose metabolism, predisposing affected women to both GDM and T2DM. Additionally, men and women differ in their risk profiles for glucose intolerance, possibly related to the influence of sex hormones on glucose metabolism.

Hypertension and Dyslipidemia

Hypertension and dyslipidemia, characterized by elevated triglycerides and low levels of high-density lipoprotein (HDL) cholesterol, are frequently observed alongside T2DM and are recognized components of the metabolic syndrome. Their co-occurrence significantly elevates the risk of developing T2DM and its associated cardiovascular complications.

Drug and Chemical Exposure

Certain pharmacological agents are known to increase the risk of diabetes, either by impairing insulin secretion or by reducing tissue sensitivity to insulin. These include glucocorticoids, thiazide diuretics, atypical antipsychotics, certain protease inhibitors, and immune checkpoint inhibitors used in cancer immunotherapy. Risk is particularly elevated in individuals with pre-existing metabolic vulnerabilities.

Gut Microbiota Imbalance

Emerging evidence supports gut dysbiosis as a risk factor for T2DM. An imbalance in intestinal microbial composition, characterized by reduced diversity and decreased abundance of beneficial bacteria, has been consistently observed in individuals with T2DM (Guo et al., 2023; Solis-Herrera et al., 2021). Disruptions in microbial metabolites and intestinal barrier integrity associated with dysbiosis have been linked to impaired glucose tolerance and increased susceptibility to T2DM.

Early Childhood Environments

Adverse childhood experiences (ACEs) are increasingly recognized as significant risk factors for the development of type 2 diabetes mellitus in adulthood. Exposure to early-life stressors such as abuse, neglect, or household dysfunction can lead to long-term dysregulation of the hypothalamic–pituitary–adrenal axis, resulting in chronic elevation of cortisol levels. This persistent stress response contributes to insulin resistance, inflammation, and metabolic imbalance. In addition, individuals with higher ACE scores are more likely to engage in unhealthy behaviors, including poor diet, physical inactivity, and substance use, which further increase diabetes risk. Together, these behavioral and biological pathways provide a strong epidemiological link between ACE exposure and the development of T2DM (Boullier & Blair, 2018; Felitti et al., 1998; Miller et al., 2011).

1.2.6 Adverse Childhood Experience (ACE)

1.2.6.1 Overview of adverse childhood experiences

Adverse childhood experiences (ACEs), also referred to as childhood maltreatment (CM), encompass a range of potentially traumatic or stress-inducing events occurring before the age of 18 years. These experiences broadly include various forms of abuse, neglect, and household dysfunction. The major categories of ACEs comprise physical, emotional, and sexual abuse; physical and emotional neglect; and household challenges such as domestic violence, parental substance abuse, mental illness, parental separation or divorce, and incarceration of a family member (**Figure 1.3**) (Finkelhor et al., 2015).

The concept of ACEs gained significant recognition in public health following the landmark CDC–Kaiser Permanente ACE Study conducted by Vincent J. Felitti and colleagues, which demonstrated a graded association between cumulative childhood adversity and adverse health outcomes in adulthood. In this original ACE study, childhood adversity was common, with more than half of participants reporting exposure to at least one ACE category and approximately one-quarter reporting exposure to two or more categories (Felitti et al., 1998). Evidence from South Asian countries indicates that children are frequently subjected to psychological aggression and physical punishment under the guise of discipline. In India, approximately 81% of parents reportedly threaten abandonment or sending children away, while nearly 75% use physical violence as a disciplinary strategy (Pednekar, 2015; Smart, 2018). Comparable trends have been observed in neighbouring countries, with 31–70% of children in rural Sri Lanka exposed to abusive parental behaviours (Siriwardhana, 2015), and around 80% of children in Pakistan experiencing parental abuse (Bureau of Statistics Punjab, Planning & Development Department, Government of the Punjab, 2016).

The situation in Bangladesh aligns with this broader regional pattern, although research in this context remains relatively limited. The Child Wellbeing Survey 2016 reported that 82% of children aged 2–14 years in Bangladesh experienced psychological aggression or physical punishment at home. Furthermore, studies by Haque et al. (2019, 2021) documented widespread exposure to both physical and psychological abuse among Bangladeshi children. Collectively, these findings suggest that ACEs represent

a significant yet underexplored public health issue in Bangladesh, with potential implications for long-term disease risk.

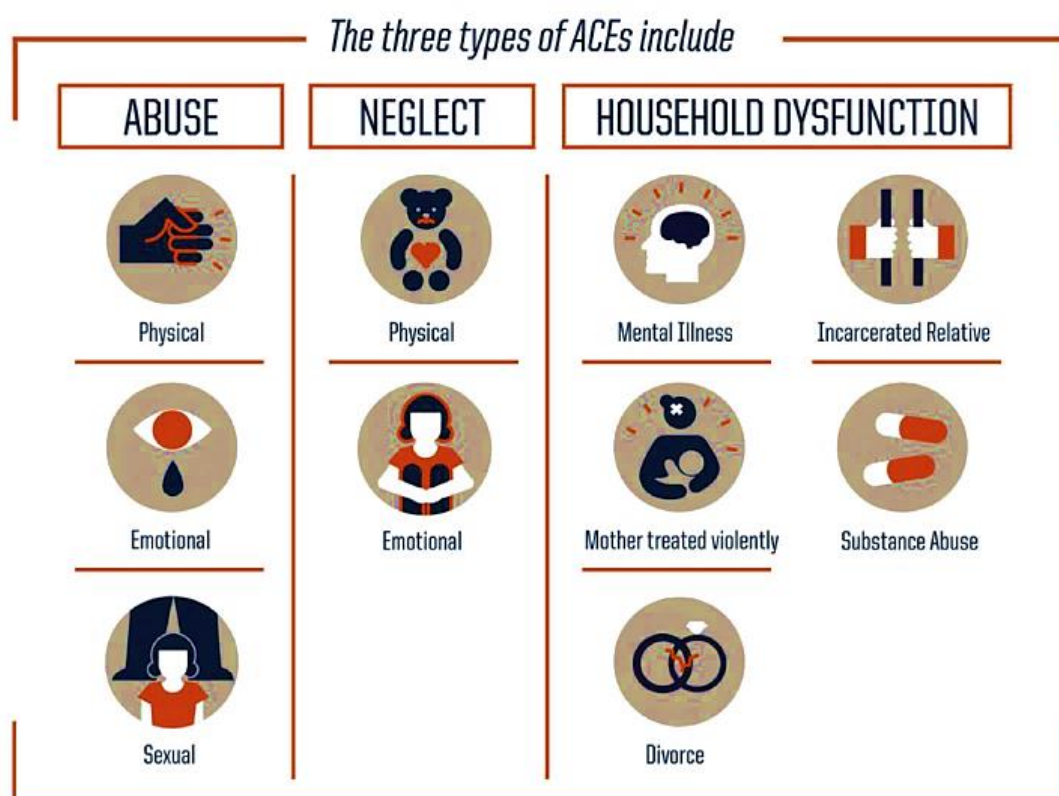


Figure 1.3: The 10 categories of ACE. Adapted from Boullier & Blair, (2018)

1.2.6.2 Health effects of ACEs

The ACE pyramid model, developed by Centers for Disease Control and Prevention, illustrates the progressive relationship between early-life adversity and adverse health outcomes across the lifespan, ultimately leading to increased risk of premature mortality. The model conceptualizes how exposure to ACEs disrupts neurodevelopment, leading to social, emotional, and cognitive impairments, adoption of health-risk behaviours, and subsequent development of chronic diseases (**Figure 1.4**) (Boullier & Blair, 2018).

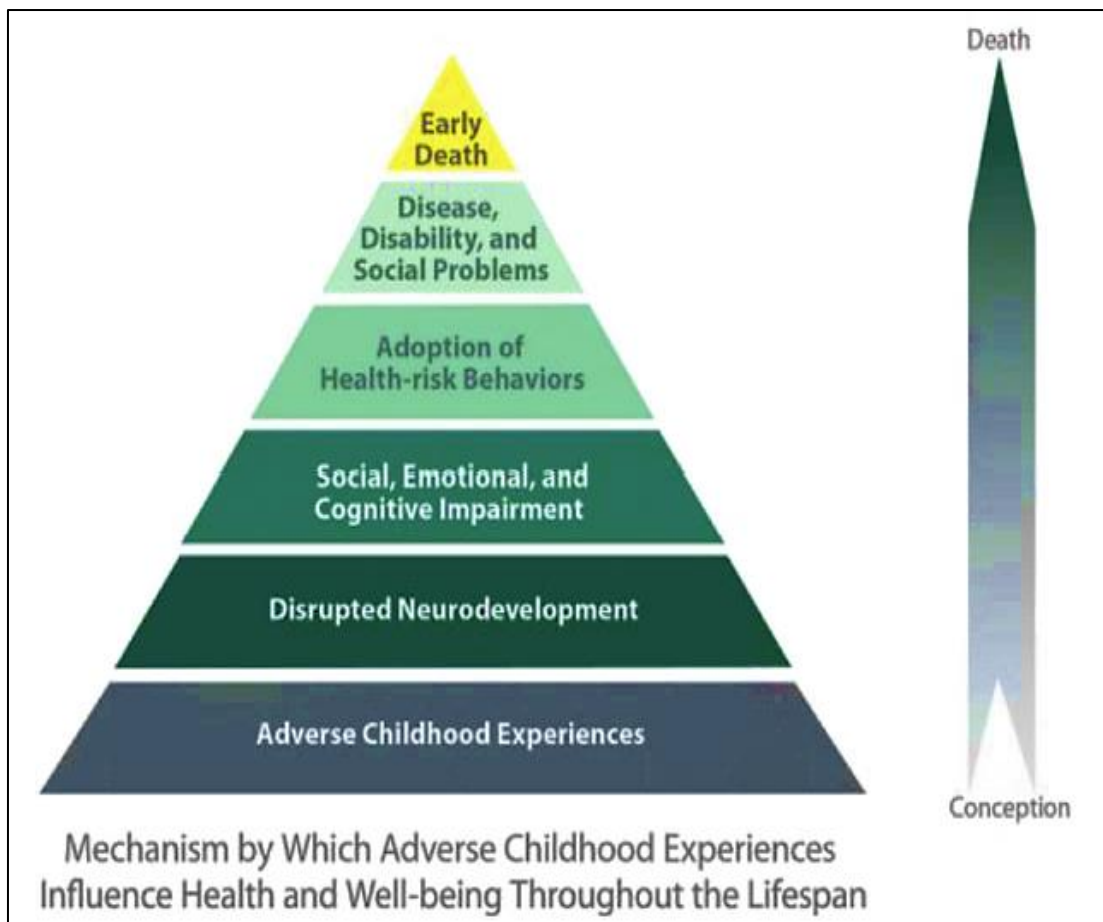


Figure 1.4: The ACE pyramid model. Adapted from Boullier & Blair, (2018)

Accumulating evidence indicates that exposure to adversity and toxic stress during critical developmental periods can profoundly influence biological systems. Early-life stress has been shown to alter molecular and genetic regulation, affecting the development and function of neurological, immune, and endocrine systems (Boullier & Blair, 2018).

The Biological Embedding of Childhood Adversity model further explains how early-life stress becomes biologically integrated, influencing long-term health. According to this framework, childhood maltreatment and family dysfunction impair normal neurodevelopment and lead to persistent alterations in emotional, behavioural, and physiological regulation (Felitti et al., 1998; Miller et al., 2011). Specifically, early adversity is proposed to “program” immune cells toward a pro-inflammatory phenotype, characterized by exaggerated cytokine responses and reduced sensitivity to

regulatory hormonal signals. This is often accompanied by dysregulation of stress-related hormonal pathways, contributing to impaired self-regulation and unhealthy lifestyle behaviours. Empirical evidence supports the association between ACE exposure and increased likelihood of risky behaviours, such as illicit drug use and high-risk sexual practices, as well as a higher burden of chronic diseases, including type 2 diabetes mellitus in adulthood (Campbell et al., 2018; Felitti et al., 1998; Huffhines et al., 2016; S. Zhu et al., 2022).

From a physiological perspective, the body responds to stress through the process of allostasis, which enables adaptation to environmental challenges in order to maintain stability. During acute stress, activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis leads to increased heart rate, pupil dilation, and cortisol release, facilitating immediate energy mobilization. While this response is adaptive in the short term, repeated or chronic activation due to persistent stress results in allostatic load, representing the cumulative “wear and tear” on physiological systems. Elevated allostatic load has been linked to metabolic dysregulation, immune dysfunction, and increased vulnerability to chronic diseases, thereby providing a mechanistic link between ACEs and long-term adverse health outcomes (Boullier & Blair, 2018; Huffhines et al., 2016).

1.2.6.3 ACE, the HPA Axis and Disease

Physiology of the HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis is a tightly regulated neuroendocrine system that plays a central role in the body’s response to both acute and chronic stress. In response to physiological or psychological stressors, the HPA axis is activated through the release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH stimulates the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH), which in turn triggers the adrenal cortex to release cortisol. This hormonal cascade facilitates a range of physiological adaptations necessary for coping with stress. Once the stressor is resolved, cortisol exerts negative feedback on the hypothalamus and pituitary gland, suppressing further release of CRH and ACTH to restore homeostasis. Activation of the HPA axis is also accompanied by stimulation of the sympathetic nervous system, leading to the release of catecholamines and pro-

inflammatory mediators such as interleukin-6, thereby initiating a cytokine response (Joseph & Golden, 2017).

Cortisol secretion follows a well-defined circadian rhythm, characterized by a rapid increase after awakening, peaking within 30–45 minutes, and gradually declining throughout the day. However, accurate assessment of glucocorticoid exposure in epidemiological studies has historically been challenging due to this circadian variability, pulsatile hormone secretion, and limitations of traditional measurement approaches such as 24-hour urinary cortisol (Joseph & Golden, 2017).

HPA axis dysregulation under chronic stress

Chronic psychological stress, defined as a sustained state in which environmental demands exceed an individual's adaptive capacity, can disrupt the normal regulatory mechanisms of the HPA axis. Prolonged activation of this system may impair the negative feedback loop, resulting in persistent elevations of cortisol, catecholamines, and inflammatory markers. This condition, often referred to as subclinical hypercortisolism, contributes to widespread physiological alterations.

One of the key metabolic consequences of sustained cortisol elevation is its effect on adipose tissue and glucose metabolism. Cortisol promotes differentiation and proliferation of adipocytes, preferentially increasing visceral fat deposition due to a higher density of glucocorticoid receptors in visceral adipose tissue compared to subcutaneous fat. Additionally, cortisol stimulates lipolysis and increases circulating free fatty acids, contributing to insulin resistance and impaired glucose metabolism. These metabolic disturbances represent critical precursors in the development of T2DM (Huffhines et al., 2016; Joseph & Golden, 2017).

Link between ACEs, HPA axis, and diabetes

Exposure to adverse childhood experiences is strongly associated with chronic activation and dysregulation of the HPA axis. Early-life stress may recalibrate the stress-response system, leading to long-term alterations in cortisol dynamics and heightened physiological reactivity to stress. This dysregulation provides a plausible biological mechanism linking ACEs with increased risk of depression, metabolic syndrome, and

type 2 diabetes (**Figure 1.5**). Therefore, HPA axis dysfunction is increasingly recognized as a key pathway connecting early-life adversity with long-term metabolic and psychological health outcomes (Huffhines et al., 2016; Joseph & Golden, 2017).

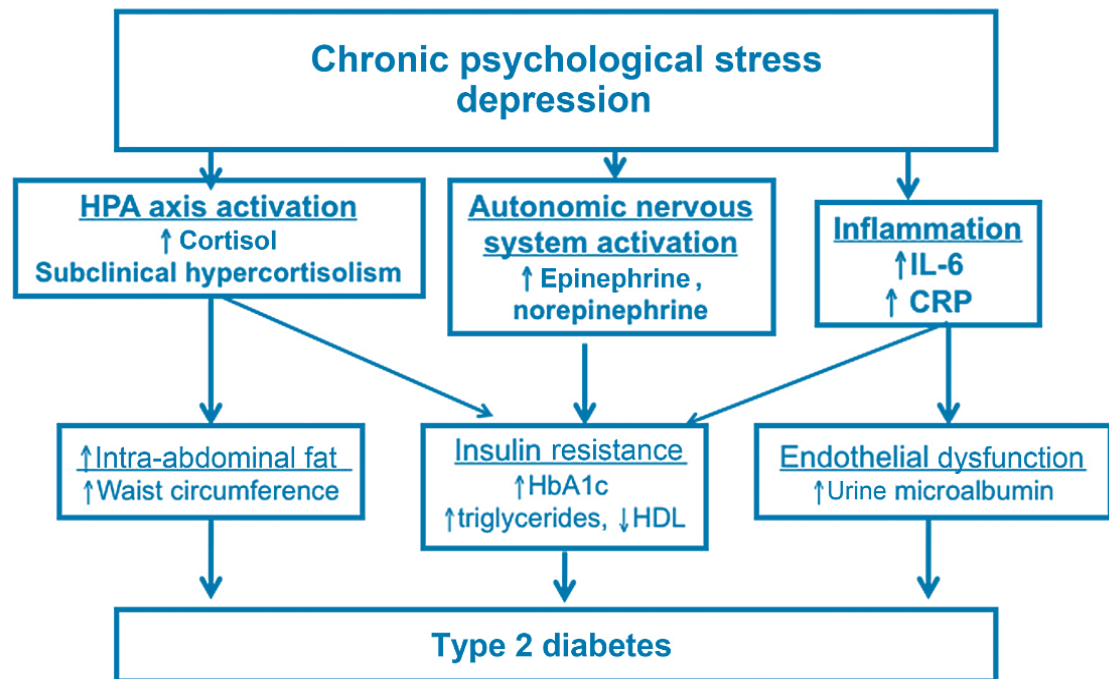


Figure 1.5: Hypothesized relationships among chronic stress, depression, cortisol, and diabetes. Adapted from Joseph & Golden, (2017)

While ACEs represent an important environmental influence on T2DM susceptibility, disease development also occurs within a background of inherited genetic susceptibility. Understanding the genetic architecture of T2DM is therefore important for determining how genetic and environmentally responsive mechanisms may jointly contribute to disease pathogenesis.

.1.2.7 Genetic Studies of T2DM

1.2.7.1 Genetic variant studies of T2DM worldwide

Type 2 diabetes mellitus (T2DM) is a multifactorial and polygenic disorder resulting from the combined influence of environmental, behavioral, and genetic factors.

Although rapid increases in T2DM prevalence are largely driven by lifestyle, ageing, urbanization, obesity, and other non-genetic exposures, genetic susceptibility also contributes importantly to individual disease risk. Evidence from family and twin studies supports a heritable component, but common T2DM is not caused by a single major gene; rather, risk is determined by many variants of small effect interacting with environmental conditions. GWAS have now identified over 500 independent loci associated with T2DM, growing from just three pre-GWAS candidates to more than 534 distinct genomic intervals by the end of 2022 (Kreienkamp et al., 2023; Prasad & Groop, 2015). A landmark expansion of this knowledge came from Mahajan et al. (2018), who combined data from 898,130 European-descent individuals across 32 GWAS with high-density imputation using the Haplotype Reference Consortium panel of 64,976 whole-genome-sequenced haplotypes. This single study identified 243 associated loci, 135 of which were newly implicated, comprising 403 distinct association signals, representing more than a doubling of the previously known T2DM risk architecture. Most of these variants are located in non-coding regions and are enriched in regulatory elements such as enhancers and promoters, particularly in pancreatic islets, adipose tissue, liver, and skeletal muscle. This indicates that genetic risk is largely mediated through altered gene regulation rather than direct protein-coding changes.

Several well-established loci illustrate the major biological pathways involved in T2DM pathogenesis. Variants in *TCF7L2* primarily influence insulin secretion, while *KCNJ11*, *SLC30A8*, *HNF1A*, *HNF1B*, *CDKAL1*, *CDKN2A/B*, and *HHEX* are involved in β -cell development and function. In contrast, *PPARG*, *IRS1*, *KLF14*, and *GRB14* are associated with insulin resistance. Obesity-related loci such as *FTO* and *MC4R* act through adiposity, whereas *GCKR*, *PNPLA3*, and *TM6SF2* highlight the role of hepatic lipid metabolism. Together, these genes demonstrate that T2DM arises from multiple interacting mechanisms, including impaired insulin secretion, insulin resistance, adiposity, and metabolic dysregulation (Kreienkamp et al., 2023; Mahajan et al., 2018; Prasad & Groop, 2015).

1.2.7.2 Genetic pathophysiology of T2DM

A major conceptual advance in T2DM genetics has been the grouping of associated loci into mechanistic clusters, which provide a more integrated understanding of disease heterogeneity. Using soft clustering approaches, Udler et al. (2018) identified five biologically meaningful clusters representing distinct pathways leading to hyperglycemia (**Figure 1.6**).

Beta Cell Cluster (Insulin Deficiency via Impaired Secretion)

The Beta Cell cluster represents T2DM driven by impaired pancreatic β -cell insulin secretion. It is characterized by reduced corrected insulin response, decreased disposition index, and elevated proinsulin levels, indicating defective insulin processing alongside reduced secretion. Key loci include *TCF7L2*, *MTNR1B*, *HNF1A*, and *SLC30A8*, all of which play critical roles in β -cell biology. Variants in this cluster are significantly enriched in pancreatic islet regulatory elements, supporting an islet-centered mechanism. Clinically, this cluster has been associated with increased risk of cardiovascular complications, reflecting the systemic impact of chronic insulin deficiency.

Proinsulin Cluster (Insulin Deficiency via Impaired Processing)

The Proinsulin cluster reflects a distinct form of β -cell dysfunction related to abnormal proinsulin biosynthesis and processing. Unlike the Beta Cell cluster, it is characterized by decreased fasting proinsulin levels, suggesting upstream defects in insulin production rather than impaired cleavage. Major loci include *ARAP1* and *SPRY2*, which influence insulin gene expression and β -cell function. This cluster also shows enrichment in regulatory elements of embryonic stem cells and neural tissues, indicating shared developmental pathways between β -cells and neuronal systems.

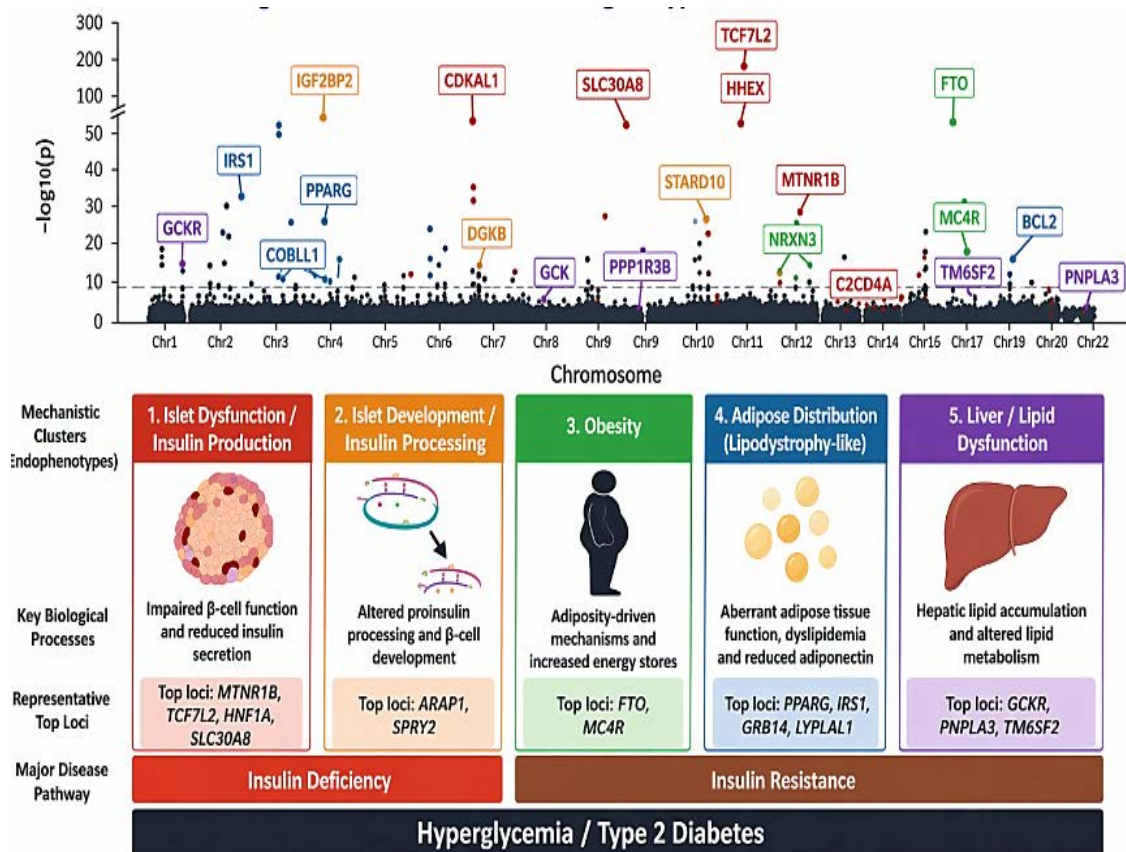


Figure 1.6: Mechanistic clustering of type 2 diabetes genetic Loci
Genome-wide association signals for type 2 diabetes are displayed in a Manhattan plot, where variants are plotted by chromosomal position (x-axis) and $-\log_{10}(p\text{-value})$ of association (y-axis). Each point represents a SNP corresponding to an independent locus included in clustering analyses. Modified from Kreienkamp et al. (2023)

Obesity Cluster (Insulin Resistance via Adiposity)

The Obesity cluster captures T2DM driven primarily by excess adiposity. It is characterized by increased body mass index, waist circumference, and body fat, with insulin resistance mediated through obesity. Key loci include *FTO* and *MC4R*, which regulate energy balance and appetite. Genetic associations in this cluster are markedly attenuated after adjustment for BMI, confirming that their effect on T2DM risk is mediated through adiposity. Variants are enriched in pre-adipose tissue regulatory elements, further supporting their role in adipose tissue biology.

Lipodystrophy Cluster (Insulin Resistance via Fat Distribution)

The Lipodystrophy cluster represents insulin resistance driven by abnormal fat distribution rather than overall obesity. It is characterized by reduced insulin sensitivity, decreased adiponectin and HDL cholesterol, and elevated triglycerides, resembling the metabolic profile of lipodystrophy. Key loci include *PPARG*, *IRS1*, *GRB14*, and *LYPLAL1*, which are involved in adipose tissue function and lipid storage. Notably, this cluster shows sex-specific effects, reflecting differences in fat distribution patterns between males and females. It is also strongly associated with cardiovascular and metabolic complications.

Liver/Lipid Cluster (Insulin Resistance via Hepatic Lipid Metabolism)

The Liver/Lipid cluster highlights the role of hepatic lipid metabolism in T2DM pathogenesis. It is uniquely characterized by decreased circulating triglycerides, reflecting increased lipid storage in the liver rather than systemic dyslipidemia. Key loci include *GCKR*, *PNPLA3*, and *TM6SF2*, which are strongly associated with non-alcoholic fatty liver disease. This cluster demonstrates that altered hepatic lipid handling can independently contribute to insulin resistance and T2DM development. Interestingly, it shows a distinct clinical profile, including differential associations with cardiovascular and renal outcomes.

1.2.7.3 Genetic studies of T2DM in Bangladesh

Bangladesh has a rapidly increasing burden of T2DM, and emerging genetic studies suggest that disease susceptibility in this population is shaped by multiple variants acting through diverse biological pathways. Several Bangladeshi case-control studies have investigated candidate genes related to insulin secretion, insulin signaling, glucose transport, mitochondrial function, inflammation, stress response, and vascular regulation. Among genes involved in glucose transport, Rashid et al. (2025) reported that the *SLC2A4* gene, which encodes the insulin-responsive glucose transporter GLUT4, may contribute to T2DM susceptibility in Bangladesh. Their study identified four variants through exon screening, including the synonymous SNP rs5435 in exon 4; although allele frequencies did not differ significantly, individuals carrying at least one C allele showed increased risk under a dominant model, and in silico analysis

suggested that this variant may alter mRNA secondary structure and potentially affect GLUT4 function. This is important because GLUT4 is central to insulin-mediated glucose uptake in muscle and adipose tissue, and impaired GLUT4 expression or translocation is a key mechanism of insulin resistance.

Genes involved in pancreatic β -cell function and insulin secretion have also been studied in Bangladesh. *KCNJ11* rs5219, which affects the ATP-sensitive potassium channel involved in glucose-stimulated insulin secretion, was associated with T2DM and related cardiovascular and renal complications in a Noakhali cohort. The TT genotype was more frequent among T2DM, cardiovascular disease, and chronic kidney disease groups, suggesting that this variant may be relevant not only for diabetes susceptibility but also for disease progression and complications (P. Saha et al., 2025). Similarly, *TCF7L2* polymorphisms, particularly rs12255372 and rs7903146, showed significant association with T2DM in Bangladeshi patients, supporting the importance of β -cell dysfunction, Wnt signaling, and incretin-related pathways in the genetic architecture of diabetes (Salaudhin et al., 2023). *CAPN10* rs3842570 has also been associated with T2DM in the Noakhali population, especially under dominant and allelic models, and was linked with anthropometric and metabolic traits such as BMI, blood pressure, cholesterol, and triglycerides (Sultana et al., 2023).

Insulin signaling pathways are another important area of Bangladeshi genetic research. A polymorphism in the microRNA-binding site of the *INSR* gene, rs1366600, was associated with increased T2DM risk, particularly under dominant inheritance, suggesting that post-transcriptional regulation of insulin receptor expression may influence diabetes susceptibility (Parvin et al., 2019). In addition, studies of the renin–angiotensin system showed that non-coding variants in renin and the (pro)renin receptor genes are associated with T2DM, hypertension, and diabetes-associated hypertension, indicating that vascular and metabolic pathways may overlap in Bangladeshi patients (Akther et al., 2021).

Beyond nuclear gene variants, mitochondrial genetic variation has also been implicated. Saha et al. (2021) investigated mitochondrial D-loop hypervariable region I variants, haplogroups, and mtDNA copy number in Bangladeshi individuals and found that G16048A and G16129A appeared protective, while haplogroups A and H were associated with T2DM risk after adjustment. They also observed lower mtDNA copy

number among T2DM patients in the highest copy-number group, supporting the role of mitochondrial dysfunction in diabetes pathogenesis.

Inflammatory and stress-response genes have also been investigated. *GATA3* rs3824662, but not *STAT4* rs10181656, was significantly associated with T2DM risk in Bangladesh, suggesting that immune regulation and T-cell differentiation may contribute to diabetes susceptibility (Huda et al., 2018). Similarly, *HSP70*-hom +2437 T/C polymorphism was associated with T2DM, particularly among female patients, and the study also suggested an interaction between genetic susceptibility and stress, highlighting the importance of gene–environment interaction in diabetes development (Moniruzzaman et al., 2020).

More recently, Mahmood et al. (2025) reported a significant association between the *TCF7L2* rs12255372 polymorphism and T2DM in a Bangladeshi population, where the minor T allele was associated with a substantially elevated risk (OR = 3.29, 95% CI: 1.78–6.05, $p < 0.001$). Both TT and GT genotypes also showed significant associations under a dominant model, further supporting *TCF7L2* rs12255372 as an important genetic risk variant for T2DM in this population. Researchers also found that the risk of T2DM is associated with genetic variation of *FTO* gene (Kar et al., 2023), vitamin D, and vitamin D-binding protein gene (codon 416 & 420) polymorphisms (Rahman et al., 2017) and *KCNJ11* gene rs5219 polymorphism (Saha et al., 2025).

Although these studies provide valuable preliminary evidence of population-specific genetic susceptibility, their findings require replication. Future studies should integrate genome-wide association analysis, epigenetic profiling, environmental exposures, and functional validation to better explain the genetic and molecular basis of T2DM in Bangladesh. In this context, the present study strategically focuses on *TCF7L2* and *SLC30A8* genes, as both are directly implicated in pancreatic β -cell function and insulin secretion. *TCF7L2* plays a crucial regulatory role in β -cell proliferation, insulin biosynthesis, and incretin-mediated insulin release, while *SLC30A8* encodes the zinc transporter ZnT8, which is essential for insulin crystallization, storage, and proper secretion in pancreatic β -cells. Variants in these genes are therefore more likely to reflect primary defects in insulin production rather than secondary metabolic or inflammatory processes. By focusing on these two biologically relevant and functionally complementary genes, this study aims to provide a more targeted

understanding of genetic susceptibility related to insulin deficiency in T2DM within the Bangladeshi population, while also facilitating integration with epigenetic and environmental factors investigated in this research.

1.2.7.4 Transcription Factor 7 like 2 (TCF7L2) gene

1.2.7.4.1 Overview of Transcription factor 7-like 2

Transcription factor 7-like 2 (*TCF7L2*), previously known as T-cell factor 4 (TCF4), is a member of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. Members of this family bind DNA through a conserved high-mobility group (HMG) domain and play important roles in Wnt/ β -catenin signaling (Weedon, 2007). *TCF7L2* was first identified by Korinek et al. (1998) through hybridization screening using TCF7 cDNA and was subsequently recognized as an important transcriptional regulator in the Wnt signaling pathway. During embryogenesis, *TCF7L2* contributes to organ development by regulating Wnt-responsive genes.

In humans, the *TCF7L2* gene is located on chromosome 10q25.2–q25.3 and spans approximately 217 kb. The gene contains 17 exons and exhibits complex tissue-specific alternative splicing, resulting in multiple transcripts and protein isoforms (**Figure 1.7**). The full-length *TCF7L2* protein consists of 596 amino acids and contains several functional domains, including an N-terminal, β -catenin-binding domain, a Groucho-binding region, an HMG box DNA-binding domain, a cysteine clamp, and a C-terminal regulatory region. The β -catenin-binding domain is encoded mainly by exon 1, whereas the HMG box DNA-binding domain is encoded by exons 10 and 11; both domains are highly conserved across species. Alternative splicing, particularly in the 3' region of the gene, generates isoforms with different transcriptional activities, which may act either as activators or repressors depending on cellular context (Duval et al., 2000b).

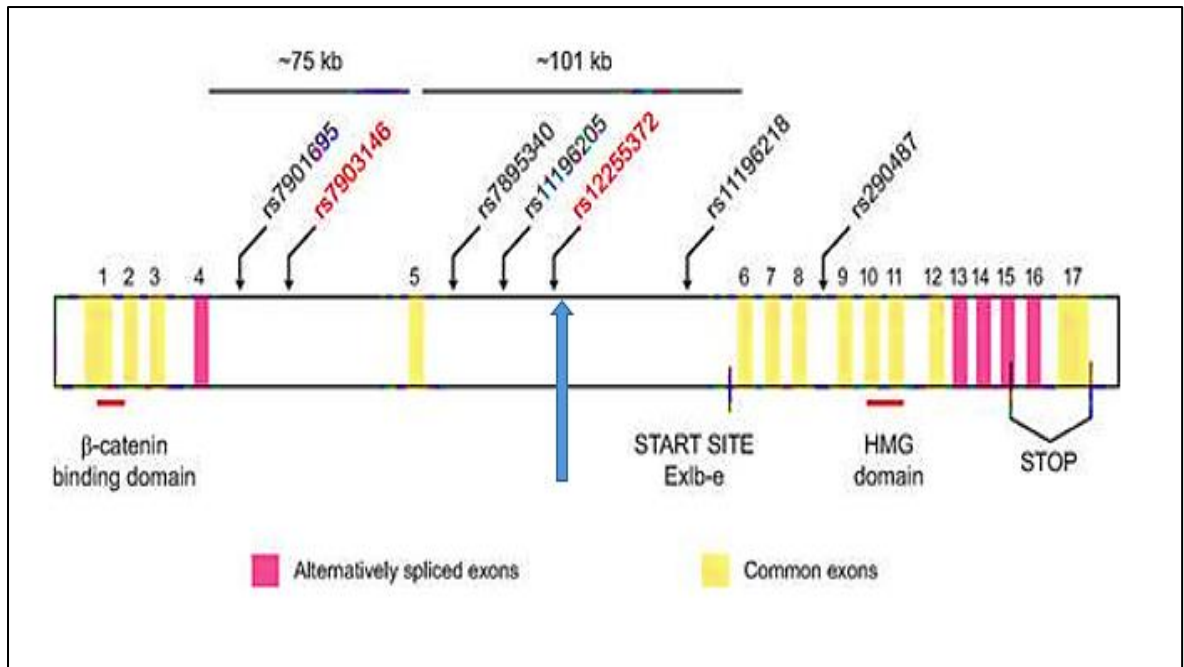


Figure 1.7: Genomic position of *TCF7L2* rs12255372. Modified from Ip et al. (2012)

1.2.7.4.2 *TCF7L2*, Wnt Signaling, and Glucose Metabolism

TCF7L2 has received increasing attention because of its involvement in several biological processes, including inflammation, metabolism, vascular development, and atherosclerosis. Experimental studies suggest that reduced *TCF7L2* expression can impair insulin secretion, adipocyte differentiation, vascular development, and lipid homeostasis, indicating its broad role in metabolic regulation (X. Chen et al., 2018; Facchinello et al., 2017).

Functionally, *TCF7L2* is a central transcriptional mediator of the canonical Wnt/ β -catenin signaling pathway, a complex cell-signaling network originally recognized for its role in embryonic development (Li et al., 2021). Upon activation of canonical Wnt signaling, β -catenin accumulates in the cytoplasm, translocates into the nucleus, and binds to *TCF7L2*. This β -catenin-*TCF7L2* complex then regulates the transcription of downstream target genes. Because Wnt signaling is active in metabolically important tissues, including pancreatic islets, intestinal endocrine cells, and adipocytes, *TCF7L2* contributes to the regulation of glucose and lipid metabolism.

TCF7L2 also influences glucose homeostasis through its effects on incretin and pancreatic hormone regulation. Insulin and glucagon exert opposing effects on blood

glucose, whereas glucagon-like peptide-1 (GLP-1) enhances glucose-dependent insulin secretion and suppresses glucagon release. Yi et al. (2005) reported that the promoters of both the GLP-1 and glucagon genes contain *TCF7L2*-binding sites. In this context, *TCF7L2* may upregulate GLP-1 expression in intestinal endocrine cells while repressing glucagon expression in pancreatic α -cells, thereby contributing to insulin secretion and blood glucose balance (Li et al., 2021; Yi et al., 2005).

1.2.7.4.3 *TCF7L2* in diabetes

Among the various candidate susceptibility genes for T2DM, single nucleotide polymorphisms (SNPs) in *TCF7L2* gene represent one of the most consistently replicated genetic associations (Tong et al., 2009). The association between *TCF7L2* and T2DM was first identified by Grant et al. (2006) through a microsatellite linkage study, which highlighted the intronic variant rs12255372 as a risk locus. Subsequent genome-wide association studies and meta-analyses have consistently confirmed *TCF7L2* as one of the strongest genetic determinants of T2DM across diverse populations, particularly variants such as rs7903146 and rs12255372. However, some studies have reported inconsistent findings in certain populations, suggesting the influence of ethnic variation and gene–environment interactions (Alsmadi et al., 2008; Guo et al., 2007).

A large meta-analysis further estimated that *TCF7L2* variants may contribute to a substantial proportion of T2DM cases globally, following a multiplicative model of inheritance (Del Bosque-Plata et al., 2021; Tong et al., 2009). Notably, the frequency of *TCF7L2* risk alleles varies considerably across different ethnic groups, including European, East Asian, and African populations, underscoring the importance of population-specific investigations (Guinan, 2012).

Despite extensive global evidence, genetic studies on *TCF7L2* in the Bangladeshi population remain limited. Most available research has focused on epidemiology and clinical aspects rather than genetic susceptibility. A study among Bangladeshi women with gestational diabetes mellitus (GDM) reported that the heterozygous CT genotype of the rs7903146 polymorphism was associated with an increased risk of GDM, although the association was not statistically significant (Hasan et al., 2016). Similarly, a recent study conducted in the Chattogram population investigating the rs12255372

(G/T) variant observed a higher frequency of the risk allele among GDM cases compared to controls, but without statistical significance, possibly due to limited sample size (Ali et al., 2023). These findings indicate the need for larger, well-designed studies to clarify the role of *TCF7L2* variants in diabetes susceptibility within the Bangladeshi population. Therefore, the present study aims to investigate the association between the rs12255372 (G/T) polymorphism of *TCF7L2* and the risk of developing T2DM in this population.

1.2.7.5 Solute Carrier 30 A8 (SLC30A8) and T2DM

1.2.7.5.1 Overview of SLC30A8

The *SLC30A8* gene, also known as solute carrier family 30 member 8 (HGNC:20303), encodes the zinc transporter protein ZnT8, which is primarily involved in pancreatic β -cell function. Structurally, *SLC30A8* comprises eight exons spanning approximately 37 kb and is located on chromosome 8q24.11, as first characterized by Chimienti et al. (2004). The gene was initially identified through RT-PCR analysis of RNA isolated from human pancreatic islets, leading to its molecular characterization and nomenclature.

Expression profiling studies have demonstrated a highly tissue-specific pattern, with predominant expression in the pancreas, particularly in the islets of Langerhans, and minimal expression in other tissues such as the amygdala, breast, lung, and sarcoma, as reported by Seve et al. (2004). This restricted expression has been consistently confirmed through expressed sequence tag (EST) database analysis and RT-PCR across multiple human tissues, indicating a specialized role of *SLC30A8* in endocrine pancreatic function (OMIM, 2025).

1.2.7.5.2 Protein of SLC30A8-ZnT8

The protein encoded by *SLC30A8*, known as ZnT8, is a protein belonging to the SLC30 family of zinc transporters and functions as a $\text{Zn}^{2+}/\text{H}^{+}$ antiporter. Structurally, human ZnT8 (HsZnT8) exists as a homodimer, with each subunit comprising a transmembrane domain (TMD) formed by six membrane-spanning helices (TM1-TM6) and a cytosolic C-terminal domain (CTD) that plays a key role in dimer stabilization. Cryo-electron

microscopy analyses have demonstrated that ZnT8 adopts distinct outward-facing and inward-facing conformations, reflecting its dynamic structural transitions during the transport cycle. The dimeric architecture is arranged such that the TMDs form the membrane-embedded region, while the CTDs extend into the cytosol and contribute extensively to subunit interaction and structural integrity.

At the molecular level, each ZnT8 subunit contains multiple Zn^{2+} binding sites that are strategically positioned to support its transport function. A highly conserved primary binding site is located within the TMD and serves as the central site for Zn^{2+} translocation, while additional binding sites are present at the interface between the TMD and CTD and within the cytosolic domain. Structural studies further reveal that conformational rearrangements within specific transmembrane helices enable alternating accessibility of the Zn^{2+} binding site from the cytosolic and luminal sides. These coordinated structural features, including the arrangement of TM helices, dimeric organization, and spatial distribution of Zn^{2+} binding sites, collectively define the architecture of ZnT8 and underpin its role as a membrane zinc transporter (Xue et al., 2020) (**Figure 1.8**).

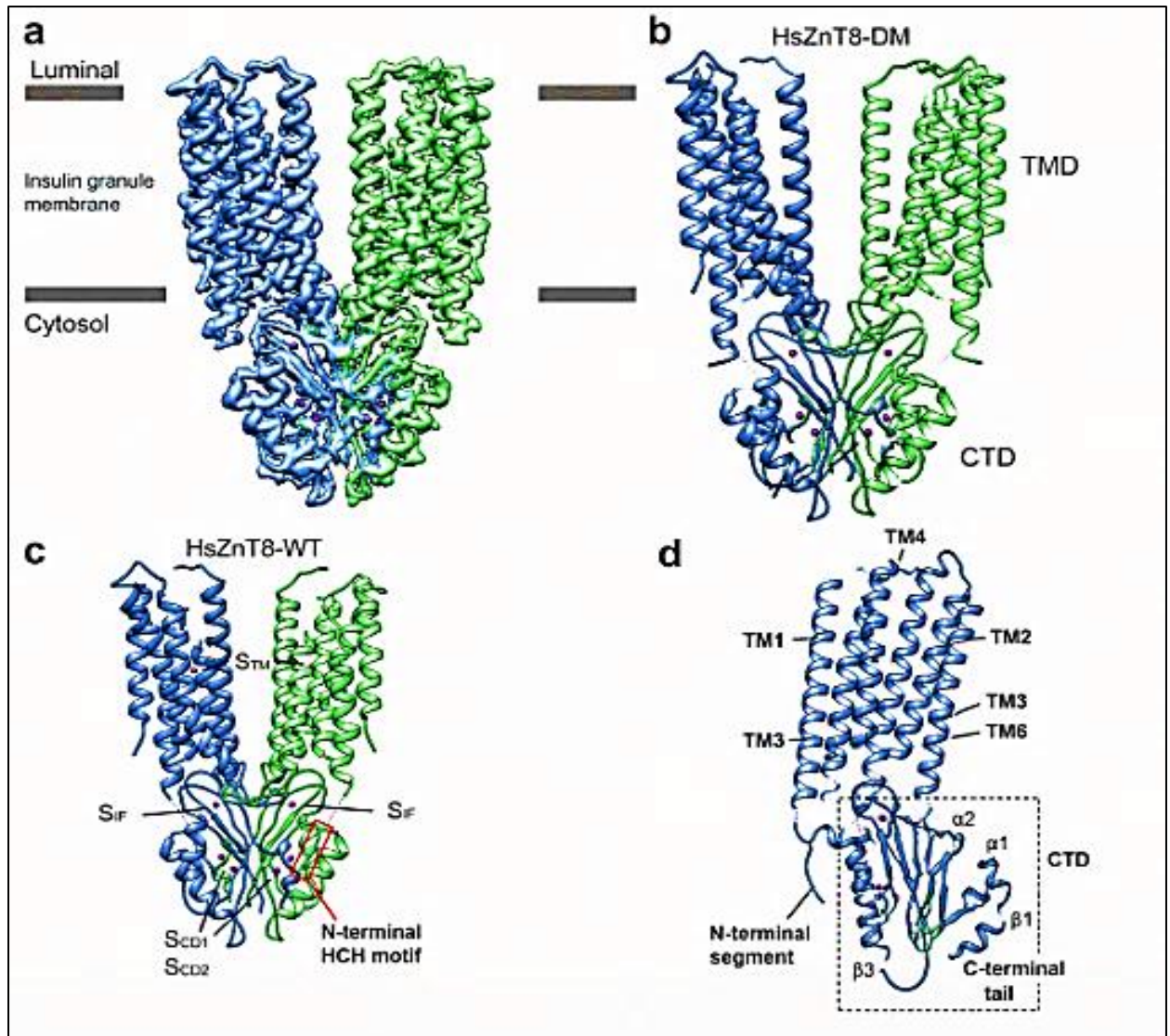


Figure 1.8: Structural overview and Zn²⁺ binding sites of human ZnT8 (HsZnT8). (a) Cryo-EM reconstruction of HsZnT8-DM in the outward-facing conformation, illustrating the dimeric architecture with two subunits (blue and green) oriented relative to the luminal and cytosolic sides of the insulin granule membrane. (b) Ribbon representation of the HsZnT8-DM dimer in side view, highlighting the transmembrane domain (TMD) and cytosolic C-terminal domain (CTD). (c) Overall view of Zn²⁺ binding sites in HsZnT8-WT, with the positions of individual binding sites indicated within the dimeric structure. (d) Ribbon representation of a single HsZnT8-DM subunit showing key structural features, including transmembrane helices (TM1–TM6) and the CTD. Adapted from Xue et al. (2020).

1.2.7.5.3 Functional Role and Loss-of-Function of *SLC30A8*

The *SLC30A8* gene encodes ZnT8, a zinc transporter that plays a central role in maintaining zinc homeostasis in pancreatic β -cells by mediating the transport of Zn²⁺

into insulin secretory granules. This zinc accumulation is essential for the crystallization and stabilization of insulin in its hexameric form, thereby ensuring proper storage and regulated secretion in response to glucose stimulation (Davidson et al., 2015; Slepchenko et al., 2013). Glucose metabolism increases the intracellular ATP/ADP ratio, leading to closure of ATP-sensitive potassium (KATP) channels and opening of voltage-dependent Ca^{2+} channels, which ultimately triggers insulin exocytosis (Idevall-Hagren & Tengholm, 2020). In addition to its structural role in insulin packaging, Zn^{2+} participates in intracellular signaling by modulating ion channels, influencing β -cell excitability and insulin release. Upon glucose stimulation, Zn^{2+} is co-secreted with insulin and exerts autocrine and paracrine effects within the islets, including modulation of glucagon secretion from α -cells (**Figure 1.9**) (Slepchenko et al., 2013, 2015).

Genetic variation in *SLC30A8* further highlights the functional importance of ZnT8 in β -cell physiology. Risk-associated variants, such as p.Arg325, have been shown to increase Zn^{2+} transport activity, resulting in elevated intragranular zinc concentrations. This may promote the formation of toxic islet amyloid polypeptide aggregates and impair β -cell viability. Additionally, increased extracellular Zn^{2+} can inhibit voltage-dependent Ca^{2+} channels and activate KATP channels, thereby stabilizing the resting membrane potential and reducing insulin secretion. Beyond intracellular effects, insulin levels are also influenced by hepatic and peripheral clearance mechanisms, which may be modulated by factors such as gut microbiota. Other zinc transporters, including ZnT3, ZnT5, and ZnT7, contribute to insulin maturation and secretion, with partial functional overlap with ZnT8, particularly within the Golgi apparatus. Interestingly, loss-of-function (LoF) variants of *SLC30A8* demonstrate a complex and somewhat paradoxical relationship with T2DM. While initial hypotheses suggested that reduced ZnT8 activity would impair insulin storage and secretion, large-scale human genetic studies have shown that LoF mutations are associated with a reduced risk of T2DM. This protective effect is thought to result from enhanced glucose-stimulated insulin secretion and improved proinsulin processing, despite reduced intracellular zinc levels. Mechanistically, decreased Zn^{2+} may alleviate inhibitory feedback on insulin release and reduce the formation of toxic amyloid aggregates. However, findings from experimental models remain inconsistent, with some studies reporting impaired glucose tolerance following complete loss of ZnT8. These discrepancies likely reflect

differences in genetic background, species-specific physiology, and the degree of functional loss. Overall, *SLC30A8* exhibits a dual role in diabetes pathophysiology, where modulation of ZnT8 activity may have therapeutic implications (Barragán-Álvarez et al., 2021; Dwivedi et al., 2019; Flannick et al., 2014).

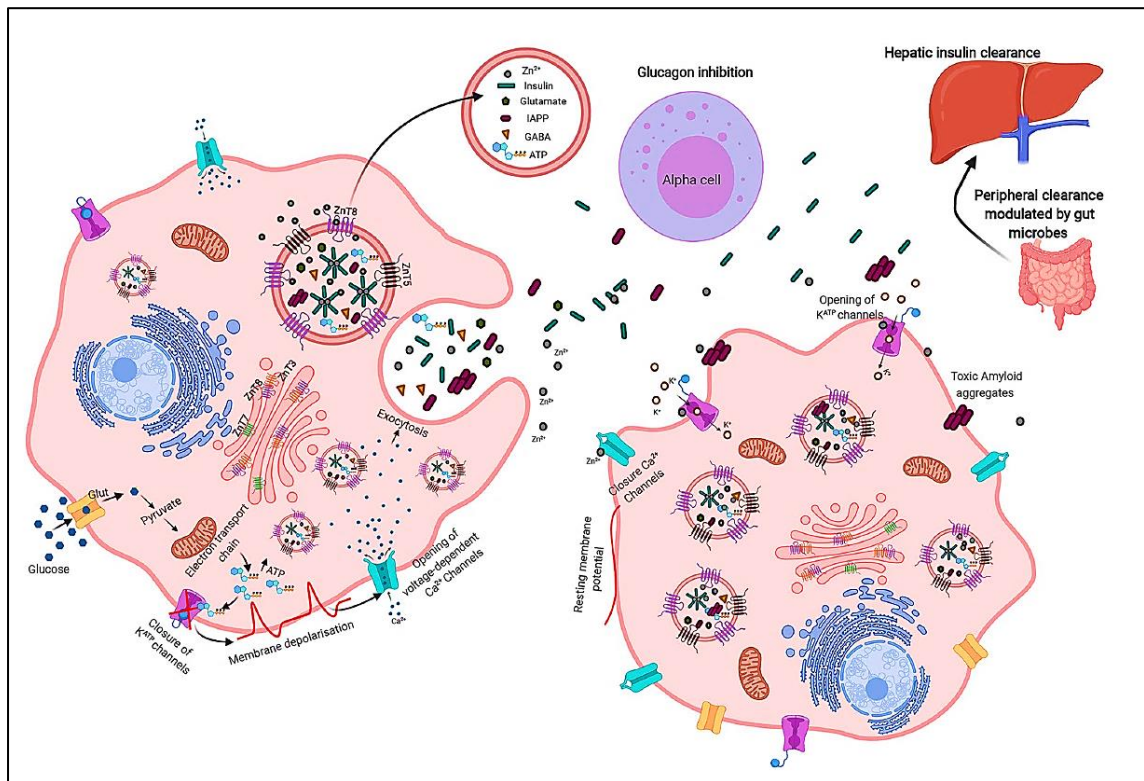


Figure 1.9: Proposed physiological effects of ZnT8 variants on pancreatic β -cell function and insulin secretion. Adapted from Barragán-Álvarez et al. (2021)

1.2.7.5.4 *SLC30A8* rs13266634 Polymorphism and Type 2 Diabetes

The most extensively studied variant of *SLC30A8* is the non-synonymous SNP rs13266634 (C>T), which results in an amino acid substitution at position 325 of the ZnT8 protein, replacing arginine with tryptophan (R325W) in the intracellular C-terminal domain (Parsons et al., 2018; Rutter & Chimienti, 2015). This variant was first identified as a T2DM risk factor in a landmark genome-wide association study (GWAS) by Sladek et al. (2007), which reported that the C allele encoding arginine was enriched in individuals with T2DM compared to healthy controls. Subsequent studies confirmed that the C allele is associated with reduced β -cell function and approximately a 14% increase in T2DM risk per risk allele carried (Rutter & Chimienti, 2015). The

risk C allele encoding arginine has been associated with an increased susceptibility to T2DM in other studies also (Drake et al., 2017; Mashal et al., 2021). At the molecular level, the arginine form (R325) of ZnT8 has been shown to exhibit greater zinc transport activity than the tryptophan form (W325), resulting in higher intragranular zinc concentrations that may promote the formation of toxic islet amyloid polypeptide aggregates and impair β -cell viability, thereby contributing to reduced insulin secretion (Barragán-Álvarez et al., 2021). Carriers of the risk C allele also show elevated proinsulin-to-insulin ratios, suggesting that altered zinc levels further affect downstream insulin processing and hepatic clearance of the hormone (Rutter & Chimienti, 2015). At the population level, the association between rs13266634 and T2DM has been examined across diverse ethnic groups worldwide. A large meta-analysis by Fan et al. (2016), which pooled data from 46 studies comprising 71,890 T2DM cases and 96,753 controls, confirmed a significant association between the rs13266634 C allele and increased T2DM risk in an overall analysis (OR = 1.16, 95% CI: 1.13–1.19, $p < 0.001$). When stratified by ethnicity, significant associations were observed in Asian (OR = 1.17, 95% CI: 1.14–1.21), European (OR = 1.14, 95% CI: 1.10–1.17), and African (OR = 1.25, 95% CI: 1.07–1.45) populations.

Despite this consistent body of evidence, several studies have reported no statistically significant association between rs13266634 and T2DM. Negative findings have been reported in Lebanese and southern Iranian population (Almawi et al., 2013; Faghih et al., 2014). These inconsistencies are likely attributable to differences in allele frequencies between ethnic groups, variation in sample sizes and statistical power, differences in study design, and the potential modifying influence of environmental factors such as diet, obesity, and physical activity (Fan et al., 2016). Adding further complexity, Flannick et al. (2014) unexpectedly found that 12 rare loss-of-function mutations in *SLC30A8* were paradoxically associated with a 65% reduction in T2DM risk, a finding that appears to contradict the increased susceptibility conferred by the common C allele. This paradox suggests that the relationship between *SLC30A8* variant activity and T2DM risk may follow a complex, non-linear pattern that is not yet fully understood and may differ across populations and age groups (Rutter & Chimienti, 2015).

Together, these findings establish rs13266634 as one of the most studied and replicated genetic risk variants for T2DM globally, while also highlighting the importance of ethnicity-specific investigation. Therefore, the present study aims to investigate the association between the rs13266634 (C>T) polymorphism of *SLC30A8* and the risk of T2DM in a Bangladeshi population, and to further examine the conformational consequences of the R325W substitution through molecular dynamics simulations to gain deeper mechanistic insight into its potential role in disease pathogenesis.

Although genetic variants contribute importantly to T2DM susceptibility, inherited variation alone cannot fully explain disease development or the influence of environmental exposures. Epigenetic mechanisms provide an additional layer of gene regulation through which environmental and metabolic factors may modify gene activity without altering the underlying DNA sequence. This provides the basis for examining epigenetic mechanisms, particularly DNA methylation, in T2DM (Cunliffe, 2015; Klengel et al., 2014).

1.2.8 Epigenetics

Epigenetics literally refers to regulatory processes “above” or “beyond” the DNA sequence. The concept was first introduced by C.H. Waddington in 1942, who used the term to explain how genetic factors interact with environmental influences during development and cellular differentiation (Waddington, 1942). In modern biology, epigenetics refers to heritable or stable changes in gene expression that occur without alteration of the primary DNA sequence. These mechanisms regulate messenger RNA and protein expression, allowing cells and organisms to respond dynamically to environmental exposures (Inbar-Feigenberg et al., 2013; Skinner, 2015).

The major epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNA-mediated regulation. Among these, cytosine-phosphate-guanine (CpG) DNA methylation is one of the most widely studied mechanisms and plays a critical role in transcriptional regulation. Histone modifications, such as acetylation and methylation, alter chromatin structure and thereby influence gene accessibility, while non-coding RNAs regulate gene expression at transcriptional and post-transcriptional levels (Benčík et al., 2025). Together, these epigenetic processes provide a molecular

link between environmental exposures and biological responses, making them highly relevant for understanding complex diseases such as type 2 diabetes mellitus.

1.2.8.1 DNA methylation

DNA methylation is one of the most extensively studied epigenetic modifications, involving the stable covalent addition of a methyl group to the fifth carbon (C5) of cytosine residues to form 5-methylcytosine (5mC) (**Figure 1.10**). The presence of 5mC was first coined by Rollin Hotchkiss during the analysis of calf thymus DNA using paper chromatography (Hotchkiss, 1948). DNA methylation plays a critical role in human development, with approximately 3–6% of cytosines methylated in the mammalian genome (Esteller, 2007). Methylation of nucleotide bases within cis-acting elements—such as promoters or enhancers—can alter DNA structure, thereby hindering the binding of trans-acting factors required to initiate gene expression (Y. Li & Neill, 2012).

In mammalian cells, DNA methylation predominantly occurs at cytosine–guanine dinucleotides (CpG sites), which are often clustered into CpG-rich regions known as CpG islands. Based on their proximity to CpG islands, the surrounding genomic regions are further classified into CpG shores (within 2 kb), CpG shelves (2–4 kb away), and open sea regions, which comprise the remaining genomic areas distant from the islands (Visone et al., 2019). These CpG islands are frequently located at gene promoter regions, particularly near transcription start sites (TSSs), and are typically unmethylated in normal conditions, allowing active gene transcription. Methylation of CpG islands within promoter regions is generally associated with stable and long-term gene silencing. In contrast, CpG sites located in repetitive elements, including centromeric and other heterochromatic regions, are usually heavily methylated, contributing to genomic stability. DNA methylation plays essential roles in silencing retroviral elements, regulating tissue-specific gene expression, genomic imprinting, and X-chromosome inactivation. Importantly, the functional impact of DNA methylation varies depending on its genomic context, as methylation in promoters, gene bodies, or intergenic regions can differentially influence gene expression (Jones, 2012).

Transfer of a methyl group from S-adenosylmethionine (SAM) to the fifth carbon of cytosine residue to form 5-methylcytosine (5mC) producing S-adenosyl-L-homocysteine (SAH) (Gómez de Cedrón et al., 2023)

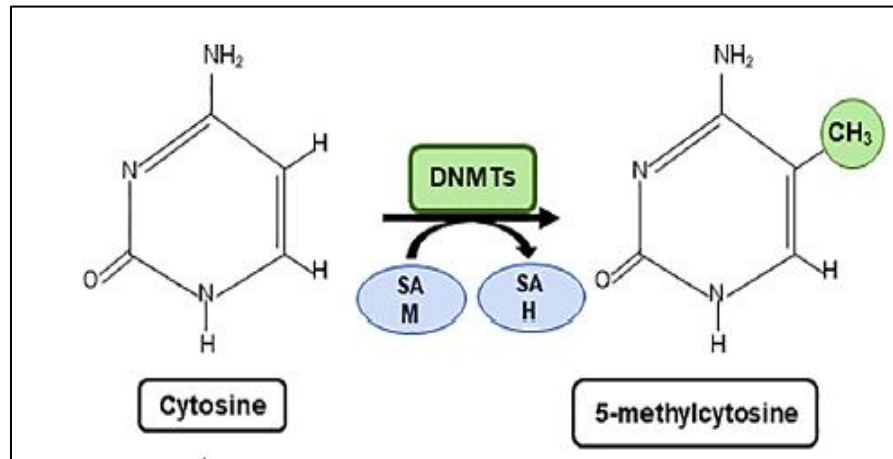


Figure 1.10: DNA methylation. Modified from Gaździcka et al. (2020)

1.2.8.2 Basic Mechanism of DNA methylation

DNA methylation is a dynamic and tightly regulated epigenetic process controlled by a coordinated set of enzymes that establish, recognize, and remove methylation marks. These enzymes are broadly classified into three functional groups: writers, readers, and erasers. Writers, primarily DNA methyltransferases, catalyze the addition of methyl groups to cytosine residues; readers recognize and bind to methylated DNA to mediate downstream regulatory effects; and erasers facilitate the removal of methylation marks through passive or active demethylation mechanisms (**Figure 1.11**) (Moore et al., 2013).

Writers of DNA methylation

DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosylmethionine (SAM) to the fifth carbon of cytosine residues, generating 5-methylcytosine (5mC). The principal enzymes involved are DNMT1, DNMT3a, DNMT3b, and DNMT3L.

DNMT1 preferentially methylates hemimethylated DNA, in which only one strand of a CpG dinucleotide is methylated. During DNA replication, DNMT1 localizes to replication foci and restores symmetrical methylation patterns by copying parental methylation marks onto the newly synthesized strand. Therefore, DNMT1 is referred to as the maintenance methyltransferase, as it preserves epigenetic information across cell divisions and contributes to cellular differentiation (Hermann et al., 2004; Pradhan et al., 1999).

In contrast, DNMT3a and DNMT3b function as *de novo* methyltransferases, establishing new methylation patterns on previously unmethylated DNA. These enzymes are capable of methylating both native and synthetic DNA substrates without preference for hemimethylated DNA, highlighting their role in initiating DNA methylation. Structurally, DNMT3a and DNMT3b are highly conserved in the PWWP domain and the C-terminal methyltransferase (MTase) domain, while their N-terminal regulatory regions differ substantially, contributing to functional divergence. The PWWP domain, particularly in DNMT3b, contains a basic surface that facilitates DNA binding (Qiu et al., 2002). The mechanisms governing the targeting of DNMT3a and DNMT3b to specific genomic loci remain incompletely understood. Current evidence suggests that recruitment is mediated through interactions with transcription factors and repressor complexes. In some cases, transcription factors recruit DNMTs to specific promoters to induce methylation, whereas in other contexts, bound transcription factors may protect CpG sites from methylation. Alternatively, DNMT3 enzymes may methylate CpG sites that are not shielded by DNA-binding proteins. DNMT3L lacks intrinsic catalytic activity but acts as a regulatory cofactor by interacting with DNMT3a and DNMT3b, thereby enhancing their methyltransferase activity and facilitating the proper establishment of DNA methylation patterns (Moore et al., 2013).

Readers of DNA Methylation

These proteins are broadly classified into three families: the methyl-CpG-binding domain (MBD) proteins, the UHRF proteins, and methylation-sensitive zinc-finger proteins. The MBD protein family, including MeCP2, MBD1, MBD2, MBD3, and MBD4, contains a conserved methyl-CpG-binding domain that enables recognition of methylated CpG sites (Nan et al., 1993). Most members of this family can directly bind methylated DNA and recruit transcriptional repression complexes through a

transcriptional repression domain (TRD), thereby facilitating gene silencing (Nan et al., 1998; Ng et al., 1999). In addition, MeCP2 has been reported to interact with DNMT1, suggesting a role in reinforcing maintenance methylation (Kimura & Shiota, 2003). The UHRF (ubiquitin-like, containing PHD and RING finger domains) protein family, including UHRF1 and UHRF2, plays a central role in maintenance methylation. UHRF1 recognizes hemimethylated DNA and recruits DNMT1 to replication sites, thereby ensuring faithful propagation of DNA methylation patterns during cell division (Bostick et al., 2007). A third group comprises methylation-sensitive zinc-finger proteins, such as Kaiso, ZBTB4, and ZBTB38. These proteins bind methylated DNA through zinc-finger domains and repress transcription in a DNA methylation-dependent manner (Filion et al., 2006).

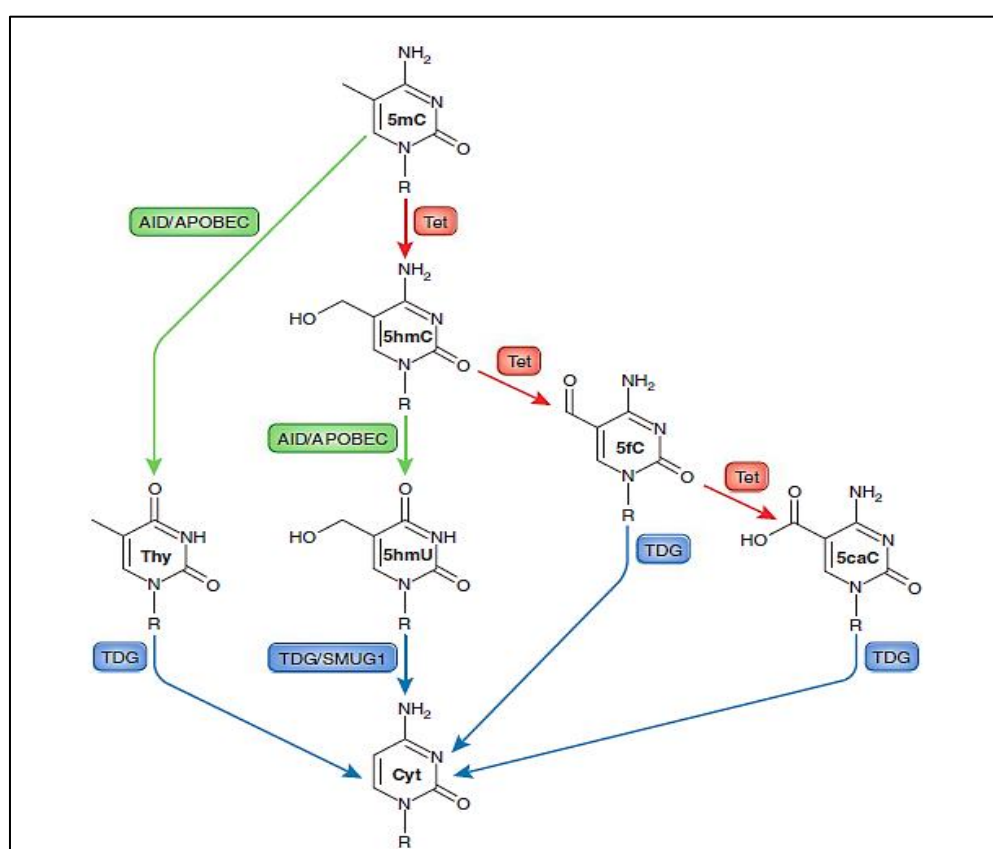


Figure 1.11: Active DNA demethylation pathways.

5-Methylcytosine (5mC) undergoes active demethylation through two pathways. AID/APOBEC enzymes (green) deaminate 5mC to thymine (Thy) and further deaminate its oxidized form 5-hydroxymethyl-cytosine (5hmC) into 5-hm-Uracil (5hmU). TET enzymes (red) oxidize 5mC stepwise into 5hmC, then 5-formyl-cytosine (5fC) and finally 5-carboxy-cytosine (5caC). All end products, Thy, 5hmU, 5fC, and 5caC, are ultimately excised by TDG and/or SMUG1 (blue) via base excision repair, restoring an unmodified cytosine (Cyt). Modified from Moore et al. (2013).

Erasers of DNA Methylation

Erasers of DNA methylation modify and remove the methyl group through both passive and active mechanisms. Passive DNA demethylation occurs during cell division when the maintenance methyltransferase DNMT1 is inhibited or dysfunctional. As DNMT1 normally copies methylation patterns onto the newly synthesized strand, its impairment results in the dilution of methylation marks over successive cell cycles, leading to a progressive reduction in global DNA methylation. In contrast, active DNA demethylation is an enzymatic process that removes 5-methylcytosine (5mC) independently of DNA replication. This process involves chemical modification of 5mC followed by replacement with unmethylated cytosine through the base excision repair (BER) pathway. One proposed mechanism involves deamination of 5mC by AID/APOBEC (activation-induced cytidine deaminase/apolipoprotein B mRNA-editing enzyme complex) enzymes, converting it into thymine and generating a G/T mismatch, which is subsequently recognized and repaired by BER.

A more widely accepted pathway is mediated by the ten–eleven translocation (TET) enzymes (TET1, TET2, and TET3), which catalyze the stepwise oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). These oxidized derivatives are then recognized and excised by thymine DNA glycosylase (TDG), initiating BER to restore unmodified cytosine. Additionally, 5hmC may undergo deamination by AID/APOBEC enzymes to form 5-hydroxymethyluracil, which is similarly processed by BER. In all active demethylation pathways, the BER machinery, particularly thymine DNA glycosylase (TDG), removes the modified base and replaces it with an unmethylated cytosine, thereby completing the demethylation process (Ito et al., 2010; Jones, 2012; Moore et al., 2013; Oswald et al., 2000).

1.2.8.3 Crosstalk of DNA Methylation with other Epigenetic Mechanisms

DNA methylation does not act alone but works together with histone modifications and microRNAs (miRNAs) to regulate gene expression (**Figure 1.12**).

Histone modifications, including methylation, acetylation, ubiquitination, and phosphorylation, alter chromatin structure and thereby control transcriptional activity.

Generally, histone marks associated with an open chromatin configuration promote transcription, whereas those leading to chromatin compaction are linked to gene repression. DNA methyltransferases, such as DNMT1, DNMT3a, and DNMT3b, interact with enzymes that mediate histone modifications, thereby reinforcing transcriptional repression. For example, DNMT1 and DNMT3a associate with the histone methyltransferase SUV39H1, which catalyzes methylation of histone H3 at lysine 9 (H3K9), a hallmark of transcriptionally inactive chromatin. In addition, DNMT1 and DNMT3b interact with histone deacetylases (HDACs), which remove acetyl groups from histone tails, resulting in chromatin condensation and reduced transcriptional accessibility (Fuks et al., 2000, 2003; Geiman et al., 2004). Methyl-CpG-binding proteins provide a critical link between DNA methylation and histone modification. Members of the MBD and UHRF protein families bind methylated DNA and recruit chromatin-modifying complexes that further strengthen gene repression. Notably, MeCP2 recruits HDACs to remove activating histone marks and can also interact with histone methyltransferases to promote repressive histone modifications such as H3K9 methylation, thereby stabilizing a transcriptionally inactive chromatin state (Fuks et al., 2003; Nan et al., 1993).

In addition to histone modifications, microRNAs (miRNAs) represent another important layer of epigenetic regulation. Mature miRNAs function through the miRNA-induced silencing complex (miRISC), which binds target mRNAs and suppresses gene expression by inhibiting translation or promoting mRNA degradation. DNA methylation can regulate miRNA expression by modifying CpG sites within miRNA gene promoters. Conversely, certain miRNAs can modulate the expression of DNMTs and histone-modifying enzymes, thereby influencing both DNA methylation and chromatin states (Benetti et al., 2008; L. Han et al., 2007; Sinkkonen et al., 2008).

Overall, these interconnected regulatory mechanisms form a complex epigenetic network in which DNA methylation, histone modifications, and miRNAs act cooperatively to fine-tune gene expression.

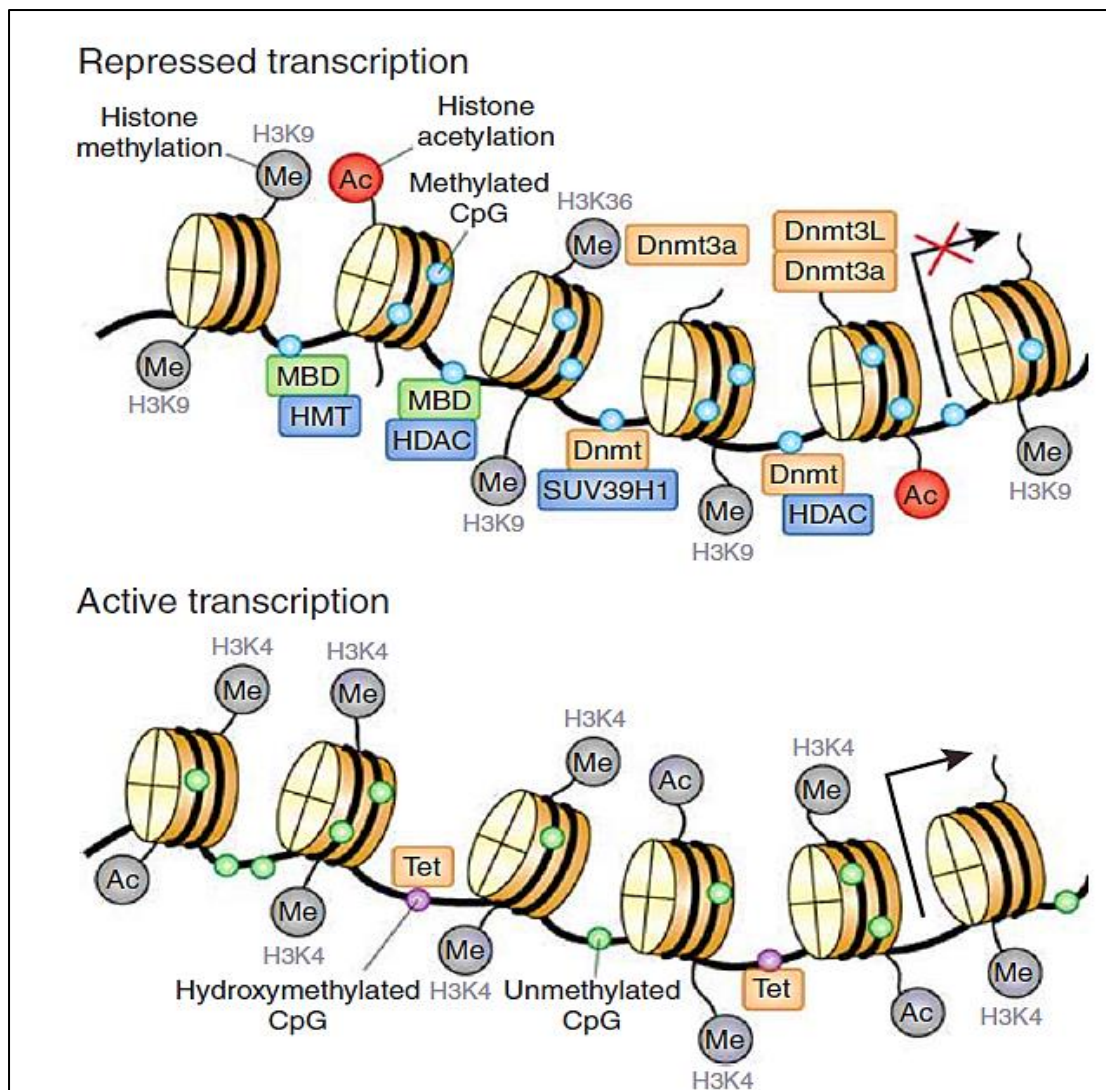


Figure 1.12: Epigenetic crosstalk in gene regulation.

Schematic illustration of the interaction between DNA methylation and histone modifications in transcriptional regulation. In repressed transcription, CpG methylation (blue) is recognized by methyl-binding proteins (green), which recruit histone deacetylases (HDACs; blue) and histone methyltransferases (HMTs; blue), leading to histone deacetylation (red loss) and H3K9 methylation (grey), resulting in compact chromatin. In contrast, active transcription is associated with unmethylated CpG sites (green), TET-mediated hydroxymethylation (purple), and activating histone marks such as H3K4 methylation (grey) and histone acetylation (red), leading to an open chromatin structure. Modified from Moore et al. (2013)

1.2.8.4 DNA methylation and environment

Environmental exposures can influence DNA methylation at both global and locus-specific levels through multiple mechanisms. At the global level, two principal pathways have been proposed. First, it may influence DNA methylation by altering DNMT and TET activity or expression, thereby affecting the overall addition or

removal of methyl groups across the genome. Second, such exposures may modify the availability of methyl donors, particularly S-adenosylmethionine (SAM), which serves as the primary substrate for methyl group transfer, ultimately influencing global methylation levels. In contrast, gene-specific DNA methylation changes are commonly explained by the transcription factor occupancy model. According to this model, transcription factors regulate the accessibility of DNA methylation machinery to specific genomic regions. Activation of transcription factors in response to environmental stimuli may promote their binding to DNA, thereby blocking DNMT access and resulting in site-specific hypomethylation (**Figure 1.13a**). Conversely, reduced or inhibited transcription factor binding may allow DNMT recruitment to these regions, leading to gene-specific hypermethylation. Thus, environmental exposures can modulate transcription factor activity as part of cellular adaptation or stress responses, thereby shaping locus-specific DNA methylation patterns (**Figure 1.13b**) (Martin & Fry, 2016).

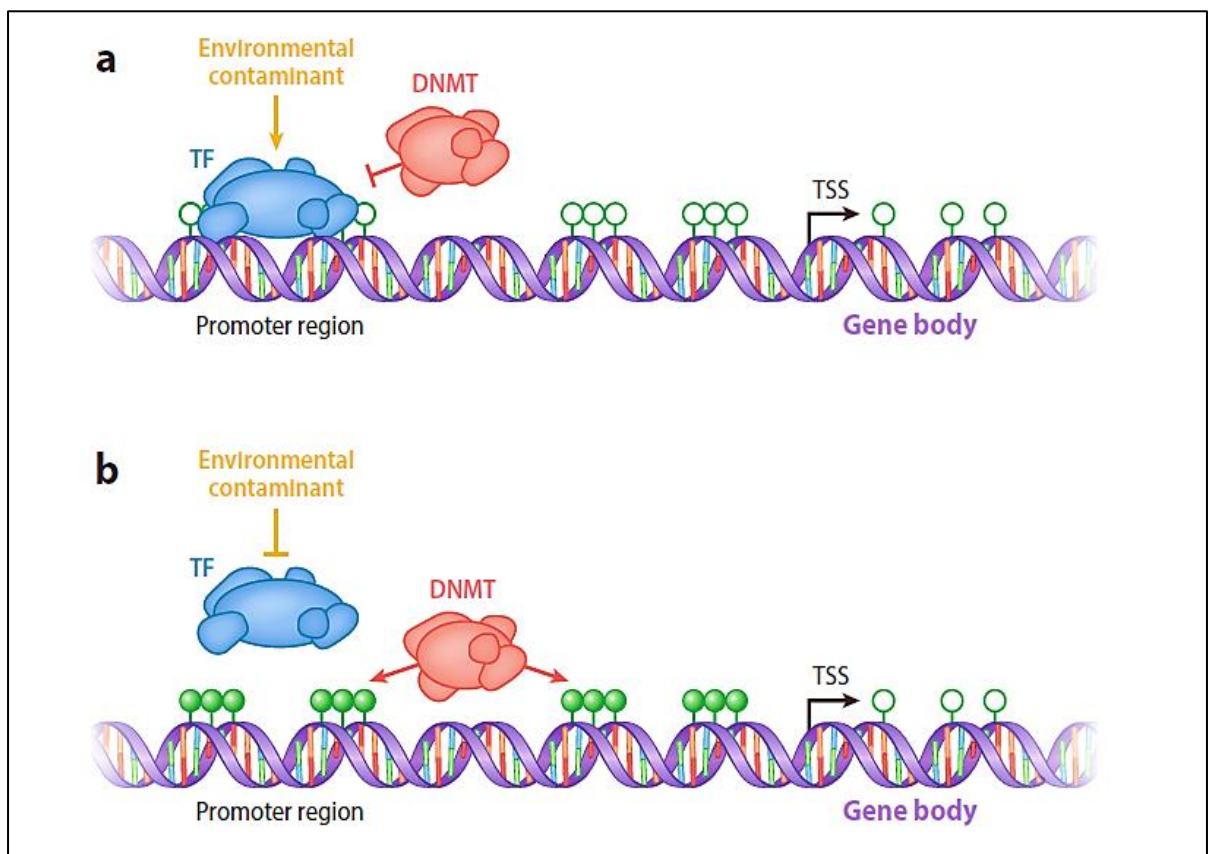


Figure 1.13: Diagram of the transcription factor occupancy theory

Modified from Martin & Fry, (2016). Here: TF, transcription factor; TSS, transcription start site; DNMT, DNA methyltransferase.

DNA Methylation and early life environmental exposure

Prenatal exposure to environmental factors has been associated with adverse health outcomes both in early life and adulthood. In infants and children, such exposures are linked to low birth weight, preterm birth, and increased susceptibility to infections. Importantly, these early-life exposures may also predispose individuals to chronic diseases later in life, including cancer, type 2 diabetes, and impaired immune function (Boekelheide et al., 2012; Windham et al., 2008).

This concept is consistent with the Developmental Origins of Health and Disease (DOHaD) framework, originally proposed through the Barker hypothesis. Barker and colleagues suggested that disturbances in the intrauterine environment can induce fetal programming changes that increase the risk of chronic disease in later life. In particular, adverse nutritional conditions during early development, often reflected by low birth weight, were proposed to increase susceptibility to metabolic abnormalities including obesity, insulin resistance, type 2 diabetes, hypertension, hyperlipidemia, coronary heart disease, and stroke (Edwards, 2019). Supporting this concept, a systematic review reported that low birth weight, small for gestational age, and preterm birth were positively associated with adult cardiometabolic disease, metabolic syndrome, and type 2 diabetes (de Mendonça et al., 2020). The thrifty phenotype hypothesis further explains this relationship by suggesting that fetal adaptation to undernutrition leads to reduced insulin secretion and increased insulin resistance, prioritizing energy supply to essential organs. While beneficial for survival in utero, these adaptations may become maladaptive in postnatal environments with abundant nutrition, increasing the risk of glucose intolerance and type 2 diabetes (Eriksson et al., 2003; Hales & Barker, 1992).

One plausible mechanism linking early-life exposure to later disease risk is epigenetic modification, particularly altered DNA methylation. Conditions that disturb metabolic programming during embryonic and fetal development may induce long-lasting changes in signaling pathways, thereby influencing placental function, fetal growth, and later metabolic health (Z. Zhu et al., 2019). Among epigenetic mechanisms, CpG methylation is especially important because it can regulate gene transcription and thereby influence disease susceptibility (Schübeler, 2015). Importantly, DNA methylation marks established during prenatal life may be modified by maternal

environmental exposures and persist over time, providing a potential biological basis for the long-term effects of early-life adversity (Heijmans et al., 2008).

Evidence supporting this mechanism is provided by the Agouti mouse model, which demonstrated a direct relationship between maternal diet, epigenetic alterations, and offspring phenotype. Maternal supplementation with methyl donors during pregnancy shifted offspring coat color from yellow to brown, accompanied by reduced susceptibility to obesity and adult-onset diabetes. These phenotypic changes were explained by epigenetic modification, highlighting how early nutritional exposures can shape later-life metabolic outcomes through altered DNA methylation (Dolinoy, 2008).

1.2.8.5 DNA methylation and type 2 diabetes

DNA methylation is one of the most extensively studied epigenetic modifications implicated in the pathogenesis of type 2 diabetes mellitus (T2DM). Growing evidence from epigenome-wide association studies (EWAS) indicates that aberrant DNA methylation patterns are associated with glycaemic traits and T2DM across different populations and tissues. Among these, the *TXNIP* gene has emerged as one of the most consistently replicated loci. In particular, reduced methylation at cg19693031, located in the 3'-UTR of *TXNIP*, has been associated with higher fasting glucose and HbA1c levels in both European and Asian populations, suggesting its potential role in glucose regulation and oxidative stress pathways (Florath et al., 2016; Z. Wang et al., 2021).

In addition to *TXNIP*, several other genes have demonstrated reproducible methylation changes in individuals with T2DM. Large-scale replication studies have identified significant differential methylation at CpG sites within *ABCG1*, *SREBF1*, *LOXL2*, and *SLC1A5*. Notably, *ABCG1* and *SREBF1* are generally found to be hypermethylated, whereas *TXNIP*, *LOXL2*, and *SLC1A5* tend to be hypomethylated in diabetic individuals compared to controls. These genes are involved in lipid metabolism, cellular transport, and metabolic signaling, indicating that epigenetic dysregulation in these pathways may contribute to insulin resistance and metabolic imbalance (Walaszczyk et al., 2018).

Longitudinal and prospective cohort studies further provide insight into the temporal relationship between DNA methylation and T2DM development. Evidence from the EPIC-Norfolk cohort demonstrates that specific methylation signatures in whole blood

can be detected years before the clinical onset of T2DM. In this study, methylation at loci near *TXNIP*, *ABCG1*, and *SREBF1* was associated with future risk of diabetes, suggesting that these epigenetic markers may precede disease manifestation and could serve as early indicators of metabolic dysfunction. Additionally, integration with genetic data identified methylation quantitative trait loci, supporting a potential causal role of certain CpG sites, such as those within *CPT1A*, in T2DM pathogenesis (Cardona et al., 2019).

Complementary findings from EWAS conducted in non-diabetic populations have revealed methylation changes associated with intermediate metabolic phenotypes, including fasting glucose and insulin levels. For instance, CpG sites mapped to genes such as *LETM1*, *IRS2*, *MAN2A2*, *FCRL6*, and *SLAMF1* have been linked with early metabolic alterations, indicating that epigenetic modifications may arise during the preclinical stages of disease development and contribute to the progression toward overt diabetes (J. Liu et al., 2019).

Targeted candidate gene studies have also explored the methylation status of genes involved in β -cell function, insulin signaling, and energy metabolism. Investigations involving genes such as *KCNJ11*, *PPAR γ* , *PDK4*, *KCNQ1*, *FTO*, *PDX1*, and *PEG3* have reported CpG-specific differences in promoter regions, although overall gene-level methylation differences were not always statistically significant. These findings highlight the importance of locus-specific epigenetic regulation rather than global methylation changes in T2DM (Van Otterdijk et al., 2017). Additional CpG sites, including those located in *CLCA4* and *LECT1*, have also been identified in association studies, further broadening the spectrum of potential epigenetic biomarkers (Arpón et al., 2019).

Systematic reviews and integrative analyses have consolidated the available evidence and identified a set of genes that consistently exhibit differential methylation across multiple studies and populations. These include *TXNIP*, *ABCG1*, *PPARGC1A*, *PTPRN2*, *TCF7L2*, *KCNQ1*, *SLC30A8*, and *FTO*, all of which are functionally linked to key pathways involved in glucose homeostasis, lipid metabolism, and pancreatic β -cell activity (Nadiger et al., 2024; Willmer et al., 2018). Furthermore, several studies have demonstrated an inverse relationship between DNA methylation and gene expression in metabolically relevant tissues, supporting the biological relevance of these epigenetic

changes. Despite these advances, most studies have been conducted in European or other non-South Asian populations, underscoring the need for population-specific investigations. Therefore, exploring DNA methylation patterns in the Bangladeshi population may provide novel insights into the epigenetic mechanisms underlying T2DM and help identify context-specific biomarkers and therapeutic targets.

1.2.8.6 Peripheral blood DNA methylation in T2DM

DNA methylation is a tissue-specific process, and ideally, studies on T2DM would utilize pancreatic β -cells or insulin-sensitive tissues such as skeletal muscle and adipose tissue, which are directly involved in glucose metabolism and insulin action. However, obtaining these tissues from human subjects is invasive and not feasible in large-scale studies. Therefore, peripheral blood is widely used as a non-invasive, easily accessible biosample for DNA methylation analysis. Although it does not fully capture tissue-specific epigenetic patterns, blood reflects systemic processes such as inflammation, immune activation, and metabolic dysregulation, which are closely linked to T2DM (Bansal & Simmons, 2018; Muka et al., 2016).

Evidence from multiple studies supports the relevance of blood-based methylation as a surrogate marker. Differential methylation has been identified in key metabolic genes across adipose tissue, skeletal muscle, and pancreatic islets in T2DM, with functional implications for β -cell proliferation and insulin secretion (Dayeh et al., 2014; Nilsson et al., 2014; Ribel-Madsen et al., 2012; Volkov et al., 2017). Importantly, overlap in methylation patterns between leukocytes and pancreatic islets has been demonstrated, with approximately 60% of age-related differentially methylated genes shared between these tissues, some of which influence insulin secretion (Bacos et al., 2016). Furthermore, altered DNA methylation profiles have been detected in peripheral blood during the pre-diabetic stage, indicating that these changes may occur early in disease development (VanderJagt et al., 2015). Collectively, these findings support the use of peripheral blood as a practical surrogate tissue for investigating DNA methylation in T2DM.

1.2.8.7 DNA methylation, T2DM and Adverse Childhood Experience (ACE)

Early life is a special period characterized by a high level of plasticity and fast development. Thus, the impact of childhood trauma is particularly deleterious, since the developmental trajectory of the brain is affected, with resultant alteration of the circuitry for threat detection, emotional regulation, and the reward system (Dunn et al., 2019; Teicher et al., 2016). Previous studies have demonstrated that the epigenetic modifications take place from the early embryo stage, and could persist across the life course, thereby leading to disease in adulthood (Cunliffe, 2015). Childhood trauma impacts health outcomes in various ways. One mechanism is by the induction of toxic stress. In fact, stress can be classified into “good stress,” “bearable stress,” and “toxic stress”, and has acute, delayed and long-term effects on the body. “Good stress” can be coped with by physiological mechanisms, encouraging healthy growth; “bearable stress” states may eventually be turned into homeostasis through successful interventions; whereas, “toxic stress,” which is characterized by prolonged or frequent activation and dysregulation of the stress response pathway, induces long-term changes and damage not only to the brain but also to the rest of body (Franke, 2014).

A growing body of evidence supports the hypothesis that ACEs, encompassing physical, emotional, and sexual abuse, neglect, and household dysfunction, confer long-term metabolic risk through epigenetically encoded alterations in HPA axis regulation. As illustrated in **Figure 1.14**, trauma exposure during critical developmental windows activates the HPA axis, triggering the translocation of the FKBP4-glucocorticoid receptor (GR) complex to the nucleus, where it functions as a transcription factor driving *FKBP5* expression. The resultant increase in FKBP51 protein shifts the equilibrium from the high-sensitivity *FKBP4*-GR complex toward the lower-sensitivity *FKBP51*-GR complex, establishing an ultra-short negative feedback loop that blunts cortisol-mediated HPA suppression. Critically, repeated or severe early-life trauma is thought to induce demethylation of CpG sites within the *FKBP5* gene, particularly in functional GRE-containing intronic regions, thereby epigenetically locking the *FKBP5* locus in a transcriptionally permissive state. This epigenetic reprogramming perpetuates GR resistance and chronic HPA axis dysregulation well into adulthood, independent of ongoing stress exposure. The downstream metabolic consequences are mechanistically direct: chronically dysregulated cortisol signaling drives visceral

adipogenesis, peripheral insulin resistance, and systemic low-grade inflammation, all core components of T2DM pathophysiology. Furthermore, ACE-associated epigenetic modifications are hypothesized to extend beyond *FKBP5* to encompass broader networks of genes governing energy metabolism and inflammatory signaling, including promoter methylation changes in cytokine genes and metabolic regulatory loci. Collectively, this framework positions epigenetic dysregulation of the stress-response machinery as a biologically plausible and empirically supported molecular bridge linking early-life adversity to the elevated T2DM risk observed in ACE-exposed populations, underscoring the importance of trauma-informed approaches in metabolic disease prevention (Ramo-Fernández et al., 2019; Womersley et al., 2022).

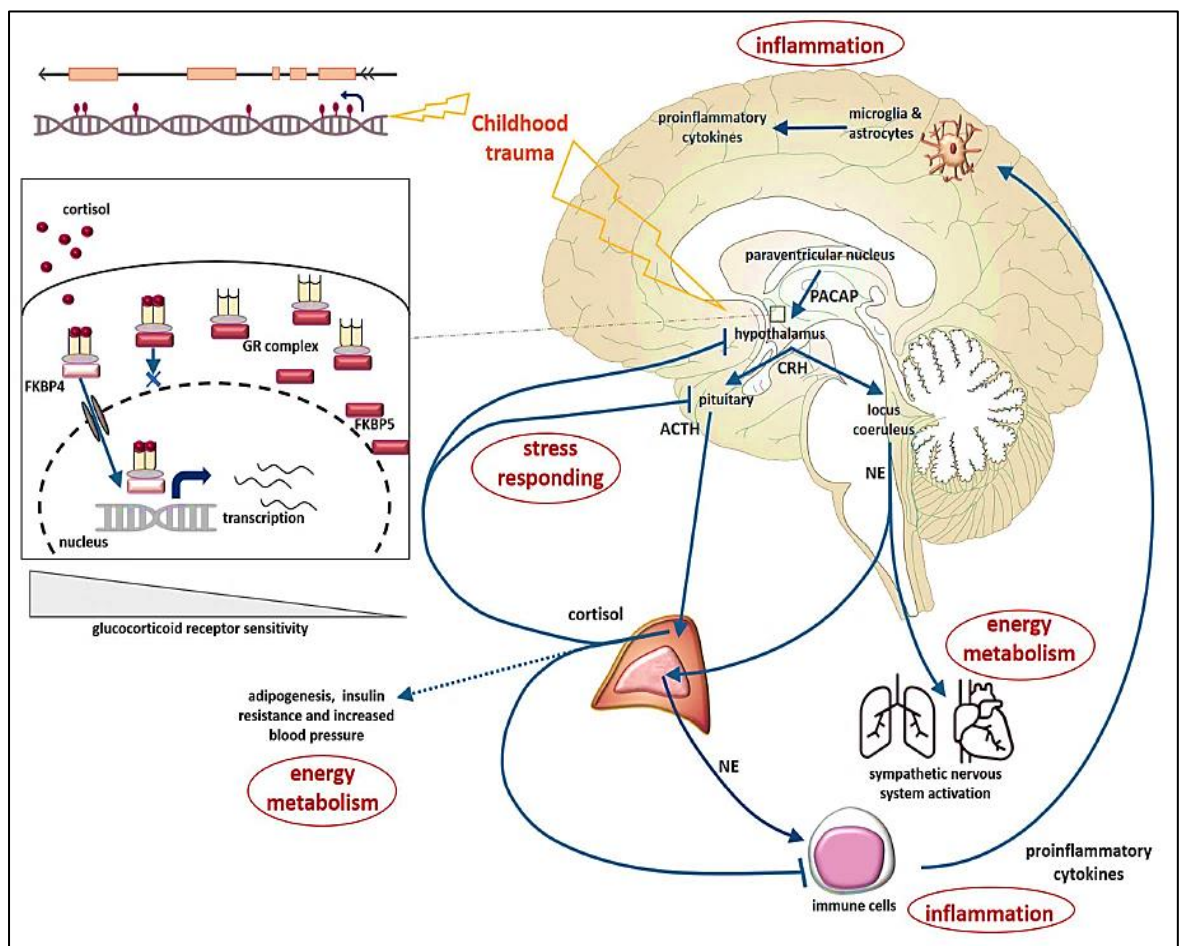


Figure 1.14: Neurobiological and Epigenetic Mechanisms Linking Childhood Trauma to Metabolic Dysregulation

The Role of HPA Axis Dysregulation, Glucocorticoid Receptor Sensitivity, and Sympathoadrenal Activation in Inflammation and Energy Metabolism. Modified from Womersley et al. (2022)

Chronic stress arising from child maltreatment sustains HPA axis hyperactivation, producing prolonged hypercortisolemia that drives insulin resistance and hyperglycemia through suppression of insulin secretion and peripheral insulin sensitivity (Geer et al., 2014). Excess glucose is preferentially stored as visceral and hepatic lipid, establishing the metabolic substrate for T2DM. Resultant visceral adiposity further amplifies this dysregulation through maladaptive adipokine signaling, sustained hyperleptinemia downregulates hypothalamic leptin receptors, impairing leptin's physiological inhibition of CRH and perpetuating HPA hyperactivity (Haleem, 2014), while concurrently reduced adiponectin concentrations worsen peripheral insulin resistance (Papargyri et al., 2018). Collectively, these interacting pathways position maltreatment-induced neuroendocrine dysregulation as a mechanistically coherent biological antecedent of T2DM, wherein chronic stress, hypercortisolemia, insulin resistance, visceral adiposity, and adipokine dysfunction converge to establish lasting cardiometabolic vulnerability (Womersley et al., 2022).

Empirical investigations across both childhood and adult populations have identified widespread epigenetic modifications associated with maltreatment exposure. Candidate gene studies have consistently reported increased methylation of *NR3C1* (encoding the glucocorticoid receptor) and *FKBP5* following childhood trauma. McGowan et al. (2009) demonstrated elevated *NR3C1* exon 1F promoter methylation in suicide victims with maltreatment histories, while Klengel et al. (2014) showed that allele-specific, trauma-dependent methylation within *FKBP5* glucocorticoid response elements significantly elevated risk for stress-related psychiatric disorders in adulthood. Maltreatment-induced epigenetic dysregulation further extends into dopaminergic pathways, with elevated *DRD2* methylation reported among women with histories of child sexual abuse, implicating broader neuromodulatory disruption beyond the HPA axis (Cicchetti et al., 2016).

Adult studies have corroborated these findings at the level of stress-regulatory gene networks. Increased *NR3C1* methylation has been associated with reduced glucocorticoid receptor expression and impaired cortisol negative feedback, sustaining chronic HPA dysregulation into adulthood (Hao et al., 2018). Both rapid nongenomic and delayed genomic feedback mechanisms — suppressing CRH/ACTH release and POMC transcription respectively — appear vulnerable to disruption by adversity-

induced epigenetic reprogramming (Megala et al., 2021). The epigenetic footprint of childhood adversity additionally encompasses COMT methylation, affecting catecholamine metabolism, and *BDNF* methylation, impairing neuroplasticity — extending dysregulation into neuroendocrine and neuroinflammatory domains and conferring broad vulnerability to stress-related and metabolic disorders across the lifespan (Hao et al., 2018; Megala et al., 2021).

The existing literature, while mechanistically rich, remains fragmented with HPA axis biology, epigenetic studies, and metabolic outcomes largely examined in isolation. This review integrates these domains to construct a coherent biological narrative linking ACEs to T2DM through epigenetically mediated stress dysregulation. By synthesizing evidence from candidate gene, genome-wide, and intergenerational studies and explicitly acknowledging null findings, this work addresses a critical gap in understanding how early-life adversity becomes metabolically embedded, providing a foundation for trauma-informed approaches to T2DM prevention and intervention.

Collectively, existing evidences indicate that genetic susceptibility, adverse early-life exposures, and epigenetic regulation may each contribute to T2DM pathogenesis (Huffhines et al., 2016; Joseph & Golden, 2017; Kreienkamp et al., 2023; Walaszczyk et al., 2018). However, these components have largely been investigated independently, and the molecular mechanisms through which early-life adversity may become biologically embedded and contribute to later metabolic disease remain incompletely understood. Evidence integrating ACE exposure, genome-wide DNA methylation, and T2DM remains limited, especially in South Asian populations, including Bangladesh. Investigating genetic susceptibility together with DNA methylation patterns and ACE-related environmental exposure may therefore provide complementary insights into the inherited and environmentally responsive mechanisms underlying T2DM.

1.3 Hypothesis

Genetic changes and epigenetic modifications, especially DNA methylation affected by environmental factors and adverse childhood experiences, play an important role in the development of type 2 diabetes mellitus among the Bangladeshi population.

1.4 Objectives of the Study

1.4.1 General Objectives of the study

To investigate single nucleotide polymorphisms (SNPs) in selected genes and DNA methylation changes associated with type 2 diabetes mellitus (T2DM), and to identify socio-contextual and environmental factors associated with T2DM and epigenetic alterations among the Bangladeshi population.

1.4.2 Specific Objectives of the Study

1. To identify socio-contextual and environmental risk factors, including adverse childhood experiences (ACEs), associated with T2DM.
2. To detect genetic variations in the form of single nucleotide polymorphisms (SNPs) in selected candidate genes among Bangladeshi patients with T2DM.
3. To determine epigenetic changes in the form of DNA methylation patterns among Bangladeshi patients with T2DM.
4. To assess the relationship between ACEs and DNA methylation changes in patients with T2DM.

1.5 Rationale of the Study

Type 2 diabetes mellitus (T2DM) is a major public health concern worldwide and a leading contributor to morbidity, mortality, and healthcare burden. South Asian countries are experiencing a rapid increase in diabetes prevalence, and Bangladesh is among the most affected countries in the region. Approximately 90% of all diabetes cases are attributable to T2DM. This increasing burden of the disease in Bangladesh has been attributed to rapid urbanization, lifestyle transition, unhealthy dietary habits, physical inactivity, obesity, and population ageing. Despite exposure to these common risk factors, not all individuals develop T2DM, suggesting that biological factors contribute to individual susceptibility. Therefore, understanding the molecular mechanisms underlying T2DM has become increasingly important for improving disease prevention and management.

Genetic factors play an important role in the development of T2DM. Numerous susceptibility loci have been identified that influence insulin secretion, insulin sensitivity, glucose metabolism, and pancreatic β -cell function. Among these, the *TCF7L2* and *SLC30A8* genes have attracted considerable attention because of their established roles in glucose homeostasis and insulin regulation. Furthermore, evidence regarding the contribution of these genetic variants to T2DM susceptibility in the Bangladeshi population remains limited. Therefore, investigation of candidate genetic polymorphisms may provide valuable insights into population-specific genetic risk factors for T2DM.

However, currently identified genetic variants explain only a portion of the heritable risk of T2DM, a phenomenon often referred to as the “missing heritability” of the disease. Increasing evidence suggests that epigenetic mechanisms may account for part of the missing heritability not explained by genetic variation alone and may help explain how environmental and socio-contextual exposures contribute to T2DM pathogenesis. Among the various epigenetic modifications, DNA methylation is one of the most extensively studied. DNA methylation can regulate gene expression without altering the underlying DNA sequence and may be influenced by environmental exposures throughout life. Emerging evidence indicates that adverse childhood experiences (ACEs), chronic psychological stress, nutritional factors, and other environmental

exposures may be associated with persistent DNA methylation changes affecting metabolic regulation, inflammatory pathways, and stress-response systems. These epigenetic alterations may contribute to the development of T2DM later in life. Despite the growing burden of T2DM in Bangladesh, studies investigating the combined roles of genetic variation, DNA methylation, and environmental exposures remain scarce. Therefore, the present study aims to characterize DNA methylation changes associated with T2DM and evaluate the potential influence of environmental exposures, particularly ACEs, on these molecular mechanisms, thereby complementing existing knowledge of genetic susceptibility to the disease. The findings are expected to improve understanding of the genetic and epigenetic basis of T2DM pathogenesis and may contribute to future strategies for risk identification and prevention.

2. Materials and Methods

2.1 Study design

A case-control study was conducted at the Department of Genetic Engineering and Biotechnology, University of Dhaka with the collaboration of Department of Laboratory Medicine, BIRDEM General Hospital and Department of Public Health and Informatics, Bangladesh Medical University.

2.2 Study Population

The study population was recruited from Sirajdikhan Upazila (sub-district) of Munshiganj District. This upazila is around 29 kilometers southeast of Dhaka, the capital city. This agro-based area is around 180 km² in size, with geographical coordinates of 23°30' and 23°41' latitude and 90°14' and 90°27' longitude (**Figure 2.1**). The case 'type 2 diabetes mellitus' (T2DM) was collected from a diabetic cohort of Sirajdikhan, Munshiganj. The diabetic cohort has been maintained for over twelve years, with each patient having a unique identification number (Barman et al., 2024). Cases were selected using computer-generated simple random sampling with the help of identifying number of diabetic cohort. At the same time, controls were age-matched neighbors of the cases without diabetes, confirmed by biochemical test.



Figure 2.1: The geographical representation of study area in circle source:

Banglapedia

Case: Diagnosed case of adult person of type 2 diabetes mellitus (30 to 65 years) with either previously having fasting blood sugar ≥ 7 mmol/L or 2 hours after glucose load ≥ 11.1 mmol/L or both, or taking doctor prescribed anti-diabetic medication (WHO, 2006).

Control: Age matched apparently healthy person, having no known disease, fasting blood sugar < 6.1 mmol/L and 2 hours after glucose load < 7.8 mmol/L (WHO, 2006).

2.3 Selection Criteria

2.3.1 Inclusion Criteria

For Case

1. Age 30 to 65 years
2. Both gender
3. Patients having fasting blood sugar ≥ 7 mmol/L or 2 hours after glucose load ≥ 11.1 mmol/L or both
4. Patients using medication for diabetes treatment
5. Permanent resident of Bangladesh

For Control

1. Age 30 to 65 years
2. Both gender
3. Person having fasting blood sugar < 6.1 mmol/L and 2 hours after glucose load < 7.8 mmol/L
4. Apparently healthy and no known disease
5. Permanent resident of Bangladesh

2.3.2 Exclusion criteria

1. Pregnancy and lactation in case of female.
2. Samples with poor DNA quality or low DNA concentration

2.4 Study Procedure

An outline of the study procedure is provided in **Figure 2.2** and the details are provided in the relevant sections.

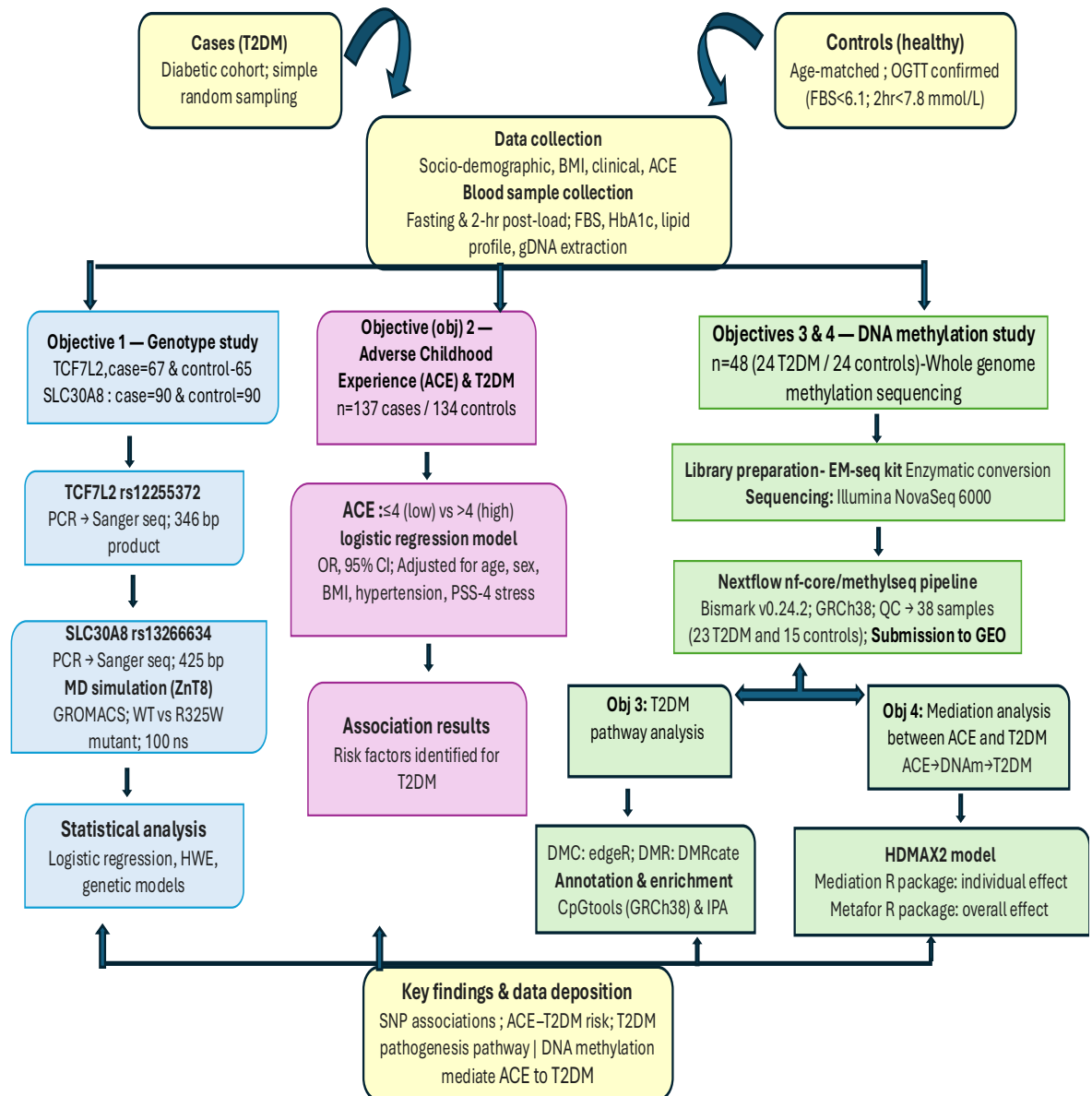


Figure 2.2: Schematic diagram of study procedure

2.5 Sample size Estimation

2.5.1 Sample size for ACE and T2DM association

Study objective 1: To identify socio-contextual and environmental risk factors, including ACEs, associated with T2DM.

The primary hypothesis under Objective 1 was an association between exposure to four or more ACEs and T2DM. Sample size was estimated in OpenEpi [version 3] for an unmatched case–control design with 1:1 allocation, assuming a two-sided α of 0.05, 80% power, an anticipated control-group exposure prevalence of 15.0%, and an odds ratio of 2.51 (95% CI: 1.12–5.63) for four or more ACEs and diabetes onset (Lown et al 2019). In the absence of local data on the prevalence of four or more ACEs, the 15.0% figure was adopted as a working assumption. Using the Fleiss formula with continuity correction, the minimum requirement was 124 participants per group; a 10% allowance for non-response and incomplete data raised the target to 137 per group. In total, 137 patients with T2DM and 134 non-diabetic controls were enrolled, with 3 controls excluded due to incomplete data.

2.5.2 Sample size for genotype Study

Study Objective 2: To detect genetic changes as single nucleotide polymorphism (SNP) in known candidate genes of T2DM patients in Bangladeshi populations

SNPs in *TCF7L2* and *SLC30A8* were analyzed in this study.

Genotype study 1: For *TCF7L2* rs12255372, the sample size was estimated using the OSSE sample size calculator assuming 80% statistical power, a 5% significance level, minor allele frequencies from Hameed et al. (2021), and a 10% dropout rate. The minimum required sample size was 62 participants per group. Finally, 67 T2DM cases and 65 controls were included between July and September 2021.

Genotype study 2: For *SLC30A8* rs13266634, the sample size was calculated using OpenEpi for SNP-based case–control association studies. Assuming 80% power, a 5% significance level, an odds ratio of 2.32 reported by Alharbi et al. (2021), and a 1:1 case–control ratio following Kelsey et al. (1996), the minimum required sample size was 90 participants in each group. Accordingly, 90 T2DM patients and 90 healthy controls were included.

2.5.3 Sample size for DNA methylation analysis

Study objectives 3 and 4: To determine epigenetic changes as DNA methylation of T2DM patients in Bangladeshi populations and to assess the relationship between ACEs and DNA methylation changes in patients with T2DM.

We conducted an exploratory study on whole genome DNA methylation for study objectives 3 and 4, to identify epigenetic alterations associated with T2DM and to examine whether DNA methylation mediates the relationship between ACE and T2DM risk. A total of 48 individuals (24 T2DM cases and 24 age-matched controls) were randomly selected from a larger case–control dataset comprising 137 T2DM cases and 134 controls using a computer-generated random sampling procedure in SPSS for genome-wide DNA methylation profiling. This sample size is consistent with discovery-stage whole-genome methylome studies given their considerable cost and computational demands, where millions of CpG measurements per sample provide sufficient statistical information for detecting differential methylation with appropriate regional aggregation and multiple-testing correction. This approach is well-precedented, as previous whole-genome methylation studies have successfully identified biologically meaningful epigenetic differences using comparable exploratory sample sizes (Song et al., 2021; van Dokkum et al., 2023; Ortiz et al., 2026). Therefore, the present analysis represents a discovery-phase exploratory study aimed at identifying methylation markers associated with T2DM and further exploring the mediating role of DNA methylation in the pathway from ACE exposure to T2DM development in later life, with findings intended to inform hypothesis generation and power calculations for future confirmatory studies in larger, population-representative cohorts.

2.6 Variables

2.6.1 Socio-demographic variables

The socio-demographic characteristics were recorded including age, sex, education attainment (classified according to years of schooling) and employment status.

2.6.2 Anthropometric variables

Body mass index (BMI) was calculated by measuring weight and height of the individual. The weight was measured using digital scales through instruction of removing heavy clothing. Meanwhile, height was measured using a standard scale in bare-footed stand position.

$$BMI = \frac{Weight (kg)}{[Height(m)]^2}$$

Using Asian-pacific cutoff points of BMI (kg/m²), individuals were categorized as 18.5-22.9 for normal-weight, 23.0-27.5 for overweight, and >27.5 for “obese” category (Lim et al., 2017).

2.6.3 Variables related to personal illness history

- History of diabetes mellitus
- History of Hypertension

2.6.4 Behavioral variables

- History of smoking – current smoker, previous smoker and non-smoker
 - Non-smoker: Who had never smoked
 - Current smoker: Who had a smoking history for a certain period (≥6 months) at present either regularly or irregularly
 - Previous smoker: A participant who had a previous history of smoking but was not currently smoking
- Alcohol intake history
- Smokeless tobacco consumption history: Betel nut, betel leaf, tobacco chewing habit

2.6.5 Clinical Variables

Blood pressure was recorded three times at 5 minutes intervals in sitting position, in the right arm, using a digital BP apparatus as per World Health Organization (WHO) guidelines (WHO, 2012). The standard protocol was followed, and the average of the last two readings was used in the analyses. Hypertensive patients were defined as those with systolic blood pressure (SBP) equal to or more than 140 mm of Hg and/or diastolic

blood pressure (DBP) equal to or more than 90 mm of Hg or those being treated for hypertension (BONITA, 2001; Booth et al., 2006).

2.6.6 Adverse Childhood Experiences (ACE) and stress variables

The ACE questionnaire, an internationally reliable and valid tool, was used to measure the childhood adversity of the study participants up to the age of 18. This tool has been extensively used in several studies (Anda et al., 2010; Felitti et al., 1998). It addresses ten individual ACEs: psychological, physical, and sexual abuse; emotional and physical neglect; parental separation; violence against mother; household substance abuse; household mental illness; and incarceration of any household member. This questionnaire is free for usage (Appendix-1). Respondents who answer "yes" to any question on this questionnaire will be considered exposed to an adverse event. A positive response to any question gets a score of one, and all positive responses are added together to generate the total ACE score, which ranges from 0 to 10. ACE questionnaire have translational and cultural validity in a previous study (Haque, Tasnim, et al., 2021). For categorization by ACE, ACE score four is the cut-point which was found a significant association with chronic diseases (Alhowaymel et al., 2023).

For estimating stress levels the participants typically experience, the four-item Perceived Stress Scale-4 (PSS-4) (Sheldon et al., 1983) was adopted. This state measure was chosen due to its adequate reliability (with a Cronbach's alpha coefficient of 0.60) (Appendix-2).

2.6.7 Biochemical variables

After collecting the aforementioned data from the participants and obtaining their written informed consent for additional laboratory tests, we invited them to attend a private healthcare facility for biochemical blood tests that would provide a reliable indication of their overall health status and assess their glycemic control level. These tests included measuring -

- Fasting Blood Sugar
- 2-hour post prandial Blood Sugar
- HbA1c and
- Fasting Lipid Profile (i.e., total cholesterol, triglyceride, HDL, and LDL).

All blood samples were sent to the Department of Laboratory Medicine, BMU, within six hours of collection while maintaining proper cold chain.

Venous blood samples were collected after an overnight fast of at least 8 hours. Fasting blood sugar (FBS) was measured using the glucose oxidase method. For 2-hour postprandial glucose assessment, participants consumed 75g of glucose solution, and blood samples were collected 2 hours post-ingestion. Serum lipid profiles (apart from LDL-cholesterol) were conducted by standard enzymatic assay. All the tests were run by an automated biochemical analyzer (Beckman Coulter AU480, Tokyo, Japan). For determining LDL-cholesterol concentrations, the Friedewald formula was adopted (Friedewald et al., 1972). The intra- and inter-assay coefficients of variation for the tests were within 2.5% and 4%, respectively.

A participant was considered having dyslipidemia if any of the following criteria were met: serum total cholesterol > 200 mg/dl, LDL-cholesterol $\geq$ 140 mg/dl, HDL-cholesterol < 40 mg/dl, triglycerides $\geq$ 150 mg/dl, and/or currently receiving treatment with lipid-lowering medications (Okamura et al., 2024).

2.7 Bio-sample collection

A registered phlebotomist collected 11.0 ml of venous blood from each participant on two occasions under strict aseptic precautions using a disposable syringe (**Figure 2.3**). Blood was drawn in two states: an overnight fasting state and 2 hours post-prandial state. From the fasting blood draw, 2 ml was transferred into a sodium fluoride glucose tube for fasting glucose estimation, 2 ml into an EDTA-containing vacutainer tube for HbA1c estimation, and 2 ml into a serum separator tube for lipid profile estimation. From the post-prandial draw, 2 ml was collected into a sodium fluoride glucose tube for glucose estimation, and 3 ml was preserved in an EDTA tube for genetic analysis and DNA extraction. Serum samples were separated from vacutainer tubes by centrifugation at 3,000 rpm for 10 minutes. HbA1c samples were analyzed within 1 hour of collection, while serum and whole blood samples were stored at -20°C and -80°C, respectively, until further analysis.

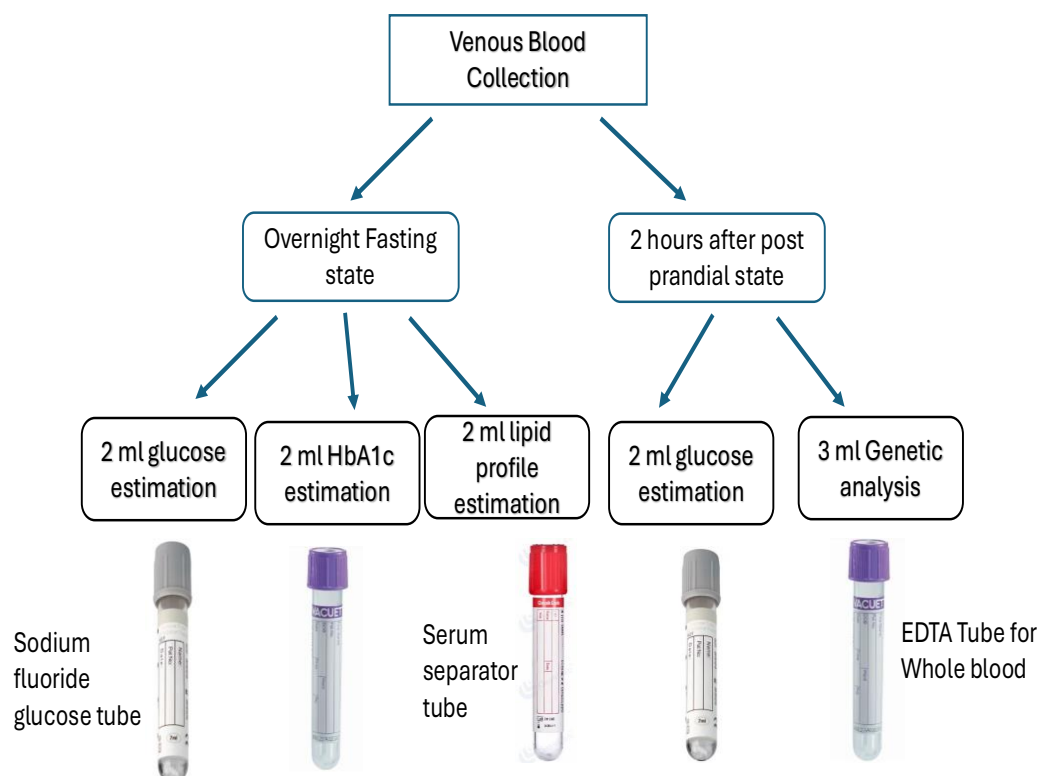


Figure 2.3: Flow Chart of Aliquot Distribution of blood sample

2.8 Genetic Analysis: DNA Extraction Procedure

a. Blood sample processing:

Whole blood sample processing and extraction of genomic DNA (gDNA) from peripheral blood were performed using a commercial extraction kit (New England Biolabs, Ipswich, MA, USA) through their extraction guideline protocol. The initial step was about extracting as much as genomic DNA possible and for that buffy coat was prepared.

b. Buffy coat preparation:

Buffy coat is a leukocyte-enriched fraction of whole blood. Buffy coat is prepared by centrifuging whole blood at $2500 \times g$ for 10 minutes at room temperature (15–25°C). After centrifugation, 3 different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is buffy coat, containing concentrated leukocytes; and the bottom layer contains concentrated erythrocytes. The buffy coat (about 300 μ l) was taken in a 1.5 ml eppendorf for genomic DNA extraction.

c. DNA extraction protocol

The following protocol was used for gDNA extraction of whole blood (**Figure 2.4**).

i. Part 1: Sample lysis

About 100 μ l of buffy coat containing sample was transferred to a 1.5 ml microfuge tube. Around 12 μ l Proteinase K, 5 μ l RNase A and 100 μ l of Blood Lysis Buffer were added to the sample. It was then incubated for 5 minutes at 56°C by using a heating block and vortex twice during the incubation.

ii. Part 2: Genomic DNA (gDNA) binding and elution:

Bind DNA to matrix:

About 400 μ l gDNA Binding Buffer was added to the sample and mixed thoroughly by pulse-vortexing for 5-10 seconds. Then the lysate/binding buffer mix (~620 μ l) to a gDNA purification column pre-inserted into a collection tube, without touching the upper column area and immediately proceeded to next step. The cap was closed and centrifuged in microfuge: first for 3 minutes at 1,000 x g to bind gDNA and then for 1 minute at maximum speed (> 12,000 x g) to clear the membrane. The flow-through and the collection tube were discarded.

Wash matrix:

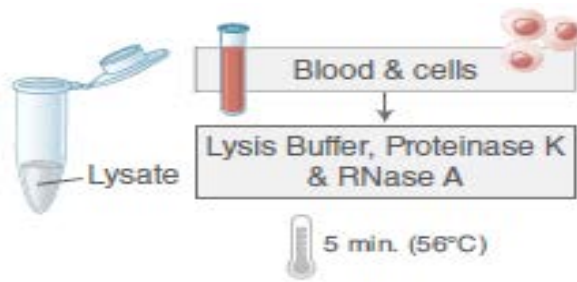
Then column was transferred to a new collection tube and added 500 μ l gDNA Wash Buffer. The cap was closed and inverted a few times so that the wash buffer reaches the cap to prevent salt contamination in the eluate. The column was reinserted into the collection tube and added 500 μ l gDNA wash buffer and close the cap. It was centrifuged immediately for 1 minute at maximum speed and discarded the collection tube and flow through.

Elute DNA:

The gDNA Purification Column was placed in a DNase-free 1.5 ml microfuge tube and 100 μ l preheated (60°C) gDNA Elution Buffer was added. Then cap was closed and incubated at room temperature for 1 minute. The tube was centrifuged for 1 minute at maximum speed (> 12,000 x g) to elute the gDNA.

PART 1: SAMPLE DISRUPTION & HOMOGENIZATION

1. Disrupt and Lyse Sample

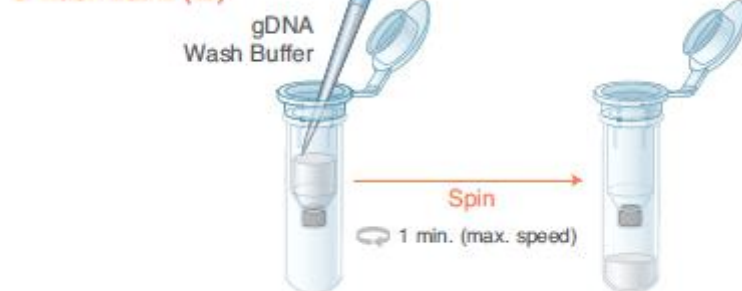


PART 2: BINDING & ELUTION

2. Bind DNA to Matrix



3. Wash Matrix (x2)



4. Elute DNA

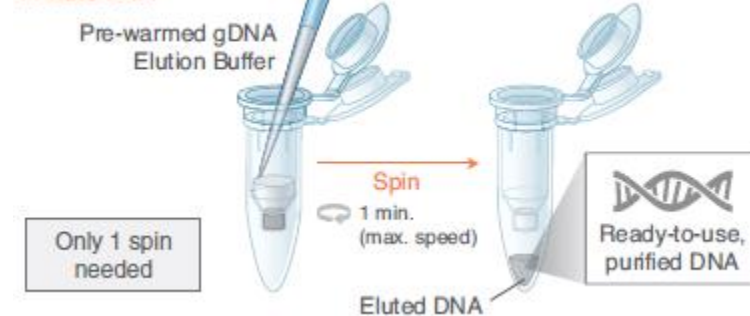


Figure 2.4: gDNA extraction method using Monarch® Genomic DNA Purification Kit

iii. DNA quality and quantity assessment

The quantity of DNA was measured using the Qubit Broad Range dsDNA kit (ThermoFisher). The quality of gDNA was seen through agarose gel electrophoresis and the purity was checked by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples yield of more than 20 ng/μl concentration and absorbance (A) A_{260}/A_{280} ratio of 1.7–2.0, were selected for PCR.

Agarose Gel electrophoresis: A 0.8% agarose gel was prepared for electrophoresis of high-molecular-weight genomic DNA. The gel was run in 1x TAE buffer. About 500 ng of 5 μL of each genomic DNA sample was mixed with 1 μL of 6x loading dye and loaded into the wells. Electrophoresis was conducted at 80 volts for 1 hour. The gel was stained with ethidium bromide and visualized using a UV transilluminator. The first lane contains a 1 kb DNA ladder serving as a size reference. Lanes 2 through 12 contain genomic DNA from study subject samples.

iv. Sample preservation:

All gDNA samples were kept in -20°C temperature in freezer for further analysis.

2.9 Genotype study

2.9.1 *TCF7L2* study

a. Primers: Polymerase chain reaction (PCR) was performed in a 25 μL reaction volume. The target region of the *TCF7L2* gene containing the rs12255372 (G/T) polymorphism was amplified using the following primers. The expected PCR product size was 346 bp.

Table 2.1: Primers of the PCR reaction of *TCF7L2* gene of the rs12255372 (G/T)

Primer	Sequence
Forward	5'-CTG GAA ACT AAG GCG TGA GG-3'
Reverse	5'-GGG TCG ATG TTG TTG AGC TT-3'

b. Preparation of PCR reaction mixture

The final PCR reaction mixture (25 μ L) was prepared in PCR tubes as follows and briefly centrifuged to mix the contents. Taq DNA polymerase used in this study was obtained from New England Biolabs (Ipswich, MA, USA).

Table 2.2: volume of reagents for PCR of the *TCF7L2* rs12255372 (G/T)

Component	Volume
Taq reaction buffer (10 $\times$)	5.0 μ L
dNTP mix (10 mM)	0.5 μ L
Forward primer (10 μ M)	0.5 μ L
Reverse primer (10 μ M)	0.5 μ L
Taq DNA polymerase (5 U/ μ L)	0.125 μ L
Template DNA	125ng
Nuclease-free water	Up to 25 μ L

c. PCR reaction

PCR amplification was performed using a GeneAtlas G02/G05 thermal cycler (ASTEC, Fukuoka, Japan). The cycling conditions (**Figure 2.5**) consisted of an initial denaturation at 95 $^{\circ}$ C for 2 minutes, followed by 35 cycles of denaturation at 95 $^{\circ}$ C for 30 seconds, annealing at 56 $^{\circ}$ C for 30 seconds, and extension at 68 $^{\circ}$ C for 30 seconds, with a final extension at 68 $^{\circ}$ C for 5 minutes.

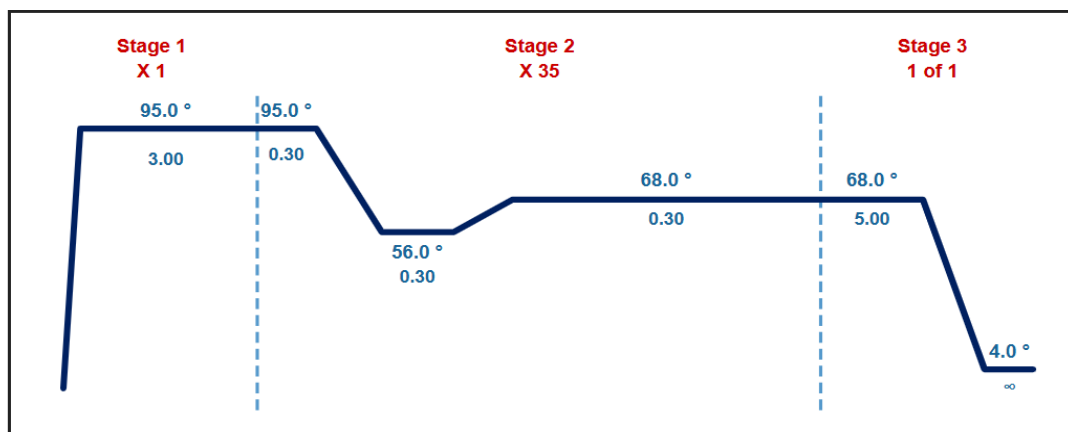


Figure 2.5: Condition for PCR reaction of *TCF7L2* rs12255372 (G/T) in thermal cycler

d. Post-PCR detection of amplified DNA by electrophoretic analysis

The amplified PCR products were separated by 1.5% agarose gel electrophoresis stained with 0.5 µg/mL ethidium bromide in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). Electrophoresis was performed and the DNA bands were visualized under ultraviolet illumination using a gel documentation system (Marne-la-Vallée, France). A 50 bp DNA ladder was loaded in the first lane to determine the size of the PCR products.

e. Sequencing reaction

The PCR products were purified prior to sequencing using ExoSAP-IT® (USB Corporation) to remove excess primers and nucleotides.

PCR purification protocol: Five microliters (5 µL) of PCR product was mixed with 2 µL of ExoSAP-IT®. The mixture was incubated at 37 °C for 15 minutes to degrade residual primers and nucleotides. The reaction was then incubated at 80 °C for 15 minutes to inactivate the enzyme.

Cycle sequencing: Sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Sequencing reactions were carried out in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, USA). In the present study, the forward primer was used for Sanger sequencing of the amplified fragment. The fluorescent-

labeled sequencing products were purified using the BigDye XTerminator® Purification Kit (Applied Biosystems) and analyzed by capillary electrophoresis using an ABI 3730xl DNA Analyzer (Applied Biosystems, USA).

Multiple sequences were aligned with the *TCF7L2* reference sequence from NCBI (Reference Sequence: NG_012631.1) using Muscle algorithm present in MEGA software (version 11) (Tamura et al., 2021) and visualized in Jalview (Waterhouse et al., 2009).

2.9.2 *SLC30A8* study

2.9.2.1 Genotype of *SLC30A8* rs13266634 (C>T)

a. Primers

Polymerase chain reaction (PCR) was performed in a 25 µL reaction volume to genotype the rs13266634 (C>T) polymorphism of the *SLC30A8* gene. The target region was amplified using the following primers.

Table 2.3: Designated primers of the PCR reaction of *SLC30A8* rs13266634 (C>T)

Primer	Sequence
Forward	5'- GACAGAAAGAGTTCCCATAGCG -3'
Reverse	5'- TAGCAGCATGTTTGAAGGTGGC -3'

b. Preparation of PCR reaction mixture

The final PCR reaction mixture (25 µL) was prepared in PCR tubes and briefly centrifuged to mix the contents.

Table 2.4: Volume of PCR reagents of *SLC30A8* rs13266634 (C>T)

Component	Volume / Amount
Taq 2× Master Mix (New England Biolabs Inc., Ipswich, MA, USA; M0270)	12.5 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Template DNA	60 ng
MilliQ PCR-grade water	Up to 25 µL (to adjust final volume)

c. PCR reaction

PCR amplification was performed using a GeneAtlas G02/G05 thermal cycler (ASTEC, Fukuoka, Japan). The cycling conditions consisted of an initial denaturation at 95 °C for 3 minutes, followed by 32 cycles of denaturation at 95 °C for 30 seconds, annealing at 65 °C for 30 seconds, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 1 minute (Figure 2.6).

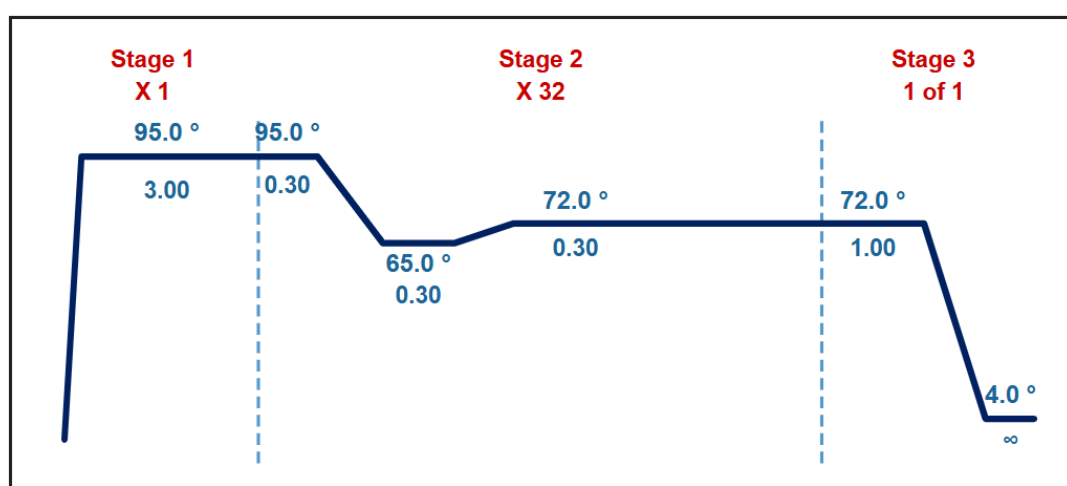


Figure 2.6: PCR cycling conditions used for amplification of the *SLC30A8* rs13266634 polymorphism.

d. Post-PCR detection of amplified DNA by electrophoretic analysis

The amplified PCR products were separated by 1.5% agarose gel electrophoresis stained with 0.5 µg/mL ethidium bromide in TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0). The DNA bands were visualized under ultraviolet illumination and photographed using a gel documentation system (Marne-la-Vallée, France). A 50 bp DNA ladder was used to estimate the size of the amplified PCR products.

e. Sequencing reaction

PCR products were purified prior to sequencing using the ExoSAP-IT PCR Product Cleanup Kit (Affymetrix Inc., Cleveland, OH, USA) to remove excess primers and nucleotides.

PCR purification protocol: Five microliters (5 µL) of PCR product was mixed with 2 µL of ExoSAP-IT. The mixture was incubated at 37 °C for 15 minutes to degrade residual primers and nucleotides. The reaction was then incubated at 80 °C for 15 minutes to inactivate the enzyme.

Cycle sequencing: Sanger sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequencing reactions were carried out in a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, USA). In the present study, the forward primer was used for sequencing of the amplified fragment. The fluorescent-labeled sequencing products were purified and analyzed by capillary electrophoresis using an ABI 3500xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Chromatogram files were converted to FASTA format and aligned with the reference sequence of *SLC30A8* rs13266634 using multiple sequence alignment.

2.9.2.2 Molecular dynamics simulation of protein ZnT8 of SLC30A8

a. Dataset for Molecular Dynamics (MD) Simulation

The three-dimensional structure of human zinc transporter 8 (ZnT8) was obtained from the Protein Data Bank (PDB ID: 6XPF), a wild-type human ZnT8 structure determined by cryo-electron microscopy at a resolution of 5.90 Å (Xue et al., 2020). The deposited structure represents a homodimer, with each ZnT8 subunit comprising a 320-residue construct. ZnT8 contains four zinc-binding sites within each subunit. The primary site

within the transmembrane domain (TMD) mediates Zn^{2+} transport, while an interfacial site between the TMD and C-terminal domain (CTD) modulates transport activity. Two additional cytosolic zinc-binding sites in the CTD are coordinated by conserved residues, including contributions from the His-Cys-His (HCH) motif of the neighboring subunit, supporting structural stability and Zn^{2+} transport (Xue et al., 2020).

b. Homology Modelling

The crystal structure lacked the first 49 N-terminal residues; however, the mutation of interest (Arg325Trp; rs13266634) is located in the cytosolic domain of chain A. The primary sequence of ZNT8_HUMAN was retrieved from the UniProt database (Accession Q8IWU4). Because several residues were missing from the 6XPF structure, homology modelling was performed for chain A (320 residues) of both the wild-type ZnT8 (WZnT8) and the mutant ZnT8 (MZnT8). Structural models were generated using the Robetta and AlphaFold2 prediction servers. The stereochemical quality of the predicted structures was assessed using the SAVES v6.1 validation server, which incorporates ERRAT, Verify-3D, and PROCHECK programs (Cohen, 1992; Colovos & Yeates, 1993; Daghestani et al., 2019; Jumper et al., 2021).

c. Molecular Dynamics (MD) Simulation Setup

Molecular dynamics simulations were performed using GROMACS v2024.4 with the CHARMM36 all-atom force field. Protein structures were prepared by removing crystallographic water molecules and adding missing hydrogen atoms. The system topology was generated with CHARMM36 parameters, with the N- and C-termini assigned their standard charged states (NH_3^+ and COO^-). Each protein was placed in a cubic simulation box with a 1.0 nm buffer distance from the edges and solvated using the TIP3P water model. Sodium and chloride ions were added to neutralize the system and achieve a physiological ionic concentration of 0.15 M. Energy minimization was carried out using the steepest descent algorithm to remove steric clashes. The minimized systems were subsequently equilibrated under the canonical (NVT) ensemble for 250 ps using the Berendsen thermostat, followed by equilibration under the isothermal-isobaric (NPT) ensemble for 250 ps at 303 K and 1 bar using the Parrinello–Rahman barostat. Following equilibration, a 100 ns production MD simulation was performed with a 1 fs time step. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method, with a 1.0 nm cutoff for

short-range van der Waals interactions. Periodic boundary conditions were applied, and bond constraints were implemented using the LINCS algorithm.

Structural stability and dynamic behavior of WZnT8 and MZnT8 were evaluated by calculating root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), radius of gyration (Rg), and solvent-accessible surface area (SASA) following previously established approaches (Daghestani et al., 2019; Swetha et al., 2016; Vijayakumar & Das, 2019). Simulation outputs were visualized and plotted using R (version 4.4.1) with the ggplot2 package (Villanueva & Chen, 2019).

2.10 DNA Methylation Study

Genomic DNA (gDNA) extracted from peripheral white blood cell were used for our Whole genome methylation sequencing. A number of studies uses peripheral whole blood as a extracted gDNA for DNA methylation study including Type 2 diabetes pathogenesis (Dayeh et al., 2016; Toperoff et al., 2012; Walaszczyk et al., 2018; X. Wang et al., 2022). A total of 48 samples were selected for the methylation study, comprising 24 T2DM cases and 24 healthy controls prior to quality-control filtering.

2.10.1 DNA Methylation Library Preparation Using NEBNext Enzymatic Methyl-seq (EM-seq)

Genome-wide DNA methylation profiling was performed using the NEBNext[®] EM-seq[™] Kit (New England Biolabs, Ipswich, MA, USA) and the NEBNext Enzymatic Methyl-seq Library Preparation Protocol (Twist Bioscience, San Francisco, CA, USA), with minor modifications (**Figure 2.7**). This method employs enzymatic conversion instead of sodium bisulfite treatment, preserving DNA integrity and reducing sequencing bias. Consequently, EM-seq enables accurate genome-wide methylation detection while requiring lower DNA input and producing higher library yields, longer reads, reduced duplication, and more uniform genomic coverage (Olova & Andrews, 2025).

Library preparation was carried out following the manufacturer's instructions (NEBNext EM-seq Kit, NEB #E7120S/L; UltraShear protocol, NEB #M7634S/L).

a. DNA input and control setup

Genomic DNA (gDNA) quality and concentration were assessed prior to library preparation. For each sample, gDNA was combined with two spike-in controls—unmethylated lambda DNA and CpG-methylated pUC19 DNA—to monitor conversion efficiency and methylation detection accuracy. The reaction mixture was prepared in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) to a final volume of 26 μ L (Table 2.5). The prepared mixture was subsequently subjected to enzymatic DNA fragmentation.

Table 2.5: Input reaction mixture for enzymatic methyl-seq library preparation

Component	Volume (μL)
Genomic DNA	24
Unmethylated lambda control DNA	1
CpG-methylated pUC19 control DNA	1
Total volume	26

b. Enzymatic DNA fragmentation

DNA fragmentation was performed using the NEBNext UltraShear enzymatic fragmentation system. To the 26 μ L DNA mixture, UltraShear Reaction Buffer and UltraShear enzyme were added to obtain a final reaction volume of 44 μ L. Fragmentation reactions were incubated in a thermal cycler according to the manufacturer's protocol to generate uniform DNA fragments suitable for downstream library preparation. The fragmentation conditions were adjusted to produce DNA fragments with an average insert size of approximately 120–150 bp, which is optimal for downstream sequencing on the Illumina NovaSeq 6000 platform

c. End repair and dA-tailing

Following fragmentation, DNA fragments underwent end repair and dA-tailing using the NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs). The End Prep Enzyme Mix repaired fragmented DNA ends and added a single adenine nucleotide to the 3' ends of the fragments. This step produced A-tailed DNA fragments compatible with subsequent adapter ligation.

d. Adapter ligation

Sequencing adapters containing methylated cytosines were ligated to the prepared DNA fragments using the NEBNext ligation reagents. The use of methylated adapters prevents artificial cytosine conversion during the enzymatic methylation conversion process and preserves accurate methylation information. Adapter-ligated DNA fragments were subsequently purified using magnetic bead-based cleanup.

e. Magnetic bead cleanup

Magnetic bead purification was performed at several stages of library preparation to remove excess adapters, enzymes, and reaction components (Table 2.6). Each cleanup involved binding DNA fragments to magnetic beads, washing with ethanol, and eluting purified DNA in a low-salt buffer.

Table 2.6: Magnetic bead cleanup steps during library preparation

Cleanup stage	Purpose
Post-ligation cleanup	Removal of excess adapters and enzymes
Post-deamination cleanup	Removal of reagents used during methylation conversion
Final cleanup	Purification of PCR-amplified libraries

f. Enzymatic methylation conversion

Cytosine conversion was achieved through sequential enzymatic reactions involving TET2 oxidation and APOBEC deamination. TET2 oxidised methylated (5-methylcytosine; 5mC) and hydroxymethylated (5-hydroxymethylcytosine; 5hmC) cytosines through intermediate 5-formylcytosine (5fC) to generate 5-carboxycytosine (5caC). The resulting 5caC residues are resistant to APOBEC-mediated deamination and therefore remain unchanged during subsequent steps.

The APOBEC enzyme selectively deaminated unmethylated cytosines in single-stranded DNA to uracil, which is read as thymine during sequencing. This enzymatic strategy enables accurate differentiation between methylated and unmethylated cytosines without the DNA degradation typically associated with bisulfite treatment.

g. PCR amplification

Following enzymatic conversion, DNA libraries were amplified using indexed PCR primers supplied in the kit. PCR amplification was performed in a thermocycler using cycling conditions recommended by the manufacturer to generate sufficient library yield while minimizing amplification bias.

h. Final library purification and quality control

PCR-amplified libraries were purified using magnetic bead-based cleanup to remove primer dimers and short fragments. Library concentration was measured using a Qubit fluorometer with the Qubit dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, USA). Libraries that met the required concentration threshold were selected for downstream sequencing on the Illumina NovaSeq 6000 platform. The purified libraries were stored at -20°C until further processing for library pooling and sequencing.

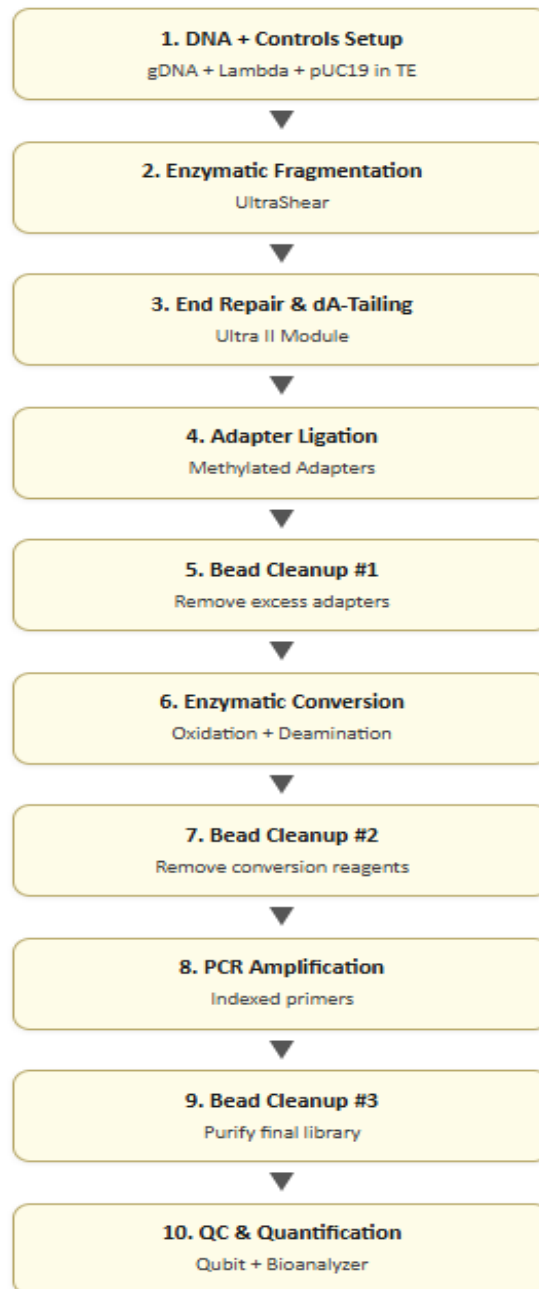


Figure 2.7: DNA methylation Library preparation workflow

2.10.2 Library Denaturation and Sequencing on Illumina NovaSeq 6000

Prepared libraries were denatured and diluted for sequencing on the Illumina NovaSeq 6000 platform following the manufacturer's protocol described in the NovaSeq 6000 System Guide (**Figure 2.8**).

a. Library pooling and normalization

Individual libraries were normalized to a final concentration of 1.5–2.5 nM using 10 mM Tris-HCl (pH 8.5) and pooled according to the required sequencing depth. Sequencing was performed on the Illumina NovaSeq 6000 platform using an S2 flow cell. For S2 sequencing, the final pooled library volume was adjusted to 150 μ L prior to denaturation and loading.

b. Library denaturation and neutralization

A fresh 0.2 N NaOH solution was prepared by diluting 1 N NaOH with nuclease-free water. The pooled library was denatured by adding NaOH, followed by brief vortexing and centrifugation at $280 \times g$ for 1 minute, and incubation at room temperature for 8 minutes. The denatured library was then neutralized using 400 mM Tris-HCl (pH 8.0) and transferred to the library tube supplied with the NovaSeq reagent cartridge. Finally, the sequencing pool consisted of 98% target-enriched libraries, 1% control material library, and 1% PhiX Control v3 Library.

c. Final loading

The denatured library pool was kept on ice and loaded into the NovaSeq 6000 sequencing system within 30 minutes. Sequencing was performed using the XP workflow on an S2 flow cell (2×150 (bp) reads). After completion of the sequencing run, raw sequencing data were generated and then base call files were converted to FASTQ format using BCLConvert (v3.10.12, DRAGEN on-instrument solution, Illumina). The FASTQ files were subsequently uploaded to Illumina BaseSpace Sequence Hub for data storage and management prior to downstream bioinformatic analysis.

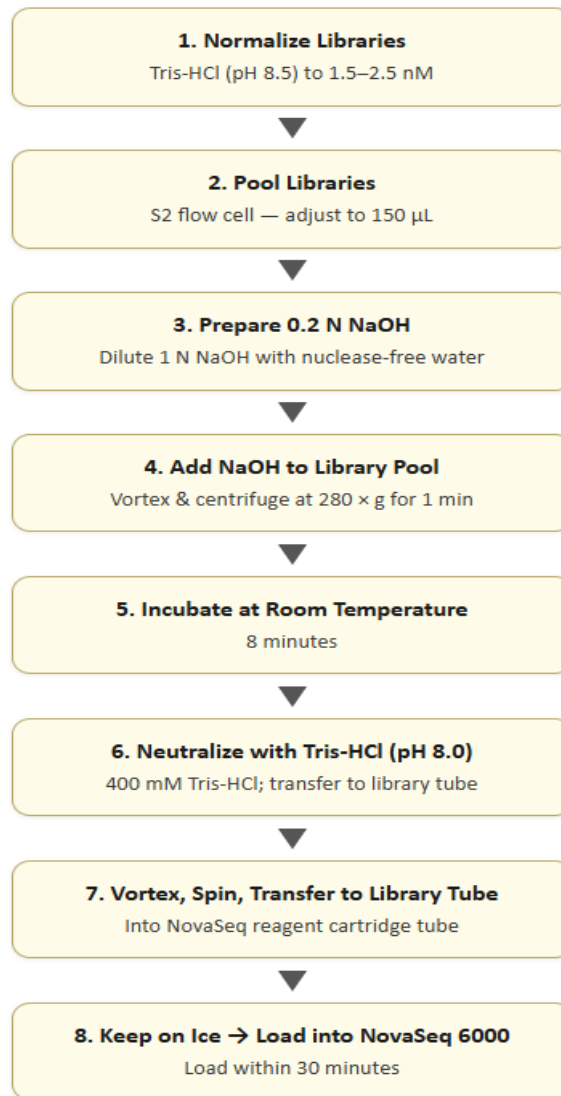


Figure 2.8: NovaSeq 6000 library denaturation and dilution workflow

2.10.3 DNA Methylation Data Processing Using nf-core/methylseq

Bioinformatic analysis of enzymatic methylation sequencing data was performed using the nf-core/methylseq pipeline (v3.0.0-g4415e90), executed via Nextflow (v24.10.3) with Singularity for environment management (**Figure 2.9**) (Ewels et al., 2020). All dependencies were automatically resolved through the nf-core infrastructure.

a. Pipeline environment and execution

Paired-end FASTQ files from the Illumina NovaSeq 6000 platform were processed against the human reference genome (GRCh38/hg38). The final pipeline command executed in the Unix environment was:

```

nextflow run nf-core/methylseq \
--non_directional \
--cytosine_report \
--no_overlap \
--input ~/samplesheet.csv \
--outdir ./results \
--genome GRCh38 \
-profile singularity \
--resume

```

The samplesheet.csv specified sample IDs, paths to paired-end FASTQ files, and associated metadata.

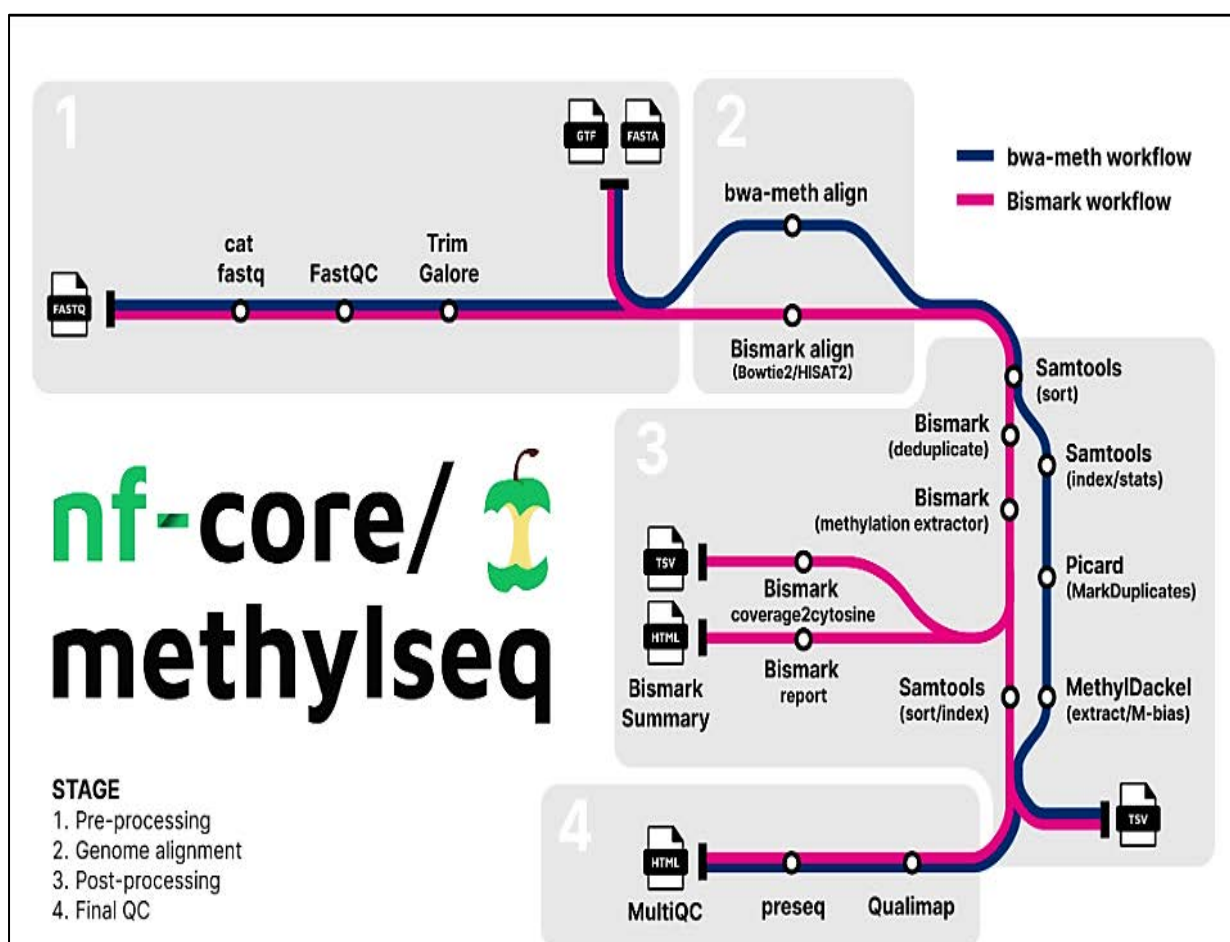


Figure 2.9: nf-core/methylseq pipeline overview. Modified from Ewels et al. (2020)

b. Quality control and trimming

Initial quality assessment of raw sequencing reads was performed using FastQC (v0.12.1), which evaluates per-base sequence quality, GC content, sequence duplication levels, and adapter contamination. Adapter trimming and removal of low-quality bases were performed using TRIMGALORE (v0.6.10), an integrated wrapper tool that utilizes Cutadapt (v4.9) and FastQC (v0.12.1). Quality control metrics from all samples were aggregated using MultiQC, providing a comprehensive summary of sequencing quality across the dataset.

c. Read alignment

Trimmed reads were aligned to GRCh38 using the BISMARK_ALIGN (v0.24.2). The pipeline handled reference indexing, alignment, and deduplication using Bismark's integrated modules. Sequencing reads were aligned to the human reference genome (GRCh38) using Bismark (v0.24.0), a three-base aligner that resolves strand ambiguity by converting reads in silico into C-to-T and G-to-A versions and performing four parallel alignments against equivalently converted reference genomes using Bowtie2 (Krueger & Andrews, 2011).

d. Methylation calling

Following alignment, CpG methylation status was extracted using the Bismark methylation extractor, which generated several output files including:

- per-base cytosine methylation calls
- summary statistics for methylation contexts (CpG, CHG, and CHH)
- genome-wide methylation coverage files in BedGraph format
- cytosine-level methylation reports for downstream visualization and analysis

e. Quality metrics and summary reports

The nf-core/methylseq pipeline generated comprehensive quality control reports summarizing:

- read trimming statistics
- alignment efficiency
- duplicate read metrics

- cytosine conversion efficiency
- methylation bias plots (M-bias)

All quality control metrics were compiled into a unified MultiQC report, enabling rapid visual assessment of sequencing performance and methylation data quality across all samples.

f. Output directory structure of nf-core/methylseq pipeline

The results generated by the nf-core/methylseq pipeline were organized into a structured directory layout to facilitate downstream analysis and data management (**Figure 2.10**). Major output directories included:

alignment/

Contains cytosine-conversion-aware aligned reads generated by Bismark.

- *.bam – sorted and deduplicated alignment files
- *.bam.bai – index files for genome browser visualization

methylation/

Contains methylation extraction outputs.

- *.CpG_report.txt.gz – cytosine-level methylation calls
- *.bedGraph.gz – genome-wide CpG methylation percentages
- *.M-bias.txt – methylation bias plots
- *.splitting_report.txt – conversion and methylation statistics

multiqc/

Provides a consolidated report of all pipeline quality metrics.

- multiqc_report.html – integrated visualization of QC results
- multiqc_data/ – raw data used to generate MultiQC plots

trimming/

Contains adapter and quality-trimmed sequencing reads generated by Trim Galore.

- *_trimmed.fq.gz – trimmed FASTQ files
- *.trimming_report.txt – trimming statistics

fastqc/

Includes initial and post-trimming FastQC reports.

- *_fastqc.html and *_fastqc.zip – detailed read quality reports

logs/

Contains execution logs, pipeline parameters, software versions, and error messages to ensure full reproducibility of the analysis workflow.

```
results/
├── alignment/
│   ├── sample1.bam
│   ├── sample1.bam.bai
│   └── sample2.bam
├── methylation/
│   ├── sample1.CX_report.txt.gz
│   ├── sample1.bedGraph.gz
│   ├── sample1.M-bias.txt
│   └── sample1.splitting_report.txt
├── trimming/
│   ├── sample1_trimmed.fq.gz
│   └── trimming_report.txt
├── fastqc/
│   ├── sample1_fastqc.html
│   └── sample1_fastqc.zip
├── multiqc/
│   ├── multiqc_report.html
│   └── multiqc_data/
├── logs/
│   ├── bismark.log
│   └── trim_galore.log
```

Figure 2.10: Directory structure of the nf-core/methylseq pipeline output

g. Quality control and sample filtering

Sequencing quality metrics were assessed using MultiQC reports generated from the nf-core/methylseq pipeline for 24 T2DM and 24 healthy control samples. Samples were evaluated based on sequencing depth, alignment efficiency, duplication rate, and the number of informative cytosine calls. Samples with low sequencing yield, poor mapping efficiency, and elevated duplication rates were considered unsuitable for reliable methylation analysis and were excluded from downstream analyses. Specifically, samples showing alignment rates below approximately 60%, high

duplication rates (>22–25%), and markedly reduced sequencing output (<60 million reads) were removed, as these conditions limit genome coverage and reduce the accuracy of CpG methylation estimation. Based on these criteria, ten samples were excluded due to insufficient sequencing depth and poor alignment performance. Samples with adequate sequencing depth (~200 million reads) and satisfactory alignment efficiency (~80 %) were retained, as they provided sufficient coverage for robust methylation analysis.

Although the exclusion of ten samples resulted in an unequal group distribution (23 diabetic versus 15 healthy controls), this imbalance reflects technical sequencing failure rather than sample selection bias, and the retained samples in both groups demonstrated uniformly high-quality metrics sufficient to support robust differential methylation analysis (Michels et al., 2013).

h. Assessment of potential selection bias in the DNA methylation study subset

For genome-wide DNA methylation analysis, participants were selected using a computer-generated random sampling procedure from the parent case–control cohort. Following sequencing and quality-control procedures, ten samples were excluded because they did not meet predefined technical quality criteria, including insufficient sequencing depth, poor alignment efficiency, and elevated duplication rates. These exclusions were made according to predefined technical quality-control criteria rather than participant-related characteristics. As the initial sample selection was random and the subsequent exclusions resulted from technical sequencing and data-quality considerations, the final analytical dataset is unlikely to have been substantially affected by systematic selection bias. Nevertheless, given the exploratory nature of the methylation study and the reduced sample size following quality-control filtering, the findings should be interpreted with appropriate caution and validated in larger independent cohorts.

i. Submission of sequencing data to GEO

All sequencing data were deposited in the NCBI Gene Expression Omnibus (GEO; accession GSE290769) following the guidelines for high-throughput sequencing datasets (<https://www.ncbi.nlm.nih.gov/geo/info/seq.html>). Raw paired-end FASTQ files were submitted in compressed fastq.gz format, accompanied by processed

alignment files (.bam), methylation reports (.cov, .bedGraph, .txt.gz), and standardised metadata spreadsheets.

Each sample was annotated with a GEO-specified metadata template covering sample title and description, organism and tissue of origin, library preparation method, sequencing platform, and paired-end read layout. The submission comprised a Series (GSE) record describing the overall study design, individual Sample (GSM) records for each biological replicate, a Data Processing Protocol specifying software versions, and a Library Strategy designated as EM-seq with genomic DNA as the library source. Data were made publicly available upon manuscript publication, with reviewer access provided via secure token during embargo.

2.11 Statistical analysis

Statistical analyses were performed using SPSS statistical software (version 24.0; IBM Corp., Chicago, USA), R and Bioconductor packages. Hardy–Weinberg equilibrium (HWE) was assessed using the HardyWeinberg package in R (version 4.4.1), where applicable. The normality of continuous variables was assessed using the Kolmogorov–Smirnov test or the Shapiro–Wilk test. Continuous variables with normal distribution were expressed as mean $\pm$ standard deviation (SD), whereas non-normally distributed variables were summarized using median, minimum, and maximum values. Comparisons between two groups were performed using the independent sample t-test for normally distributed variables and the Mann–Whitney U test for skewed distribution. Categorical variables were presented as frequency and percentage (%) and compared using the chi-square test.

Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to estimate the strength of association between the measured variables and T2DM. Variables found to be significant in bivariate analysis, including age, gender, body mass index (BMI), hypertension status, family history of diabetes, ACEs, perceived stress scale (PSS-4), serum lipid profiles including triglycerides, and high-density lipoprotein (HDL) cholesterol, were included as potential confounding factors in the multivariable logistic regression model.

For conventional statistical analyses, a two-sided p -value < 0.05 was considered statistically significant. For genome-wide DNA methylation and pathway analyses,

multiple-testing thresholds and analysis-specific significance criteria were applied as described in the respective subsections.

2.11.1 ACE observational study

For the ACE observational study, both univariable and multivariable logistic regression analyses were conducted to examine the association of the ACE and other independent predictors with T2DM as the outcome variable. ORs and 95% CIs were calculated for each predictor in both models.

2.11.2 Genotype studies

For genetic analyses, genotype and allele frequencies were calculated for each polymorphism, including variants in the *TCF7L2* and *SLC30A8* genes. Hardy–Weinberg equilibrium (HWE) was evaluated in the control group to assess the consistency of genotype distribution. To assess the association of the *TCF7L2* rs12255372 (G/T) and *SLC30A8* rs13266634 (C/T) variants with T2DM as the outcome variable, binary logistic regression was applied under additive, dominant, recessive, and over-dominant genetic models, where applicable. Covariates found significant in bivariate analysis were entered as confounders in the multivariable model. The interaction effect of the dominant model with age, sex, BMI, and family history of diabetes was further examined by logistic regression for rs12255372 (G/T) of *TCF7L2*.

2.11.3 DNA methylation study

Three complementary tools were applied at different stages of the methylation analysis pipeline.

- i. methylKit R package** - descriptive quality assessment, sample clustering, principal component analysis, and Pearson correlation
- ii. edgeR Bioconductor Package** - to identify differentially methylated cytosines (DMCs)
- iii. DMRcate Bioconductor Package** - to identify differentially methylated regions (DMRs)

i. Descriptive analysis and mapping: methylKit R package

Descriptive analysis of epigenome-wide DNA methylation data was performed using the methylKit R package (v1.24.0) (Akalin et al., 2012). Cytosine-level methylation reports generated by Bismark were imported and symmetric CpG sites across strands were merged. CpG loci were filtered to retain sites with $\geq 1\times$ coverage while removing extreme coverage values above the 99.9th percentile to minimize PCR bias, and filtered CpGs were unified across samples for comparative analysis. Sample relationships were assessed using Pearson correlation, hierarchical clustering, and principal component analysis.

ii. Identification of differentially methylated cytosines (DMC): EdgeR Bioconductor Package

Genome-wide CpG methylation data were analyzed using the edgeR package (v3.42.4) in R within the Bioconductor framework, following the negative binomial generalized linear model approach for methylation sequencing data (Y. Chen et al., 2018). Bismark coverage files were imported using readBismark2DGE(), retaining separate methylated and unmethylated read counts at each CpG locus to preserve inherent data variability and enable inference conditional on read coverage. CpG sites on unplaced scaffolds, mitochondrial DNA, and the Y chromosome were excluded, retaining only autosomal and X-chromosomal loci. The resulting dataset comprised approximately 57.5 million CpGs across 76 paired-end libraries derived from 38 samples. Coverage filtering retained sites exceeding median coverage thresholds and showing a minimum 12% deviation from balanced methylation, ensuring that only biologically informative and reliably covered loci were carried forward, finally yielding 398,253 CpGs for downstream analysis. A design matrix incorporating case–control status and clinically relevant covariates including age, sex, BMI, adverse childhood experiences, hypertension, and family history of diabetes, was constructed to account for potential confounding, in accordance with established EWAS analytical guidelines (Michels et al., 2013). Quasi-likelihood generalized linear models were fitted and tested using glmQLFit() and glmQLFTest(), which provide robust type I error control through empirical Bayes moderation of dispersion (Y. Chen et al., 2018). Differentially

methyated CpGs were identified at $p < 0.0001$ and $FDR < 0.20$, thresholds appropriate for exploratory genome-wide discovery and modest sample size (Michels et al., 2013).

Given the exploratory nature of the genome-wide methylation analysis and the modest sample size, $FDR < 0.20$ was used as a broader threshold for DMC discovery. DMCs meeting $FDR < 0.05$ were distinguished as findings meeting a more stringent statistical significance threshold, whereas those with $FDR \geq 0.05$ but < 0.20 were considered exploratory findings. For downstream gene annotation and pathway enrichment analysis, DMCs identified at the broader $FDR < 0.20$ threshold were included and classified as hypermethyated or hypomethyated.

iii. Identification of differential methyated regions (DMR)

Differentially methyated regions (DMRs) associated with T2DM were identified using the DMRcate Bioconductor package (v2.12.0). Regional methylation analysis was performed in addition to single-CpG analysis because aggregating neighbouring CpGs increases statistical power to detect biologically meaningful methylation differences compared with individual CpG tests alone (Lee et al., 2016; Michels et al., 2013). The filtered DGEList object containing 398,253 CpG sites across 38 samples after coverage-based quality filtering was converted to a BSseq object for regional methylation analysis. CpG-level test statistics were then computed using a covariate-adjusted design matrix incorporating case–control status together with age, sex, BMI, adverse childhood experience (ACE), hypertension, and family history of diabetes. Regional differential methylation was detected using the sequencing.annotate() and dmrcate() functions, which apply Gaussian kernel smoothing to CpG-level statistics and aggregate spatially correlated CpG loci to identify candidate DMRs. A smoothing bandwidth of 1 kb ($\lambda = 1000$) was used, and regions were required to contain at least three CpGs. Statistical significance was defined using a false discovery rate (FDR) < 0.05 , and significant DMRs were extracted as genomic ranges for downstream functional annotation. Region-based methylation analysis improves detection power in genome-wide methylation studies by leveraging the spatial correlation structure of neighbouring CpG sites (Hansen et al., 2012; Peters et al., 2021).

iv. Gene annotation and Pathway enrichment analysis

Annotation of DMCs and DMRs was performed using CpGtools with the GRCh38 human genome assembly and a whole-genome background. Gene assignment was conducted using the *CpG_to_gene.py* function, which maps CpG sites to putative target genes following the “basal plus extension” model implemented in GREAT (McLean et al., 2010; Schröder et al., 2017; Wei et al., 2021). In this framework, the basal regulatory domain was defined as ± 5 kb from the transcription start site (TSS) to represent promoter regions, while distal regulatory elements were extended up to 1 Mb from the TSS to capture potential enhancer associations (Montesino-Goicolea et al., 2020). Based on this annotation strategy, hypermethylated DMCs mapped to 273 promoter genes, whereas hypomethylated DMCs corresponded to 195 promoter genes. Annotation of DMRs further indicated that methylation alterations predominantly affected cis-regulatory domains capable of influencing gene expression across long genomic distances (Fleischer et al., 2014; Zhang et al., 2015), resulting in 121 hypermethylated and 35 hypomethylated genes. Genes annotated within ± 5 kb of the TSS of DMCs identified at the exploratory threshold, together with genes associated with hyper- and hypomethylated DMRs, were subsequently subjected to pathway enrichment analysis using Ingenuity Pathway Analysis (IPA) to identify significantly enriched canonical pathways and upstream regulatory networks.

Ingenuity Pathway Analysis (IPA; QIAGEN, Redwood City, CA, USA) was used for downstream bioinformatic analysis, including canonical pathway, disease/function, upstream regulator, and molecular network analyses. IPA utilizes a curated knowledge base to identify functional relationships and construct gene interaction networks. Pathway enrichment was assessed using right-tailed Fisher’s exact test based on a hypergeometric distribution and expressed as $-\log(\text{P-value})$, with $-\log(\text{P}) > 2$ considered significant. Upstream regulators were prioritized using overlap p-values ($p < 0.05$), while regulator effects and molecular networks were evaluated using consistency and network scores. Pathways were further ranked by the proportion of dataset genes mapped to each pathway (Krämer et al., 2014; S. Thomas & Bonchev, 2010; Yu et al., 2014)

2.11.4 DNA methylation mediation analysis of the association between ACEs and T2DM

i. Measures

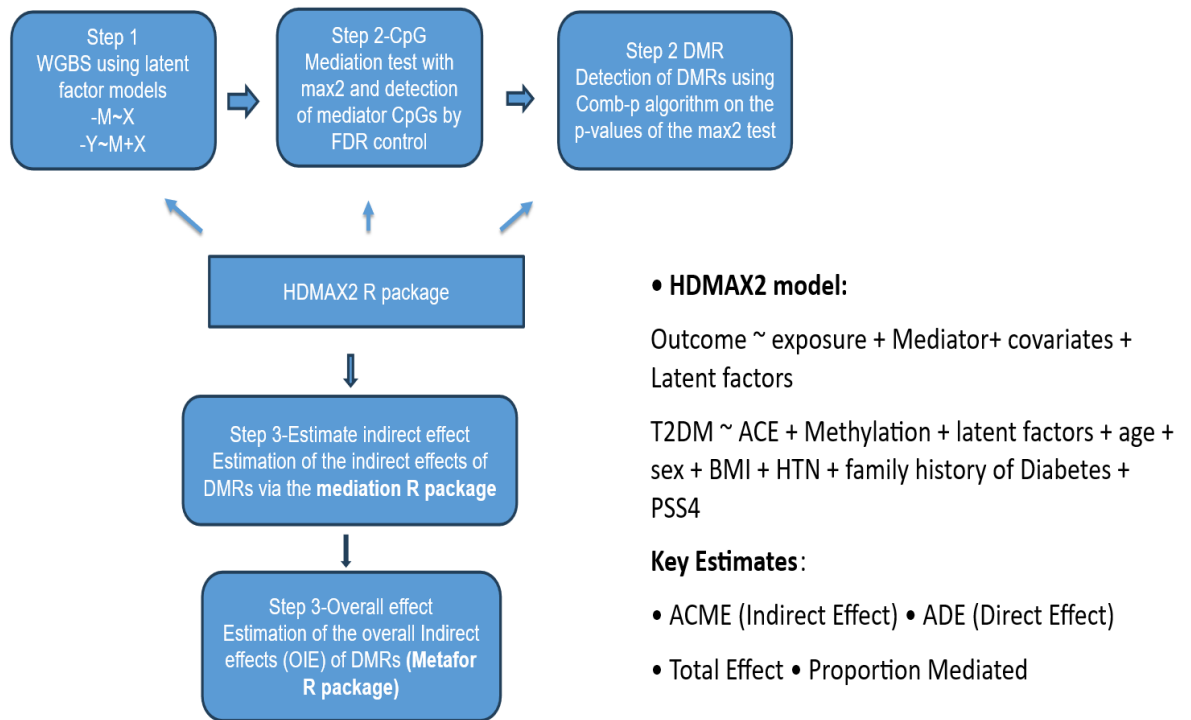
ACE score was dichotomized as ≤ 4 (low) and > 4 (high) per the threshold established by the original ACE study (Felitti et al., 1998). Type 2 diabetes diagnosis served as the outcome variable, with cases drawn from a diabetic cohort and age-matched healthy controls confirmed biochemically via fasting blood glucose (< 6.1 mmol/L) and 2-hour post-load glucose (< 7.8 mmol/L) by oral glucose tolerance test (WHO, 2006). Covariates included age, sex, BMI, hypertension, first-degree family history of diabetes, and perceived stress assessed by the PSS-4. Full selection criteria are described in Barman et al. (2024).

ii. Statistical Software and Packages

All statistical analyses were performed in R version 4.2.2 (R Core Team, 2022) on a Linux platform. High-dimensional mediation analysis was conducted using the `hdmax2` package v2.0.0.9000 (Pittion et al., 2025), and causal mediation analysis using the `mediation` package v4.5.1 (Tingley et al., 2014) and `metafor` package (4.9-31). Data manipulation and visualization were performed using `dplyr` v1.1.0 (Wickham, 2016), `ggplot2` v4.0.1 (Villanueva & Chen, 2019), and `cowplot` v1.1.1 (Wilke, 2020).

iii. Data Filtering

CpG methylation data from Bismark coverage files were merged across 38 samples, initially yielding 57,956,460 CpG sites. A three-stage quality control pipeline was applied, including group-level coverage filtering, missingness filtering ($\leq 20\%$) with group-wise mean imputation, and variance filtering based on the median variance threshold, resulting in a final dataset of 6,295,480 CpG sites for downstream analyses.



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Figure 2.11: Workflow of the high-dimensional mediation analysis. Modified from Jumentier et al. (2023)

iv. High-Dimensional mediation Testing

High-dimensional mediation analysis was conducted using HDMAX2 (Jumentier et al., 2023; Pittion et al., 2025), which applies latent factor mixed models to account for unmeasured confounding in high-dimensional methylation data. Two latent factors were specified to capture unmeasured sources of variation beyond the measured covariates beyond measured covariates (age, sex, BMI, hypertension, family history of diabetes, and perceived stress) (van Iterson et al., 2017). Given the limited sample size ($n = 38$), this conservative specification helps ensure stable parameter estimation and is supported by simulation studies demonstrating reliable factor recovery for datasets of 30–50 samples with two latent factors (Preacher & MacCallum, 2002). In the HDMAX2 framework, ACE was modeled as the exposure (X), T2DM as the outcome (Y), and CpG methylation levels as mediators while adjusting for covariates and latent factors. CpG sites identified by the HDMAX2 test were subsequently aggregated into

differentially methylated regions using the comb-p method, resulting in 11 candidate DMRs. Formal causal mediation analysis was then performed using the mediation R package (v4.5.1) (Tingley et al., 2014) to estimate the average causal mediated effect (ACME), average direct effect (ADE), and the proportion mediated, with statistical inference obtained through bootstrap resampling (1,000 iterations). All mediation models incorporated the same covariates and latent factors used in the association analysis to maintain consistent adjustment for confounding. Finally, overall indirect, direct, and proportion-mediated effects across the 11 DMRs were summarized using meta-analysis implemented in the metafor R package (Viechtbauer, 2010). (**Figure 2.11**)

2.11.5 Availability of code repository

To ensure transparency, reproducibility, and accessibility of the bioinformatics analyses performed in this study, a comprehensive computational pipeline was developed and documented in a publicly available GitHub repository entitled *Methylation_T2DM*. The repository contains the workflow, analysis scripts, methodological documentation, and supporting resources used for genome-wide DNA methylation data processing, differential methylation analysis, functional annotation, enrichment analysis, and mediation analysis. The complete repository is available at: https://github.com/epigen-bioinformlab/Methylation_T2DM.

2.12 Ethical consideration

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki for research involving human participants. Ethical approval was obtained from the Institutional Review Board of the University of Dhaka (Ref. No. 152/Biol. Scs. 2021) as well as the Ethical Review Committee (ERC), Diabetic Association of Bangladesh (Ref no-BADAS-ERC/EC/22/394) prior to the initiation of the study. Written informed consent was obtained from all participants before enrollment. Participants were fully informed about the study objectives, procedures, potential risks, and their right to withdraw at any time or decline to answer any question without any consequences.

Because the assessment of adverse childhood experiences (ACE) involves sensitive personal information, particular care was taken to ensure participant safety, confidentiality, and voluntary participation (Kvale & Brinkmann, 2015). Although recalling childhood adversity may cause temporary discomfort, previous studies indicate that such inquiries rarely result in significant psychological harm (DePrince & Freyd, 2004). All interviewers received prior ethical training to appropriately manage sensitive discussions, and participants were informed about the nature of the questionnaire. During data collection, approximately 11 ml of venous blood was collected aseptically from the antecubital vein by a trained phlebotomist for biochemical and molecular analyses. Participant confidentiality and data privacy were strictly maintained throughout the study.

3. Results

3.1 Baseline characteristics of the study participants

A total of 271 participants were included in the study, comprising 134 healthy controls and 137 individuals with type 2 diabetes mellitus (T2DM).

3.1.1 Socio-demographic characteristics

The socio-demographic characteristics of the study participants are presented in **Table 3.1**.

The age distribution showed that higher proportion of participants in both groups belonged to the 41–50 years category (controls: 39.6%; T2DM: 38.0%). However, a relatively increased number of participants aged above 50 years was observed in the T2DM group (38.0%) compared to controls (26.1%), although these differences did not reach statistical significance. Meanwhile, there was also no statistical difference between T2DM and controls in terms of mean (SD) age [46.89 (7.82) vs. 45.66 (8.68), respectively]. Females constituted the majority in both groups, accounting for 61.2% of controls and 69.3% of T2DM subjects, with no statistically significant difference.

Most participants were married, with comparable proportions in controls (93.3%) and T2DM subjects (89.1%) ($p > 0.05$). Household structure was also similar between groups, with the majority belonging to single-family households (controls: 77.6%; T2DM: 75.2%; $p > 0.05$). Educational status and employment status did not differ significantly between the groups. Monthly income was also comparable between controls (mean: 30242.5 BDT) and T2DM subjects (30357.7 BDT), with no statistically significant difference.

Table 3.1: Socio-demographic data of the study participants

Socio-demographic data	Healthy (n=134)	Control	T2DM (n=137)	p-value
Age group				
30-40	46 (34.3)\$		33 (24.1)	0.066
41-50	53 (39.6)		52 (38.0)	
> 50	35 (26.1)		52 (38.0)	
Gender				
Male	52 (38.8)		42 (30.7)	0.159
Female	82 (61.2)		95 (69.3)	
Marital Status				
Married	125 (93.3)		122 (89.1)	0.286
Single*	9 (6.7)		15 (10.9)	
Household status				
combined family	30 (22.4)		34 (24.8)	0.670
Single family	104 (77.6)		103 (75.2)	
Educational Status				
Non-institutional	28 (20.9)		22 (16.1)	0.296
Class 1 to 9	58 (43.3)		72 (52.6)	
SSC pass	29 (21.6)		21 (15.3)	
HSC pass and above	19 (14.2)		22 (16.1)	
Employment status				
Unemployed	84 (62.7)		93 (67.9)	0.375
Employed	50 (37.3)		44 (32.1)	
Monthly Income (BDtk)				
Mean (SD)	30242.5 (37955.0) \$\$		30357.7 (24283.2)	0.976#
Median	20000		25000	0.288##

*single=unmarried/separated/widow; \$ figure within parenthesis denoted frequency and percentage except \$\$ mean (SD); p-value derived from chi-square test except # from independent sample t-test and ## Mann-Whitney U test

3.1.2 Anthropometric and behavioral characteristics of the study participants

The anthropometric and behavioral characteristics are summarized in **Table 3.2**. The mean body mass index (BMI) was slightly higher in T2DM subjects ($26.3 \pm 4.0 \text{ kg/m}^2$) compared to controls ($25.9 \pm 4.1 \text{ kg/m}^2$), although the difference was not statistically significant ($p > 0.05$). The distribution of BMI categories was also similar between the two groups, with a higher proportion of participants categorized as obese I. A highly significant difference was observed in physical activity levels ($p < 0.0001$). Regular physical exercise was reported by 32.1% of T2DM subjects compared to only 9.0% of controls. Conversely, a higher proportion of controls (88.1%) reported no physical exercise compared to T2DM subjects (47.4%). Smoking and smokeless tobacco status did not differ significantly between the groups ($p > 0.05$).

Table 3.2: Anthropometric and behavioral data of the study participants

Anthropometric and behavioral data	Healthy Control (n=134)	T2DM (n=137)	p-value
BMI (kg/m^2)**	25.9 (4.1)	26.3 (4.0)	0.828
BMI Category#			
underweight (<18.5)	6 (4.5) *	1 (0.7)	
Normal weight (18.5-22.9)	25 (18.7)	29 (21.2)	
Overweight (23-24.9)	22 (16.4)	26 (19.0)	0.326
Obese I (25-29.9)	59 (44.0)	55 (40.1)	
Obese II (>30)	22 (16.4)	26 (19.0)	
Physical Exercise			
Regular	12 (9.0)	44 (32.1)	
Sometimes	4 (3.0)	28 (20.4)	<0.0001
No	118 (88.1)	65 (47.4)	
Smoking			
Yes	20 (14.9)	16 (11.7)	0.477
No	114 (85.1)	121 (88.3)	

Smokeless tobacco			
Regular	10 (7.5)	13 (9.5)	
Irregular	5 (3.7)	11 (8.0)	0.251
No	119 (88.8)	113 (82.5)	

*Figure within parenthesis denoted percentage of frequency except ** mean (SD); p-value derived from chi-square test except ** where independent sample t test done.; # Asian WHO BMI classification

3.1.3 Clinical characteristics of the study participants

Clinical characteristics of the study participants are presented in **Table 3.3**. Both systolic and diastolic blood pressure were significantly higher among T2DM subjects compared to controls. The prevalence of hypertension was significantly higher in T2DM subjects (64.2%) compared to controls (31.3%) ($p < 0.0001$). Dyslipidemia was also more prevalent among T2DM subjects (89.8%) compared to controls (61.2%) ($p < 0.0001$). A significantly higher proportion of T2DM subjects had a family history of diabetes (81.0%) compared to controls (59.7%), suggesting a strong genetic predisposition. The mean duration of diabetes among T2DM subjects was 6.95 ± 3.93 years. Regarding treatment modalities, the majority were on oral hypoglycemic agents (71.5%), while smaller proportions were receiving insulin therapy (2.2%), combined therapy (8.0%), or were managing diabetes through lifestyle modification alone (18.3%).

Table 3.3: Clinical data of the study participants

Clinical Data	Healthy (n=134)	Control	T2DM (n=137)	p-value
SBP	119.11 (17.66)**		126.99 (16.59)	<0.0001
DBP	78.03 (10.31)		81.53 (9.98)	0.005
Hypertension				
Yes	42 (31.3)*		88 (64.2)	<0.0001
No	92 (68.7)		49 (35.8)	
Family history of HTN				
Yes	89 (66.4)		101 (73.7)	0.232
No	45 (33.6)		36 (26.3)	
Dyslipidemia				
Yes	82 (61.2)		123 (89.8)	<0.0001
No	52 (38.8)		14 (10.2)	
Family history of DM				
Yes	80 (59.7)		111 (81.0)	
No	54 (40.3)		26 (19.0)	<0.0001
Duration of Diabetes (years)			6.95 (3.93)	
Drug history of diabetes				
Oral			98 (71.5)	
Insulin			3 (2.2)	
Both oral and insulin			11 (8.0)	
Diet and lifestyle change			25 (18.3)	

*Figure within parenthesis denoted percentage of frequency except ** mean (SD); p-value derived from chi-square test except ** where independent sample t test done

3.1.4 Biochemical characteristics of the study participants

Biochemical parameters are presented in **Table 3.4**. Fasting blood glucose levels and 2-hour postprandial glucose levels were significantly higher in T2DM subjects

compared to controls ($p < 0.0001$). HbA1c levels were also significantly higher in T2DM subjects compared to control. Regarding lipid profile, total cholesterol levels were comparable between the two groups ($p = 0.755$). However, triglyceride levels were significantly higher in T2DM subjects [188.0 (166.5) mg/dl] compared to controls [133 (87.5) mg/dl] ($p < 0.0001$).

Table 3.4: Biochemical data of the study participants

Biochemical Data	Healthy Control (n=134)	T2DM (n=137)	p-value
Fasting Blood sugar (mmol/L)			
median (IQR)	5.3 (0.6)	9.7 (6.1)	<0.0001
Range (mmol/L)	4.0 – 6.0	4.4 – 24.1	
2-hr postprandial blood sugar (mmol/L)			
median (IQR)	6.4 (1.3)	14.4 (8.8)	<0.0001
Range	4.5 – 7.7	6.2 – 38.2	
HbA1c (%),			
median (IQR)	5.6 (0.4)	8.7 (3.75)	<0.0001
Range	4.3 – 6.5	5 -14.7	
Serum lipid profile			
Total cholesterol (mg/dl)			
median (IQR)	182 (46.5)	184 (65)	0.755
Range	102 – 420	62.0 – 350.0	
Triglyceride (mg/dl)			
median (IQR)	133 (87.5)	188.0 (166.5)	<0.0001
Range	41.0 – 488.0	23.0 – 1321.0	
HDL (mg/dl)			
median (IQR)	44.0 (15.0)	42.0 (14.0)	0.048
Range	19.0 -103.0	19.0 – 86.0	
LDL (mg/dl)			
median (IQR)	104 (34.5)	100 (45.0)	0.062
Range	32.0 – 287.0	20.0 - 225.0	

Median (IQR) as data were in skewed distribution, p value derived from Mann-Whitney U test.

3.2 Adverse Childhood Experiences (ACE) Study

Sample Size

After sample size calculation, the minimum required sample size was 137 participants per group. Although 137 participants were initially recruited in each group, three controls were excluded because of incomplete data. Therefore, the final analysis included 137 T2DM cases and 134 healthy controls.

3.2.1 Distribution of adverse childhood experiences and perceived stress among study participants

The distribution of adverse childhood experiences (ACEs) and perceived stress levels among study participants is presented in **Table 3.5**. Among the ACE domains, emotional neglect was significantly higher in T2DM participants compared to controls (45.3% vs. 32.8%, $p = 0.049$). Similarly, exposure to domestic violence was significantly more frequent in the T2DM group (69.3%) than in controls (55.2%) ($p = 0.023$). Although other ACE components such as emotional abuse and sexual abuse were more common among T2DM participants, these differences did not reach statistical significance. The overall ACE score was significantly higher in T2DM participants [median (IQR): 4.0 (2.0)] compared to controls [3 (3.0)] ($p = 0.002$). Perceived stress levels were also significantly elevated in the T2DM group, with a median PSS-4 score of 9 compared to 6 in controls ($p < 0.0001$), indicating a higher psychological stress burden among diabetic individuals.

Table 3.5: Adverse Childhood Experiences (ACE) and perceived stress among Study participants

Measures	Healthy Control (n=134)	T2DM (n=137)	p-value
Adverse Childhood Experiences			
Physical neglect	126 (94.0)	127 (92.7)	0.845
Physical abuse	97 (72.4)	107 (78.1)	0.342
Emotional neglect	44 (32.8)	62 (45.3)	0.049
Emotional abuse	41 (30.6)	58 (42.3)	0.06
Parental separation	6 (4.5)	8 (5.8)	0.817
Domestic Violence	74 (55.2)	95 (69.3)	0.023
Household Member with substance use	13 (9.7)	17 (12.4)	0.605
Mental illness	15 (11.2)	17 (12.4)	0.903
Household member incarcerated	6 (4.5)	13 (9.5)	0.168
Sexual abuse	25 (18.7)	39 (28.5)	0.079
ACE, Median (Interquartile range)	3 (3.0)	4.0 (2.0)	0.002
Minimum- Maximum/range	0 – 8.0	0 – 7.0	
Perceived Stress scale-4			
Median (Interquartile range)	6 (6)	9 (5)	<0.0001
Minimum – maximum	0 – 16	1 - 16	

Values are expressed as frequency (%) or median (interquartile range). P-values were calculated using chi-square test or the Mann–Whitney U test where applicable

3.2.2 Association between adverse childhood experiences (ACEs) and T2DM

The association between ACEs, perceived stress, and T2DM risk was evaluated using logistic regression models is presented in **Table 3.6**. In the adjusted model, female sex was significantly associated with increased T2DM risk (OR = 1.93, 95% CI: 1.02–3.66). Clinical factors such as hypertension (OR = 3.92, 95% CI: 2.12–7.26) and dyslipidemia (OR = 5.14, 95% CI: 2.42–10.93) showed strong independent associations

with T2DM. A positive family history of diabetes was also significantly associated with increased risk (OR = 2.95, 95% CI: 1.54–5.65). Perceived stress (PSS-4 score) was significantly associated with T2DM, with each unit increase in stress score increasing the odds of T2DM (OR = 1.16, 95% CI: 1.07–1.26).

Participants with higher ACE scores (≥ 4) had more than two-fold increased odds of T2DM compared to those with lower ACE scores (< 4) (adjusted OR = 2.24, 95% CI: 1.20–4.06). The model demonstrated good explanatory power (Nagelkerke $R^2 = 0.396$), indicating that these variables collectively contribute substantially to T2DM risk.

Table 3.6: Association between adverse childhood experiences and T2DM development

Factors	OR _{unadj}	95% CI	OR _{adj}	(95% CI)
Age	1.018	0.99–1.05	1.02	0.98–1.05
Sex				
Male	Ref.		Ref.	
Female	1.43	0.87–2.37	1.93*	1.02–3.66
Hypertension				
No	Ref.		Ref.	
Yes	3.93***	2.37–6.52	3.92***	2.12–7.26
Dyslipidemia				
No	Ref.		Ref.	
Yes	5.57***	2.90–10.70	5.14***	2.42–10.93
Family history of DM				
No	Ref.		Ref.	
Yes	2.88***	1.66–4.99	2.95**	1.54–5.65
PSS-4	1.17***	1.09–1.25	1.16***	1.07–1.26
ACE group				
score < 4	Ref.		Ref.	
score ≥ 4	2.28**	1.40–3.71	2.24**	1.2–4.06

p-value * < 0.05 , ** ≤ 0.001 , *** < 0.0001 ; Notes: unadj – unadjusted, adj – adjusted, OR – odds ratio, CI – confidence interval, ref – reference category, Nagelkerke $R^2 = 0.396$

3.3 Genotype study

3.3.1 Genomic DNA Extraction and Quality Assessment

Genomic DNA (gDNA) was successfully extracted from peripheral blood leukocytes of the study subjects. The quality and integrity of the extracted DNA were assessed by agarose gel electrophoresis, as shown in **Figure 3.1**. High-molecular-weight DNA bands were distinctly visible in Lanes 2, 3, 6, 7, 8, and 9, confirming the successful extraction of intact, high-quality genomic DNA suitable for downstream molecular analyses. However, Lanes 4 and 5 were excluded from further analysis due to the presence of faint gDNA bands, indicating insufficient DNA yield. Similarly, Lanes 10, 11, and 12 were excluded due to evidence of gDNA shearing, which compromises the integrity of the extracted material. Panel B presents the inverse image of the gel for enhanced visualization of the DNA bands.

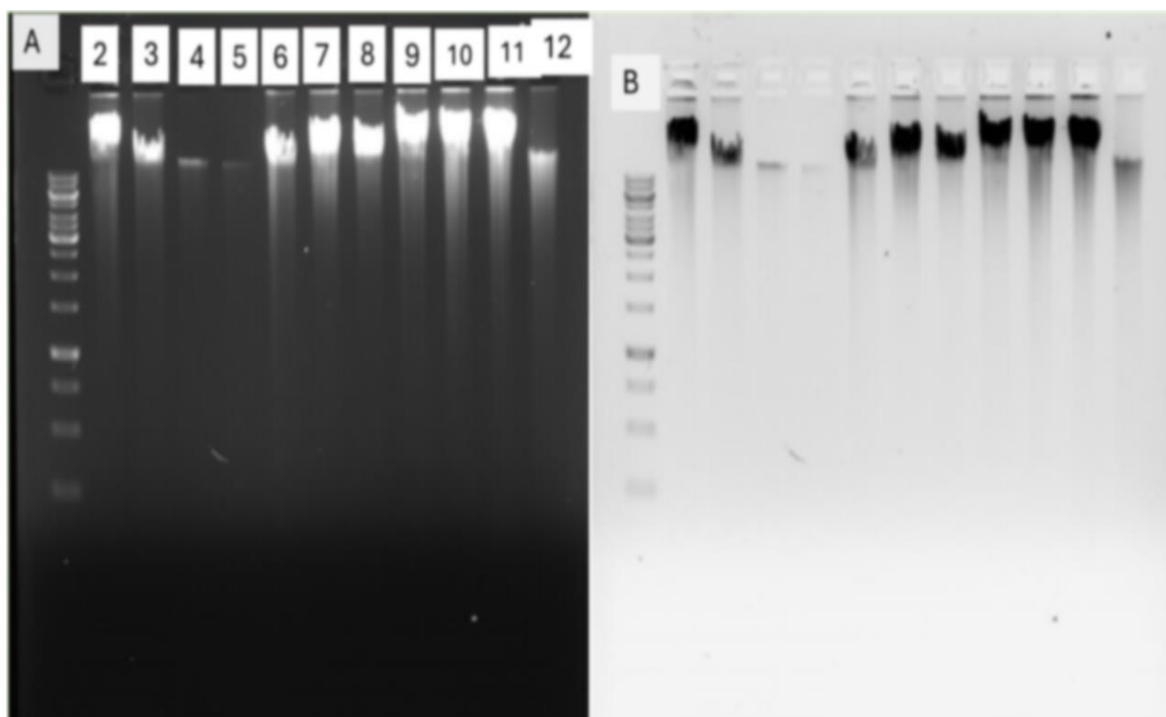


Figure 3.1: Agarose gel electrophoresis of high-molecular-weight genomic DNA (gDNA)

gDNA extracted from peripheral blood leukocytes of study subjects, (A) Lane 1: 1 kb DNA ladder; Lane 2 to 12: gDNA of study subjects. (B) inverse figure of gel.

3.3.2 Genotype study 1: *TCF7L2* rs12255372 study

Sample size

Based on the sample size calculation, a minimum of 62 participants were required in each group. Finally, 67 T2DM cases and 65 controls were included

3.3.2.1 PCR Amplification of *TCF7L2* rs12255372

A specific segment of the *TCF7L2* gene harboring the rs12255372 polymorphism was amplified by polymerase chain reaction (PCR) using site-specific primers. The amplified PCR products were resolved by agarose gel electrophoresis to confirm successful amplification prior to genotyping. As shown in **Figure 3.2**, a distinct band of 346 bp was observed in Lanes 2 through 7, confirming the specific and consistent amplification of the target region across all samples. Lane 1 contained the 50 bp DNA ladder, which served as a molecular size reference. No non-specific amplification products or primer-dimer artifacts were observed, indicating the high specificity of the PCR conditions employed.

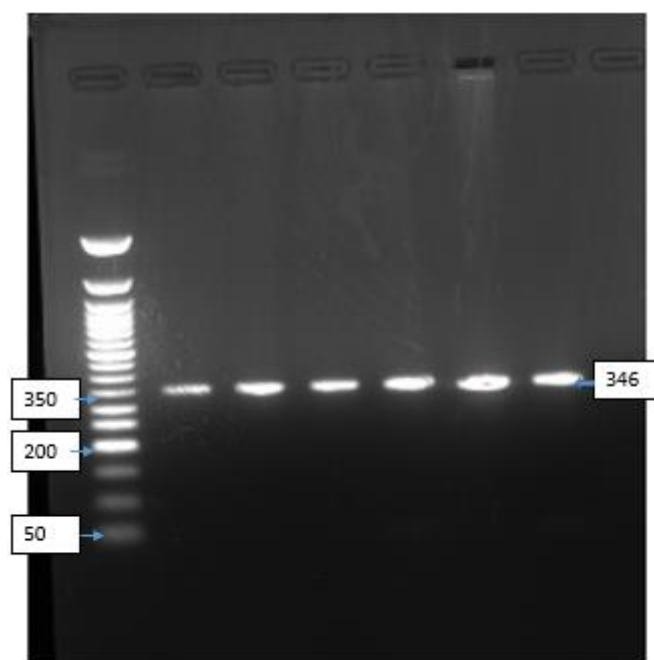


Figure 3.2: Agarose gel electrophoresis of PCR products of *TCF7L2* rs12255372

These were 346 bp in length found in the 2nd to 7th lane and in 1st lane was 50bp DNA ladder.

3.3.2.2 Genotyping of *TCF7L2* rs12255372 by Sanger Sequencing

The genotyping of the *TCF7L2* rs12255372 (G>T) single nucleotide polymorphism was performed by Sanger sequencing, and the resulting chromatograms were analyzed to determine the genotype of each study subject. As illustrated in **Figure 3.3**, three distinct genotypes were identified among the study population: the homozygous wild type (GG), the heterozygous variant (GT), and the homozygous mutant (TT). The chromatogram peaks corresponding to each genotype were clearly distinguishable, confirming the successful and accurate genotyping of the rs12255372 locus in all included subjects. The genotype distribution among controls did not deviate significantly from Hardy–Weinberg equilibrium ($p > 0.05$) [Supplementary table S1].

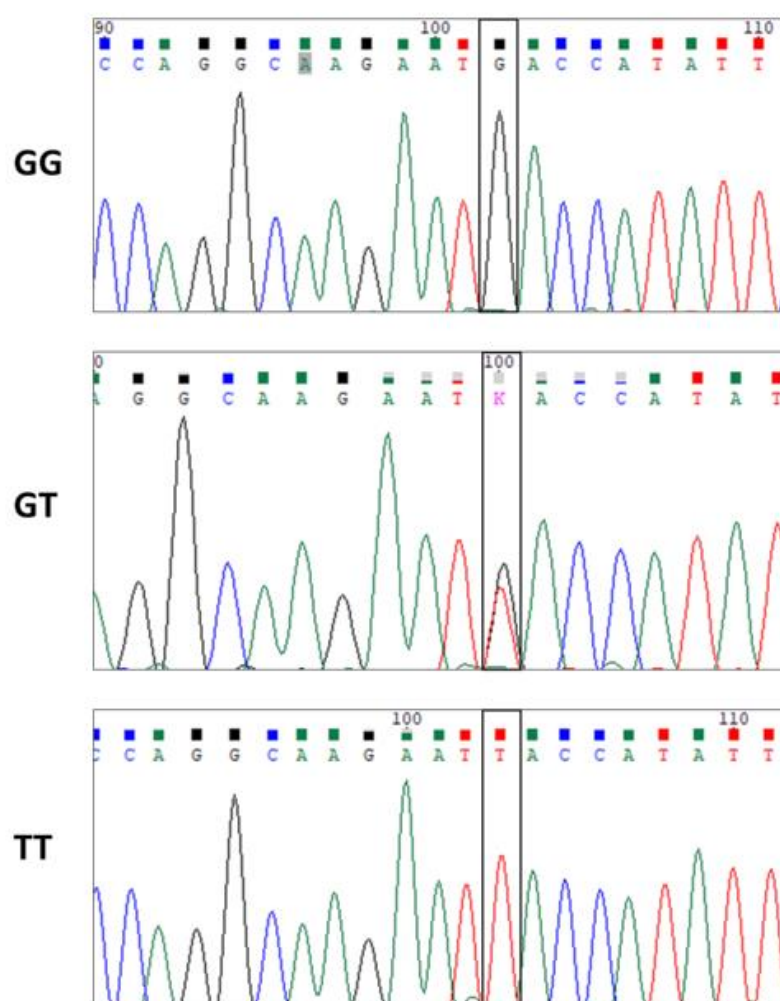


Figure 3.3: Chromatogram of genotypes of *TCF7L2* rs 12255372 (G>T)
(wild type GG, heterozygous GT and mutant TT)

3.3.2.3 Genotypic and allelic distribution of *TCF7L2* rs12255372 polymorphism

The genotypic and allelic frequencies of the *TCF7L2* rs12255372 (G>T) polymorphism were determined in T2DM and control subjects and are presented in **Table 3.7** under additive, dominant, and recessive genetic models. A statistically significant difference in genotype distribution was observed between cases and controls in the additive model ($p < 0.05$), with a higher frequency of heterozygous (GT) and mutant (TT) genotypes among T2DM patients. Under the dominant model, the combined GT–TT genotype was significantly more prevalent in T2DM subjects compared to controls (52.2% vs. 30.8%, $p = 0.012$). However, no significant association was found under the recessive model. Allelic analysis revealed that the minor T allele was significantly more frequent in T2DM patients than in controls (29.1% vs. 16.9%, $p < 0.05$), suggesting its potential role in increasing susceptibility to T2DM.

Table 3.7: Genotypic and allelic frequency of *TCF7L2* rs12255372 in type 2 diabetic and control subjects

Model	Genetic variable	T2DM (n=67) n (%)	Control (n=65) n (%)	p-value
Genotypes (Additive model)	GG (wild type)	32 (47.8)	45 (69.2)	0.043
	GT (Heterozygous)	31 (46.3)	18 (27.7)	
	TT (Mutant)	4 (6.0)	2 (3.1)	
Dominant model	GG	32 (47.8)	45 (69.2)	0.012
	GT-TT	35 (52.2)	20 (30.8)	
Recessive model	GG-GT	63 (94.0)	63 (96.9)	0.425
	TT	4 (6.0)	2 (3.1)	
Alleles	G allele (Major)	95 (70.9)	108 (83.1)	0.020
	T allele (Minor)	39 (29.1)	22 (16.9)	

Figure within parenthesis denoted frequency percentage and p value derived from chi-squared test

3.3.2.4 Distribution of demographic, clinical, and biochemical variables according to *TCF7L2* rs12255372 genotypes (GG, GT, and TT)

The demographic, clinical, and biochemical characteristics of the study participants were stratified according to *TCF7L2* rs12255372 genotypes (GG, GT, and TT) and are summarized in **Table 3.8**. A significant difference in age was observed among genotype

groups ($p = 0.025$), with higher mean age in TT genotype carriers. Hypertension was also significantly more prevalent among individuals with GT and TT genotypes compared to GG ($p = 0.033$). In addition, fasting blood glucose ($p = 0.019$) and 2-hour postprandial blood glucose levels ($p = 0.048$) showed significant differences across genotypes, indicating poorer glycemic control in variant allele carriers. However, no significant associations were observed for sex distribution, family history of diabetes or hypertension, body mass index, or lipid profile parameters (total cholesterol, triglycerides, HDL, and LDL cholesterol).

Table 3.8: Distribution of demographic, clinical, and biochemical variables of study participants according to *TCF7L2* rs12255372 genotypes (GG, GT, and TT)

Variables	Genotypes			p-value
	GG (n=77)	GT (n=49)	TT (n=6)	
Age, mean (SD)	43.6 (9.6)	46.7 (10.1)	53.5 (9.6)	0.025
Sex, n (%)				
Male	32 (41.6)	19 (38.8)	3 (50.0)	0.856
Female	45 (58.4)	30 (61.2)	3 (50.0)	
Family history of diabetes	50 (64.9)	33 (67.3)	5 (83.3)	0.649
Hypertension	25 (32.5)	26 (53.1)	4 (66.7)	0.033
Family history of hypertension	54 (70.1)	36 (73.5)	4 (66.7)	0.893
Body Mass Index	25.8 (3.1)	26.4 (4.2)	25.5 (2.2)	0.614
Fasting blood glucose (mmol/L), mean (SD)	6.9 (3.0)	8.8 (5.1)	9.2 (3.8)	0.019
2 hours postprandial blood glucose (mmol/L), mean (SD)	9.5 (5.2)	12.0 (7.0)	12.6 (6.8)	0.048
Total Cholesterol (mg/dl), mean (SD)	187.6 (47.5)	190.4 (53.3)	193.5 (32.2)	0.930
Triglyceride (mg/dl), mean (SD)	219.7 (224.2)	192.0 (104.6)	180.2 (64.8)	0.665
HDL (mg/dl), mean (SD)	41.6 (12.0)	39.8 (10.4)	42.7 (7.7)	0.631
LDL (mg/dl), mean (SD)	100.8 (32.3)	111.0 (38.3)	91.8 (42.2)	0.196

p-values were obtained using the chi-square test for categorical variables and the independent samples t-test for continuous variables

3.3.2.5 Association of *TCF7L2* rs12255372 genetic models and other factors with T2DM

To identify independent risk factors for type 2 diabetes mellitus and to evaluate the contribution of *TCF7L2* rs12255372 genotypes, logistic regression analyses were performed, and the results are presented in **Table 3.9**. In univariate analysis, family history of diabetes (OR = 4.4, $p < 0.0001$), hypertension (OR = 4.3, $p < 0.0001$), family history of hypertension (OR = 2.2, $p = 0.044$), and triglyceride levels ($p = 0.036$) were significantly associated with T2DM. After adjustment, family history of diabetes (OR = 3.8, $p = 0.003$) and hypertension (OR = 3.1, $p = 0.006$) remained significant predictors. Regarding genetic association, individuals with the GT genotype had a significantly increased risk of T2DM compared to the GG genotype in both unadjusted (OR = 2.4, $p = 0.019$) and adjusted models (OR = 2.4, $p = 0.043$), whereas the TT genotype did not show a statistically significant association. Under the dominant model, carriers of the variant allele (GT–TT) exhibited a significantly higher risk of T2DM compared to GG genotype in both unadjusted (OR = 2.5, $p = 0.013$) and adjusted analyses (OR = 2.3, $p = 0.041$). These findings indicate that the presence of the T allele, particularly in the heterozygous state, is associated with increased susceptibility to T2DM.

Table 3.9: Multivariable regression analysis of significant factors of T2DM with genetic models of *TCF7L2*

variables	OR _{unadj} (95% CI)	p-value	OR _{adj} (95% CI)	p-value
Age	1.0 (0.9-1.0)	0.597		
Sex				
Male	Ref			
Female	2.0 (1.0 - 4.0)	0.057		
BMI	1.0 (0.9-1.1)	0.342		
Family history of HTN	2.2 (1.0 - 4.8)	0.044	1.1 (0.5 – 2.8)	0.759
Family history of DM	4.4 (2.0 - 9.8)	<0.0001	3.8 (1.5 - 9.2)	0.003
Hypertension	4.3 (2.0-9.0)	<0.0001	3.1 (1.4 - 7.0)	0.006
Triglyceride	1.0 (1.0-1.0)	0.036	1.0 (1.0 - 1.0)	0.083
Additive model*				
GG (wild type)	Ref		Ref	
GT (Heterozygous)	2.4 (1.1 - 5.0)	0.019	2.4 (1.0 - 5.5)	0.043
TT (Mutant)	2.8 (0.5 -16.3)	0.249	1.9 (0.3 - 12.7)	0.489
Dominant model*				
GG	Ref		Ref	
GT-TT	2.5 (1.2-5.0)	0.013	2.3 (1.0 – 5.2)	0.041

*Each genetic model enters in separate logistic regression models. OR-odds ratio; CI: Confidence Interval; unadj-unadjusted; adj-adjusted

3.3.3 Genotype study 2: *SLC30A8* rs13266634 study

Sample size

To achieve this study objective, the sample size calculation indicated a minimum requirement of 90 participants in each group. Accordingly, 90 T2DM patients and 90 healthy controls were included in the analysis.

3.3.3.1 Genotyping of *SLC30A8* rs13266634 polymorphism

Agarose gel electrophoresis image **Figure 3.4 (a)** showing PCR-amplified products of the *SLC30A8* rs13266634 gene fragment, with bands observed at approximately 429 bp in lanes S1-S7, indicating successful amplification of the target region. Lane L represents the 50 bp DNA ladder. Panel **Figure 3.4(b)** shows representative Sanger sequencing chromatograms illustrating the three genotypes: homozygous wild type (CC), heterozygous (CT), and homozygous mutant (TT), confirming the presence of the C>T polymorphism.

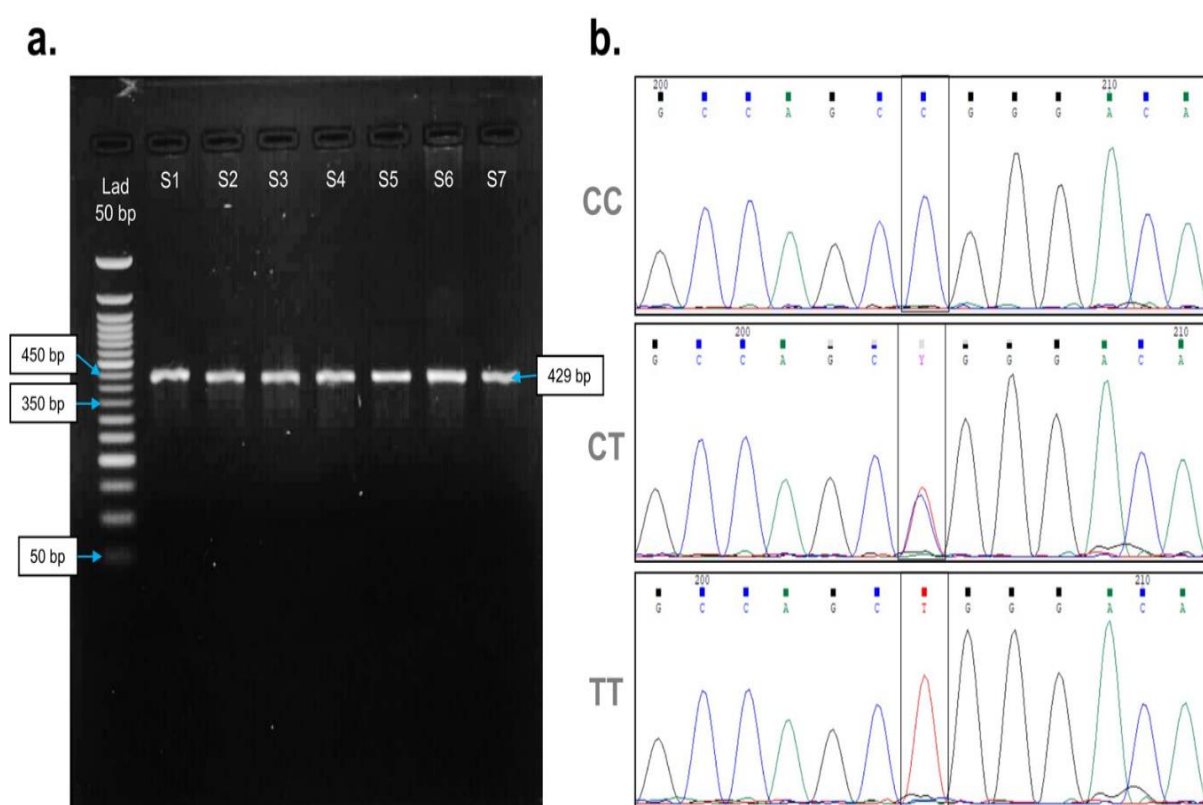


Figure 3.4: Agarose gel electrophoresis (a) and sequencing chromatogram (b) of *SLC30A8* rs13266634 (C>T) polymorphism

3.3.3.2 Genotypic and allelic frequency of *SLC30A8* rs13266634 (C>T) polymorphism

The distribution of *SLC30A8* rs13266634 genotypes and alleles among T2DM patients and controls is presented in **Table 3.10**. A statistically significant difference in genotype distribution was observed under the additive model ($p = 0.023$), with the CC genotype being more frequent in T2DM patients (66.7%) compared to controls (50.0%), while

the TT genotype was less frequent in T2DM (3.3%) than in controls (12.2%). Under the dominant model, the combined CT+TT genotype was significantly lower in T2DM subjects (33.3%) compared to controls (50.0%) ($p = 0.023$). Similarly, in the recessive model, the TT genotype was significantly less frequent among T2DM patients ($p = 0.026$). Allelic analysis showed that the C allele was significantly more prevalent in T2DM patients (81.7%) compared to controls (68.9%), whereas the T allele frequency was lower in T2DM (18.3%) than in controls (31.1%) ($p = 0.005$). Genotype distribution in controls was consistent with Hardy–Weinberg equilibrium ($p > 0.05$) [Supplementary Table S1].

Table 3.10: Genotypic and allelic frequency of *SLC30A8* rs13266634 (C>T) polymorphism among type 2 diabetes and control subjects

Genetic Model	Genetic variable	Healthy Control (n=90)	Type 2 diabetes (n=90)	p value**
Additive Model	CC	45 (50.0)*	60 (66.7)	0.023
	CT	34 (37.8)	27 (30.0)	
	TT	11 (12.2)	3 (3.3)	
Dominant Model	CT + TT	45 (50.0)	30 (33.3)	0.023
	CC	45 (50.0)	60 (66.7)	
Recessive Model	TT	11 (12.2)	3 (3.3)	0.026
	CT + CC	79 (87.8)	87 (96.7)	
Over-dominant model	CC+TT	56 (62.2)	63 (70.0)	0.270
	CT	34 (37.8)	27 (30.0)	
Allele	C Allele	124 (68.9)	147 (81.7)	0.005
	T Allele	56 (31.1)	33 (18.3)	

* Figure within parentheses denotes frequency percentage and ** P -value derived from chi-squared test

3.3.3.3 Distribution of clinical and biochemical variables according to *SLC30A8* genotypes

The distribution of demographic and clinical variables across *SLC30A8* genotypes is shown in **Table 3.11**. No significant differences were observed for age, sex distribution,

family history of diabetes or hypertension, body mass index, or lipid profile parameters across genotype groups. However, fasting blood glucose levels differed significantly among genotypes ($p = 0.019$), with higher levels observed in the CC genotype group compared to CT and TT genotypes. Although 2-hour postprandial glucose and triglyceride levels showed trends across genotypes, these differences did not reach statistical significance.

Table 3.11: Distribution of demographic, clinical, and biochemical variables according to *SLC30A8* rs13266634 genotypes

Variables	CC (n=105)	CT (n=61)	TT (n=14)	p-value
Age, mean (SD)	47.0 (9.5)	45.0 (7.8)	44.3 (7.3)	0.284
Sex, n (%)				
Male	39 (36.5)	30 (50.0)	4 (28.6)	0.148
Female	66 (63.5)	31 (50.0)	10 (71.4)	
Family history of diabetes	71 (68.3)	38 (61.3)	9 (64.3)	0.654
Hypertension	45 (43.3)	30 (48.4)	6 (42.9)	0.803
Family history of hypertension	71 (68.3)	41 (66.1)	10 (71.4)	0.917
Body Mass Index	25.9 (3.7)	26.0 (4.4)	27.8 (3.7)	0.214
Fasting blood glucose (mmol/L), mean (SD)	8.6 (5.0)	7.2 (3.2)	5.8 (1.8)	0.019
2 hours postprandial blood glucose (mmol/L), mean (SD)	11.7 (7.1)	10.0 (5.6)	8.6 (4.1)	0.114
Total Cholesterol (mg/dl), mean (SD)	203.5 (60.8)	191.8 (41.6)	196.8 (41.7)	0.393
Triglyceride (mg/dl), mean (SD)	243.6 (217.2)	195.1 (114.9)	144.6 (103.8)	0.071
HDL (mg/dl), mean (SD)	43.2 (12.7)	42.5 (11.3)	47.5 (16.5)	0.412
LDL (mg/dl), mean (SD)	109.6 (41.1)	110.2 (30.1)	116.6 (30.8)	0.804

p-values were obtained using the chi-square test for categorical variables and the independent samples t-test for continuous variables

3.3.3.4 Association of *SLC30A8* rs13266634 polymorphism with T2DM risk

Logistic regression analysis evaluating the association between *SLC30A8* rs13266634 polymorphism and T2DM risk is presented in **Table 3.12**. In the additive model, the TT

genotype was significantly associated with reduced risk of T2DM in both unadjusted (OR = 0.205, $p = 0.02$) and adjusted models (OR = 0.196, $p = 0.029$). Under the dominant model, individuals with the CC genotype had a significantly increased risk of T2DM compared to CT+TT genotypes in the adjusted model (OR = 2.253, $p = 0.025$). The recessive model showed a trend toward increased risk for CT+CC genotypes, although this did not remain statistically significant after adjustment. These findings indicate that the T allele may have a protective effect against T2DM, while the C allele is associated with increased susceptibility. The adjusted models demonstrated moderate explanatory power (Nagelkerke R^2 ranging from 0.315 to 0.346).

Table 3.12: Association of *SLC30A8* rs13266634 genetic models with T2DM

Genetic Model	OR _{Unadj} (95% CI)	p value	OR _{Adj} (95% CI)	p value
Additive Model				
CC	Ref.		Ref.	
CT	0.596 (0.315-1.125)	0.110	0.522 (0.248-1.101)	0.088
TT	0.205 (0.054–0.774)	0.02	0.196 (0.045- 0.850)	0.029
Dominant Model				
CT + TT	Ref.		Ref.	
CC	2.0 (1.095-3.653)	0.024	2.253 (1.109- 4.576)	0.025
Recessive Model				
TT	Ref.		Ref.	
CT + CC	4.038 (1.087-15.002)	0.037	3.901 (0.940-16.189)	0.061
Over-dominant model				
CC+TT	Ref.		Ref.	
CT	0.706 (0.380-1.313)	0.271	0.64 (0.31-1.31)	0.222
Allelic Model				
C Allele	2.01 (1.23–3.29)	0.005		
T Allele	Ref.			

The significant factors related to type 2 diabetes of age group, hypertension, serum triglyceride and serum HDL cholesterol were entered in binary logistic regression model. Nagelkerke R^2 in

adjusted model for additive model=0.346, dominant model=0.336 and recessive model=0.330; over -dominant model=0.315; Here, Unadj=unadjusted; adj=adjusted; CI=confidence interval; Ref.= Reference

3.3.3.5 Three-dimensional structure of ZnT8 (chain A) and validation of homology model

The structural model of ZnT8 (chain A) was analyzed to identify the location of the mutation site p.R325. The Ramachandran plot demonstrated that all residues were within allowed regions, confirming the structural reliability and stability of the modeled protein (**Figure 3.5**).

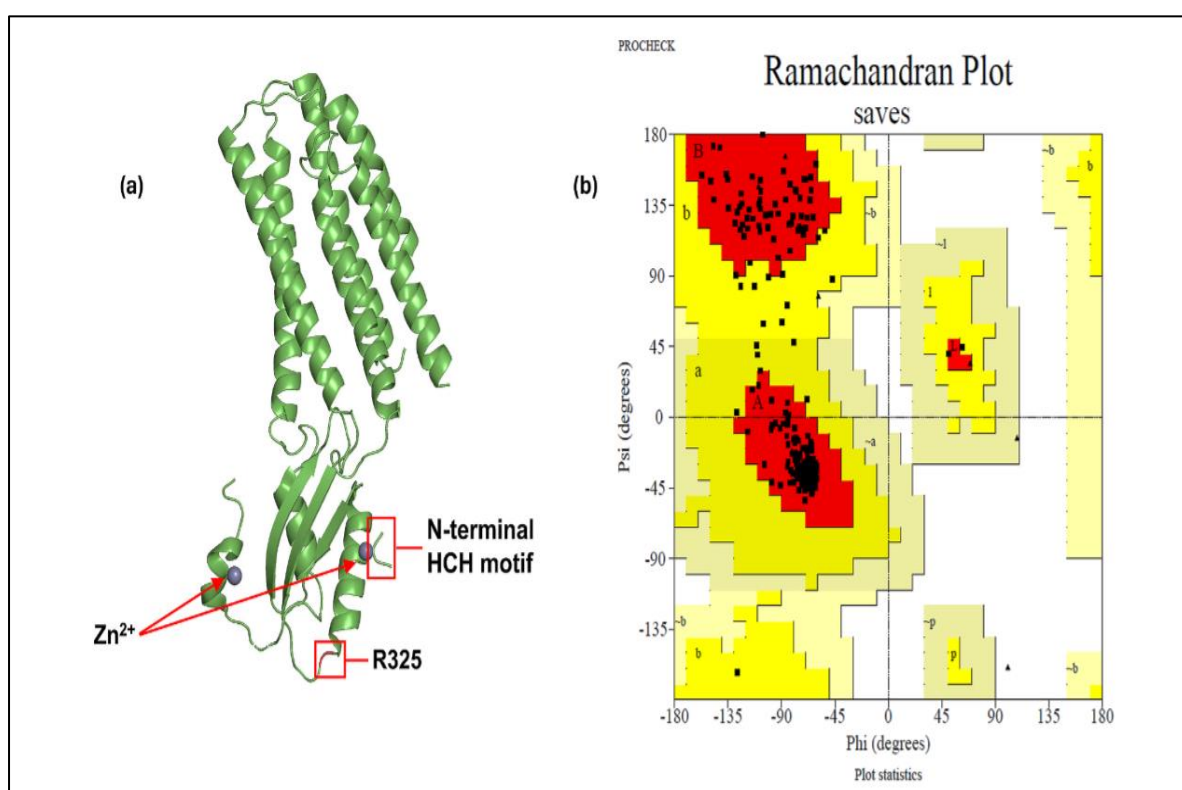


Figure 3.5: (A) ZnT8 chain A (PDB ID: 6XPF) showing the position of p.R325. (B) Ramachandran plot of the homology model showing no residues in disallowed regions.

3.3.3.6 Molecular dynamics simulation analysis of wild-type and variant ZnT8

To assess the effects of the rs13266634 (C>T) polymorphism on ZnT8 protein in terms of structural and dynamic stability, molecular dynamics (MD) simulations were

performed for both wild-type ZnT8 (WZnT8) and variant ZnT8 (MZnT8) for 100 ns and the results are presented in **Figure 3.6**.

The structural stability of WZnT8 and MZnT8 was evaluated by analyzing the backbone root mean square deviation (RMSD) of C α atoms relative to their initial conformations. RMSD reflects the degree of structural deviation over time, where higher values indicate greater instability (Dash et al., 2020). The wild-type protein showed a slightly higher mean RMSD (0.83 Å; SD: 0.144) compared to the variant (0.82 Å; SD: 0.130). During the initial 20 ns, the variant exhibited higher RMSD (~1 Å), followed by stabilization around ~0.8 Å. The relatively lower RMSD in MZnT8 suggests reduced conformational deviation, whereas higher fluctuations in WZnT8 indicate greater structural flexibility (**Figure 3.6a**).

Protein flexibility at the residue level was assessed using root mean square fluctuation (RMSF). Regions with higher RMSF values indicate greater flexibility, typically associated with loop or coil regions, whereas lower values reflect stable secondary structures like alpha helices and beta sheets (Dash et al., 2020). The wild-type protein showed a higher average RMSF (0.42 Å) compared to the variant (0.34 Å), indicating reduced flexibility in MZnT8. At the mutation site, RMSF decreased from 0.50 Å (arginine) to 0.45 Å (tryptophan), suggesting reduced local flexibility (**Figure 3.6b**). As this site lies near the zinc-binding region at C-terminal domain (CTD)–transmembrane domain (TMD) interface, coordinated by His137 and His345 (Xue et al., 2020), this rigidity may limit the conformational adaptability required for efficient zinc ion capture and transport, potentially impairing zinc homeostasis in the cell.

The radius of gyration (Rg), an indicator of protein compactness (Lobanov M, Bogatyreva NS, 2008), was also analyzed. Stable Rg values indicate consistent folding, whereas fluctuations suggest structural instability (Dash et al., 2020). The variant protein showed a lower mean Rg (2.63 Å; SD: 0.067 Å) compared to the wild type (2.80 Å; SD: 0.089 Å). Both proteins stabilized after ~20 ns, with average values of ~2.60 Å (variant) and ~2.84 Å (wild type), indicating that the variant adopts a more compact structure (**Figure 3.6c**).

Solvent-accessible surface area (SASA) analysis further supported this observation, with the variant protein showing lower average SASA (201.924 nm²) compared to the wild type (210.834 nm²), indicating reduced solvent exposure. This suggests tighter

structural packing in MZnT8. In contrast, the relatively higher SASA and Rg values in the wild type indicate a less compact structure, potentially allowing greater exposure of functional residues involved in zinc binding and transport (**Figure 3.6d**).

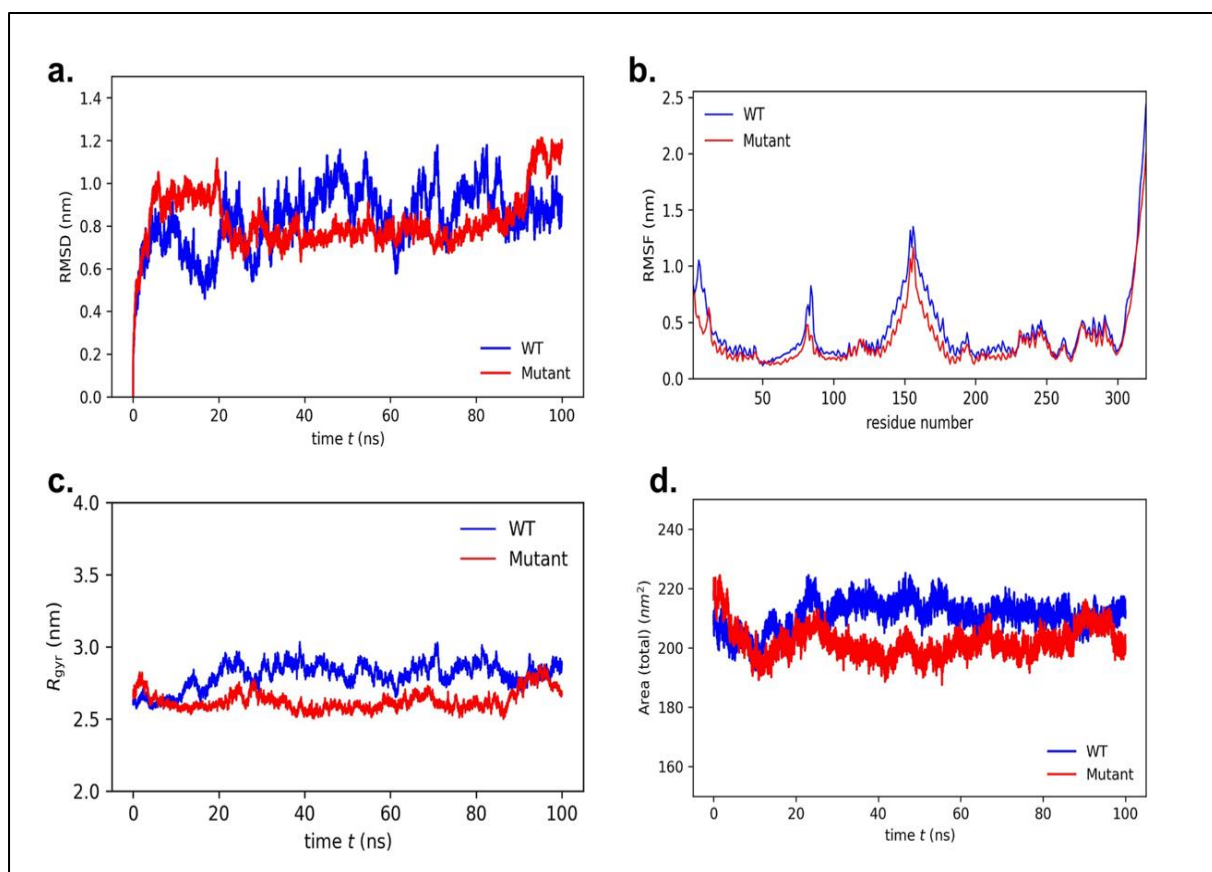


Figure 3.6: Trajectory analysis of wild-type ZnT8 and variant ZnT8 (320 residues)

These are based on (a) root-mean-square deviation (RMSD), (b) root-mean-square fluctuation (RMSF), (c) radius of gyration (Rg), and (d) solvent-accessible surface area (SASA). Since the first 49 residues were missing from the total 369 residues of ZnT8, molecular dynamics simulation was performed on 320 residues, with the arginine-to-tryptophan (p.R325W) substitution observed at position p.R276W.

3.4 DNA Methylation Study

Selection of Participants for Genome-Wide DNA Methylation Analysis

Participants for the exploratory genome-wide methylation study were selected using computer-generated random sampling from the parent case–control cohort, yielding 24 T2DM cases and 24 age-matched controls. This sample size is consistent with discovery-stage whole-genome methylome studies, where dense per-sample CpG coverage provides sufficient statistical power for differential methylation analysis with regional aggregation and multiple-testing correction (Song et al., 2021; van Dokkum et al., 2023; Ortiz et al., 2026). Following sequencing quality control, 10 samples were excluded for failing predefined technical criteria (sequencing depth, alignment efficiency, duplication rate), unrelated to participants' clinical or demographic characteristics, leaving 38 participants (23 cases, 15 controls) for final analysis. As the initial selection was random and subsequent exclusions were based solely on technical quality criteria rather than participant characteristics, substantial selection bias was considered unlikely.

3.4.1 Bismark Alignment Summary from MultiQC Report

The MultiQC summary of Bismark alignment across forty-eight samples (24 T2DM and 24 controls) is illustrated in **Figure 3.7**. It showed that most libraries achieved satisfactory mapping efficiency, with alignment rates generally around ~80% in high-quality samples. However, a subset of samples exhibited suboptimal performance, including reduced alignment rates (<60%), elevated duplication levels (>22–25%), and lower sequencing output (<60 million reads). These samples also showed decreased numbers of informative cytosine calls, indicating limited genome coverage.

Based on these quality-control criteria, 10 samples were excluded from further analysis due to insufficient sequencing depth and poor alignment performance. The remaining thirty-eight samples (23 T2DM cases and 15 controls) demonstrated consistently high alignment efficiency and adequate sequencing depth (~200 million reads), supporting their suitability for downstream differential methylation analysis.

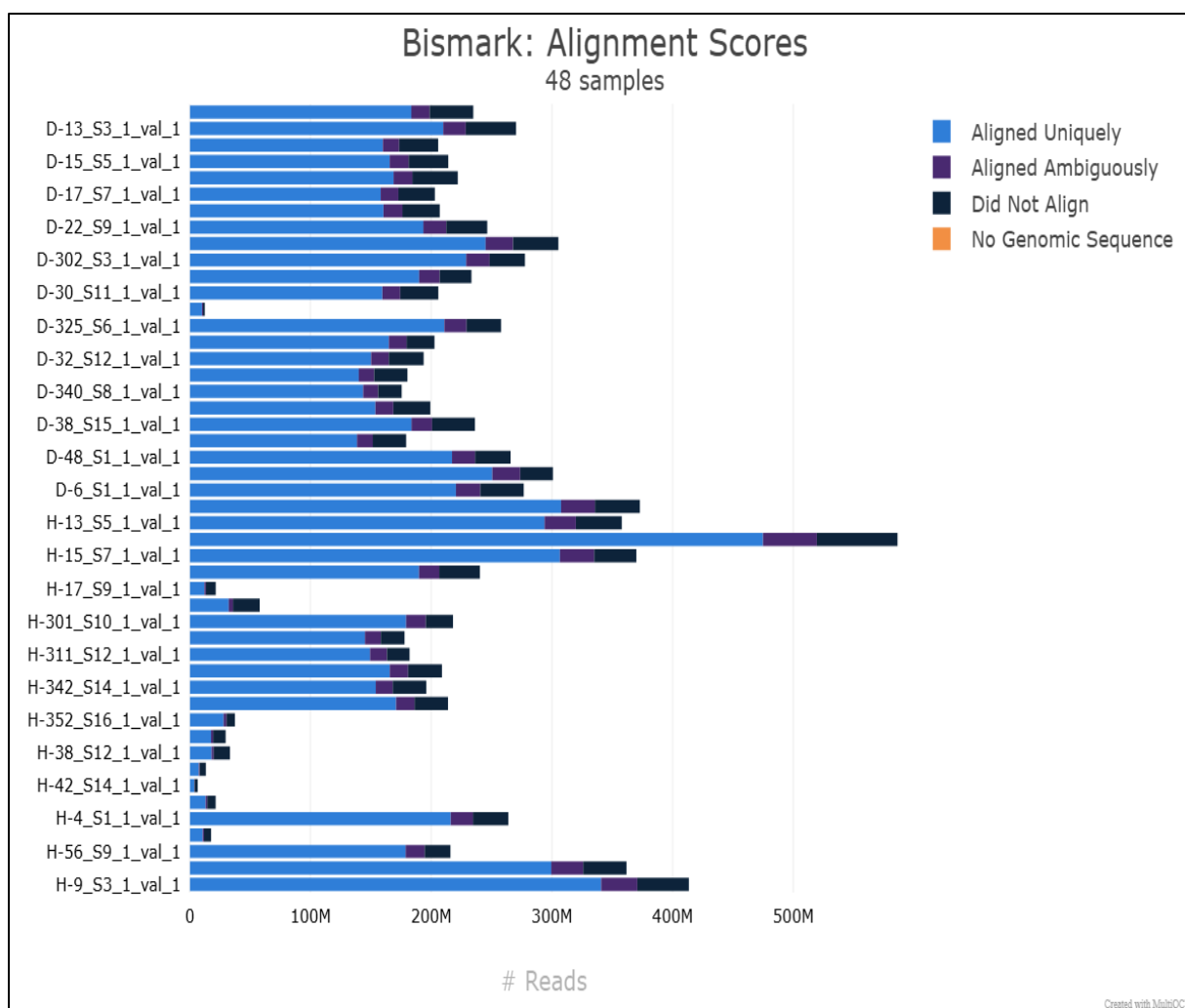


Figure 3.7: Bismark alignment summary of forty-eight samples from MultiQC report

3.4.2 Quality assessment and methylation characteristics of Whole-genome DNA methylation data in T2DM and control groups

Genome-wide DNA methylation profiling at single-base-pair resolution was performed using whole-genome enzymatic methyl sequencing on whole-blood samples from 23 patients with type 2 diabetes mellitus and 15 healthy control subjects. The sequencing quality metrics and summary statistics of the generated dataset are presented in **Table 3.13**.

The total average raw read count across all samples was 254.6 million. After filtering low-quality reads, ambiguous bases, and adapter sequences, the average number of clean reads per sample was 204.5 million. After removal of duplicate reads (average duplicate reads: 34,873,452), the average unique clean read count was 169,614,188 for

mapping to the human reference genome (hg38). In the T2DM group, mapping rates ranged from 76.06% to 83.30%, while in the healthy control group, mapping rates ranged from 78.61% to 82.88%, indicating satisfactory and comparable alignment efficiency across both groups. The enzymatic cytosine conversion rate was uniformly high and consistent across all 38 samples at 99.53%, confirming the high efficiency of enzymatic cytosine conversion during library preparation.

The overall average global methylated cytosine percentage (total mC%) across all samples was 80.89%, with an average of 123,105,974 methylated CpG sites and 29,068,880 unmethylated CpG sites per sample. The overall average genome coverage was $8.21\times$ per base, with an average CpG depth of $1.79\times$. These findings confirm the high quality and consistency of the DNA methylome dataset generated from whole blood, making it suitable for reliable identification of differentially methylated sites and regions between the type 2 diabetes mellitus and control groups.

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Table 3.13: Summary statistics of whole-genome DNA methylation data

Groups	Clean reads	Mapping rate (%)	Cytosine conversion rate (%)	Total mC (%)	Coverage (X)
Diabetic	181306251	79.7	99.53	81.69	8.77
Diabetic	155813614	78.15	99.53	80.49	7.54
Diabetic	173614656	77.68	99.53	81.14	8.4
Diabetic	137372616	77.75	99.53	82.12	6.65
Diabetic	140478960	77.35	99.53	81.07	6.8
Diabetic	139715482	76.06	99.53	81.12	6.76
Diabetic	133064525	77.9	99.53	81.1	6.44
Diabetic	137866593	77.48	99.53	81.58	6.67
Diabetic	161796214	78.52	99.53	81.43	7.83
Diabetic	206912303	80.25	99.53	79.86	10.01
Diabetic	130563705	77.49	99.53	81.76	6.32
Diabetic	126516530	77.53	99.53	80.8	6.12
Diabetic	118367881	77.64	99.53	82.27	5.73
Diabetic	130270577	77.16	99.53	80.62	6.3
Diabetic	152279668	77.77	99.53	81.11	7.37
Diabetic	117518348	77.31	99.53	80.44	5.69
Diabetic	180835671	81.75	99.53	80.84	8.75
Diabetic	214066650	83.3	99.53	80.26	10.36
Diabetic	190996022	82.47	99.53	81.32	9.24
Diabetic	164430827	81.42	99.53	81.01	7.96
Diabetic	140084918	81.36	99.53	80.51	6.78
Diabetic	176342012	81.8	99.53	81	8.53
Diabetic	123990222	81.82	99.53	79.9	6
Control	172371884	81.92	99.53	79.79	8.34
Control	241686990	82.77	99.53	81.79	11.69
Control	264808991	82.43	99.53	80.41	12.81
Control	247794721	82.48	99.53	80.88	11.99
Control	238148301	82.12	99.53	79.99	11.52
Control	385885334	80.97	99.53	81.32	18.67
Control	246179794	82.88	99.53	81.81	11.91
Control	154293456	79	99.53	82.04	7.47
Control	149669814	82.83	99.53	80.11	7.24
Control	150713926	82.18	99.53	79.95	7.29
Control	123414408	81.57	99.53	80.62	5.97
Control	123538629	82.02	99.53	79.84	5.98
Control	138893022	79.32	99.53	82.3	6.72
Control	130626657	78.61	99.53	81.21	6.32
Control	143108985	79.9	99.53	78.23	6.92

Footnotes:

- Clean reads: Number of high-quality sequencing reads after trimming and filtering.
- Mapping rate (%): Percentage of clean reads successfully aligned to the reference genome.
- Cytosine conversion rate (%): Efficiency of bisulfite treatment, calculated based on unmethylated cytosines converted to uracil.

- *Total mC (%)*: Percentage of total cytosines methylated across CpG, CHG, and CHH contexts.
- *Coverage (X)*: Average sequencing depth per base across the genome.

3.4.3 Heatmap of association significance between DNA methylation derived principal component scores and baseline phenotypic traits

To provide a comprehensive overview of the associations between DNA methylation derived principal components and all baseline phenotypic traits simultaneously, a heatmap of $-\log_{10}(\text{p-values})$ was constructed using methylkit R package and is presented in **Figure 3.8**. The heatmap summarizes the statistical significance of Pearson correlations between the first five principal components (PC1–PC5) and a panel of clinical and biochemical variables, with darker color intensities corresponding to more significant associations. The strongest signal was observed for PC3 in association with the Adverse Childhood Experiences (ACE) score ($-\log_{10}p = 2.16$, $p = 0.007$), followed by PC2 with Body Mass Index (BMI) ($-\log_{10}p = 1.46$, $p = 0.034$), and PC4 with Fasting Blood Sugar (FBS) ($-\log_{10}p = 1.33$, $p = 0.046$). All other trait–component combinations, including age, total cholesterol, LDL, HDL, 2-hour postprandial blood sugar, and HbA1c, exhibited lower $-\log_{10}p$ -values, indicating non-significant associations with any of the first five principal components. These findings suggest that the methylation variation captured by the principal components is more specifically linked to psychosocial stress exposure, adiposity, and glycemic status rather than to general demographic or lipid-related parameters.

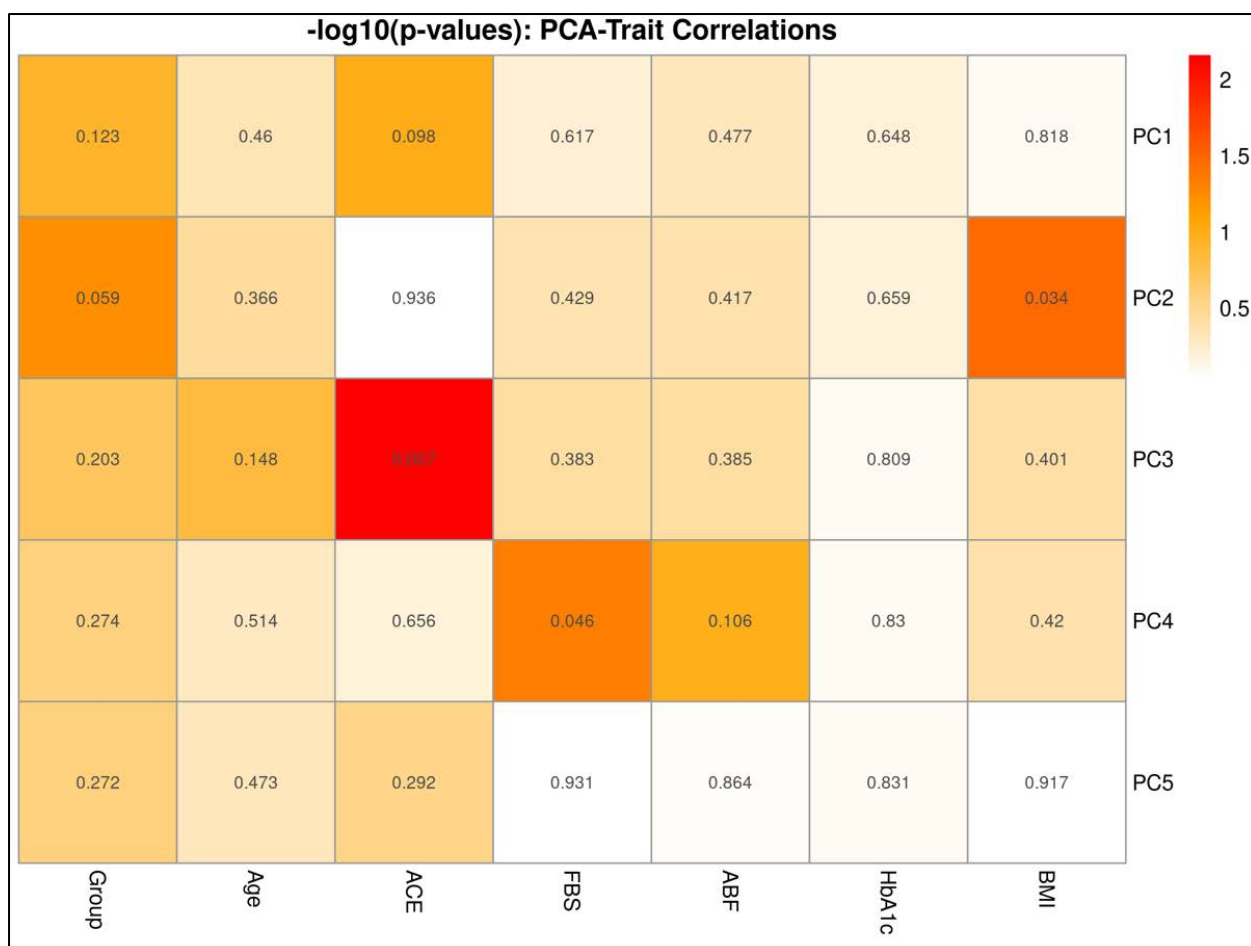


Figure 3.8: Heatmap showing $-\log_{10}(\text{p-values})$ of Pearson correlations between principal component scores (PC1–PC5) and baseline phenotypic traits

Darker shades indicate stronger statistical significance. Here, Group = type 2 diabetes mellitus and healthy controls; ACE = adverse childhood experience; FBS = fasting blood sugar (mmol/L); ABF = 2-hour postprandial blood sugar (mmol/L); HbA1c (%); BMI = body mass index (kg/m²).

3.4.4 Correlation between DNA methylation-derived principal components and significant phenotypic traits in type 2 diabetic and healthy control subjects

To examine the relationship between genome-wide DNA methylation patterns and phenotypic characteristics of the study subjects, Pearson correlation analyses were performed between principal component scores derived from methylation beta values and a panel of baseline traits using methylkit R package, as shown in **Figure 3.9**. Among the first five principal components, three showed statistically significant associations with specific phenotypic traits. A significant positive correlation was observed between PC3 and Adverse Childhood Experiences (ACE) score ($r = 0.43$, $p =$

0.006), suggesting that early-life psychosocial stress may leave a detectable imprint on DNA methylation patterns captured by PC3. This association was primarily driven by higher ACE scores among T2DM subjects compared to controls. A significant negative correlation was observed between PC2 and Body Mass Index (BMI) ($r = -0.34$, $p = 0.034$), with higher BMI values predominantly concentrated in the T2DM group, indicating a potential link between adiposity-related methylation signatures and the PC2 axis. A third significant association was identified between PC4 and fasting blood sugar (FBS) ($r = 0.33$, $p = 0.046$), with elevated FBS values largely observed among T2DM subjects, suggesting a relationship between glycemic status and methylation variation captured by PC4.

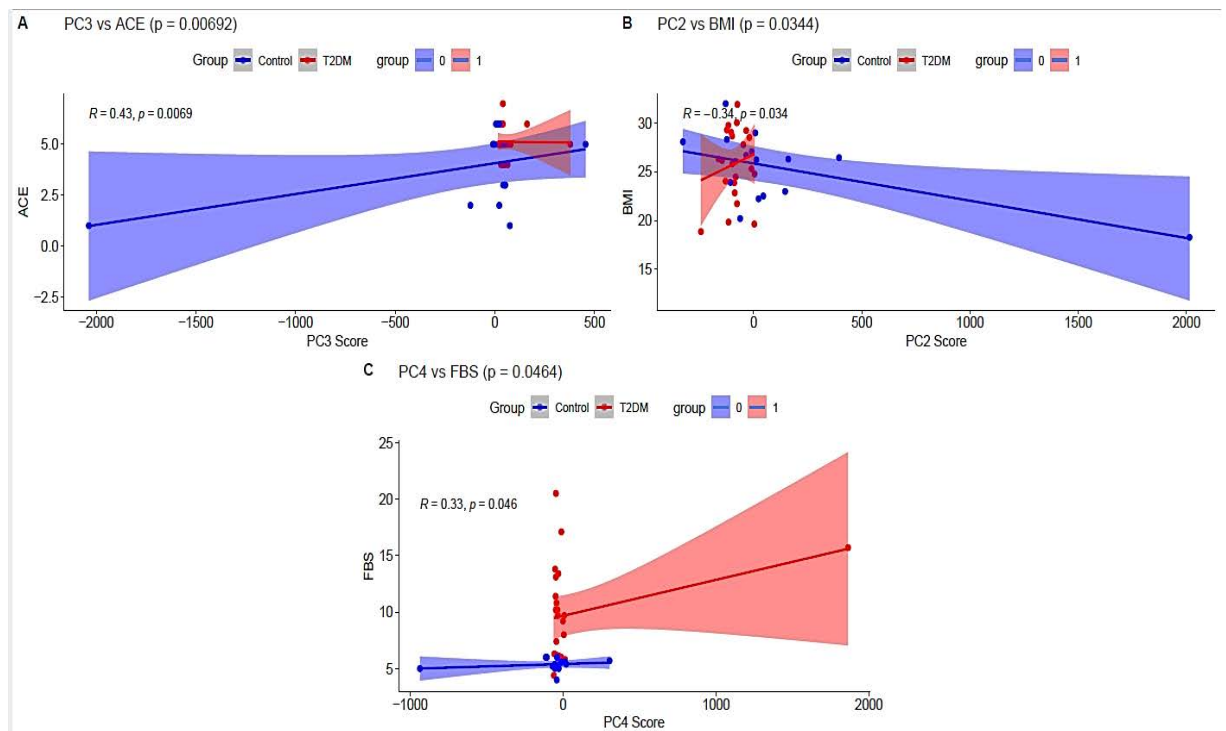


Figure 3.9: Scatter plots showing the Pearson correlations between principal component scores (PC) derived from DNA methylation data and phenotypic traits

(A) PC3 versus Adverse Childhood Experiences (ACE) score, (B) PC2 versus Body Mass Index (BMI), and (C) PC4 versus Fasting Blood Sugar (FBS). Control subjects are shown in blue and T2DM subjects in red. Shaded regions represent 95% confidence intervals.

3.4.5 Genomic element and CpG island context annotation of differentially methylated CpG sites

To characterize the genomic landscape of the identified differentially methylated CpG sites using edgeR package, annotation was performed with respect to both gene-centric elements and CpG island context, as shown in **Figure 3.10**. With respect to gene structure (Panel A), the majority of DMCs were located within intronic regions (45%), followed by intergenic regions (42.5%), exonic regions (8.9%), and promoter regions (3.6%). The predominance of DMCs within intronic and intergenic regions rather than classical promoter regions suggests that diabetes-associated methylation changes in whole blood may primarily exert regulatory functions through non-coding and distal regulatory elements rather than through canonical promoter-mediated gene silencing mechanisms. With respect to CpG island context (Panel B), the vast majority of DMCs (95.8%) were located in open sea regions, which are CpG-poor genomic regions located outside CpG islands and their flanking shore and shelf regions. Only 3.68% of the DMCs were found within CpG island shores and a minimal fraction (0.36%) occurred directly within CpG islands. These DMC sites were selected by edgeR package. These findings indicate that diabetes-associated differential methylation events in the present Bangladeshi cohort are predominantly enriched in CpG-sparse genomic regions, which are increasingly recognized as tissue- and disease-specific regulatory hotspots in the epigenomic literature.

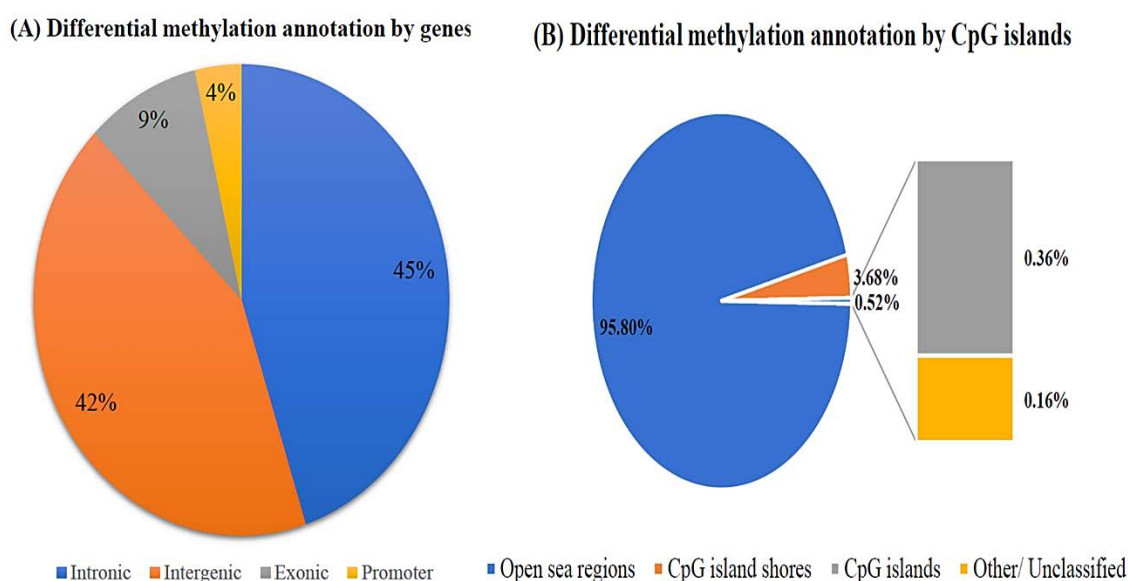


Figure 3.10: Genomic and CpG island distribution of differentially methylated CpG sites (DMCs).

(A) Proportional distribution of DMCs across genomic elements including promoter, exonic, intronic, and intergenic regions. (B) Distribution of DMCs relative to CpG island context, including CpG islands, shores, and open sea regions.

3.4.6 Genome-wide identification and chromosomal mapping of differentially methylated CpG sites in type 2 diabetes mellitus

Differential methylation analysis was performed using edgeR, and the results are presented in **Figure 3.11**. A total of 608 differentially methylated CpGs were identified at $FDR \leq 0.2$ between T2DM and healthy controls. The volcano plot (**Figure 3.11A**) reveals a broad distribution of methylation changes, with effect sizes ranging from approximately -10 to $+10$ logFC and statistical significance values reaching $-\log_{10}(FDR)$ of approximately 2.0. Overall, a predominant pattern of hypermethylation was observed, with 381 CpGs (62.7%) showing increased methylation and 227 CpGs (37.3%) showing decreased methylation in diabetic subjects compared to controls.

Chromosome-wise analysis (**Figure 3.11B**) revealed heterogeneous methylation patterns across the genome. The highest proportions of hypermethylation were observed in smaller chromosomes, particularly chromosome 21 (90.0%, 9/10 sites) and chromosome 22 (80.0%, 8/10 sites). Several larger chromosomes also showed substantial hypermethylation enrichment, including chromosome 3 (76.1%, 35/46 sites), chromosome 16 (75.0%, 15/20 sites), and chromosome 15 (72.2%, 13/18 sites). In contrast, chromosome 18 displayed a predominance of hypomethylation (53.8% hypomethylated vs. 46.2% hypermethylated), while chromosomes 10, 12, and 13 showed relatively balanced distributions between hyper- and hypomethylated sites.

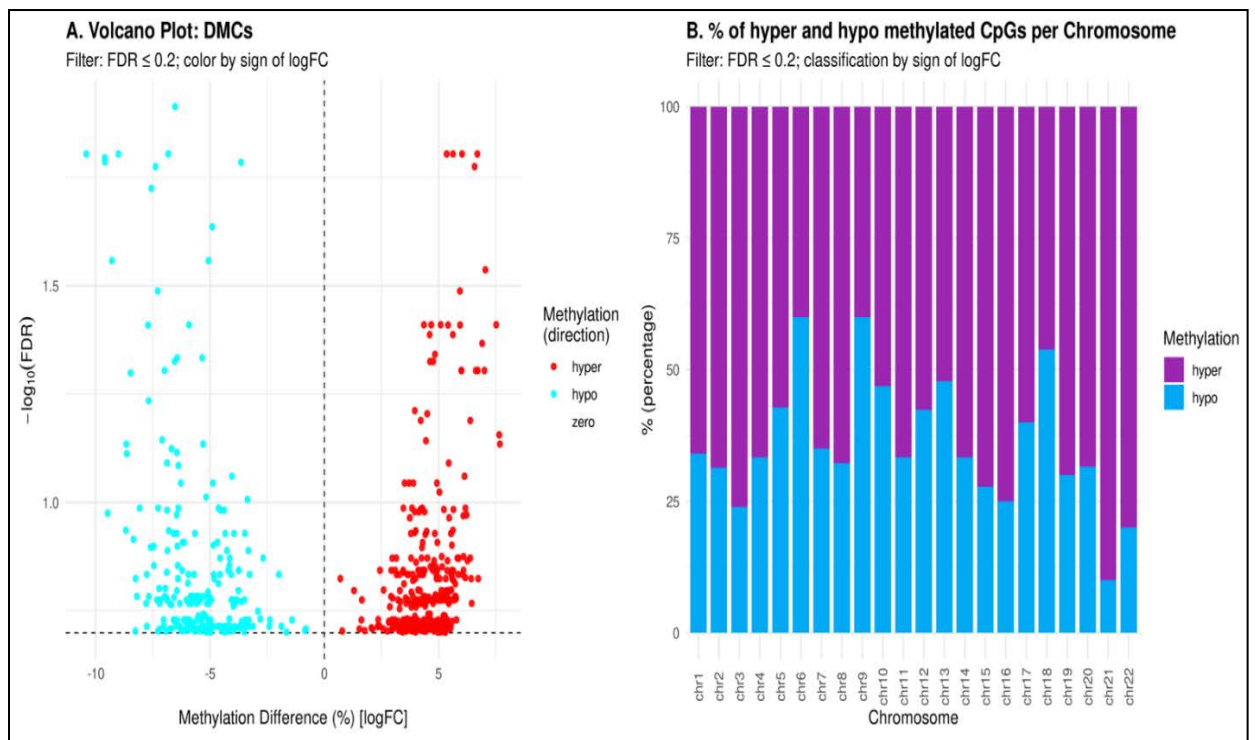


Figure 3.11: Volcano plot and Stacked bar plot of differentially methylated CpGs

(A) Volcano plot displaying the distribution of differentially methylated CpGs ($FDR \leq 0.2$) identified by edgeR tool, according to log fold change (logFC) and $-\log_{10}(FDR)$. Hypermethylated sites ($\log FC > 0$) are shown in red and hypomethylated sites ($\log FC < 0$) in cyan. The horizontal dashed line represents the $FDR = 0.2$ significance threshold. (B) Stacked bar plots showing the proportion of hypermethylated (purple) and hypomethylated (blue) CpGs across autosomes (chr1–chr22).

3.4.7 Distribution of differentially methylated cytosines across chromosomes in type 2 diabetes mellitus

The genome-wide distribution and statistical significance of differentially methylated cytosines (DMCs) identified between T2DM patients and healthy controls using edgeR, are illustrated in the Manhattan plot in **Figure 3.12**. Each point on the plot represents a single CpG site, with its chromosomal position on the x-axis and its $-\log_{10}(p\text{-value})$ on the y-axis. A genome-wide significance threshold of $p < 5 \times 10^{-8}$ ($-\log_{10}p \approx 7.3$, indicated by the dotted gray line) was applied to identify the most robustly differentially methylated loci. The plot demonstrates a widely scattered distribution of significant methylation signals across multiple chromosomes, with notable enrichment peaks observed on chromosomes 1, 2, 4, 5, 6, 7, 10, and 22, several of which surpass the genome-wide significance threshold. The dispersed nature of these peaks across diverse

chromosomal locations indicates that the epigenetic alterations associated with T2DM in whole blood are not confined to a single genomic region but are distributed across the genome, reflecting the complex and multifactorial epigenetic landscape of this disease.

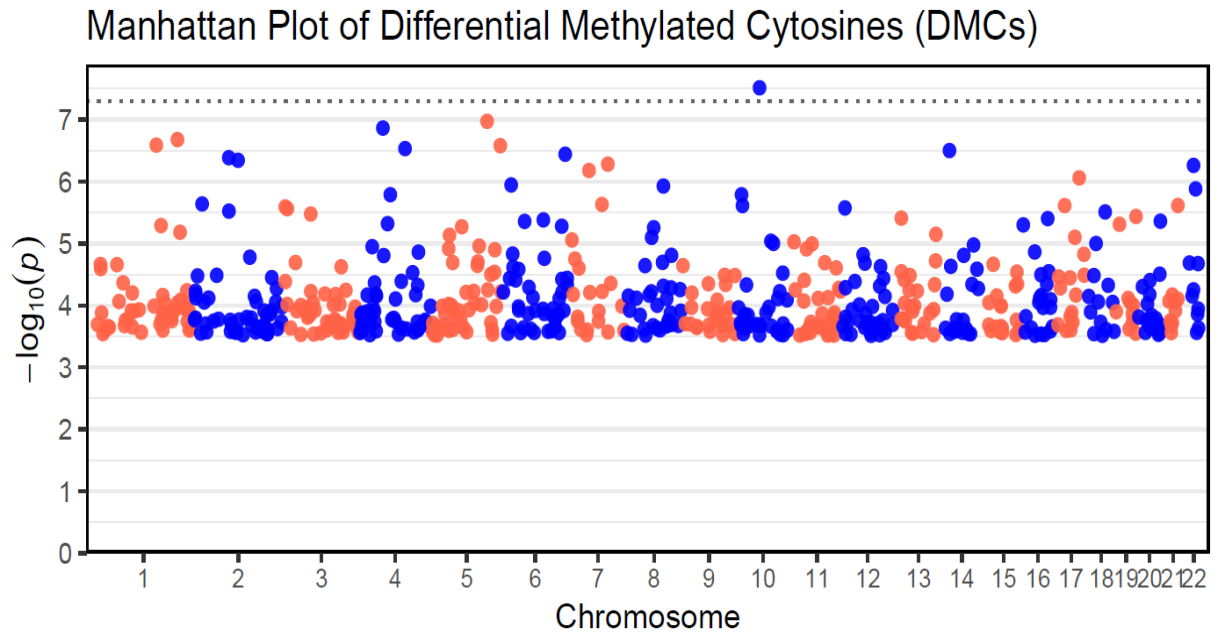


Figure 3.12: Manhattan plot of differentially methylated cytosines in T2DM across chromosomes 1–22.

The x-axis represents chromosomal positions displayed in alternating tomato and blue colors and the y-axis represents $-\log_{10}(\text{p-value})$.

3.4.8 Top 20 significant hypermethylated differentially methylated CpG (DMC) sites

The top 20 most significantly hypermethylated CpG sites identified by edgeR analysis between type 2 diabetes mellitus patients and healthy controls are presented in **Table 3.14**. These sites are ranked by statistical significance, with logFC values ranging from 4.36 to 7.52, indicating robust and statistically reliable hypermethylation signals. The most substantially hypermethylated site was located on chromosome 21 at position 41,150,408, annotated to the *BACE2* gene (logFC = 7.52, FDR = 0.039), followed by a site on chromosome 22 annotated to *SNU13* (logFC = 7.04, FDR = 0.029), and a site on chromosome 4 annotated to *ANKRD17-DT/COX18* (logFC = 7.00, FDR = 0.050). Other notable hypermethylated genes included *CUX1* and *SH2B2* on chromosome 7 (logFC = 6.57), *G2E3* on chromosome 14 (logFC = 6.69), *CAMK1D* on chromosome 10 (logFC = 5.93), *GRM3* on chromosome 7 (logFC = 4.36), and *KCTD16* on chromosome 5 (logFC = 5.63). The hypermethylated sites were distributed across multiple chromosomes, with representation on chromosomes 1, 2, 3, 4, 5, 6, 7, 10, 14, 18, 19, 20, 21, and 22, indicating that hypermethylation associated with type 2 diabetes mellitus in the present study is a genome-wide phenomenon rather than being confined to specific chromosomal regions.

Table 3.14: Top significant 20 hypermethylated differentially methylated CpG sites (DMCs)

Chr	Locus	logFC	logCPM	F	p-value	FDR	Promoter	Enhancer
chr5	143929003	5.63	2.39	34.26	1.06E-07	0.016		<i>KCTD16</i>
chr1	214317412	6.03	2.29	34.27	2.10E-07	0.016		<i>PTPN14</i>
chr4	119262395	5.35	2.35	31.46	2.93E-07	0.016		
chr14	30525667	6.69	2.26	36.21	3.15E-07	0.016		<i>G2E3</i>
chr7	102126760	6.57	2.26	31.91	5.23E-07	0.017		<i>CUX1; SH2B2</i>
chr22	41690311	7.04	2.28	30.12	1.31E-06	0.029	<i>SNU13</i>	
chr10	12276672	5.93	2.26	32.75	1.63E-06	0.033		<i>CAMK1D</i>
chr2	28000159	5.09	2.29	28.41	2.30E-06	0.039		<i>RBKS</i>
chr7	86530135	4.36	2.49	25.86	2.35E-06	0.039		<i>GRM3</i>
chr21	41150408	7.52	2.29	28.23	2.43E-06	0.039		<i>BACE2</i>
chr10	14691665	4.68	2.30	28.22	2.44E-06	0.039		<i>FAM107B</i>
chr3	3846718	5.41	2.29	27.80	2.57E-06	0.039		<i>LRRN1</i>
chr3	9213135	5.94	2.26	30.29	2.74E-06	0.039		<i>SRGAP3-AS4/AS2</i>
chr2	98709486	4.61	2.27	29.20	3.00E-06	0.041		<i>MGAT4A</i>
chr18	50259724	5.62	2.31	26.19	3.09E-06	0.041		<i>MYO5B; MBD1</i>
chr3	71074330	6.90	2.26	26.92	3.34E-06	0.043		<i>FOXP1-AS1</i>
chr19	52951289	4.84	2.29	24.77	3.66E-06	0.046		<i>ZNF816; ZNF321P</i>
chr20	59268097	4.76	2.34	26.49	4.37E-06	0.047		<i>EDN3; ZNF831</i>
chr6	57128567	4.62	2.28	26.76	4.39E-06	0.047		<i>ZNF451; ZNF451- AS1</i>
chr4	73184486	7.00	2.31	26.22	4.78E-06	0.050		<i>ANKRD17-DT; COX18</i>

Here, Chr = chromosome; logFC = log fold change; logCPM = log counts per million; FDR = false discovery rate; Promoter and enhancer refer to gene annotations based on basal and extended genomic regions respectively.

3.4.9 Top 20 significant hypomethylated differentially methylated CpG (DMC) sites

The top 20 most significantly hypomethylated CpG sites identified by edgeR analysis between type 2 diabetes mellitus patients and healthy controls are presented in **Table 3.15**. Notably, the hypomethylated sites demonstrated generally larger effect sizes compared to the hypermethylated sites, with logFC values ranging from -3.64 to $-$

10.41. The most substantially hypomethylated site was located on chromosome 1 at position 158,645,089, annotated to *SPTA1* and *OR6Y1* (logFC = -10.41, FDR = 0.016), followed by a site on chromosome 6 without gene annotation (logFC = -9.61, FDR = 0.016), and a site on chromosome 2 annotated to *INPP4A* (logFC = -9.60, FDR = 0.016). Other significantly hypomethylated genes included *APOL1* and *MYH9-DT* on chromosome 22 (logFC = -7.38), *KCNS2* and *OSR2* on chromosome 8 (logFC = -9.29), *MRLN* and *CCDC6* on chromosome 10 (logFC = -6.53), and *PRKG2-AS1* and *CFAP299* on chromosome 4 (logFC = -7.29).

Table 3.15: Top 20 hypomethylated differentially methylated sites

Chr	Locus	logFC	logCPM	F	p-value	FDR	Promoter	Enhancer
chr10	59797228	-6.53	2.45	37.90	3.07E-08	0.012		<i>MRLN</i> ; <i>CCDC6</i>
chr4	60921725	-9.01	2.32	35.58	1.37E-07	0.016		
chr1	158645089	-10.41	2.27	35.34	2.58E-07	0.016		<i>SPTA1</i> ; <i>OR6Y1</i>
chr5	178805470	-6.83	2.28	35.27	2.63E-07	0.016		<i>ZNF354A</i> ; <i>ZNF354B</i>
chr6	164107809	-9.61	2.30	31.63	3.62E-07	0.016		
chr2	98418824	-9.60	2.31	33.80	4.11E-07	0.016		<i>INPP4A</i>
chr2	122860056	-3.64	2.50	31.59	4.53E-07	0.016		
chr22	36267087	-7.38	2.27	32.87	5.48E-07	0.017		<i>APOL1</i> ; <i>MYH9-DT</i>
chr7	52402839	-7.56	2.29	32.27	6.63E-07	0.019		
chr17	65478153	-4.90	2.30	31.40	8.71E-07	0.023		
chr6	21490961	-5.07	2.42	28.15	1.13E-06	0.028		
chr8	98936198	-9.29	2.29	28.76	1.18E-06	0.028		<i>KCNS2</i> ; <i>OSR2</i>
chr4	80396338	-7.29	2.29	29.46	1.63E-06	0.033		<i>PRKG2-AS1</i> ; <i>CFAP299</i>
chr17	27173905	-5.92	2.30	28.22	2.44E-06	0.039		
chr12	7192173	-7.71	2.29	27.96	2.67E-06	0.039		
chr13	17861011	-5.34	4.03	27.74	3.90E-06	0.046		
chr16	71325397	-6.44	2.28	24.81	3.96E-06	0.046		
chr6	106498336	-6.55	2.30	26.23	4.15E-06	0.047		<i>QRSL1</i> ; <i>CRYBG1</i>
chr1	171058403	-6.99	2.30	29.43	5.13E-06	0.050		<i>MROH9</i> ; <i>FMO3</i>

Here, Chr = chromosome; logFC = log fold change; logCPM = log counts per million; FDR = false discovery rate; Promoter and enhancer refer to gene annotations based on basal and extended genomic regions respectively.

3.4.10 Hypermethylated Differentially methylated regions identified in type 2 diabetic subjects

Differentially methylated region analysis using DMRcate identified a total of 62 hypermethylated DMRs across the genome in type 2 diabetes mellitus patients compared to healthy controls, and the top 20 of these regions are presented in **Table 3.16**. These regions were ranked by minimum smoothed FDR and maximum methylation difference. Each DMR contained a minimum of 3 CpG sites, with the number of CpGs per region ranging from 3 to 4 across the top 20 DMRs. The number of CpGs per DMR ranged from 3 to 4, with widths spanning from 5 bp to 785 bp, and maximum methylation differences up to 2.05, reflecting the variable extent of contiguous hypermethylated CpG clustering. The maximum methylation differences ranged from 0.81 to 2.05, with the largest difference observed in the unannotated region on chromosome 9. Among the biologically notable hypermethylated DMRs, regions annotated to *CNTNAP2*, *OLFM3*, *TRPC7*, *ACTR3*, *ITPKB*, and *PDE11A*. The minimum smoothed FDR values for these regions ranged from 0.0002 to 0.04, confirming their statistical reliability.

Table 3.16: Top 20 hypermethylated differentially methylated regions (DMRs) identified type 2 diabetes mellitus patients compared to healthy control subjects

Seqnames	Start	End	Width	No CpGs	Min smoothed FDR	Maxdiff	Meandiff	Promoter	Enhancer
chr9	31416909	31417693	785	3	0.0002	2.05	0.29		
chr7	146215470	146215566	97	3	0.03	1.93	0.86		<i>CNTNAP2</i>
chr12	74537902	74537916	15	3	0.04	0.81	0.72		<i>LINC02882</i>
chr4	133060129	133060186	58	3	0.03	1.00	-0.23		
chr7	20835350	20835354	5	3	0.03	1.13	1.06	LINC01162	
chr6	91088459	91088580	122	3	0.02	1.38	1.27		
chr2	210860501	210860601	101	3	0.01	1.53	0.48		<i>CPS1</i>
chr2	18973619	18974341	723	3	0.001	1.62	0.69		<i>LINC01376</i>

chr1	102078776	102078982	207	3	0.04	1.43	1.15	<i>OLFM3</i>
chr4	60875825	60876271	447	3	0.02	1.27	0.63	LINC02271; LINC02496
chr17	14559466	14559716	251	3	0.04	1.38	1.02	
chr2	126084580	126084599	20	3	0.04	1.52	0.88	LINC01941
chr2	5592263	5592472	210	3	0.02	1.56	0.91	LINC01248
chr10	78658803	78659284	482	3	0.002	1.98	0.21	
chr5	136217111	136217648	538	3	0.02	1.54	0.33	<i>TRPC7-AS1; TRPC7</i>
chr2	113892012	113892051	40	3	0.02	1.74	0.93	<i>ACTR3-AS1;</i> <i>ACTR3</i>
chr4	117271825	117271941	117	3	0.02	1.90	0.31	LINC01378
chr1	226704728	226704813	86	3	0.02	2.01	0.47	<i>ITPKB; ITPKB-IT1</i>
chr4	37993861	37993877	17	4	0.03	0.99	0.71	<i>TBC1D1</i>
chr2	177744407	177744812	406	4	0.01	1.66	0.69	<i>PDE11A-AS1; PDE11A</i>

Here, Width = genomic span of the DMR in base pairs; No CpGs = number of CpG sites within the DMR; Maxdiff = maximum methylation difference; Meandiff = mean methylation difference; Basal Genes and Extended Genes refer to gene annotations based on basal and extended genomic regions respectively

3.4.11 Hypomethylated differentially methylated regions identified in type 2 diabetic subjects

Differentially methylated region analysis using DMRcate identified a total of 170 hypomethylated DMRs across the genome in type 2 diabetes mellitus patients compared to healthy controls, and the top 20 of these regions are presented in **Table 3.17**. These regions were ranked by minimum smoothed FDR and maximum methylation difference, with all regions achieving statistical significance at minimum smoothed FDR ≤ 0.05 . The top 20 hypomethylated DMRs, ranked by minimum smoothed FDR and maximum methylation difference, are presented in Table 3.17. These regions spanned widths ranging from 5 bp to 588 bp and included 3–4 CpGs per region. Several hypomethylated DMRs overlapped with or were in proximity to biologically relevant genes such as *PRKAR2B-AS1*, *TET2*, *HDAC9*, *RAPGEF4*, and *PCCA*. The maximum methylation difference observed among these regions reached up to -2.44 .

Table 3.17: Top 20 hypomethylated differentially methylated regions (DMRs) of type 2 diabetes mellitus patients compared to healthy control subjects

Seqnames	Start	End	Width	No CpGs	Min smoothed FDR	maxdiff	meandiff	Promoter	Enhancer
chr2	136862947	136863270	324	3	0.003	-1.50	-0.90		<i>THSD7B</i>
chr10	21636422	21636597	176	3	0.004	-1.48	-0.80		<i>SKIDA1</i>
chr7	107129148	107129703	556	4	0.0002	-2.44	-0.84	<i>PRKAR2B-AS1</i>	
chr11	85554245	85554249	5	3	0.04	-0.96	-0.82		<i>DLG2</i>
chr12	64658573	64658582	10	3	0.04	-0.76	-0.71		<i>GNS</i> ; <i>RASSF3</i>
chr12	109272336	109272360	25	3	0.002	-1.03	-0.94		<i>FOXM4</i> ; <i>ACACB</i>

chr13	66596619	66596625	7	3	0.01	-1.53	-1.30	<i>PCDH9-AS2</i>
chr13	100451060	100451606	547	3	0.03	-0.91	-0.86	<i>PCCA-DT; PCCA-ASI</i>
chr14	33495075	33495282	208	3	0.04	-1.25	-1.08	
chr14	67342448	67342477	30	3	0.03	-0.81	-0.74	<i>PALSI; ATP6VID</i>
chr2	131382838	131382870	33	3	0.02	-1.04	-0.87	<i>LINC01120</i>
chr2	172864972	172865152	181	3	0.03	-1.02	-0.86	<i>RAPGEF4-ASI; MAP3K20</i>
chr2	232372727	232373127	401	3	0.05	-1.20	-0.45	<i>DIS3L2; ALPP</i>
chr3	37296106	37296122	17	3	0.01	-1.41	-1.09	<i>GOLGA4; APRG1</i>
chr4	58426141	58426151	11	3	0.03	-1.17	-1.04	<i>LINC02494</i>
chr4	105121040	105121248	209	3	0.02	-1.03	-0.99	<i>TET2</i>
chr4	176575716	176576303	588	3	0.01	-1.60	0.26	<i>HAFML</i>
chr7	17967966	17968333	368	3	0.02	-1.08	-0.43	<i>SNX13; HDAC9</i>
chr10	7841140	7841146	7	3	0.03	-0.93	-0.78	
chr9	138001450	138001579	130	3	0.05	-1.20	-0.39	

Width = genomic span of the DMR in base pairs; No CpGs = number of CpG sites within the DMR; Maxdiff = maximum methylation difference; Meandiff = mean methylation difference; Promoter and enhancer refer to gene annotations based on basal and extended genomic regions respectively

3.4.12 Pathway Analysis of canonical pathways associated with differentially methylated cytosines in type 2 diabetes mellitus

Bar plots showing top 10 enriched canonical pathways identified by Ingenuity Pathway Analysis (IPA) for (a) hypermethylated and (b) hypomethylated differentially methylated cytosines (DMCs). The x-axis represents statistical significance ($-\log_{10}$ p-value), and the dashed red line indicates the significance threshold ($p < 0.05$). In hypermethylated DMCs (**Figure 3.13a**), the most significantly enriched pathways included SUMOylation of transcription cofactors ($-\log_{10} p = 2.89$) involving genes such as *MBD1*, *PHC2*, *RNF2*, and *SAFB*, as well as regulation of Toll-like receptor signaling (*TLR1*, *TLR6*, *FGB*) and circadian gene expression (*BMAL1*, *NPAS2*, *TBLIX*). Additionally, pathways related to fatty acyl-CoA biosynthesis and fatty acid activation (*ACSBG2*, *ACSL5*, *ELOVL7*) and insulin secretion signaling were enriched. In hypomethylated DMCs (**Figure 3.13b**), enriched pathways included pexophagy ($-\log_{10} p = 2.5$), WNK renal signaling, DYRK1A signaling, and Type II Diabetes Mellitus signaling, along with mitophagy and triacylglycerol degradation. These pathways are associated with mitochondrial quality control, metabolic regulation, and cellular stress responses. Overall, these findings indicate that cytosine-level methylation changes influence genes involved in transcriptional regulation, immune signaling, mitochondrial function, and metabolic homeostasis in T2DM. The full table of IPA is placed in appendix.

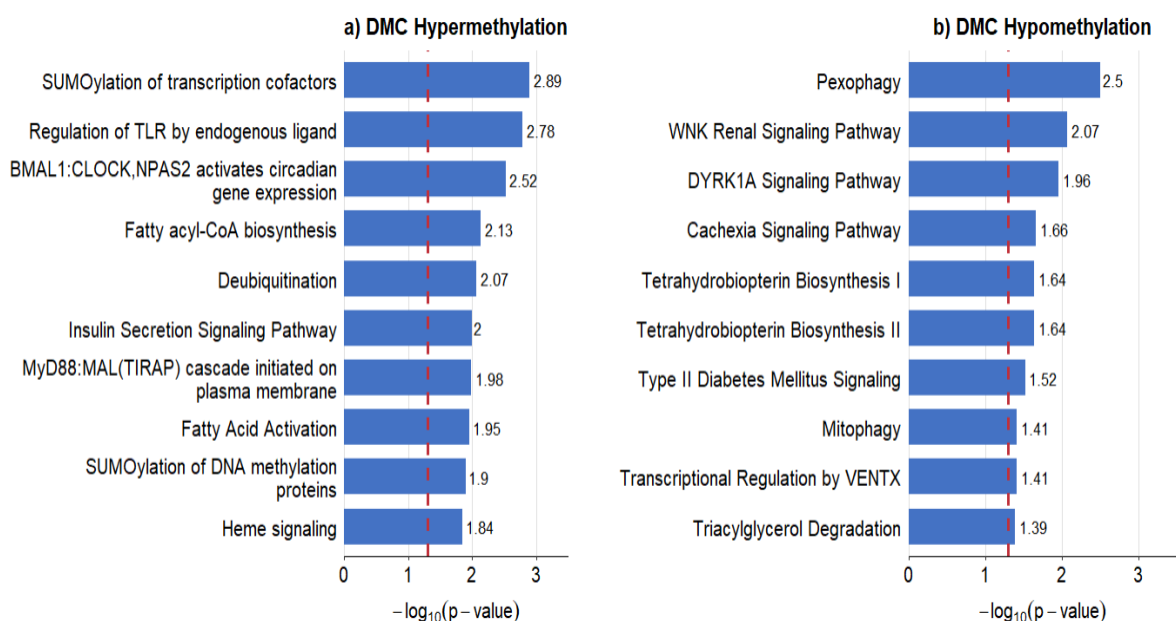


Figure 3.13: Ingenuity Pathway Analysis of hypermethylated (a) and hypomethylated (b) differentially methylated cytosines (DMCs) showing enriched canonical pathways

The x-axis represents statistical significance expressed as $-\log_{10}(\text{p-value})$ and the y-axis lists the identified pathways

3.4.13 Pathway Analysis of canonical pathways associated with differentially methylated regions in type 2 diabetes mellitus

Bar plots showing Top 10 enriched canonical pathways identified by Ingenuity Pathway Analysis (IPA) for (a) hypermethylated and (b) hypomethylated differentially methylated regions (DMRs). In hypermethylated DMRs (**Figure 3.14a**), the most significantly enriched pathway was neurotransmitter receptors and postsynaptic signal transmission ($p=8.51 \times 10^{-5}$) involving genes such as *GLRA3* and *NBEA*. Other enriched pathways included eumelanin biosynthesis (MIF), platelet homeostasis (*PDE11A*, *TRPC7*), and dTMP de novo biosynthesis (*DHFR2*), along with pathways related to synaptogenesis, RHOA signaling, and SNARE signaling. In hypomethylated DMRs (**Figure 3.14b**), enriched pathways included O-linked glycosylation ($-\log_{10} p = 3.45$) involving *ADAMTSL3*, *B3GALNT2*, *B3GNT2*, *MUC20*, and *THSD7B*, as well as activation of NMDA receptors (*DLG2*, *ERBB4*, *PRKAR2B*, *RPS6KA2*), TGFBR3 signaling (*PSEN1*, *TCF12*, *TNRC6B*), and thiamin salvage (*TPK1*). Additional pathways such as NOTCH1 signaling and Sonic Hedgehog signaling highlight involvement of developmental and regulatory processes. Overall, region-level methylation changes affect genes involved in neuronal signaling, metabolic regulation, and cellular communication, indicating coordinated epigenetic alterations in T2DM. The full table of IPA is placed in appendix.

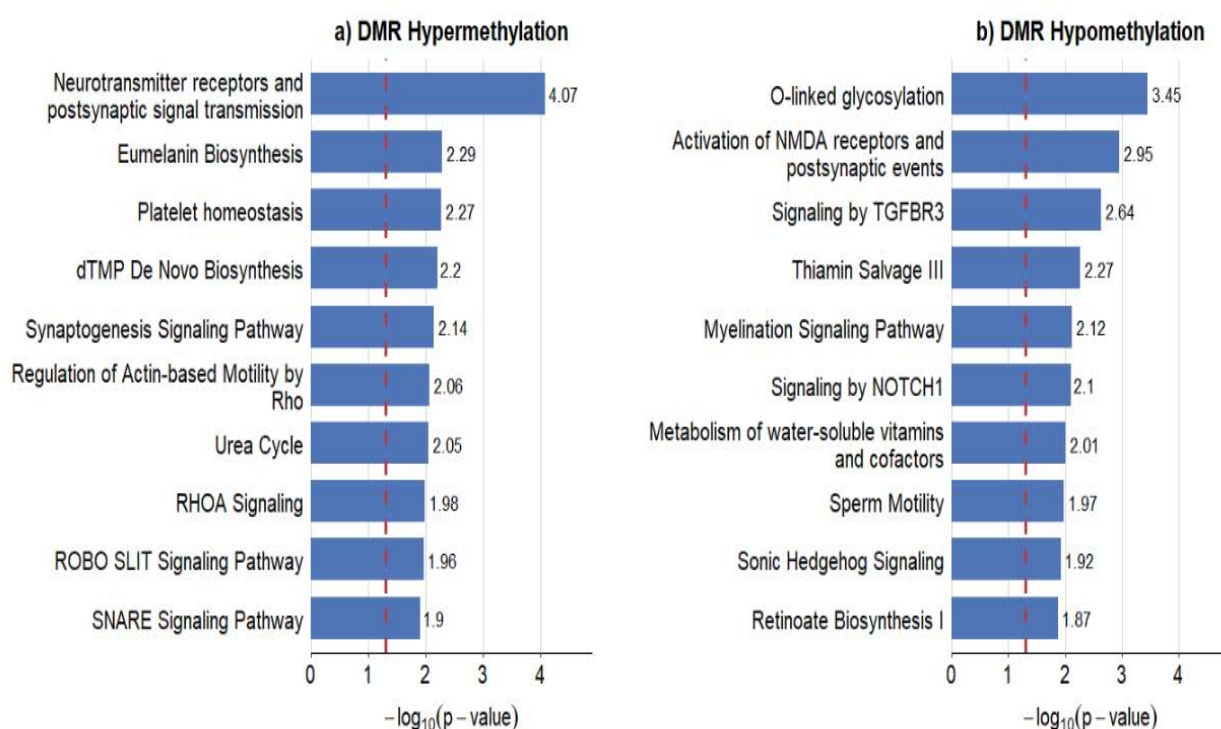


Figure 3.14: Ingenuity Pathway Analysis of hypermethylated (a) and hypomethylated (b) differentially methylated regions (DMRs) showing enriched canonical pathways

The x-axis represents statistical significance expressed as $-\log_{10}(\text{p-value})$ and the y-axis lists the identified pathways

3.4.14 Comparative canonical pathway enrichment across hypermethylated and hypomethylated differentially methylated cytosines and regions in type 2 diabetes mellitus

To provide a comprehensive and integrated view of the canonical pathway enrichment patterns across all four methylation categories simultaneously, a comparative heatmap of $-\log_{10}(\text{p-values})$ was generated and is presented in **Figure 3.15**. This analysis enables direct visual comparison of pathway enrichment between hypermethylated and hypomethylated events at both the cytosine level (DMC) and regional level (DMR), providing systems-level insight into the coordinated epigenetic dysregulation associated with type 2 diabetes mellitus. Notably, neurotransmitter receptors and postsynaptic signal transmission exhibited the highest enrichment, particularly in hypermethylated DMRs, suggesting involvement of neuroendocrine signaling mechanisms. Pathways such as fatty acyl-CoA biosynthesis, insulin secretion signaling,

and mitochondrial L-carnitine shuttle were enriched across both cytosine- and region-level analyses, highlighting consistent epigenetic alterations in metabolic pathways. In contrast, hypomethylated DMRs showed stronger enrichment in pathways such as O-linked glycosylation, activation of NMDA receptors, NOTCH1 signaling, and vitamin metabolism, indicating activation of signaling and regulatory pathways. Meanwhile, hypermethylated DMCs were more prominently associated with SUMOylation, Toll-like receptor signaling, and transcriptional regulation pathways.

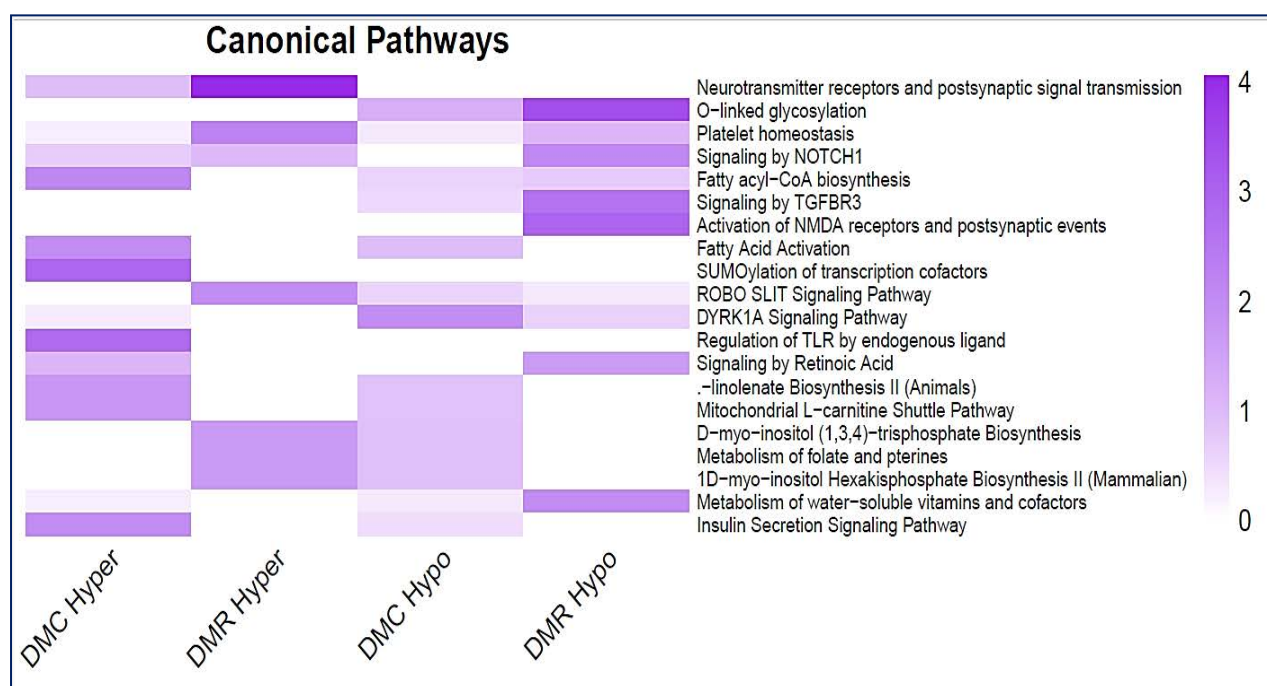


Figure 3.15: Heatmap showing enrichment scores ($-\log_{10}(\text{p-value})$) of canonical pathways across four methylation categories

DMC_Hyper (hypermethylated differentially methylated cytosines), DMR_Hyper (hypermethylated differentially methylated regions), DMC_Hypo (hypomethylated differentially methylated cytosines), and DMR_Hypo (hypomethylated differentially methylated regions). Pathway analysis was performed using Ingenuity Pathway Analysis (IPA). Color intensity represents statistical significance (darker purple = higher significance; scale 0–4). Pathways with $-\log_{10}(\text{p-value}) \geq 1.3$ ($p < 0.05$) are shown. DMC = differentially methylated cytosine; DMR = differentially methylated region; Hyper = hypermethylation; Hypo = hypomethylation.

3.4.15 Enrichment of Type 2 Diabetes Mellitus signaling canonical pathway

The Type II Diabetes Mellitus canonical pathway is presented in **Figure 3.16**, consisting of two major components: pancreatic β -cell insulin secretion (left panel) and insulin signaling in peripheral tissues such as adipocytes, hepatocytes, and skeletal muscle cells (right panel). In the left panel, the pathway illustrates glucose entry into pancreatic β -cells followed by glycolysis, mitochondrial metabolism, and ATP production. This leads to membrane depolarization, activation of voltage-gated calcium channels, and calcium influx, ultimately resulting in insulin granule exocytosis. Within the nuclear region, transcriptional regulators of insulin gene expression are depicted, including *PDX1*, *MAFA*, and *CEBPB*, with *CEBPB* highlighted in this pathway. The signaling component *PRKDI* is also mapped within the intracellular signaling network associated with oxidative stress pathways. In the right panel, the pathway represents insulin signaling following insulin binding to its receptor (INSR), leading to downstream activation of signaling cascades involving adaptor proteins and kinases. These signaling events regulate glucose transport through translocation of glucose transporters such as *GLUT4* to the plasma membrane. The figure also shows interactions with inflammatory mediators (e.g., TNF) and free fatty acids influencing insulin signaling pathways. The gene *ADIPOR1* is mapped within this signaling framework, while *ACSBG2* is associated with lipid metabolism-related processes shown in the pathway.

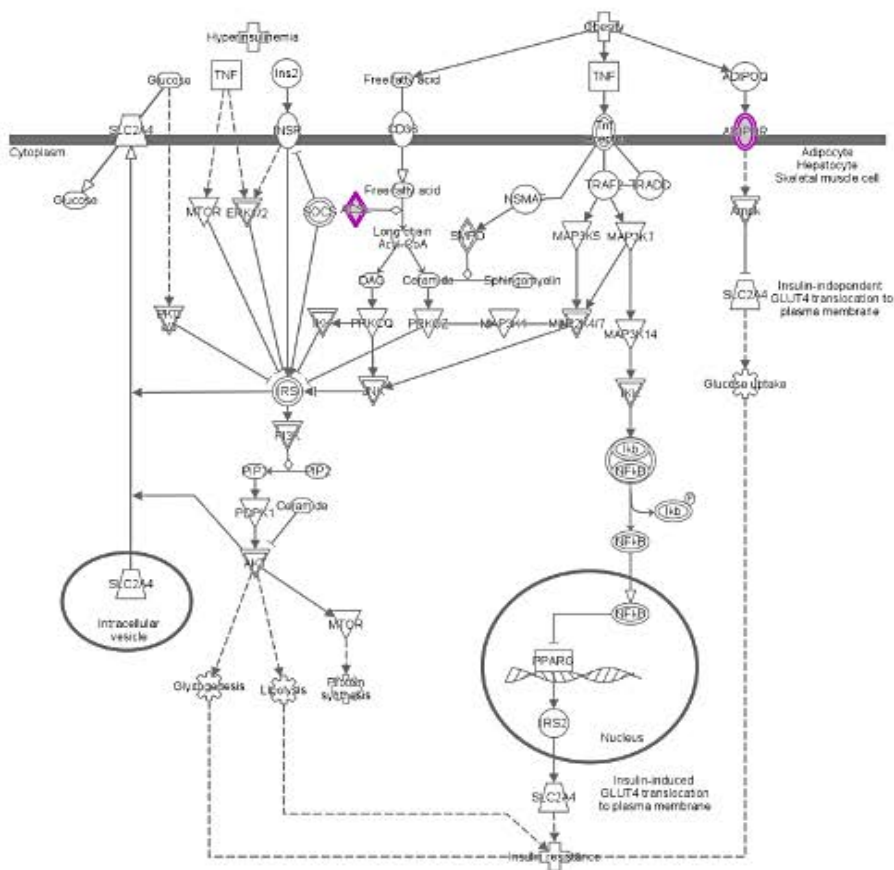


Figure 3.16: Type 2 diabetes mellitus canonical signaling pathway

Its highlighting genes associated with hypomethylated differentially methylated cytosines. The pathway shows a statistical significance of $-\log_{10}(\text{p-value}) = 1.52$ ($p \approx 0.03$).

3.5 Adverse childhood experiences (ACE), Type 2 diabetes mellitus and mediation by DNA methylation

3.5.1 Hypermethylated differentially methylated CpG sites linking ACE to T2DM

The top 20 hypermethylated CpG loci emerging from the HDMAX2 association screening across 6,295,480 quality-controlled CpG sites in study subjects are presented in **Table 3.18**. Genomic annotations include promoter and enhancer associations based on CpGtools annotation. p-values correspond to the statistical significance of mediation association between ACE (exposure), DNA methylation (mediator), and T2DM (outcome). Absolute methylation differences range from 39 to 53, indicating biologically substantial gains in methylation among individuals with high ACE scores (>4) relative to low ACE (≤ 4). Several loci map to enhancer regions of genes, including *RUNX1T1*, *CNTN4*, and *SATB1*. These hypermethylated sites were subsequently subjected to comb-p regional aggregation to identify candidate differentially methylated regions (DMRs) for causal mediation analysis.

Table 3.18: Top hypermethylated DMCs identified by HDMAX2 in the ACE–T2DM pathway

Chr	Start	End	abs_meth_diff	Promoter	Enhancer	p-value
chr9	74262353 17070403	74262354	53			4.24E-05
chr2	2 14989086	170704033	52		<i>SP5</i> <i>RAET1E; RAET1E-</i>	5.65E-06
chr6	8	149890869	50		<i>AS1</i>	5.46E-06
chr2	91571464	91571465	49			1.97E-05
chr14	19825759 20961048	19825760	48		<i>OR4M1; OR4K2</i>	8.68E-05
chr2	9	209610490	47		<i>UNC80; MAP2</i>	8.18E-05
chr3	2036331	2036332	45		<i>CNTN4</i>	4.57E-05
chr5	55776823	55776824	44	<i>SLC38A9</i>		1.76E-05
chr8	92114782	92114783	42		<i>RUNX1T1</i>	1.45E-06
chr8	120032048	120032049	42		<i>DEPTOR-AS1</i>	4.31E-05
chr16	6590728	6590729	41		<i>LINC01570</i>	6.68E-05
chr2	91571340	91571341	40			5.28E-05
chr3	196791400	196791401	40		<i>PAK2; SENP5</i>	7.38E-05
chr4	18445334	18445335	40			8.85E-06
chr5	164084696	164084697	40		<i>LINC03000</i>	2.63E-05

chr9	34163756	34163757	40	<i>DCAF12; UBAP1</i>	6.39E-05
chr9	121703887	121703888	40		1.9E-05
chr3	18520513	18520514	39	<i>SATB1</i>	3.43E-05
chr3	79420979	79420980	39	<i>ROBO1</i>	3.12E-05
chr3	166637606	166637607	39		7.82E-05

CpG sites are ranked by absolute methylation difference (abs_meth_diff). Chromosomal coordinates (hg38), absolute methylation difference, nearest promoter and enhancer-associated gene annotations, and unadjusted p-values are reported. adjusting for age, sex, BMI, hypertension, first-degree family history of diabetes, perceived stress (PSS-4), and two latent factors capturing unmeasured confounding.

3.5.2 Hypomethylated differentially methylated CpG sites linking ACE to T2DM

The top 20 hypomethylated CpG loci identified in the HDMAX2 scan, with absolute methylation differences ranging from -8 to -31, reflecting loss of methylation associated with high ACE exposure. Notable loci include *MEF2C-AS1*, *MYRFL*, *RB1CC1*, *TRIM44*, and *COL12A1*. Hypomethylation at these sites may reflect epigenetic erosion consequent to chronic stress exposure in early life, potentially disrupting gene regulatory networks relevant to metabolic disease susceptibility. These hypomethylated sites were subsequently subjected to comb-p regional aggregation.

Table 3.19: Top hypomethylated DMCs identified in the ACE-T2DM pathway

Chr	Start	End	abs_meth_diff	Promoter	Enhancer	p-value
chr12	69897912	69897913	-31		<i>MYRFL</i>	4.24E-05
chr5	88869643	88869644	-27		<i>MEF2C-ASI; GSCAR</i>	5.65E-06
chr4	34722504	34722505	-24			5.46E-06
chr6	75084621	75084622	-16		<i>COL12A1</i>	1.97E-05
chr8	54720203	54720204	-15		<i>RPI</i>	8.68E-05
chr10	65843421	65843422	-15			8.18E-05
chr4	123806738	123806739	-14			4.57E-05
chr22	47990922	47990923	-13			1.76E-05
chr1	170170800	170170801	-11	<i>LINC01681</i>		1.45E-06
chr2	199081569	199081570	-11		<i>SATB2-ASI</i>	4.31E-05
chr4	123737917	123737918	-11			6.68E-05
chr17	77882009	77882010	-11			5.28E-05
chr21	13265503	13265504	-11		LINC01674	7.38E-05
chr2	78651669	78651670	-9			8.85E-06
chr4	139267106	139267107	-9		<i>ELF2; NAA15</i>	2.63E-05
chr11	12918649	12918650	-9		<i>LINC00958; TEAD1</i>	6.39E-05
chr11	35914970	35914971	-9		<i>TRIM44</i>	1.9E-05
chr3	95506225	95506226	-8		<i>MTHFD2P1</i>	3.43E-05
chr8	53148914	53148915	-8		<i>RB1CC1</i>	3.12E-05
chr10	61398151	61398152	-8		<i>TMEM26-ASI</i>	7.82E-05

CpG sites are ranked by absolute methylation difference (negative values denoting hypomethylation). Chromosomal coordinates (hg38), absolute methylation difference (abs_meth_diff), nearest promoter and enhancer-associated gene annotations, and unadjusted p-values are reported. adjusting for age, sex, BMI, hypertension, first-degree family history of diabetes, perceived stress (PSS-4), and two latent factors capturing unmeasured confounding.

3.5.3 Individual Causal Mediation Analysis of Differentially Methylated Regions (DMRs) Linking Adverse Childhood Experiences to Type 2 Diabetes Risk

Region-specific causal mediation estimates for the 11 candidate differentially methylated regions (DMRs) identified via comb-p aggregation of HDMAX2-significant CpG sites are presented in **Table 3.20**. Among the identified regions, DMR 1, DMR 4, and DMR 5 showed statistically significant mediation effects. These regions exhibited relatively higher average causal mediation effects (ACME), indicating their potential contribution to the indirect pathway linking ACE and T2DM. Several other DMRs, including DMR 3, DMR 9, and DMR 10, demonstrated moderate mediation effects but did not reach statistical significance. The variation observed across DMRs

suggests heterogeneity in the contribution of different methylation regions to the mediation pathway between ACE exposure and T2DM.

Table 3.20: Differentially methylated regions (DMRs) mediating the association between ACE and T2DM identified

DMR ID	Chr	Start	End	CpG Count	ACME	ADE	TE	PM	p-val
DMR 1	8	127663958	127664258	2	0.32	-0.08	0.24	1.35	0.01
DMR 2	2	91571329	91571641	39	0.15	0.29	0.44	0.34	0.12
DMR 3	20	17971793	17971989	6	0.15	0.15	0.31	0.50	0.08
DMR 4	2	218593576	218593577	1	0.40	-0.20	0.21	1.95	0.02
DMR 5	14	48627121	48627122	1	0.25	-0.25	-0.001	-295.7	0.03
DMR 6	13	108383683	108383911	3	0.19	0.17	0.36	0.53	0.14
DMR 7	5	87412920	87412929	3	0.067	0.31	0.38	0.17	0.47
DMR 8	21	13265419	13265504	2	0	0.50	0.50	0	0.95
DMR 9	4	6223592	6223593	1	0.29	-0.29	0.001	601.7	0.06
DMR 10	4	136559196	136559207	2	-0.09	0.36	0.27	-0.35	0.06
DMR 11	X	89204936	89204949	2	0.09	0.06	0.16	0.59	0.57

Here, ACME-average causal mediated effect, ADE-average direct effect, TE-total effect,

PM- proportion mediated Analyses were performed using the *mediation* R package (v4.5.1)

3.5.4 Overall Causal Mediation Effects of DNA Methylation in the ACE-T2DM Pathway

This table demonstrated pooled causal mediation estimates synthesizing individual DMR-level effects across 11 candidate differentially methylated regions using random-effects meta-analysis (*metafor* package). The pooled total effect of high ACE exposure (>4) on T2DM risk was OR = 1.30 (95% CI: 1.17-1.45; $p = 8.25 \times 10^{-7}$), confirming a significant overall association. The overall indirect (epigenetically mediated) effect was OR = 1.11 (95% CI: 1.04-1.19; $p = 0.001$), indicating that DNA methylation changes at the 11 DMRs collectively and significantly mediate part of this risk. The overall direct effect, representing ACE pathways to T2DM independent of these methylation changes, was OR = 1.12 (95% CI: 0.99-1.25; $p = 0.05$), which was marginal and did not reach conventional statistical significance. Notably, 41.1% of the total ACE–T2DM association was accounted for by DNA methylation. These findings suggest that DNA methylation partially mediates the association between ACE and T2DM, highlighting a potential epigenetic pathway linking early-life stress to metabolic disease risk.

Table 3.21: Meta-Analytic Summary of Overall Causal Mediation Effects of DNA Methylation Across 11 DMRs in the ACE–T2DM Pathway

Effect	Estimate	95% CI	Log Odds	Odds	95% CI OR	p value
	Log Odds	Odds		Ratio		
Total (OTE)	0.26	[0.16, 0.37]		1.30**	[1.17, 1.45]	8.25×10^{-7}
Direct (ODE)	0.11	[-0.002, 0.23]		1.12**	[0.99, 1.25]	0.05
Indirect (OIE)	0.11	[0.04, 0.17]		1.11**	[1.044, 1.19]	0.001
Proportion	-	-		41.1%*	-	-
Mediated						

Here, OTE- overall total effect, ODE- overall direct effect, OIE- overall indirect (mediated) effect, OR-Odds ratios, CI-confidence intervals; ** values are odds ratio except * signifies proportion mediated

4. Discussion

4.1 Discussion

Our study was designed to investigate the complex interplay among genetic susceptibility, socio-environmental determinants, and epigenetic alterations in the development of type 2 diabetes mellitus (T2DM) in a Bangladeshi population. Using a comprehensive case–control framework, this research integrated candidate gene polymorphism analysis, psychosocial exposure assessment, genome-wide DNA methylation profiling, and high-dimensional mediation analysis to explore both biological and environmental contributors to T2DM.

T2DM is a multifactorial metabolic disorder characterized by chronic hyperglycemia resulting from impaired insulin secretion, insulin resistance, or both. Although conventional risk factors such as obesity, aging, family history, and lifestyle behaviors are well established, these factors alone cannot fully explain the heterogeneity of disease occurrence across populations (Hu et al., 2019; Inolopú et al., 2019; Temelkova-Kurktschiev & Stefanov, 2012). Increasing evidence suggests that the interaction between inherited genetic susceptibility and environmentally induced epigenetic modifications may contribute substantially to T2DM pathogenesis (C. Ling & Groop, 2009). In this context, the present study attempted to examine T2DM from a multidimensional perspective encompassing genetic, environmental, and epigenomic determinants.

To investigate the genetic contribution to T2DM susceptibility, this study focused on two well-recognized candidate genes, *TCF7L2* and *SLC30A8*, both of which have been repeatedly implicated in glucose homeostasis and β -cell function across different ethnic populations (Muruganantham & Veerabathiran, 2025). The *TCF7L2* rs12255372 polymorphism and the *SLC30A8* rs13266634 variant were analyzed using PCR amplification followed by Sanger sequencing–based genotyping. In addition, molecular dynamics simulation was performed for the ZnT8 protein encoded by *SLC30A8* to evaluate the structural and dynamic consequences of the rs13266634 substitution on protein stability and function.

Beyond genetic predisposition, the present study also explored the contribution of socio-contextual stressors, particularly adverse childhood experiences (ACE), to the development of T2DM. ACE exposure was assessed using the standardized ACE questionnaire, while perceived stress was evaluated using the PSS-4 scale. Previous studies have demonstrated that early-life adversity can exert long-term metabolic effects through chronic activation of the hypothalamic–pituitary–adrenal (HPA) axis and subsequent epigenetic dysregulation (Anacker et al., 2014; Yam et al., 2015). Therefore, the inclusion of ACE-related variables in the present study provided an opportunity to examine psychosocial determinants alongside molecular mechanisms in T2DM.

To investigate epigenetic alterations associated with T2DM, genome-wide DNA methylation profiling was performed using the NEBNext Enzymatic Methyl-seq (EM-seq) platform. Unlike conventional bisulfite sequencing, EM-seq preserves DNA integrity and minimizes sequencing bias, thereby allowing more accurate detection of methylated cytosines. Peripheral blood-derived genomic DNA was used as a minimally invasive biospecimen, consistent with previous epigenetic studies demonstrating that blood methylation patterns may reflect systemic metabolic and inflammatory alterations relevant to T2DM pathogenesis.

Although DNA methylation is highly tissue-specific, peripheral blood has become a widely accepted surrogate tissue in T2DM epigenetic studies because it reflects systemic inflammatory, immune, and metabolic processes associated with diabetes (Bansal & Simmons, 2018; Muka et al., 2016). Previous studies have also demonstrated overlap between leukocyte and pancreatic islet methylation profiles, with approximately 60% of age-related differentially methylated genes shared between these tissues (Bacos et al., 2016). Therefore, although whole blood may not fully capture tissue-specific pancreatic epigenetic alterations, it remains a practical and biologically informative tissue for investigating systemic epigenetic dysregulation in T2DM.

Comprehensive bioinformatic processing of methylation data was carried out using the nf-core/methylseq pipeline, incorporating quality control, read trimming, alignment with Bismark, methylation extraction, and downstream differential methylation analyses. Differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) were identified using edgeR and DMRcate Bioconductor packages

after rigorous quality filtering and covariate adjustment. Functional annotation and pathway enrichment analyses were subsequently conducted using CpGtools and Ingenuity Pathway Analysis (IPA) to identify biologically relevant pathways and regulatory networks associated with T2DM. An additional strength of the present study was the application of high-dimensional mediation analysis using the HDMAX2 framework to explore whether DNA methylation acts as a biological mediator linking ACE exposure to T2DM risk. Overall, the present study combined epidemiological, genetic, psychosocial, and epigenomic approaches to provide a broader understanding of T2DM pathogenesis in the Bangladeshi population.

On genetic susceptibility, a significant association was observed between the *TCF7L2* rs12255372 polymorphism and development to T2DM in current study. After adjustment for major confounding variables, including age, sex, and body mass index (BMI), individuals carrying the heterozygous GT genotype in the additive model and GT-TT genotypes in the dominant model demonstrated a significantly increased risk of developing T2DM. These findings are consistent with several previous studies conducted in diverse ethnic populations, supporting the role of *TCF7L2* as one of the most consistently replicated genetic susceptibility loci for T2DM worldwide. Similar associations have been reported in Indian, Japanese, Iranian, and other South Asian populations, where carriers of the risk allele showed significantly higher odds of developing T2DM even after adjustment for metabolic covariates (Bodhini et al., 2007; Chandak et al., 2007; Hayashi et al., 2007; Shokouhi et al., 2014).

The minor T allele was found to be significantly associated with T2DM risk, conferring an approximately two-fold increased risk to disease development. The frequency of the T allele among diabetic individuals was comparable to frequencies previously reported in neighboring South Asian as well as other international populations, including Indian, Cameroonian, Czech, and Iranian cohorts (Alami et al., 2012; Hameed et al., 2021; Hayashi et al., 2007). Interestingly, the heterozygous GT genotype was found more frequently among T2DM patients than healthy controls, whereas the homozygous TT genotype did not show statistical significance. This finding may partly reflect the relatively lower frequency of homozygous carriers together with the modest sample size of the present study. The comparatively high heterozygosity observed in the Bangladeshi population may additionally reflect the long-standing genetic admixture

of the Bengal delta region, which historically experienced migration and interaction among multiple ancestral populations (Bengali Nation, 2021).

The present study further demonstrated that the diabetogenic effect of the rs12255372 variant may interact with established metabolic and familial risk factors. Individuals carrying the *TCF7L2* risk genotype together with a positive family history of diabetes exhibited greater susceptibility to T2DM, and this association became more pronounced with increasing age, higher BMI, and female sex. These findings support the concept that genetic susceptibility acts synergistically with environmental and metabolic exposures in the pathogenesis of T2DM. Similar observations have been reported previously, where the combined influence of *TCF7L2* variants, obesity, and family history contributed substantially to disease variability and progression (Berumen et al., 2019; Dedoussis et al., 2007). Therefore, the present findings reinforce the multifactorial nature of T2DM and highlight the importance of gene–environment interaction in determining disease susceptibility within the Bangladeshi population.

Biologically, the observed association is plausible considering the central role of *TCF7L2* in glucose metabolism and pancreatic β -cell function. The rs12255372 polymorphism is located within intron 4 of the *TCF7L2* gene on chromosome 10q25.3 and belongs to a non-coding regulatory region strongly linked with T2DM susceptibility across multiple ethnicities (Duval et al., 2000a). Although intronic variants do not directly alter protein sequence, they may influence transcriptional activity, mRNA stability, and alternative splicing patterns (G. Wang & Cooper, 2007). *TCF7L2* is an important transcription factor within the Wnt signaling pathway and plays a crucial role in pancreatic β -cell proliferation, insulin secretion, incretin hormone regulation, and glucose homeostasis (Chiang et al., 2012; Jin, 2008). Previous functional studies have demonstrated that *TCF7L2* variants may impair insulin secretion and alter tissue-specific splicing isoforms, particularly in pancreatic islets and adipose tissue (Mondal et al., 2010; Weise et al., 2010). Prokunina-Olsson et al. identified pancreas-specific splice variants associated with glucose-stimulated insulin secretion, suggesting that altered transcriptional regulation of *TCF7L2* may contribute to β -cell dysfunction and subsequent T2DM development (Prokunina-olsson et al., 2009).

Overall research findings support the role of the *TCF7L2* rs12255372 variant as an important genetic susceptibility factor for T2DM in the Bangladeshi population. While *TCF7L2* primarily influences glucose homeostasis through transcriptional regulation and incretin-mediated insulin secretion, T2DM susceptibility is also strongly affected by genes directly involved in pancreatic β -cell secretory function. Therefore, the current study further evaluated the *SLC30A8* rs13266634 polymorphism, which encodes the zinc transporter ZnT8 involved in insulin granule biology.

Analysis of the *SLC30A8* rs13266634 (C>T) polymorphism demonstrated a significant association with T2DM susceptibility in the Bangladeshi population. Individuals carrying the homozygous TT polymorphic genotype exhibited a protective effect against T2DM, with approximately 80% lower odds of disease development under the additive model. In contrast, the homozygous wild-type CC genotype was significantly more prevalent among T2DM patients and was associated with increased disease risk under the dominant model. Furthermore, the major C allele emerged as an important susceptibility factor, conferring higher risk of T2DM compared with the minor T allele. These findings suggest that the rs13266634 polymorphism may influence genetic susceptibility to T2DM and further support the role of β -cell dysfunction in diabetes pathogenesis.

Our study findings on *SLC30A8* are consistent with several previous studies across diverse ethnic populations. Studies from Jordanian, Turkish, Indian, and Bangladeshi populations have similarly demonstrated that the C allele and CC genotype are associated with increased susceptibility to T2DM, whereas the TT genotype appears to exert a protective effect (Erkoc et al., 2023; Mashal et al., 2021; Mitu et al., 2024; Yamini et al., 2022). Particularly, a previous Bangladeshi study from the Noakhali region reported significantly higher frequencies of the C allele among diabetic individuals, reinforcing the relevance of this variant within native Bangladeshi populations (Mitu et al., 2024). However, some studies failed to identify a significant association between *SLC30A8* polymorphism and T2DM susceptibility (Almawi et al., 2013; Faghih et al., 2014), indicating that ethnic heterogeneity, environmental interactions, sample size variation, and population-specific genetic architecture may influence the observed associations.

The biological relevance of this association is supported by the functional role of ZnT8 in pancreatic β -cell physiology. The rs13266634 polymorphism results in a non-synonymous amino acid substitution (p.Arg325Trp) within ZnT8, a zinc transporter predominantly expressed in pancreatic β -cells and responsible for transporting zinc ions into insulin secretory granules. Zinc plays an essential role in insulin crystallization, storage, and secretion (Barragán-Álvarez et al., 2021; Rutter et al., 2016; Sladek et al., 2007). The risk-associated wild-type p.R325 protein exhibits higher zinc transport activity, which may promote excessive intragranular zinc accumulation, amyloid aggregation, calcium channel inhibition, and impaired insulin secretion. In contrast, the variant p.W325 protein represents a partial loss-of-function form associated with reduced zinc accumulation within insulin granules. This reduced zinc concentration may prevent toxic amyloid formation, preserve insulin monomer stability, and facilitate glucose-stimulated insulin secretion, thereby maintaining β -cell function and protecting against T2DM development. Experimental evidence from transgenic mouse models further supports this mechanism, where expression of the variant ZnT8 protein improved glucose homeostasis and insulin secretion compared with the wild-type protein (Barragán-Álvarez et al., 2021; L. Li et al., 2017).

To further understand the structural consequences of the p.R325W substitution, molecular dynamics simulations were incorporated into the present analysis. The variant MZnT8 protein demonstrated lower root mean square deviation and root mean square fluctuation values compared with the wild-type ZnT8 protein, suggesting greater structural rigidity and reduced conformational flexibility (Sala et al., 2021). These findings are biologically meaningful because the positively charged arginine residue in the wild-type protein likely facilitates dynamic conformational rearrangements necessary for efficient zinc ion transport, whereas replacement with the bulkier hydrophobic tryptophan residue results in a more compact and conformationally restricted structure. Reduced radius of gyration and solvent-accessible surface area observed in the mutant protein further indicated a tighter molecular conformation with potentially impaired membrane interaction and zinc-binding efficiency (Gupta & Vadde, 2020; Puthenveetil et al., 2022). Conversely, the wild-type ZnT8 protein exhibited a comparatively expanded conformation with higher SASA and Rg values, supporting more effective membrane interaction and zinc transport capability.

Taken together, the genetic association findings together with the structural and functional analyses provide comprehensive evidence supporting the role of the *SLC30A8* rs13266634 polymorphism in T2DM susceptibility within the Bangladeshi population. The protective effect associated with the TT genotype appears to be linked with altered ZnT8 structural dynamics, reduced zinc transport efficiency, and subsequent modulation of insulin granule physiology. Overall, the study highlights *SLC30A8* as an important T2DM susceptibility gene and suggests that ZnT8 may represent a promising therapeutic target for future diabetes prevention and precision medicine strategies.

Following the genetic analyses of *TCF7L2* and *SLC30A8* polymorphisms, we further explored the contribution of socio-demographic, metabolic, and psychosocial factors to T2DM, with particular emphasis on adverse childhood experiences (ACEs). Multivariable regression analysis demonstrated that female sex, hypertension, dyslipidemia, family history of diabetes, and higher perceived stress scores were significantly associated with T2DM. Among these factors, hypertension and dyslipidemia showed particularly strong associations, highlighting the close interrelationship between metabolic syndrome components and diabetes risk. A major finding of this study was the significant association between ACE exposure and T2DM in adulthood. In the unadjusted model, individuals with an ACE score of more than 4 had 2.28 times higher risk of developing T2DM than those with an ACE score of less than 4 (OR_{unadj} = 2.28, 95% CI: 1.40–3.71). Furthermore, after adjustment for major confounding variables including age, sex, hypertension, dyslipidemia, family history of diabetes, and perceived stress, individuals experiencing four or more ACEs during childhood had more than two-fold greater risk of T2DM later in life.

The present findings are consistent with previous epidemiological studies investigating the relationship between ACEs and diabetes risk. The landmark ACE study conducted by Felitti et al. (1998) first demonstrated that individuals with higher ACE scores had significantly greater likelihood of developing T2DM and other chronic diseases later in life. Similarly, Thomas et al. reported in a longitudinal British birth cohort that exposure to childhood adversity increased the risk of obesity and subsequent hyperglycemia during adulthood (C. Thomas et al., 2008). In another large retrospective cohort study from the United Kingdom, Chandan et al. demonstrated that childhood maltreatment

was associated with significantly increased incidence of T2DM, hypertension, cardiovascular disease, and mortality, even after adjustment for important covariates (Chandan et al., 2020). The consistency between the current findings and previous international studies strengthens the evidence that ACEs represent an important but often underrecognized risk factor for T2DM across different populations and sociocultural settings.

Several biological mechanisms may explain the observed association between ACEs and T2DM. Chronic exposure to childhood adversity may lead to persistent activation of the hypothalamic–pituitary–adrenal (HPA) axis, resulting in long-term dysregulation of cortisol secretion and stress-response pathways (Dempster et al., 2021; Diz-Chaves et al., 2016; Kalmakis et al., 2015). Sustained HPA axis activation may subsequently promote hyperglycemia, elevated circulating free fatty acids, dyslipidemia, and insulin resistance, all of which contribute to the pathogenesis of T2DM. In addition, prolonged exposure to stress-related stimuli may induce chronic low-grade inflammation characterized by increased production of pro-inflammatory cytokines such as TNF- α and IL-6, further exacerbating insulin resistance and metabolic dysfunction (L. Chen et al., 2015; Gill et al., 2020). These findings support the hypothesis that toxic stress during critical developmental periods may biologically embed itself within neuroendocrine and immune systems, thereby increasing long-term vulnerability to metabolic disease.

In recent years, increasing evidence has further suggested that the long-term health effects of ACEs may be mediated through epigenetic mechanisms (Kaufman et al., 2018; Palma-Gudiel et al., 2015; Yang et al., 2013). Gene–environment interactions occurring during early developmental stages may alter gene expression through DNA methylation and histone modifications, leading to persistent changes in stress-response and metabolic regulatory pathways. Experimental studies by Weaver et al. (2006) demonstrated that inadequate maternal care in rodents resulted in hypermethylation of the glucocorticoid receptor gene promoter, thereby altering HPA axis responsiveness. Similarly, (Jiang et al., 2019) reported that epigenetic silencing of stress-response genes associated with childhood trauma may contribute to chronic diseases including T2DM. These observations support the hypothesis that ACE-associated epigenetic dysregulation may represent an important molecular bridge linking early-life adversity

with adult metabolic disease. Therefore, the present study further investigated genome-wide DNA methylation alterations in individuals with T2DM to better understand both the epigenetic mechanisms underlying diabetes pathogenesis and the potential contribution of ACE-related epigenetic remodeling to disease susceptibility.

Genome-wide DNA methylation profiling was performed using whole blood samples obtained from patients with T2DM and healthy controls through whole-genome enzymatic methyl sequencing. The findings demonstrated widespread differential methylation across the genome, with predominance of hypermethylation across both differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) in T2DM subjects compared with controls. The overall average global methylated cytosine percentage across all samples was 80.89%. A total of 608 differentially methylated CpGs were identified between T2DM patients and healthy controls, of which approximately 62.7% were hypermethylated in diabetic subjects. Similar widespread methylation alterations have been reported in previous epigenome-wide association studies (EWASs) of T2DM across different populations. Toperoff et al. (2012) demonstrated significant differential methylation patterns in peripheral blood DNA of individuals with T2DM, while Fraszczyk et al. (2022) identified 76 differentially methylated CpG sites associated with incident T2DM across multiple European cohorts. Likewise, Chung et al. (2026) reported 111 CpG sites significantly associated with T2DM and related cardiometabolic traits in Korean individuals. Similarly, Seo et al. (2023) reported 106 differentially methylated probes in Korean individuals with T2DM involving genes related to glucose metabolism, oxidative stress, and metabolic signaling pathways.

Another important observation of the present study was the genomic distribution of DMCs. Most methylation changes were located within intronic and intergenic regions rather than promoter regions, while most DMCs were enriched within open sea regions rather than classical CpG islands. These findings are consistent with the established epigenomic landscape, where methylated cytosines predominantly occur throughout the bulk of the genome, whereas unmethylated CpGs are characteristically concentrated within CpG islands (Caiafa & Zampieri, 2005). Since open sea regions constitute the major proportion of the methylated genome, enrichment of DMCs within these regions suggests that disease-associated methylation alterations may preferentially occur at

dispersed and environmentally responsive regulatory sites rather than exclusively within promoter-associated CpG islands. Therefore, the predominance of open sea methylation changes observed in the present study likely reflects dynamic epigenetic remodeling associated with complex metabolic disease processes.

The traditional view that DNA methylation primarily regulates gene expression through CpG island promoter silencing has progressively evolved. Increasing evidence indicates that disease-associated methylation changes frequently occur within intronic and intergenic regions harboring distal regulatory elements such as enhancers, transcription factor binding sites, and orphan CpG islands which impart to cause transcriptional regulation (Angeloni & Bogdanovic¹, 2021; Pachano et al., 2020). Supporting this concept, Toperoff et al. (2012) identified T2DM-associated methylation signatures at isolated non-promoter CpG sites located within intronic regions of diabetes-related genes, where methylation changes overlapped with enhancer chromatin signatures and methylation-sensitive transcription factor binding regions. Collectively, these findings support the observation of the present study that T2DM-associated epigenetic dysregulation extends beyond canonical promoter CpG islands and may involve broader genome regulatory networks influencing chromatin organization and long-range transcriptional regulation.

The pathway enrichment analyses identified several biologically relevant canonical pathways associated with both DMCs and DMRs. Among hypermethylated DMRs, the most significantly enriched pathway involved neurotransmitter receptors and postsynaptic signal transmission ($p=8.51 \times 10^{-5}$), including genes such as *GLRA3*, *GLRB*, and *NBEA*. This finding is particularly intriguing because it suggests that epigenetic dysregulation in T2DM may extend beyond peripheral glucose metabolism and involve neuroendocrine regulatory mechanisms. Conversely, hypomethylated DMRs showed enrichment of pathways related to activation of NMDA receptors and postsynaptic signaling ($p=0.001$) involving genes such as *DLG2*, *ERBB4*, *PRKAR2B*, and *RPS6KA2*.

Hypermethylated DMRs involving *GLRA3*, *GLRB*, and *NBEA* indicate potential suppression of inhibitory glycinergic neurotransmission, whereas hypomethylated DMRs involving NMDA receptor-related signaling genes suggest enhanced excitatory glutamatergic signaling. Glycine receptors are major mediators of inhibitory

neurotransmission within the central nervous system, while NMDA receptors regulate excitatory neurotransmission, synaptic plasticity, calcium influx, and neuronal metabolic responses. Therefore, simultaneous dysregulation of these pathways may disturb the excitatory–inhibitory balance within hypothalamic and brainstem neuroendocrine networks involved in systemic glucose regulation. Emerging evidence indicates that the hypothalamus and brainstem function as integrative neuroendocrine hubs that sense glucose, insulin, leptin, and nutrient-derived signals to coordinate autonomic output, feeding behavior, hepatic glucose production, pancreatic hormone secretion, and systemic energy balance. Disruption of these central regulatory circuits has been increasingly implicated in obesity, insulin resistance, and T2DM (Gallardo et al., 2025).

In addition, communication through the gut–brain–liver and brain–pancreas axes provide an important mechanistic link between altered neuronal signaling and peripheral metabolic regulation. The gut–brain axis is mediated through hormonal and neural signals generated in response to nutrients, which act through circulation as well as through vagal and spinal afferent neurons to influence hypothalamic and brainstem centres involved in energy and glucose homeostasis (Wachsmuth et al., 2022). This gut-derived information is integrated mainly by the hypothalamus, which coordinates feeding behaviour, energy expenditure, and glucose metabolism, while gut–brain–liver signaling pathways may additionally regulate hepatic glucose production. Furthermore, Borgmann et al. demonstrated that distinct gut-innervating sensory neurons differentially regulate feeding and glucoregulatory responses, with GLP1R-positive vagal afferent activation improving glucose tolerance and GPR65-positive vagal afferent stimulation increasing hepatic glucose production, thereby highlighting the importance of gut-derived neuronal signaling in metabolic regulation (Borgmann et al., 2021). The brain–pancreas axis is further supported by Rosario et al., who mapped efferent neuronal circuits linking the hypothalamus to pancreatic islets and demonstrated that hypothalamic regions, including the arcuate nucleus, ventromedial nucleus, and lateral hypothalamic area, differentially regulate insulin secretion, glucagon secretion, and glucose tolerance through autonomic outputs to the pancreas (Rosario et al., 2016). Therefore, methylation-mediated disruption of neurotransmitter receptor and synaptic signaling pathways observed in the present study may impair neuroendocrine communication between the gut, brain, liver, and pancreas, thereby

contributing to β -cell dysfunction, insulin resistance, hepatic glucose dysregulation, and altered glucose homeostasis in T2DM.

Glycine receptor-related genes, particularly *GLRA3* and *GLRB*, may represent an additional mechanistic pathway linking neurotransmitter signaling to T2DM pathogenesis. Glycine, acting through glycine receptors (GlyRs), has increasingly been recognized as an important regulator of metabolic homeostasis (Razak et al., 2017). Reduced circulating glycine levels have been consistently associated with insulin resistance, obesity, and T2DM across multiple populations (Yan-Do & MacDonald, 2017). Structurally, GlyRs are pentameric ligand-gated chloride channels composed of α and β subunits; *GLRA3* encodes the $\alpha 3$ subunit and *GLRB* encodes the β subunit. Although GlyRs are classically known for inhibitory neurotransmission in neurons through chloride influx and membrane hyperpolarization, they are also expressed outside the nervous system, including in the pancreas. Importantly, available single-cell transcriptomic evidence shows that *GLRA3* can be detected in β -cells, while *GLRB* has been reported in β -cells or ductal cells (Y. J. Wang et al., 2016).

In human pancreatic β -cells, GlyR activation does not necessarily act as an inhibitory signal; because β -cells have a higher intracellular chloride concentration than neurons, opening of GlyRs can depolarize β -cells, increase intracellular Ca^{2+} , and promote insulin secretion (Yan-Do & MacDonald, 2017). Therefore, methylation alterations involving *GLRA3* and *GLRB* in the present study may affect GlyR expression or signaling activity, potentially disrupting glycine-mediated β -cell depolarization, calcium entry, and insulin secretion. This interpretation is further supported by recent human islet epigenetic data showing that altered DNA methylation in pancreatic islets is linked to gene expression changes, β -cell dysfunction, and impaired insulin secretion in T2DM; notably, reduced expression and increased methylation of *GLRA1*, another glycine receptor subunit gene, were reported in T2DM islets (Rönn et al., 2023).

Pathway enrichment analysis revealed significant hypermethylation-associated enrichment of fatty acyl-CoA biosynthesis ($p = 0.007$) and fatty acid activation ($p = 0.01$) pathways among differentially methylated CpGs, implicating epigenetic dysregulation of lipid metabolic processes as a contributing mechanism in T2DM pathogenesis. Genes contributing to these pathways, including *ACSBG2*, *ACSL5*, and *ELOVL7*, are functionally involved in fatty acid activation, elongation, and lipid

utilization. *ACSBG2* belongs to the acyl-CoA synthetase superfamily and catalyzes activation of long-chain and very-long-chain fatty acids through CoA esterification, a key step in lipid biosynthesis and metabolic reprogramming (X. Ling et al., 2026). Similarly, *ACSL5* is a member of the ACSL family that converts long-chain fatty acids into fatty acyl-CoA derivatives, which are subsequently directed toward β -oxidation or complex lipid synthesis (Deng et al., 2025). *ELOVL7* participates in elongation of saturated long-chain and very-long-chain fatty acids, an essential process for lipid homeostasis, complex lipid synthesis, and membrane biogenesis (Ferrero et al., 2025). Therefore, hypermethylation of these genes may suggest altered regulation of fatty acid activation and elongation pathways in T2DM.

Fatty acid activation is a critical metabolic process in which free fatty acids are converted into fatty acyl-CoA derivatives before entering β -oxidation, triglyceride synthesis, phospholipid remodeling, or other lipid metabolic pathways. Disturbance of these pathways may promote accumulation of lipid intermediates such as fatty acyl-CoA, diacylglycerol, and ceramides, which can impair insulin signaling and contribute to insulin resistance, lipotoxicity, mitochondrial dysfunction, oxidative stress, and β -cell impairment. Shetty and Kumari (2021) reported that fatty acids may actively contribute to T2DM pathogenesis by influencing insulin receptor signaling, glucose transporter activity, membrane fluidity, permeability, and inflammatory responses. This interpretation is further supported by Boden, who proposed that increased circulating free fatty acids represent an important mechanistic link between obesity and insulin resistance by inhibiting insulin-stimulated glucose uptake, glycogen synthesis, and insulin signaling, as well as by promoting hepatic glucose production (Boden, 2001). Collectively, these findings suggest that hypermethylation of fatty acid activation and elongation genes may contribute to T2DM pathogenesis through dysregulated lipid handling, impaired insulin action, and metabolic stress.

One of the important enriched pathways identified among hypomethylated DMCs was the type II diabetes mellitus signaling pathway, involving genes associated with both impaired insulin secretion and insulin resistance. Hypomethylation of *PKC* and *CEBPB* may indicate altered signaling pathways contributing to β -cell dysfunction and impaired insulin secretion. In addition, ACS-related fatty acid activation pathways together with hypomethylation of *ADIPOR2* suggest dysregulation of lipid metabolism and insulin

sensitivity contributing to insulin resistance. One of the important genes involved in the hypomethylated type II diabetes mellitus signaling pathway in the present study was *PKC*, a lipid-dependent serine/threonine kinase family that plays a central role in insulin signaling, glucose metabolism, and β -cell function. Previous studies have shown that increased intracellular lipid intermediates, particularly diacylglycerol (DAG), can activate *PKC* isoforms during conditions of lipid oversupply and obesity, thereby contributing to insulin resistance. Activated PKC has been reported to impair insulin signaling through serine phosphorylation of insulin receptor substrate-1 (*IRS-1*), resulting in reduced downstream PI3K/Akt signaling and diminished glucose uptake (Moeschel et al., 2004; Schmitz-peiffer & Biden, 2008). Therefore, methylation alterations involving PKC observed in the present study may reflect epigenetic dysregulation of lipid-mediated insulin signaling pathways contributing to impaired glucose homeostasis in T2DM.

In line with this interpretation, *CEBPB* encodes CCAAT/enhancer-binding protein beta (C/EBP β), a transcription factor involved in inflammatory regulation, adipogenesis, insulin sensitivity, and metabolic homeostasis. Recent functional genomic studies demonstrated that T2DM-associated enhancer variants can alter *CEBPB* binding and transcriptional regulation of *RASGRP1*, a key regulator of AKT signaling and GLUT4 translocation. Impaired *RASGRP1* signaling reduced cellular glucose uptake and promoted insulin resistance, whereas *Rasgrp1* knockout mice developed obesity, impaired glucose tolerance, and insulin resistance under high-fat diet conditions (Ning et al., 2026). In addition, chronic activation of C/EBP β has been associated with obesity-induced inflammation and dysregulated glucose metabolism (Ren et al., 2023). Thus, methylation alterations involving CEBPB in the present study may indicate epigenetic dysregulation of inflammatory and insulin signaling pathways contributing to insulin resistance and T2DM pathogenesis.

ADIPOR2 encodes adiponectin receptor 2 (AdipoR2), a receptor predominantly expressed in the liver and involved in adiponectin-mediated regulation of insulin sensitivity, fatty acid oxidation, lipid utilization, and glucose homeostasis. Reduced *ADIPOR2* expression has been associated with insulin resistance, obesity, oxidative stress, and impaired glucose metabolism (Khodyrev et al., 2015). In consistent with our findings, epigenetic studies have demonstrated that adipogenic and adiponectin-related

genes undergo dynamic DNA methylation changes during adipocyte differentiation and metabolic remodeling, suggesting an important role of epigenetic regulation in adipose metabolic homeostasis (Fang et al., 2025; Takada et al., 2014). Furthermore, previous studies using placental tissue from maternal obesity demonstrated that altered methylation of the *ADIPOR2* promoter was associated with reduced receptor expression and impaired adiponectin signaling (Nogues et al., 2019). Although the study was performed in obesity-related placental tissue, the findings support the broader role of epigenetic dysregulation of *ADIPOR2* in metabolic dysfunction, insulin resistance, and impaired glucose homeostasis. Therefore, methylation changes of *ADIPOR2* observed in the present study may indicate altered adiponectin-mediated metabolic signaling contributing to T2DM pathogenesis.

In addition, hypermethylated DMCs demonstrated enrichment of Toll-like receptor signaling pathways, suggesting involvement of chronic low-grade inflammation in the diabetic epigenome. Activation of innate immune pathways through Toll-like receptors can induce NF- κ B signaling, cytokine production, macrophage activation, and insulin resistance (Qian & Cao, 2013; Shah, 2007). Since the present study utilized whole blood DNA, the identified methylation alterations likely reflect systemic immune and inflammatory remodeling associated with T2DM.

Hypomethylated DMCs also showed enrichment of pathways related to mitophagy, pexophagy, and mitochondrial quality control. Mitochondrial dysfunction is considered a central feature of insulin resistance and β -cell failure. Increased mitochondrial stress may activate compensatory pathways involved in removal of damaged organelles and maintenance of cellular bioenergetic homeostasis (Filomeni et al., 2015; Schirmacher, 2020). Thus, altered methylation of genes involved in mitochondrial quality-control pathways may represent adaptive responses to chronic metabolic stress in T2DM.

Differentially methylated region (DMR) analysis identified widespread regional methylation alterations in T2DM patients. Among the hypermethylated DMRs, biologically notable regions were annotated to genes including *CNTNAP2*, *OLFM3*, *TRPC7*, *ACTR3*, *ITPKB*, and *PDE11A*. In contrast, hypomethylated DMRs were more numerous and included biologically relevant genes such as *TET2*, *HDAC9*, *RAPGEF4*, *PCCA*, and *PRKAR2B-AS1*. Collectively, these findings indicate that both hypermethylated and hypomethylated DMRs in T2DM are distributed across multiple

genomic regions involving genes with potential biological relevance to metabolic and cellular regulatory processes.

Among the hypomethylated DMR-associated genes identified in the present study, *HDAC9* is particularly noteworthy because histone deacetylases (HDACs) are major epigenetic regulators involved in chromatin remodeling, histone acetylation, transcriptional regulation, inflammation, and metabolic homeostasis. Increasing evidence suggests that aberrant HDAC activity contributes to diabetes pathogenesis. Studies in foetal endothelial cells from gestational and type 2 diabetic mothers demonstrated persistent global DNA hypermethylation together with elevated *HDAC2* expression even under normoglycemic culture conditions, suggesting long-lasting epigenetic reprogramming consistent with the concept of metabolic memory (Sultan & Almalki, 2023). Christensen et al. further highlighted the role of *HDACs* in regulation of β -cell development, insulin transcription, inflammatory signaling, and insulin resistance, supporting the involvement of *HDAC*-mediated epigenetic mechanisms in diabetes (Christensen et al., 2011). In a clinically relevant human setting, Sathishkumar et al. demonstrated significantly increased *HDAC3* activity and mRNA expression in peripheral blood mononuclear cells from patients with T2DM, which positively correlated with fasting glucose, HbA1c, insulin resistance, and circulating inflammatory mediators including TNF- α and IL-6 (Sathishkumar et al., 2016). Collectively, these findings support the role of altered *HDAC*-related epigenetic regulation in chronic inflammation and metabolic dysfunction in T2DM.

Another hypomethylated DMR-associated gene identified in the present study was *TET2*, an important epigenetic regulator involved in active DNA demethylation. *TET2* converts 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), which can initiate DNA demethylation and influence transcriptional regulation (Ding & Huang, 2014). In diabetes, this mechanism is biologically relevant because hyperglycemia has been shown to alter *TET2*-mediated demethylation pathways in diabetic tissues. For example, in diabetic retinopathy, *TET2*-mediated hypomethylation of the *ROBO4* promoter was associated with endothelial dysfunction and retinal vascular abnormalities under hyperglycemic conditions (L. Zhou et al., 2019). Similarly, persistent *TET2* activation and *MMP-9* promoter hypomethylation after glucose normalization have been linked to diabetic metabolic memory, indicating that

hyperglycemia-induced epigenetic changes may continue even after glycemic improvement (Mishra & Kowluru, 2016). In diabetic wound healing, *TET2*-interacting lncRNA *TETILA* was shown to promote locus-specific demethylation of the *MMP-9* promoter and contribute to inflammatory activation (Zhao et al., 2023). Therefore, hypomethylation involving *TET2* may suggest altered active DNA demethylation machinery, which could contribute to persistent epigenetic dysregulation in T2DM.

Epigenetic mechanisms linking ACE with T2DM were further explored in this study. Particular emphasis was given to DNA methylation as a potential biological mediator connecting early-life psychosocial stress with later metabolic disease development. Using high-dimensional mediation analysis (HDMAX2), genome-wide DNA methylation profiling was performed across approximately 6.3 million quality-controlled CpG sites. The analysis identified numerous differentially methylated loci associated with ACE exposure, including both hypermethylated and hypomethylated CpG sites.

Among the top hypermethylated loci, substantial gains in methylation ranging from 39 to 53 percentage points were observed in regulatory regions of genes including *SLC38A9*, *RUNX1T1*, *CNTN4*, *SATB1*, and *ROBO1*. In contrast, several hypomethylated CpG loci demonstrated methylation losses of up to 31 percentage points and were mapped to genes such as *MEF2C-AS1*, *MYRFL*, *RB1CC1*, *TRIM44*, *COL12A1*, and *ELF2*.

Among these genes, *SLC38A9* is particularly relevant to metabolic regulation because of its role in nutrient sensing and mTORC1 signaling which is a central regulator of cellular metabolism, nutrient sensing, autophagy, and cell growth. Previous studies demonstrated that *SLC38A9* participates in sensing intracellular amino acid availability, particularly glutamine and arginine, and subsequently activates mTORC1 signaling under nutrient-sufficient conditions. Dysregulation of the mTOR pathway has been strongly implicated in insulin resistance, impaired glucose homeostasis, β -cell dysfunction, and the pathogenesis of T2DM (Rebsamen et al., 2015; Rebsamen & Superti-Furga, 2016). Thus, hypermethylation of *SLC38A9* may indicate epigenetic suppression of nutrient-sensing pathways associated with chronic psychosocial stress exposure. In addition to nutrient-sensing pathways, altered methylation was also observed in *RUNX1T1*, a gene linked with adipogenesis and metabolic syndrome.

Previous studies reported that *RUNX1T1* participates in adipocyte differentiation and metabolic regulation through interaction with the *FTO*-associated m6A modification pathway. Genetic variation in *RUNX1T1* has also been associated with increased waist circumference, obesity risk, and metabolic abnormalities (Y. Zhou et al., 2021). Altered methylation of *RUNX1T1* may reflect disruption of metabolic regulatory pathways associated with stress-related metabolic dysfunction.

Beyond these metabolic regulatory genes, *CNTN4* suggests a possible involvement of neuronal signaling pathways in ACE-related epigenetic changes. *CNTN4* is a neuronal cell adhesion molecule involved in neural development, synaptic organization, and neuronal communication. Previous studies have linked *CNTN4* with several psychological, psychiatric, and cardiometabolic traits including BMI, smoking behavior, and blood pressure (Morris et al., 2020). Similarly, *ROBO1* further supports the contribution of neuronal guidance and stress-response pathways in the epigenetic effects of early-life adversity. *ROBO1* is a neuronal guidance receptor involved in axon guidance, neuronal projection, and cellular signaling. A previous epigenetic study on elderly individuals exposed to severe childhood trauma and indentured child labor demonstrated significant DNA methylation alterations in genes related to neuronal development and axon guidance pathways, including *ROBO1* (Marinova et al., 2017). These findings suggest that early-life adversity may induce long-lasting epigenetic changes affecting neurodevelopmental and stress-response pathways that persist into later life. In addition, altered SLIT/ROBO signaling has also been implicated in diabetic neuropathy and neuronal dysfunction in diabetic neuropathy (Kwiatkowska et al., 2026).

Among the hypomethylated loci, *RB1CC1* provides another biologically relevant link, as it is involved in cellular stress adaptation and autophagy regulation. Previous studies showed that *RB1CC1* expression increases under cytotoxic and cellular stress conditions and may participate in stress-adaptive regulatory pathways through interaction with the RB1 signaling network (Chano et al., 2002). More recent evidence has further demonstrated that *RB1CC1* (also known as FIP200) plays an important role in autophagy initiation, particularly during nutrient deprivation and cellular stress, through regulation of the ULK1 autophagy complex (Popelka & Klionsky, 2022). Interestingly, epigenetic studies in youths with emerging psychopathology also

identified correlations between *RB1CC1* methylation and gene expression changes, suggesting its potential involvement in stress-related neuro-epigenetic regulation (Spindola et al., 2019). Therefore, altered methylation of *RB1CC1* observed in the present study may reflect dysregulation of stress-response, autophagy, and cellular adaptive pathways associated with chronic psychosocial stress exposure. Finally, *TRIM44* adds a vascular and inflammatory dimension to these findings, linking ACE-related methylation changes with diabetes-associated endothelial dysfunction and metabolic stress. Previous studies showed that *TRIM44* expression increases under high-glucose and diabetic conditions and promotes angiogenesis, endothelial dysfunction, and VEGFA-mediated vascular injury (N. Han et al., 2025).

Further regional aggregation of these HDMAX2-significant CpG sites using comb-p analysis identified 11 candidate differentially methylated regions (DMRs), among which DMR 1, DMR 4, and DMR 5 showed statistically significant mediation effects within the causal mediation modelling framework. These regions exhibited comparatively higher average causal mediation effect (ACME) estimates, suggesting that they may contribute to the potential indirect pathway linking ACE with T2DM. However, the variability in mediation effect sizes observed across the identified DMRs suggests that epigenetic mediation of ACE-associated T2DM risk is unlikely to be driven by a single genomic region alone. Rather, the findings support the presence of a distributed epigenetic regulatory architecture involving multiple loci with modest but biologically meaningful contributions. This pattern is consistent with the multifactorial and polyepigenetic nature of complex metabolic diseases such as T2DM.

At the meta-analytic level, pooled mediation analysis across all 11 DMRs provided statistical evidence consistent with a potential mediating role of DNA methylation in the association between ACE and T2DM. The total effect of high ACE exposure on T2DM risk was significant (OR = 1.30, 95% CI: 1.17–1.45), indicating an overall association between childhood adversity and diabetes susceptibility. Importantly, the indirect effect mediated through DNA methylation changes was also statistically significant (OR = 1.11, 95% CI: 1.04–1.19; $p = 0.001$), accounting for approximately 41.1% of the total ACE–T2DM association. This finding suggests that DNA methylation may represent a potential biological mechanism through which early-life psychosocial stress contributes to adult metabolic disease risk. In contrast, the overall

direct effect independent of the identified methylation changes was marginal and did not reach clear statistical significance (OR = 1.12; $p = 0.05$). The present findings support a potential mediating role of DNA methylation in the association between childhood adversity and metabolic disease susceptibility. These observations are consistent with growing evidence indicating that chronic early-life stress can induce persistent epigenetic remodeling in genes involved in neuroendocrine regulation, immune signaling, inflammation, and cardiometabolic pathways (Hao et al., 2018; Kaufman et al., 2018; Womersley et al., 2022). Although classical HPA-axis regulatory genes such as *NR3C1* or *FKBP5* (Normann & Buttenschøn, 2019) were not identified among the significant loci in the present study, many of the differentially methylated genes identified were functionally related to stress-response pathways, neuronal signaling, metabolism, autophagy, endothelial dysfunction, and cellular adaptation. Previous studies have suggested that adverse childhood experiences (ACE) may induce long-lasting epigenetic alterations through dysregulation of stress-response systems and downstream inflammatory and metabolic pathways, ultimately contributing to obesity, metabolic syndrome, cardiovascular disease, and diabetes susceptibility (Hao, NA, et al., 2018; Kaufman et al., 2018; Megala et al., 2021; Womersley et al., 2022).

The findings of the present study are consistent with the concept of “biological embedding,” whereby psychosocial stress experienced during early life may become molecularly integrated through persistent epigenetic alterations (Douglas-Palumberi et al., 2014). Several previous studies have shown that ACE exposure is associated not only with differential methylation of stress-related genes but also with accelerated epigenetic aging and altered metabolic phenotypes later in life. For example, cumulative ACE exposure and childhood physical or emotional abuse were associated with DNA methylation age acceleration in adolescents, suggesting that chronic stress may influence long-term cellular aging processes through epigenetic mechanisms (Tang et al., 2020). Furthermore, previous epigenetic studies identified methylation alterations in pathways related to neurogenesis, axonal guidance, inflammation, immune regulation, and metabolic signaling among individuals exposed to childhood trauma (Kaufman et al., 2018). Therefore, the present findings may reflect broader biological consequences of chronic psychosocial stress rather than direct methylation changes confined only to canonical HPA-axis genes. Collectively, these observations support the developmental origins of health and disease (DOHaD) framework and suggest that

early-life adversity may contribute to T2DM susceptibility through persistent epigenetic dysregulation of neuroendocrine, inflammatory, and metabolic pathways (Edwards, 2019). However, as the mediation analysis was based on observational data, these findings should be interpreted as statistical evidence consistent with a potential mediating pathway rather than proof of causality; longitudinal studies are required to establish temporal sequence and confirm causal relationships.

A major strength of the present study was the generation of high-quality whole-genome methylation data using high-resolution enzymatic methyl sequencing in a Bangladeshi population with T2DM. The sequencing data showed satisfactory mapping efficiency, high cytosine conversion rates, and adequate genome coverage across the retained samples, supporting the reliability of the downstream methylation analyses. Although ten samples were excluded because of poor sequencing quality, the remaining samples have acceptable alignment rates and sequencing depth. The average global methylated cytosine level was approximately 80.9%, which is comparable to previously reported methylation levels in mammalian genomes and suggests preservation of the expected genome-wide methylation pattern.

Another important strength of this study was the application of HDMAX2 for high-dimensional mediation analysis while accounting for latent confounding factors, which improved the robustness of the identified associations. In addition, combining comb-p regional aggregation with formal causal mediation analysis allowed evaluation of both site-specific and region-level methylation effects. The study also integrated genome-wide methylation data with psychosocial, anthropometric, and biochemical variables, providing a broader understanding of ACE-associated epigenetic dysregulation in T2DM. Importantly, epigenetic studies in Bangladeshi populations remain very limited. Therefore, the present findings contribute valuable population-specific evidence regarding the epigenetic architecture of T2DM in an underrepresented South Asian population.

4.2 Conclusion

In conclusion, the current study provides evidence that T2DM in the Bangladeshi population is influenced by a complex interaction among genetic susceptibility, psychosocial stress exposure, and epigenetic dysregulation. The genetic analyses identified significant associations between the *TCF7L2* rs12255372 and *SLC30A8* rs13266634 variants and T2DM, supporting the contribution of inherited genetic factors to disease development in this population. The study also identified a significant association between adverse childhood experiences (ACE) and increased risk of T2DM in adulthood, supporting the growing evidence that early-life psychosocial stress may contribute to long-term metabolic dysfunction. Chronic stress exposure during critical developmental periods may induce persistent neuroendocrine, inflammatory, and metabolic alterations that increase later susceptibility to diabetes. These findings highlight the importance of considering psychosocial and environmental determinants alongside conventional metabolic risk factors in understanding T2DM pathogenesis.

Genome-wide DNA methylation profiling revealed widespread differential methylation involving pathways related to neuronal signaling, lipid metabolism, inflammation, mitochondrial homeostasis, autophagy, and insulin signaling. Pathway enrichment analyses suggested potential dysregulation of neuroendocrine communication between the gut, brain, liver, and pancreas, together with altered glycinergic and glutamatergic signaling pathways that may influence glucose homeostasis and β -cell function. In addition, methylation changes involving genes associated with fatty acid activation, adiponectin signaling, immune activation, and metabolic stress further support the role of epigenetic remodeling in T2DM pathogenesis. Importantly, mediation analyses provided statistical evidence consistent with a potential mediating role of DNA methylation in the association between ACE exposure and T2DM, with the indirect effect accounting for approximately 41.1% of the total ACE–T2DM association. These findings suggest a potential epigenetic pathway through which early-life adversity may contribute to long-term metabolic risk.

Finally, this study provides novel insight into the molecular and environmental architecture of T2DM in the Bangladeshi population, representing one of the first comprehensive investigations to integrate genetics, ACE exposure, genome-wide

methylation profiling, and mediation analysis in a South Asian cohort. Further validation in larger longitudinal and functional studies is required to establish temporal relationships and clarify causality. Nevertheless, these findings contribute important population-specific evidence regarding gene-environment-epigenetic interactions in T2DM pathogenesis. Notably, the identification of epigenetically altered pathways related to neuroendocrine communication between the gut, brain, liver, and pancreas provides new insights into biological mechanisms underlying T2DM and may help guide future research into novel therapeutic targets and intervention strategies. Given the increasing burden of T2DM and the prevalence of childhood adversity in Bangladesh, policies aimed at reducing ACEs through child protection programs, mental health support services, family-centered interventions, and trauma-informed healthcare may contribute to long-term prevention of diabetes and other non-communicable diseases. Collectively, these findings support a more integrated approach to T2DM prevention and precision medicine that combines genetic, epigenetic, metabolic, and psychosocial perspectives for high-risk populations.

4.3 Limitations and Recommendations of the Study

Despite several important findings, the present study has certain limitations that should be considered while interpreting the results. First, the study was conducted using a relatively modest sample size, particularly in the genome-wide methylation and mediation analyses following quality-control filtering. Since the genome-wide methylation analysis was exploratory in nature, replication of the findings in larger independent cohorts is necessary to confirm the observed epigenetic associations.

Second, the study employed a case–control design, which limits the ability to establish temporal or causal relationships among ACE, DNA methylation alterations, and T2DM. While mediation analysis suggested that DNA methylation may partially mediate the association between ACE exposure and T2DM, definitive causal inference cannot be established without longitudinal follow-up studies.

Third, genome-wide methylation profiling was performed using peripheral whole blood rather than metabolically active tissues such as pancreatic islets, adipose tissue, liver, or skeletal muscle. Although peripheral blood is widely used in epigenetic epidemiology and reflects systemic inflammatory and metabolic alterations, it may not fully capture tissue-specific epigenetic changes occurring in the pancreas or other metabolically active organs directly involved in T2DM pathogenesis.

Fourth, the present study primarily focused on DNA methylation and did not incorporate additional multi-omics approaches such as transcriptomics, proteomics, metabolomics, or chromatin accessibility analyses. Consequently, the functional consequences of the identified methylation alterations on gene expression and downstream biological activity could not be directly confirmed.

Finally, the study population was limited to Bangladeshi participants recruited from a specific geographical and clinical setting, which may affect the generalizability of the findings to other ethnic or population groups. Future studies involving larger multicenter cohorts, longitudinal follow-up, and integrative functional analyses will be important to validate and extend the present findings.

5. References

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Appendix-I**ACE Questionnaire**

Prior to your 18th birthday:

1. Did a parent or other adult in the household often or very often... Swear at you, insult you, put you down, or humiliate you? or Act in a way that made you afraid that you might be physically hurt? If Yes, add 1 point ____
2. Did a parent or other adult in the household often or very often... Push, grab, slap, or throw something at you? or Ever hit you so hard that you had marks or were injured? Yes, add 1 point ____
3. Did an adult or person at least 5 years older than you ever... Touch or fondle you or have you touch their body in a sexual way? or Attempt or actually have oral, anal, or vaginal intercourse with you? If Yes, add 1 point ____
4. Did you often or very often feel that ... No one in your family loved you or thought you were important or special? or Your family didn't look out for each other, feel close to each other, or support each other? If Yes, add 1 point ____
5. Did you often or very often feel that ... You didn't have enough to eat, had to wear dirty clothes, and had no one to protect you? or Your parents were too drunk or high to take care of you or take you to the doctor if you needed it? If Yes, add 1 point ____
6. Were your parents ever separated or divorced? If Yes, add 1 point ____
7. Was your mother or stepmother: Often or very often pushed, grabbed, slapped, or had something thrown at her? or Sometimes, often, or very often kicked, bitten, hit with a fist, or hit with something hard? or Ever repeatedly hit over at least a few minutes or threatened with a gun or knife? Yes, add 1 point ____
8. Did you live with anyone who was a problem drinker or alcoholic, or who used street drugs? If Yes, add 1 point ____
9. Was a household member depressed or mentally ill, or did a household member attempt suicide? If Yes, add 1 point ____
10. Did a household member go to prison? If Yes, add 1 point ____

With add up "Yes" answers, final ACE Score: _____

Appendix-II

Perceived Stress Scale 4 (PSS-4)

	Never 0	Almost Never 1	Sometimes 2	Fairly Often 3	Very Often 4
1. In the last month, how often have you felt that you were unable to control the important things in your life?	☐	☐	☐	☐	☐
2. In the last month, how often have you felt confident about your ability to handle your personal problems?	☐	☐	☐	☐	☐
3. In the last month, how often have you felt that things were going your way?	☐	☐	☐	☐	☐
4. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?	☐	☐	☐	☐	☐

Scoring for the Perceived Stress Scale 4:

Questions 1 and 4

0 = Never

1 = Almost Never

2 = Sometimes

3 = Fairly Often

4 = Very Often

Questions 2 and 3

4 = Never

3 = Almost Never

2 = Sometimes

1 = Fairly Often

0 = Very Often

Lowest score: 0

Highest score: 16

Cohen, S., Kamarck, T., & Mermelstein, R. (1983). A global measure of perceived stress. *Journal of Health and Social Behavior*, 24, 385-396.

Appendix-III

Ethical Clearance: University of Dhaka

ডিন অফিস
জীববিজ্ঞান অনুষদ
ঢাকা বিশ্ববিদ্যালয়, ঢাকা-১০০০, বাংলাদেশ



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Ref. No. 152/Biol. Scs.

November 27, 2021

Ethical Review Committee

Professor Dr. ABM Md. Khademul Islam
Department of Genetic Engineering and Biotechnology
University of Dhaka

Sub: Ethical Clearance.

Dear Dr. Khademul Islam,

With reference to your application on the above subject, this is to inform you that your research proposal entitled "Elucidating genetic and epigenetic mechanisms of type 2 diabetes mellitus pathogenesis" has been reviewed and approved by the Ethical Review Committee of the Faculty of Biological Sciences, University of Dhaka.

I wish for the success of your research project.

Professor Dr. Mihir Lal Saha
Dean (Acting), Faculty of Biological Sciences
University of Dhaka

Appendix-IV

Ethical Clearance: Diabetic Association of Bangladesh



বাংলাদেশ ডায়াবেটিক সমিতি
DIABETIC ASSOCIATION OF BANGLADESH

Memo No. BADAS-ERC/EC/22/394

Date: October 3, 2022

Subject: Ethical Clearance

The Ethical Review Committee (ERC) of the Diabetic Association of Bangladesh (BADAS) has approved the under mentioned protocol:

- Title of the Research Work : "Association of DNA methylation of candidate genes in type 2 diabetes mellitus".
- Principal Investigator : **Dr. Nilima Barman**
Associate Professor
Department of Laboratory Medicine
BIRDEM General Hospital
122, Kazi Nazrul Islam Avenue
Shahabag, Dhaka-1000, Bangladesh.
- Co- Investigators : **1. Dr. Abul Basar Mir Md. Khademul Islam**
Professor, Department of Genetic Engineering and Biotechnology,
University of Dhaka-1000, Bangladesh
2. Dr. Md. Nasir Uddin
Director
Bangladesh Institute of Tropical & Infectious Diseases (BITID)
Fouzderhat, Chottogram-4317,
Bangladesh
3. Dr. Md. Abdullah Yusuf
Associate Professor
Department of Microbiology
National Institute of Neurosciences and Hospital, Dhaka,
Bangladesh

K. M. S. Aziz
(Dr. KMS Barman)
Chairman
Ethical Review Committee
Diabetic Association of Bangladesh

Appendix-V**Supplementary Table of Hardy Weinburg Equilibrium****Supplementary Table S1.** Hardy–Weinberg equilibrium testing of genotype distributions in controls and T2DM cases.

Variant	Group	N	Genotype counts (Major/Heterozygous/Minor)	P value (χ^2 test)	P value (Exact test)
TCF7L2 rs12255372 (G/T)	Control	65	45 / 18 / 2	0.783	1.000
	T2DM	67	32 / 31 / 4	0.447	0.392
SLC30A8 rs13266634 (C/T)	Control	90	45 / 34 / 11	0.350	0.323
	T2DM	90	60 / 27 / 3	0.770	1.000

Genotype frequencies were tested for departure from Hardy–Weinberg equilibrium (HWE) separately in controls and cases using both a chi-square test (with Yates' continuity correction) and an exact test (Haldane/Wigginton method). N = number of individuals genotyped in each group.

Supplementary Table of Ingenuity Pathway Analysis (IPA)

Supplementary Table S2: Enriched canonical pathways identified in hypermethylated differentially methylated cytosines (DMCs) through Ingenuity Pathway Analysis (IPA)

Ingenuity Canonical Pathways	-log(p-value)	p-value	Genes
SUMOylation of transcription cofactors	2.89	0.001	<i>MBD1,PHC2,RNF2,SAFB</i>
Regulation of TLR by endogenous ligand	2.78	0.002	<i>FGB,TLR1,TLR6</i>
BMAL1:CLOCK,NPAS2 activates circadian gene expression	2.52	0.003	<i>BMAL1,NPAS2,TBL1X</i>
Fatty acyl-CoA biosynthesis	2.13	0.007	<i>ACSBG2,ACSL5,ELOVL7</i>
Deubiquitination	2.07	0.008	<i>IDE,INO80,NEDD8,OTUD7B,PSMD14,TAF9B,USP15,USP34,CACNA1C,CREB3L3,EIF1,EIF4G1,PDHX,PLCL2,SPCS3,VTI1B</i>
Insulin Secretion Signaling Pathway	2	0.01	
MyD88:MAL(TIRAP) cascade initiated on plasma membrane	1.98	0.01	<i>FGB,TLR1,TLR6</i>
Fatty Acid Activation	1.95	0.01	<i>ACSBG2,ACSL5</i>
SUMOylation of DNA methylation proteins	1.9	0.01	<i>PHC2,RNF2</i>
Heme signaling	1.84	0.01	<i>BMAL1,NPAS2,TBL1X</i>
γ -linolenate Biosynthesis II (Animals)	1.75	0.01	<i>ACSBG2,ACSL5</i>
Mitochondrial L-carnitine Shuttle Pathway	1.75	0.01	<i>ACSBG2,ACSL5</i>
S-methyl-5-thio- α -D-ribose 1-phosphate Degradation	1.67	0.02	<i>APIP</i>
Chondroitin Sulfate Biosynthesis	1.64	0.02	<i>B3GAT1,CHST10,CHST9</i>
Circadian Clock	1.6	0.02	<i>BMAL1,NPAS2,TBL1X</i>
Nucleotide salvage	1.6	0.02	<i>GMMPR2,UCK1</i>
Glycogen metabolism	1.53	0.02	<i>PYGL,UGP2</i>
rRNA modification in the nucleus and cytosol	1.44	0.04	<i>RRP7A,SNU13,WDR46</i>
Processing of Capped Intron-Containing Pre-mRNA	1.42	0.04	<i>CWC25,FIP1L1,POLR2D,PPIG,PUF60,SNRPC,SNU13</i>
Neurotransmitter receptors and postsynaptic signal transmission	0.948	0.11	<i>GLRB</i>

Supplementary Table S3: Enriched canonical pathways identified in hypomethylated DMCs through Ingenuity Pathway Analysis (IPA)

Canonical Pathway	-log(p-value)	p_value	Genes
Pexophagy	2.5	0.003	<i>ATM,PEX5</i>
WNK Renal Signaling Pathway	2.07	0.008	<i>ANAPC10,ELOC,PRKD1,PRKN</i>
DYRK1A Signaling Pathway	1.96	0.01	<i>ELOC,ID2,NFATC3,PRKN,VEGFC</i>
Cachexia Signaling Pathway	1.66	0.02	<i>CASP5,CEBPB,EIF2B5,IL1RAP,MFN1,PRKD1,TGFBR3</i>
Tetrahydrobiopterin Biosynthesis I	1.64	0.02	<i>SPR</i>
Tetrahydrobiopterin Biosynthesis II	1.64	0.02	<i>SPR</i>
Type II Diabetes Mellitus Signaling	1.52	0.03	<i>ACSBG2,ADIPOR1,CEBPB,PRKD1</i>
Mitophagy	1.41	0.04	<i>MFN1,PRKN</i>
Transcriptional Regulation by VENTX	1.41	0.04	<i>ANAPC10,CEBPB</i>
Triacylglycerol Degradation	1.39	0.04	<i>DAGLA,PLB1</i>
Lipid particle organization	1.34	0.04	<i>CIDEA</i>
Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde	1.34	0.04	<i>KMO</i>
M-decay: degradation of maternal mRNAs by maternally stored factors	1.3	0.05	<i>EIF4A2,ELOC</i>
DNA damage-induced 14-3-3 σ Signaling	1.3	0.05	<i>ATM,CASP5</i>
Transport of inorganic cations/anions and amino acids/oligopeptides	1.28	0.05	<i>SLC24A5,SLC8A1,SLC8B1</i>
Ceramide Degradation	1.27	0.05	<i>ASAH2</i>
IL-17 Signaling	1.25	0.06	<i>CCL20,CEBPB,DEFB108B,VEGFC</i>
Meiotic recombination	1.24	0.06	<i>ATM,TEX15</i>
O-linked glycosylation	1.23	0.06	<i>DAG1,GALNT5,THBS1</i>
Protein Ubiquitination Pathway	1.22	0.06	<i>ANAPC10,DNAJC6,ELOC,PRKN,USP40</i>
Platelet homeostasis	0.31	0.48	<i>SLC8A1</i>

Supplementary Table S4: Enriched canonical pathways identified in hypermethylated differentially methylated regions (DMRs) through IPA

Canonical Pathway	-log(p-value)	p_value	Genes
Neurotransmitter receptors and postsynaptic signal transmission	4.07	0.00008	<i>GLRA3,NBEA</i>
Eumelanin Biosynthesis	2.29	0.005	<i>MIF</i>
Platelet homeostasis	2.27	0.005	<i>PDE11A,TRPC7</i>
dTMP De Novo Biosynthesis	2.2	0.006	<i>DHFR2</i>
Synaptogenesis Signaling Pathway	2.14	0.007	<i>ACTR3,CNTNAP2,STXBP4</i>
Regulation of Actin-based Motility by Rho	2.06	0.008	<i>ACTR3,PIP4K2A</i>
Urea Cycle	2.05	0.008	<i>CPS1</i>
RHOA Signaling	1.98	0.01	<i>ACTR3,PIP4K2A</i>
ROBO SLIT Signaling Pathway	1.96	0.01	<i>ACTR3,CNTNAP2</i>
SNARE Signaling Pathway	1.9	0.01	<i>RIMS1,STXBP4</i>
Urea cycle	1.9	0.01	<i>CPS1</i>
RAC Signaling	1.88	0.01	<i>ACTR3,PIP4K2A</i>
Superpathway of Citrulline Metabolism	1.69	0.02	<i>CPS1</i>
Metabolism of folate and pterines	1.67	0.02	<i>DHFR2</i>
1D-myo-inositol Hexakisphosphate	1.67	0.02	<i>ITPKB</i>
Biosynthesis II (Mammalian)			
D-myo-inositol (1,3,4)-trisphosphate	1.67	0.02	<i>ITPKB</i>
Biosynthesis			
GABA synthesis, release, reuptake and degradation	1.62	0.02	<i>RIMS1</i>
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	1.56	0.03	<i>ITPKB</i>
RHOA Signaling	1.5	0.03	<i>ACTR3,PIP4K2A</i>
D-myo-inositol (1,4,5)-Trisphosphate	1.49	0.03	<i>PIP4K2A</i>
Biosynthesis			
Signaling by NOTCH1	1.03	0.09	<i>NBEA</i>

Supplementary Table S5: Enriched canonical pathways identified in hypomethylated differentially methylated regions (DMRs) through Ingenuity Pathway Analysis (IPA)

Canonical Pathway	-log (p-value)	p_value	Molecules/Genes
O-linked glycosylation	3.45	0.0003	<i>ADAMTSL3,B3GALNT2,B3GNT2,MUC20,THSD7B</i>
Activation of NMDA receptors and postsynaptic events	2.95	0.001	<i>DLG2,ERBB4,PRKAR2B,RPS6KA2</i>
Signaling by TGFBR3	2.64	0.002	<i>PSEN1,TCF12,TNRC6B</i>
Thiamin Salvage III	2.27	0.005	<i>TPK1</i>
Myelination Signaling Pathway	2.12	0.007	<i>CNTN6,ERBB4,FGFR2,HDAC9,PRKAR2B,PSEN1</i>
Signaling by NOTCH1	2.1	0.007	<i>HDAC9,PSEN1,TLE3</i>
Metabolism of water-soluble vitamins and cofactors	2.01	0.009	<i>ACACB,SLC25A16,TPK1</i>
Sperm Motility	1.97	0.01	<i>ERBB4,FGFR2,PDE2A,PRKAR2B,SYK</i>
Sonic Hedgehog Signaling	1.92	0.01	<i>CCNB1,PRKAR2B</i>
Retinoate Biosynthesis I	1.87	0.01	<i>ALDH8A1,DHRS3</i>
MITF-M-dependent gene expression	1.84	0.01	<i>ATP6V1D,CCNB1,TNRC6B</i>
Palmitate Biosynthesis I (Animals)	1.8	0.01	<i>CBR4</i>
Geranylgeranyldiphosphate Biosynthesis	1.8	0.01	<i>GGPS1</i>
Biotin-carboxyl Carrier Protein Assembly	1.8	0.01	<i>ACACB</i>
Integration of energy metabolism	1.69	0.02	<i>ACACB,GNGT1,PRKAR2B</i>
Trans, trans-farnesyl Diphosphate Biosynthesis	1.67	0.02	<i>GGPS1</i>
Activation of gene expression by SREBF (SREBP)	1.67	0.02	<i>ACACB,GGPS1</i>
Retinol Biosynthesis	1.67	0.02	<i>DHRS3,LIPG</i>
Vasopressin regulates renal water homeostasis via Aquaporins	1.65	0.02	<i>GNGT1,PRKAR2B</i>
Signaling by Retinoic Acid	1.65	0.02	<i>ALDH8A1,DHRS3</i>

Appendix VI

Consent paper of the respondents in Bangla**সম্মতিপত্র**

আমি নিজের ইচ্ছায় এই গবেষণা কার্যক্রমে অংশগ্রহণ করছি, যার নাম
"Association of DNA methylation of candidate genes in type 2 diabetes mellitus"। আমাকে এই গবেষণা সম্পর্কে বিস্তারিত তথ্যাদি বলা হয়েছে।

আমি রক্ত পরীক্ষার পরে এ সংক্রান্ত রক্তের তথ্য শনাক্ত হলে, পরীক্ষার ফলাফল আমাকে বুঝিয়ে বলা হবে এবং উপযুক্ত চিকিৎসার ব্যবস্থা করা হবে। সেই সাথে ভবিষ্যতে নিজেকে এবং অন্যকে কীভাবে আক্রান্ত হওয়া থেকে রক্ষা করা যায়, সেই বিষয়ে আলোচনা করা হবে।

রক্ত নেয়ার আগে আমার সব ধরনের প্রশ্ন করার সুযোগ ছিল এবং সমস্ত প্রশ্নের উত্তরও আমাকে যথাযথভাবে দেয়া হয়েছে।

সবকিছু বিবেচনা করে, আমি স্বেচ্ছায়, সজ্ঞানে কোন রকম প্রলোভিত/ প্ররোচিত না হয়ে এই গবেষণা কার্যক্রমে অংশগ্রহণ করার জন্য সম্মতি দিচ্ছি।

রোগীর নাম:

স্বাক্ষর/ টিপসহি:

তারিখ:

Appendix VII**Questionnaire****Section A: General Information**

Household Information:

Sample ID	
Date of Interview	
Interviewer's Name:	
Respondent's Name:	
Respondent's Father's/Husband's Name:	
Address	
Household Number:	
Mobile Number:	
Date of Birth	
Age:	
Sex:	

A. Socio-economic Measurement:

A1. Occupation (Based on respondent's statement or observation): (If not applicable, skip to A2)	1. Wage 2. Farmer 3. Business 4. Service 5. Housewife 6. Student 7. Others
A2. What is your monthly income?	Amount: Taka
A3. Gender (Circle as appropriate, leave blank if not applicable):	1. Male 2. Female
A4. What is your educational qualification?	1. Illiterate 2. Primary 3. Secondary 4. Higher Secondary 5. Graduate/Post-Graduate 88. Decline to answer

Section B: Hypertension and Diabetes Related Data

B1 | Have you ever been diagnosed with hypertension? | Yes/No | 1/2 | (If no, skip to B5)

B2 | What type of hypertension do you have? | Primary (Essential) Hypertension, Secondary Hypertension, Others (Specify) | 1, 2, 99 |

B3 | Do you take medication for hypertension? | Yes/No | 1/2 | (If no, skip to B5)

B4 | If yes, what kind of medication do you take? | Diuretics, Beta Blockers, Calcium Channel Blockers, ACE Inhibitors, ARBs, Combined (Specify) | 1, 2, 3, 4, 5, 6 |

B5 | Have you ever been diagnosed with diabetes? | Yes/No | 1/0 | (If no, skip to C1)

B6 | If yes, what kind of diabetes do you have? | Type 1, Type 2, Gestational, Others (Specify) | 1, 2, 3, 4 |

B7 | Do you take medication for diabetes? | Yes/No | 1/0 | (If no, skip to C1)

B8 | If yes, what kind of medication do you take? | Insulin, Oral medications, Both | 1, 2, 3 |

B9 | Do you take any other medication? | Specify | 1, 2, 3 |

B10 | Specify your fasting blood sugar level | mmol/L |

B11 | Specify your 2 hours after glucose load blood sugar level | mmol/L |

Section C: Childhood Adversity and Trauma

C1 | During your childhood (before age 18), did any of the following happen to you? |

Yes/No/Prefer not to say | 1, 2, 3

C2 | Were you physically abused, beaten, kicked, or hurt in any way by an adult? |

Yes/No/Prefer not to say | 1, 2, 3

C3 | Were you ever emotionally abused, ridiculed, or humiliated by an adult? |

Yes/No/Prefer not to say | 1, 2, 3

C4 | Were you ever sexually abused or molested by an adult? | Yes/No/Prefer not to

say | 1, 2, 3

C5 | Did you witness domestic violence in your household? | Yes/No/Prefer not to say |

1, 2, 3

C6 | Did any household member suffer from mental illness or commit suicide? |

Yes/No/Prefer not to say | 1, 2, 3

C7 | Did any household member abuse alcohol or drugs? | Yes/No/Prefer not to say | 1,

2, 3

C8 | Were any household members ever imprisoned? | Yes/No/Prefer not to say | 1, 2,

3

C9 | Were your parents ever separated or divorced? | Yes/No/Prefer not to say | 1, 2, 3

C10 | Did you experience any form of deprivation such as hunger, lack of proper

clothing, or shelter? | Yes/No/Prefer not to say | 1, 2, 3

Section D: Physical Examination

Measurement	SBP (mm of Hg)	DBP (mm of Hg)
F1 First reading		
F2 Second reading		
F2 Third reading		
F4 Average (if different readings)		
F5 Pulse Rate (if any abnormality, record here)		
F6 Height (in cm)		
F7 Weight (in kg)		

Section : Laboratory Tests

Test	Result
G1 Fasting Blood Glucose	
G2 2 hrs after Glucose load	
G3 Total Cholesterol	
G4 Triglycerides	
G5 HDL Cholesterol	
G6 LDL Cholesterol	
G7 HbA1C	
G7 Genotyping of TCF7L2	
G7 Genotyping of SLC30A8	
G8 DNA Methylation Study	

Appendix VIII

Published articles of PhD Thesis

Title of PhD: “Elucidating genetic and epigenetic mechanisms of type 2 diabetes mellitus pathogenesis”

1. Barman N, Atiqul Haque M, Firoz M, Abdullah Yusuf M, Islam AB. Association of transcription factor 7-like 2 rs12255372 polymorphism with susceptibility of type 2 diabetes mellitus in Bangladeshi population. *Molecular Genetics and Genomics*. 2023 Sep;298(5):1201-9.
2. Barman N, Islam AB, Haque MA. Association between adverse childhood experiences and type 2 diabetes mellitus in later life: A case-control study. *PLOS Global Public Health*. 2024 Jun 25;4(6):e0002715.
3. Barman N, Haque MA, Ridwan M, Ghosh D, Islam AB. Loss-of-function variant of SLC30A8 rs13266634 (C> T) protects against type 2 diabetes by stabilizing ZnT8: Insights from epidemiological and computational analyses. *Journal of Genetic Engineering and Biotechnology*. 2025 Dec 1;23(4):100565.