Association of *LEPR* gene rs1137100 (Lys109Arg) & rs1137101 (Gln223Arg) Polymorphisms with Type 2 Diabetes Mellitus in Young Bangladeshi Population: A Pilot Study

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Mathematics and Natural Sciences
Brac University
December 2024

Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Ethics Statement

The study received ethical approval from the Institutional Review Board (IRB) of the National Institute of Biotechnology (NIB), which was conducted following the Declaration of Helsinki. Prior to data collection, participants were provided with a detailed explanation of the study and, their written consent was obtained. Throughout the data collection and analysis phases, the privacy and confidentiality of the respondents were strictly maintained.

Abstract

Background: Youth-onset Type 2 Diabetes Mellitus (T2DM) is an emerging global public health concern. Polymorphisms in the *LEPR* (leptin receptor) gene, specifically rs1137100 (Lys109Arg) and rs1137101 (Gln223Arg) have been widely linked to an increased risk of T2DM due to their roles in insulin resistance, energy homeostasis, and obesity. However, genetic risk factors for T2DM in young Bangladeshi populations remain unexplored. Objective: In this study, we tried to determine the required sample sizes and provide initial data for investigating the association of *LEPR* gene polymorphisms (rs1137100 and rs1137101) with youth-onset T2DM in full-scale research. Methods: A case-control pilot study was conducted involving 56 Bangladeshi participants (18 T2DM cases, 38 NGT controls) aged 18-29 years. Clinical and biochemical data were gathered. Blood samples were collected for DNA extraction and genotyping using PCR-RFLP and sequencing. Statistical analysis was performed using the t-test and chi-square test to show any significant difference between the groups. Results: The T2DM group showed higher waist-hip ratios, triglycerides, and altered glycemic profiles compared to controls. In rs1137100 polymorphism, the AA genotype was more frequent in T2DM, while AG and GG genotypes were predominant in controls. In rs1137101, the AG genotype was more common in T2DM. However, there were no significant associations between *LEPR* polymorphisms and T2DM risk. To ensure adequate statistical power, 138 participants/group (rs1137100) and 27,450/group (rs1137101) are required for future studies. Conclusion: *LEPR* polymorphisms showed genotype variations but no significant risk association, demanding large-scale studies.

Keywords: *LEPR*; SNPs; rs1137100; rs1137101; Youth-onset T2DM; Genetic association.

Dedication

I dedicate this thesis to all women in STEM, shattering stereotypes and encouraging future generations with their unparalleled resilience, intelligence, and unwavering dedication to advancing science and technology.

Acknowledgement

First and foremost, praises and thanks to Almighty Allah for His unceasing blessings, which allowed me to complete my thesis. I also want to express my gratitude to my parents and sibling for their encouragement and support, without which I could not have finished my thesis.

I am deeply grateful to my internal supervisor, Dr. Dr. Fahim Kabir Monjurul Haque, Associate Professor, Department of Mathematics and Natural Sciences, Brac University for his constant support, brilliant insights, and invaluable guidance which have been crucial in materializing this experiment. It was a great opportunity to get Dr. Fahim as a supervisor and I will always be grateful to him for accepting me as a thesis student.

I would also like to extend my appreciation to my external supervisor, Dr. U.S. Mahzabin Amin, Senior Scientific Officer, Molecular Biotechnology Division (MOBD), National Institute of Biotechnology. The study would have been impossible without her insightful feedback, helpful recommendations and the resources and general support provided by MOBD.

I would also like to address the contributions and guidance provided by the research fellows, scientific officers, and my fellow thesis mates for their constant support. Just as importantly, I also extend my appreciation to my amazing friends and juniors for providing me with all the mental support and motivation I ever needed.

Lastly, I am incredibly appreciative of the constant support Dr. Md. Firoze H. Haque, Associate Professor, and Chairperson of the Department of Mathematics and Natural Sciences, Brac University provides to all his students. I am also grateful to Dr. Iftekhar Bin Naser and Dr. Munima Haque Chowdhury for their active support.

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List of Acronyms

ADA	American Diabetes Association
AMPK	AMP-activated protein kinase
BSMMU	Bangabandhu Sheikh Mujib Medical University
BDNF	Brain-derived neurotrophic factor
BBB	Blood-brain barrier
BMI	Body mass index
DM	Diabetes Mellitus
DBP	Diastolic blood pressure
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-related kinase
GLUT4	Glucose transporter type 4
HNF1A	Hepatocyte Nuclear Factor 1 Alpha
JAK2	Janus kinase 2
JAK-STAT	Janus kinase-signal transducer and activator of transcription
KATP	ATP-sensitive potassium
MC4R	Melanocortin 4 Receptor
NIB	National Institute of Biotechnology
NGT	Normal glucose tolerance
OGTT	Oral Glucose Tolerance Test
PHF2	PHD Finger Protein 2
PI3K	Phosphoinositide 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PCR	Polymerase Chain Reaction
Akt	Protein kinase B
RFLP	Restriction Fragment Length Polymorphism
SNP	single nucleotide polymorphism
SLC30A8	Solute Carrier Family 30 Member 8
SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
SBP	Systolic blood pressure
TBE	Tris-borate EDTA
TCF7L2	T Cell Factor 7 Like 2
T2DM	Type 2 Diabetes Mellitus
WFS1	Wolfram Syndrome 1
WAT	White adipose tissue

Chapter 1

Introduction

Diabetes mellitus (DM) is an escalating global health concern, with an estimated 537 million cases globally, which is predicted to surge to 783 million cases by 2045 (Ginzburg, 2023). With impacting over 400 million individuals, diabetes is currently among the top ten leading causes of mortality worldwide (Mishra et al., 2023). Type 2 diabetes mellitus (T2DM) is a category of diabetes mellitus that is more broadly classified alongside type 1 diabetes and gestational diabetes (Harreiter & Roden, 2023). Almost 90% of cases of diabetes are caused by type 2 diabetes, and its incidence is notably rising in younger populations (Ahmad et al., 2022). Type 2 DM is defined as a chronic metabolic illness where insulin resistance and insufficient insulin production lead to elevated blood sugar levels ("Type 2 Diabetes", 2024). Type 2 diabetes diagnosed in people between the ages of 15 and 39 is referred to as youth onset (Xie et al., 2022). Since the 2000s, younger individuals (under 40), adolescents, and even children have seen the most significant relative increases in T2DM prevalence and incidence, even though T2DM was traditionally perceived to primarily affect the middle-aged and elderly (Viner et al., 2017).

1.1 Diabetes and the young: Epidemiology

The increasing incidence and prevalence rates of type 2 diabetes on a global scale have raised concerns about the epidemiological profile of T2DM within the youth. The rate of youth-onset T2DM is rising at an alarming pace and, if prevailing patterns persist, projections indicate a fourfold increase in this incidence by the year 2050 (Hannon & Arslanian, 2015). The prevalence of T2DM is estimated to be as high as 5300 cases per 100,000 adolescents as stated by a comprehensive analysis conducted in 2013 (Fazeli-Farsani et al., 2013). From 2002 to

2018, there has been a twofold rise in the incidence of T2DM in youth, and by 2060, this is expected to be 220,000 cases up from 28,000 in 2017 (Huff, 2024). Nevertheless, the disproportionately high prevalence of patients from particular ethnic backgrounds, such as Native Americans (Fagot-Campagna et al., 2000), Indigenous Australians (Haynes et al., aa2016), Black and Hispanic patients (TODAY Study Group, 2013), Pima Indian patients (Pavkov et al., 2007), Canadian First Nationals (Dean et al., 1998), and South Asian patients (Khanolkar et al., 2016), is the most striking aspect of the young-onset T2DM phenotype. According to recent research, T2DM presently causes more than 50% of newly diagnosed diabetes cases among individuals aged 10–18 years, especially within ethnic minority groups, drawing attention to an unsettling trend in high-risk populations (Craig & Huang, 2009; Hannon & Arslanian, 2015).

1.1.1 Global prevalence and incidence of youth-onset T2DM

In a previous report of the International Diabetes Federation Diabetes Atlas, the incidence of type 2 diabetes among people aged 20 to 29 years, varied greatly, ranging from less than 1% in Europe to 2.5% in the Caribbeans (Cho et al., 2018). In Canada, 80–95% of diabetes cases among the cohort aged 20–29 have type 2 diabetes and, the incidence of T2DM in this age range is reported to be 50–150 per 100,000 (Basak, 2023; Kanagalingam et al., 2024). A study conducted in British Columbia demonstrated that incidence rates of T2DM were 2.2 times higher among South Asian adolescents with diabetes (86.4%) compared to their White counterparts (Ke et al., 2015). Another study in British Columbia, Canada in 2018 reported that the prevalence of T2DM among individuals aged 1-20 years rose by 2.3 times over the ten-year period, from 0.009% in 2002–2003 to 0.021% in 2012–2013 (Amed et al., 2018). Over the preceding two decades, T2DM prevalence in the United States, among adolescents aged 10-19 years has doubled with notable differences: for example, among non-Hispanic white

youth, the rate is approximately 0.2 per 1000, whereas among Black and American Indian youth, it is 2 per 1000 (Bloomgarden & Rapaport, 2023). According to forecasts, there might be 220,000 cases by 2060 as the annual incidence of T2DM in youth has increased from 9 to 18 cases per 100,000 (Basak, 2023; Bloomgarden & Rapaport, 2023). Between 1990 and 2000, the number of African-American and Caribbean-Hispanic adolescents in New York with early-onset T2DM surged ninefold (Grinstein et al., 2003). The prevalence of T2DM among younger individuals (10–19 years old) was estimated to be 34 cases per 100,000 in 2001 by the SEARCH Study. In contrast, in 2009, this number climbed to 46 cases per 100,000, reflecting a 31% rise in the course of nine years (Dabelea et al., 2014).

In the United Kingdom, during the period spanning from 2003 to 2006, the incidence of type 2 Diabetes among individuals below the age of 30 escalated from 5% to 12% (Harron et al., 2011). According to a Jamaican clinic-based study (Tulloch-Reid et al., 2010), 22% of those diagnosed with diabetes prior to reaching the age of 25 exhibited characteristics typical of type 2 diabetes. However, the situation in Europe is comparatively less alarming. The occurrence of T2DM in adolescents is less than 0.5 cases per 100 thousand people (Oester et al., 2016). Even while T2DM remains relatively infrequent in adolescents in Europe, the UK has a substantially greater frequency than the rest of the European nations—roughly 10 cases per 100 thousand people. This phenomenon can be partially attributed to the higher population of South Asian individuals residing in the UK than the rest of Europe, a group that is more likely to develop type 2 diabetes (Khanolkar et al., 2016). A 2007 Australian research found that among young Indigenous persons aged 0 to 19, T2DM is six times more common than in non-Indigenous people (Craig et al., 2007).

1.1.2 Prevalence and incidence of youth-onset T2DM in the Asian region

As of 2002, 10% of diabetes patients in Asian nations were under 30 years old, a significant divergence from Western patterns, where diabetes predominantly affects older persons

(Dhanwal et al., 2014). In the context of China, according to Fu et al. (2013), the prevalence of T2DM among individuals aged 0-18 years escalated from 4.1 cases per 100,000 in 1995 to 10 cases per 100,000 by 2010. The majority of evidence pertaining to the epidemic of T2DM in the youth has been derived from pediatric data collected in Japan and the United States. In Japan, the yearly incidence of type 2 diabetes in children is around 2.62 per 100,000, exhibiting a considerable increase over the last three decades (between 1975 and 2000), reaching a peak of about 3.0 per 100,000 (Urakami et al., 2007). Between 1980 and 1990, the number of Japanese children with T2DM exceeded that of Type 1 Diabetes Mellitus, indicating a noticeable increase in T2DM occurrence (Kitagawa et al., 1998). Recent investigations have revealed that the prevalence of type 2 diabetes among Japanese school-aged children is now seven times more prevalent than type 1 diabetes, with a fourfold increase in incidence observed in the cohort aged 6 to 15 (Bloomgarden, 2004; Rosenbloom et al.,1999). Furthermore, another study by Pinhas-Hamiel and Zeitler (2005) in Japan, indicated that over 80% of all newly diagnosed cases of diabetes in children and adolescents were classified as type 2 diabetes. Research conducted in South Asia, mostly in India and Bangladesh, throughout the 1990s found that around 1% of individuals between the ages of 5 and 19 had type 2 diabetes and, this frequency had doubled in the subsequent twenty years. Their data implies that depending on the country, the prevalence of type 2 diabetes in South Asians between the ages of 20 and 30 ranges from the lowest 1% to the highest 7% (Praveen et al., 2015). According to the MASALA study, 32% of South Asians transitioned from a state of normal glucose tolerance to prediabetes or T2DM within a five-year time frame (Gujral et al., 2020). A study conducted in India documented an incidence of 21.6 per 1,000 person-years for T2DM, identifying obesity and a sedentary lifestyle as significant risk factors (Gupta et al., 2023). Additionally, another investigation in India discovered that 12% of cases among people under the age of 18 had T2DM (Bhatia et al., 2004). T2DM was identified in 25.3% of cases of diabetes in those under

25 years old, according to the Indian Council of Medical Research (ICMR) Registry of Diabetes in the Young (Praveen et al., 2016). As reported in a comprehensive nationwide survey in Pakistan, adults with T2D had a prevalence of 16.98% with younger people having a higher prevalence (Aamir et al., 2019). The Global Burden of Disease Study highlights that low- and middle-income nations, including Pakistan, are seeing an increase in the incidence of early-onset Type 2 Diabetes (T2D) among people aged 15 to 39 (Xie et al., 2022). Talukder et al. (2024) mentioned that the prevalence of T2D in rural areas of Bangladesh is lower (7.4%) when compared to urban areas (10.8%). Between 2011 and 2018, the incidence of T2DM in children and adolescents in Dhaka increased by 12% per year, from 0.22 to 0.57 per 100,000 (Zabeen et al., 2021).

1.1.3 Risk factors of youth-onset T2DM

Both modifiable factors, such as obesity and hypertension, and non-modifiable ones, like age and family history, play a significant role in the increased risk of developing type 2 diabetes in youth (Misra et al., 2014; Zeliger, 2023). Unhealthy eating habits, reduced physical activity, and poor diets also contribute to this heightened risk (Ahmmed et al., 2023; Aktar et al., 2023; Hitt et al., 2023; B. Liu et al., 2023; Misra et al., 2014). In addition to these factors, genetic influences are involved; researchers have identified rare single nucleotide polymorphisms (SNPs) associated with obesity and impaired β-cell function in early-onset cases (Kwak et al., 2024).

1.2 Symptoms of T2DM

According to estimates, between 30% and 80% of type 2 diabetes (T2DM) patients go untreated, depending on the nation (Espino, 2010). A growing number of young people diagnosed with T2DM, manifest a variety of symptoms that are different from those usually

observed in adults. However, symptoms of type 2 diabetes are frequently mild or non-existent whereas type 1 diabetes has a higher likelihood of exhibiting symptoms (Espino, 2010).

Table 1.1: Symptoms of T2DM in youth

Symptom Category	Symptom	Description	Reference
Classical symptoms	Increased Thirst and Urination	Young people frequently describe frequent urination (polyuria) and excessive thirst (polydipsia) as their first symptoms.	(Huff, 2024)
	Unexplained Weight Loss	Many young people lose weight inexplicably, even while their hunger has grown.	(Bollam et al., 2022)
	Fatigue	The body's incapacity to efficiently use glucose results in persistent fatigue.	(Weghuber et al., 2019)
Additional symptoms	Acanthosis Nigricans	A skin disorder where dark, velvety patches frequently develop around the armpits and neck, indicative of insulin resistance.	(Huff, 2024; Weghuber et al., 2019)
	Growth Impairment	Due to metabolic abnormalities, certain young people may have delayed puberty or limited development.	(Bollam et al., 2022)
	Psychological Symptoms	Youth with type 2 diabetes have higher rates of anxiety and depression.	(Weghuber et al., 2019)

Nevertheless, the type of diabetes cannot usually be identified at diagnosis, thus clinical presentation and plasma glucose levels should be used to inform first treatment choices (Espino, 2010).

1.3 Diagnostic criteria for T2DM - WHO

The World Health Organization (WHO) has developed particular standards for the diagnosis of T2DM in young people. In order to distinguish type 2 diabetes from type 1 diabetes which is more prevalent in children and adolescents, these criteria are designed to make early identification and management easier.

Table 1.2: Diagnostic criteria for T2DM according to WHO

Diagnostic Test	Criteria	Reference
Fasting blood glucose	126 mg/dL (7.0 mmol/L) or above	(Espino, 2010)
Oral glucose tolerance test (OGTT)	A two-hour plasma glucose level of 200 mg/dL (11.1 mmol/L) or above	(Espino, 2010)
HbA1c level	6.5% (48 mmol/mol) or above	(Espino, 2010)
Random plasma glucose	200 mg/dL (11.1 mmol/L) or above, together with symptoms like polyuria or polydipsia	(Espino, 2010)

Furthermore, some screening recommendations were made to facilitate the detection at the earliest stage: (1) Children who are overweight (BMI ≥85th percentile) and who have other risk factors, such as family history or membership in high-risk ethnic groups, should be screened starting at age 10 or the start of puberty (Rao & Jensen, 2020); (2) Autoantibody testing is advised for all individuals to distinguish between T1DM and T2DM (Rao & Jensen, 2020).

1.4 Diabetes and the young: Pathophysiology

The hallmark aetiologies of both late-onset and early-onset type 2 diabetes include insulin resistance, β-cell dysfunction, and metabolic dysregulation. There is strong empirical evidence

showing that in younger T2DM patients, the deterioration of β-cells occurs three to four times more quickly than in older ones (Craig & Huang, 2009; Hannon & Arslanian, 2015). According to estimates, the yearly rate of decline in β-cell functionality, as assessed by an oral glucose tolerance test, can almost reach 7% in late-onset type 2 diabetes (Kahn et al., 2011; Matthews et al., 1998). Conversely, with minimal improvement in insulin resistance, this rate can escalate to between 20-30% annually in cases of young-onset T2DM (Bacha et al., 2013; TODAY Study Group, 2013). In a different study, it was discovered that while insulin or metformin treatment improved β-cell functionality in adults, it deteriorated over a 12-month period in youths with T2DM when measured through the oral glucose tolerance test and hyperglycaemic clamps after modifying for insulin sensitivity (Nadeau et al., 2018). In comparison to late-onset type 2 diabetes, the initial and subsequent phases of the insulin response were decreased by 75% and 55%, respectively. This is opposite to the typically expected outcomes associated with late-onset diabetes mellitus when the disease has been active for over ten years (Bagust & Beale, 2003; Gungor et al., 2005).

Several common and rare genetic variants including ***HNF1A***, ***MC4R***, ***WFS1, SLC30A8***, and ***TCF7L2*** are linked to an increase in the risk of developing T2DM at a young age (Kwak et al., 2023, 2024). The ProDiGY Consortium's studies have identified genetic markers like **rs7903146** in the ***TCF7L2*** gene and **rs10992863** in the ***PHF2*** gene as the genetic causes of youth-onset T2DM (Srinivasan et al., 2021; SRINIVASAN et al., 2018). Additionally, specific changes in genes related to glucose metabolism and insulin sensitivity, such as **rs1501299** and **rs2241767** in ***ADIPOQ*** (Zusi et al., 2023) and **c.2357T>C, p.*(*Leu786Pro*)*** in ***LEPR*** (Chaves et al., 2022) have shown an association with increasing the likelihood of developing T2DM in younger people.

In youth-onset type 2 diabetes (T2D), insulin resistance mainly impacts the muscles and liver, which results in increased glucose production by the liver and compromised glucose uptake

(Abdallah & Mihailescu, 2023). Over time, the pancreas struggles to produce enough insulin due to the gradual failure of β-cells, making high blood sugar levels worse (Abdul-Ghani, 2013). In addition to this, inflammation and disruptions in key metabolic processes, like the citric acid cycle and mitochondrial function, are common in T2DM (Diamanti et al., 2022). The more aggressive nature of T2D in younger people, the higher the likelihood of serious complications. Research indicates that, after diagnosis of approximately 13.3 years, 54.8% of patients develop kidney problems (nephropathy), 32.4% experience nerve damage (neuropathy), and 13.7% face vision issues (retinopathy) (Ovsyannikova & Zubareva, 2022). Moreover, having T2DM at a young age also raises the chances of developing heart disease and certain types of cancer, such as colorectal and breast cancer (Scherübl, 2021).

1.4.1 Mechanism of insulin resistance (IR)

Insulin resistance (IR), which is defined as the reduced capacity of insulin to control glucose metabolism in important organs such as the liver, adipose tissue, and skeletal muscle, is a major contributing factor to the development of T2DM. Two essential proteins for insulin signaling are insulin receptor (IR) and insulin receptor substrate (IRS). Changes in these proteins can interfere with the action of insulin, for example, IRS serine phosphorylation (Pei et al., 2022). By inducing cellular stress and upsetting insulin signaling pathways, elevated free fatty acids and inflammatory cytokines from adipose tissue contribute to insulin resistance (Chandrasekaran & Weiskirchen, 2024; A. Singh et al., 2022). According to F.-J. Liu et al. (2022), impaired mitochondrial activity causes oxidative stress, which aggravates insulin resistance and glucose intolerance, together with misfolded protein accumulation in the endoplasmic reticulum causes stress responses that impede insulin transmission.

1.4.2 Mechanism of β-cell dysfunction

There are several factors contributing to β-cells failure in type 2 diabetes (T2D), but dedifferentiation, identity loss, and decreased insulin production are the main ones. Chronic hyperglycemia, inflammation, and hereditary effects are among the variables that aggravate this dysfunction and cause a steady reduction in beta-cell activity. Dedifferentiation is the name for the process by which beta cells lose their specific identity and become non-functioning progenitor-like cells (Patel & Remedi, 2024). Environmental stresses, such as oxidative stress and inflammation, change transcription factor activity and gene expression, which is what causes this identity loss (Ghasemi Gojani et al., 2024). Due to a compromised stimulus-secretion coupling, insulin secretion patterns are disturbed, especially at elevated glucose levels (Skelin Klemen et al., 2024). According to Dutta et al. (n.d.), chronic hyperglycemia downregulates vital transcription factors including PDX1 and MAFA, which are necessary for the synthesis of insulin. By modifying histone alterations and transcriptional regulation, pro-inflammatory agents such as BMP-2 suppress beta cell activity and encourage dedifferentiation (Urizar et al., 2023).

1.5 Obesity, T2DM, Leptin, and *LEPR* gene: A Complex Interplay

The development of T2DM is of a multifaceted nature, where there is a complicated interplay between obesity, leptin, *LEPR* gene, and type 2 diabetes. Multiple research has shown links between obesity and type 2 diabetes. Obesity is a major risk factor for beta cell dysfunction and insulin resistance, two crucial aspects of type 2 diabetes. Adipose tissue alterations brought on by obesity include altered lipid metabolism and inflammatory responses, which all contribute to insulin resistance (Mocciaro & Gastaldelli, 2022). Research shows that the switch from obesity to type 2 diabetes is accelerated by insulin resistance in adipose tissue, happening far more quickly in youth than in adulthood (Oranika et al., 2023). Among 25% of affected

young Hispanic individuals, obesity is a key driver of type 2 diabetes putting them at a higher risk for insulin resistance (Huff, 2024; Kwak et al., 2024). Another noteworthy observation is that over 80% of those with young-onset T2DM are obese in comparison with around 50% of individuals with late-onset T2DM (Candler et al., 2018; Lascar et al., 2018). Moreover, the risk of developing type 2 diabetes shoots up significantly in severely obese adolescents, with hazard ratios showing a sharp rise in risk with elevated BMI (Twig et al., 2020). Obesity-related variables put beta-cell function under pathogenic stress, causing beta-cell hyperresponsiveness and insulin resistance, which ultimately impairs glucose homeostasis and increases the risk of diabetes (B. Yang et al., 2023). In obesity, insulin resistance induces elevation of insulin production resulting in the exhaustion and failure of beta cells (Jesuino et al., 2022). This beta cell failure can further be intensified by the impact of environmental factors like diet (Watada, 2020).

Additionally, obesity is found to have an association with leptin resistance, which decreases energy expenditure but increases appetite (Rangareddy et al., 2023). According to Cho and Batchelder (2023), leptin resistance is linked to metabolic dysfunctions such as insulin resistance and dyslipidemia. Studies show that lower levels of leptin mRNA expression are linked to type 2 diabetes, indicating it is a potential biomarker that may have diagnostic significance (Al-Harithy & Alomari, 2021). Besides, according to genetic studies, leptin levels and type 2 diabetes are positively correlated, and common genetic loci have been found (Wang et al., 2020). Patients with type 2 diabetes in multiple studies frequently have elevated blood leptin levels, which may be related to insulin resistance and complications from diabetes (Ashraf et al., 2022; Katsiki et al., 2018; Moonishaa et al., 2017). Moreover, the intricacy of leptin's involvement in type 2 diabetes is shown by the inverse correlation found between T2DM risk and soluble leptin receptor levels (Sun et al., 2010).

On the other hand, leptin resistance caused by mutations in the *LEPR* gene affects energy balance and appetite control mechanisms, which is a major factor in obesity and related health issues (Da Silva et al., 2023). A higher incidence of obesity, T2DM, and insulin resistance in a number of populations is observed to be associated with the single nucleotide polymorphisms (SNPs) in the leptin and leptin receptor (*LEPR)* gene, suggesting a major role of *LEPR* (Bains et al., 2020; Dasgupta et al., 2015; Meshkani et al., 2016). Thus, the interaction of leptin, obesity, insulin resistance, and *LEPR* polymorphisms highlights the complex process of T2DM development (Riccioni et al., 2004).

1.6 Leptin: An Overview

Initially discovered in 1994 (Zhang et al., 1994), leptin is considered as an anorexigenic hormone (H.-K. Park & Ahima, 2015). It is mainly secreted by white adipose tissue (WAT) and is vital for regulating appetite and energy expenditure (Suma H Y et al., 2023) as it signals the hypothalamus about nutritional status (Lebl et al., 2024; Polyzos & Mantzoros, 2022). Leptin regulates appetite and energy expenditure primarily through the hypothalamus (Elmquist et al., 1999).

1.6.1 Structural features and function

The leptin gene (*LEP*) is located on human chromosome 7, particularly in the region 7q22.1-7q35, which might be associated with obesity through linkage disequilibrium (W.-D. Li et al., 2003). With a molecular weight of 16 kDa, leptin is an endogenous hormone consisting of 167 amino acids (Denver et al., 2011). According to Saxton et al. (2023) and Danielsson et al. (2020), leptin's structure is a four-helix bundle with a distinctive perforated lasso topology. This topology is crucial for the protein's biological activity and receptor engagement. The primary structural component is its linear sequence of amino acids, which comprises a signal peptide of 41 amino acids that is cleaved to produce a circulating form of 146 amino acids

(Meraj & Sabiullah, 2019). Four alpha helices that make up the secondary structure create a helical bundle that is necessary for receptor binding (Haglund et al., 2012; Rock et al., 1996). Molecular dynamics simulations demonstrate that intrinsically disordered regions exist in the conformational dynamics of leptin, especially between residues 25 and 42, where several secondary structures can be adopted (Chimal-Vega et al., 2015). Its interaction with the leptin receptor (LepR), which affects signaling pathways and may have therapeutic implications, depends on this dynamic characteristic (Simien & Haglund, 2023). In order to preserve structural integrity and promote receptor contacts, the tertiary structure entails the three-dimensional arrangement of these helices, maintained by an intrachain-disulfide linkage between cysteine residues (Haglund et al., 2012; Rock et al., 1996). The function of leptin in energy management and signaling pathways is supported by its structural complexity.

Leptin inhibits hunger by influencing hypothalamic neurons as well as it preserves energy equilibrium by indicating total body fat where more body fat is correlated with higher levels of leptin (Polyzos & Mantzoros, 2022). Studies on obesity-related disorders such as type 2 diabetes and cardiovascular disease have linked leptin to these problems (H. J. Singh et al., 2022). According to (Manglani et al., 2024), leptin is linked to proinflammatory cytokines that worsen insulin resistance. It has been determined that a major biomarker for type 2 diabetes is reduced leptin mRNA levels (Al-Harithy & Alomari, 2021). Lebl et al. (2024) and H. J. Singh et al. (2022) claim that excessive eating and severe obesity are caused by a leptin deficiency and that leptin insufficiency is also associated with impaired immune responses.

1.6.2 Epigenetic regulation of leptin expression

Epigenetic mechanisms, including DNA methylation and histone modifications, intricately regulate leptin expression. This regulation affects leptin levels, which are crucial in the pathophysiology of type 2 diabetes (T2DM).

DNA Methylation: Lower expression of leptin in T2DM patients is associated with altered methylation patterns in adipose tissue and pancreatic islets (Roy, 2021). According to research, the expression of the leptin gene (LEP) is dynamically influenced by DNA methylation at specific enhancer elements and the promoter region, especially in response to metabolic disorders and obesity (Sadashiv et al., 2021; Taege et al., 2022).

Histone Modifications: According to Wróblewski et al. (2019), alterations in histone acetylation and deacetylation affect the expression of the leptin gene and cause metabolic dysregulation. Furthermore, via epigenetic processes involving histone acetylation regulated by the AKT/p300 pathway, leptin affects the expression of other genes, including *BDNF* (C. Li et al., 2021).

MicroRNA Regulation: In metabolic diseases, distinct miRNAs affect the production and sensitivity of leptin, and leptin is influenced by these miRNAs (Wróblewski et al., 2019). This implies that leptin has a dual role in metabolic health by actively regulating epigenetic modifications in addition to responding to them (Wróblewski et al., 2019).

1.6.3 Leptin & its receptors

According to reports, the effects of leptin (LEP) and leptin receptor (LEPR) significantly influence body weight, energy metabolism, and food intake (H.-K. Park & Ahima, 2015; Y. Yang & Niu, 2018). When leptin attaches to its receptor (LEPR), internal signaling pathways are triggered, which impact immunological responses and gene expression (Mukherjee et al., 2023; Suma H Y et al., 2023). The long isoform of leptin, or OB-Rb, is the main receptor for the hormone and is involved in a number of physiological reactions, including energy expenditure and appetite regulation. According to Barrios-Correa et al. (2018), the long isoform of the leptin receptor is essential for controlling hunger and energy metabolism. Arora (2023)

stated that other isoforms like OB-R also play a role in the variety of effects of leptin, including reproductive health.

1.6.3.1 Leptin Receptors (LEPR)

A member of the class I cytokine receptor family, the leptin receptor (LEPR) is the receptor through which leptin operates. The brain, liver, pancreas, and adipose tissue are among the areas where LEPR is expressed (Zhang et al., 1994). Although the receptor has many isoforms, intracellular signaling is mostly dependent on the long form (LEPRb) (Tartaglia et al., 1995). When leptin attaches itself to LEPRb, the receptor is activated and further downstream signaling is started (Elmquist et al., 1999). The only isoform that can trigger JAK-STAT (Janus kinase-signal transducer and activator of transcription) signaling is LEPRb, which has three tyrosine residues (Y986, Y1079, and Y1141) (Lee et al., 1996). Leptin receptors control insulin sensitivity and glucose metabolism in the peripheral tissues for instance liver, muscles, and pancreas, affecting how cells react to insulin (Sahu, 2003).

1.6.3.2 Effect of leptin and its receptor on β-cells

Through its receptors, leptin profoundly affects the function of pancreatic β-cells, mainly by modifying the activity of KATP (ATP-sensitive potassium channels). By raising KATP channel conductance, it suppresses insulin production by causing membrane hyperpolarization and decreased electrical activity in β-cells (Cochrane & Shyng, 2019, 2020). AMP-activated protein kinase (AMPK) signaling mediates the leptin-induced trafficking of KATP channels to the plasma membrane in this mechanism (Holz et al., 2013; S.-H. Park et al., 2013). Moreover, leptin receptors in β-cells alter β-cell mass and long-term insulin gene expression through other signaling pathways, in addition to their acute effects on insulin production (Marroquí et al., 2012). The interaction between glucose and leptin concentrations is important because appropriate leptin levels promote KATP channel translocation, which in turn regulates β-cell

excitability by enhancing AMPK activation (S.-H. Park et al., 2013). Notwithstanding these discoveries, the precise methods via which leptin signaling may be interfered with in ailments like as obesity and type 2 diabetes are still being investigated, underscoring the intricacy of β-cell control in metabolic disorders.

1.6.4 Signalling pathways activated by leptin

Upon binding with its receptor, leptin triggers the activation of many intracellular signaling cascades. Understanding the hormone's metabolic effects is essential, especially in light of type 2 diabetes.

JAK-STAT Pathway: The most researched pathway in leptin signaling is the "Janus kinase-signal transducer and activator of transcription (JAK-STAT)" pathway (Myers et al., 2010; Tartaglia, 1997) which affects hunger and metabolism-related gene expression (J. Liu et al., 2018b). **JAK2** (Janus kinase 2) is recruited and activated when leptin binds to LEPR, causing conformational changes in the receptor (Bjorbaek, 2004). As a result, **STAT3** (Signal transducer and activator of transcription 3) is phosphorylated and moves to the nucleus to control the transcription of target genes related to insulin sensitivity, glucose metabolism, and energy balance (A. S. Banks et al., 2000).

PI3K/Akt Pathway: Another important signaling pathway that leptin activates is the "Phosphoinositide 3-kinase (PI3K)" and "Protein kinase B (Akt)" pathway. When the leptin receptor is activated, PI3K is called upon to produce PIP3, or phosphatidylinositol (3,4,5)-trisphosphate, which is an important lipid secondary messenger that triggers Akt activation to forward downstream signaling (Z. Liu et al., 2023). Particularly in peripheral organs like the liver and muscles, the PI3K-Akt pathway is essential for controlling insulin sensitivity and glucose absorption (Huang et al., 2018). Besides, it Contributes to thermogenesis and mitochondrial oxidation (J. Liu et al., 2018b). This pathway's activation promotes the

translocation of glucose transporter type 4 (GLUT4) to the cell membrane, which facilitates the absorption of glucose by cells (Świderska et al., 2020). Furthermore, by encouraging insulin-stimulated glucose elimination and preventing hepatic glucose synthesis, this signaling is crucial for regulating insulin sensitivity (Saltiel & Kahn, 2001).

The AMPK Pathway: Additionally, "AMP-activated protein kinase (AMPK)", an essential modulator of cellular energy balance, is activated by leptin (Hardie, 2018). By enhancing fatty acid oxidation and glucose absorption in skeletal muscle, leptin-induced AMPK activation improves energy balance and supports metabolic health (Minokoshi et al., 2002). Furthermore, leptin-mediated AMPK activation inhibits important gluconeogenic enzymes, which lowers the liver's synthesis of glucose (Steinberg & Kemp, 2009).

MAPK Pathway: Leptin signaling is also mediated via the "Mitogen-activated protein kinase (MAPK)" pathway (H.-K. Park & Ahima, 2014). Processes like cell division, proliferation, and survival are regulated by this route (Bardwell, 2006). Inhibition of ERK (extracellular signal-related kinase), a member of the MAPK family, inhibits leptin-induced sympathetic activation of brown adipose tissue, pointing towards the involvement of MAPK pathway leptin's control of food intake and energy expenditure (Rahmouni et al., 2009). In contrast to the JAK-STAT and PI3K-Akt pathways, the precise function of MAPK in glucose metabolism is less well characterized (Gehart et al., 2010).

1.6.5 Leptin Resistance

A condition known as leptin resistance (LR) occurs when the brain shows less sensitivity to leptin even when leptin levels are raised. Due to this disorder, leptin's capacity to control appetite and energy expenditure is compromised, which may result in an increase in food consumption and the development of issues connected to obesity (Izquierdo et al., 2019; J. Liu et al., 2018a). A number of factors, including decreased leptin receptor signaling pathways,

elevated SOCS3 expression, endoplasmic reticulum stress, and reduced leptin transport across the blood-brain barrier, are involved in leptin resistance (Friedman & Halaas, 1998), which is a major factor connecting obesity and type 2 diabetes.

JAK-STAT Inhibition: In leptin resistance, JAK-STAT pathway is compromised which can lead to increased appetite, decreased energy expenditure, and insulin resistance in the setting of T2DM (Xu et al., 2018). The inability of JAK2 kinase to activate downstream enzymes like STAT3 is a crucial characteristic of leptin resistance. This disrupts the transcription of genes that suppress appetite, known as anorexigenics. This leads to an increase in appetite and a decrease in energy expenditure, which in turn causes weight gain and insulin resistance, two major contributors of T2DM onset (Ghasemi et al., 2019).

PI3K/Akt pathway disruption: By interfering with the leptin receptor's regular function and downstream signaling cascades like the PI3K-Akt pathways, inflammatory cytokines impede leptin signaling. Insulin resistance in type 2 diabetes is associated with hyperphagia (competitive eating) and weight gain, which are both exacerbated by this interference that lowers the hypothalamic sensitivity to leptin (Acosta-Martinez & Cabail, 2022). Because of its function in this pathway, leptin can assist in maintaining insulin sensitivity. However, when leptin resistance occurs, which is frequently observed in obesity, this signaling is suppressed, which leads to insulin resistance and type 2 diabetes (Bjørbæk et al., 1997).

AMPK pathway disruption: Disruption of AMPK signaling can result in dysregulated glucose homeostasis and increased hepatic glucose output in especially under leptin resistance condition in T2DM (Minokoshi et al., 2002).

Increased SOCS3 expression: Chronic rise of leptin in obesity results in increased production of SOCS3 (suppressor of cytokine signaling 3, a negative regulator of leptin signaling), which phosphorylates JAK2 and STAT3. As a result, there is a suppression of the leptin signal, which

aggravates insulin resistance and leptin resistance (Mori et al., 2004). It has been demonstrated that SOCS3 overexpression in the hypothalamus affects insulin sensitivity and glucose homeostasis, two processes intimately related to the etiology of type 2 diabetes (Howard & Flier, 2006).

Endoplasmic reticulum (ER) stress: Due to the malfunctioning of the endoplasmic reticulum (ER), misfolded proteins can accumulate and cause the unfolded protein response (UPR), which leads to ER stress interfering with regular cellular processes (Baba et al., 2021). Obesity and leptin resistance are two metabolic conditions that are associated with this. Although the UPR aims to restore ER equilibrium, if stress continues, it may result in cell death (Fernandes-da-Silva et al., 2021). Leptin resistance is linked to persistent ER stress in which the hormone is unable to efficiently activate its signaling pathways (Preninka et al., 2022). Systemic leptin resistance can be further intensified by ER stress by upregulating SOCS3 and through a dysfunctional hypothalamic response to leptin which inhibits leptin signaling (Baba et al., 2021; Ozcan et al., 2009).

Reduced leptin transport: Reduced leptin transport across the blood-brain barrier (BBB) is seen in leptin-resistant people, which may be linked to impaired hypothalamic signaling, hyperphagia, and decreased energy expenditure. According to W. Banks (2008), this process makes obesity, insulin resistance, and the development of type 2 diabetes worse.

1.7 *LEPR* gene

The class I cytokine receptor family member leptin receptor, which is essential for controlling hunger and energy balance, is encoded by the *LEPR* gene. The *LEPR* gene which is around 1.75 megabases long with 20 exons and 19 introns, is located on the short arm of the chromosome first (1p31) (Liefers et al., 2004; Paracchini et al., 2005). The N-terminal,

cytokine receptor homology (CRH) I, and fibronectin type III domains are the primary three domains that make up the molecular structure of the gene and are crucial to its activity (Peelman et al., 2014; Tartaglia et al., 1995; Zabeau et al., 2004). The N-terminal domain is crucial for the receptor to engage with leptin, start the signaling cascade, and, also in ligand binding. In order to initiate downstream signaling pathways that control hunger and energy expenditure, the CRH domain helps the receptor communicate with intracellular pathways, which is essential for signal transduction (K. S. Park et al., 2006; Pérez-Montarelo et al., 2013). According to El Fessikh et al. (2023) and Wasim et al. (2016), the Fibronectin Type III domain is involved in receptor flexibility and stability, which affects the receptor's structural integrity and function.

1.7.1 *LEPR* gene polymorphisms

All of the physiologic actions of the leptin hormone are inhibited when there is a mutation in the leptin or its receptor gene. Individuals with this mutation experience delayed growth and puberty in addition to the onset of obesity. Notably, in several groups, common genetic variations (such as SNPs) in the *LEPR* gene locus have been linked to changes in leptin levels, obesity, hyperinsulinemia, and type 2 diabetic mellitus (T2DM). Obesity, insulin resistance, and diabetes in animals (Zhang et al., 1994) and, in rare instances, morbid obesity and "hyperinsulinemia" in humans (Montague et al., 1997) are caused by mutations in the leptin gene that result in leptin insufficiency. Variations in the stability and flexibility of the receptor, which impact its signaling capacities, have been connected to obesity through polymorphisms including Lys109Arg, Gln223Arg, and Lys656Asn (Carrillo-VÃ¡zquez, 2013; El Fessikh et al., 2023). In multiple association studies, these three non-synonymous SNPs have been evaluated (Loos et al., 2006; K. S. Park et al., 2006; Rosmond et al., 2000; Takahashi-Yasuno et al., 2003; van Rossum et al., 2003). According to molecular dynamics simulations, these

SNPs may modify the structural integrity of the receptor, affecting how it interacts with leptin and perhaps resulting in disorders such as leptin resistance (Carrillo-VÃ¡zquez, 2013; Da Silva et al., 2023).

1.7.2 Polymorphism of *LEPR* gene rs1137100

The single-nucleotide polymorphism in rs1137100 is the substitution of adenine (A) for guanine (G)in the gene's nucleotide 326 (326A>G), which results in the substitution of lysine (K) for the amino acid arginine (R) in the protein receptor's codon 109 (Lys109Arg) in exon 4. The capacity of leptin to bind to the receptor may be impaired due to the substitution of arginine for lysine in the extracellular portion of the receptor (Mărginean et al., 2016). The N-terminal and CRH I domains of the LEPR protein undergo substantial alterations due to the Lys109Arg (K109R) mutation, which results in decreased stability and increased flexibility of the protein. According to in silico investigations, this substitution breaks hydrophobic and hydrogen bonding interactions, which may affect receptor function (El Fessikh et al., 2023). Variations in fat distribution and cholesterol metabolism have been noted in persons with this polymorphism, despite the fact that it is not directly linked to non-alcoholic fatty liver disease (Chen et al., 2006).

1.7.3 Polymorphism of *LEPR* gene rs1137101

On the other hand, the SNP point in rs1137101 is the substitution of adenine (A) for guanine (G) in nucleotide 668 of the gene (668A>G), which results in the substitution of glutamine (Q) for arginine (R) in the codon 223 of the protein receptor (Gln223Arg) in exon 6. Defects in leptin binding and signaling may result from this amino acid alteration in the receptor's extracellular domain ("An Integrated Map of Genetic Variation from 1,092 Human Genomes," 2012; Peelman et al., 2004; Tartaglia et al., 1995). The leptin receptor's conformation modifications as a result of the Gln223Arg (Q223R) mutation impair its signaling properties.

According to molecular dynamics research, the Arg variant modifies hydrogen bonding patterns and enhances receptor flexibility, potentially disrupting leptin signaling pathways (Daghestani et al., 2019; El Fessikh et al., 2023). Higher risks of type 2 diabetes and obesity are associated with the Arg allele (Daghestani et al., 2019; SV, 2018).

1.7.4 Role of LEPR rs1137100 and rs1137101 in T2DM susceptibility

Numerous individual genetic association studies on polymorphisms located in the *LEPR* gene in various ethnic communities have been carried out by various research groups in order to assess the possible implications of *LEPR*'s molecular variations in T2D risk (Y. Yang & Niu, 2018). These research findings, however, are debatable and unclear. Many obese people do not have the homozygote genotype of the rare LEPR polymorphism (Klok et al., 2007; Komşu-Ornek et al., 2012). Nonetheless, only two missense single nucleotide polymorphisms (SNPs) were extensively studied for their roles in T2DM risk: Q223R (rs1137101) and K109R (rs1137100) where for each SNP, a sufficient number of single studies (i.e., > 5) were attained (Y. Yang & Niu, 2018).

Studies conducted in South India and Iraq have shown a strong correlation between the rs1137100 polymorphism and T2DM susceptibility, suggesting the association of the variant with an increased risk of T2DM in a number of groups (Amhe et al., 2022; Veerabathiran et al., 2023). Obesity, one known risk factor for type 2 diabetes was linked to the rs1137100 variant in a case-control study involving adolescents (Kochetova et al., 2022). The *LEPR* gene variation rs1137100 was shown to correspond with elevated inflammatory markers in young people with metabolically unhealthy obesity, indicating a possible link to the severity of type 2 diabetes (Abaturov & Nikulina, 2021).

The significance of genetic background in disease susceptibility was highlighted by the discovery that the RR genotype in case of rs1137101 polymorphism considerably increased the

incidence of type 2 diabetes in a study of the Slavonic population (Hussein & Hassan, 2017). Higher insulin resistance is shown by higher homeostatic model assessment-insulin resistance (HOMA-IR) scores in those with the Arg allele in the *LEPR* gene variation rs1137101 (Ashraf et al., 2022). A meta-analysis demonstrated a substantial correlation of the rs1137101 variant with the risk of T2DM (M. M. Yang et al., 2016). Another research conducted in Sri Lankan populations validated the positive relationship between the rs1137101 polymorphism and obesity (Illangasekera et al., 2020).

1.8 Complications of T2DM

1.8.1 Acute complications

A number of acute complications that youth with type 2 diabetes (T2DM) suffer can have a serious negative influence on their health. The three most prominent side effects are severe hypoglycemia, hyperglycemic hyperosmolar state (HHS), and diabetic ketoacidosis (DKA).

Diabetic Ketoacidosis (DKA): High blood sugar and ketone production are hallmarks of DKA, a dangerous disease that affects around 29% of young people with type 2 diabetes at the time of diagnosis (Rewers, 2018). Studies show that isolated DKA is present in 58% of young people with type 2 diabetes who are hospitalized for hyperglycemic crises; DKA may also be worsened by severe hyperglycemia or hyperosmolality in other individuals (Schmitt et al., 2020). This makes DKA particularly alarming. According to Schmitt et al. (2020), acute kidney damage (AKI) is also common in these patients, particularly in those with high hyperglycemia.

Hyperglycemic Hyperosmolar State (HHS): Although less prevalent, HHS can happen to young people with type 2 diabetes and frequently results in severe dehydration and electrolyte abnormalities. Because of HHS's high case fatality rates, awareness of the organization has grown (Rewers, 2018).

Severe Hypoglycemia: According to Rewers (2018), it is the reason behind a sizable portion of hospital admissions for diabetes patients. Patients who aspire for lower A1c objectives without the necessary education are more susceptible to severe hypoglycemia, which can result in coma or seizures (Rewers, 2018).

Other consequences include cardiovascular problems, which are becoming major dangers since T2DM in young people progresses so quickly (Kehler et al., 2015). To avoid serious consequences and hospital admissions, these acute problems require close observation and care (Rewers, 2018).

1.8.2 Chronic complications

In general, the chronic complications related to T2DM, mostly caused by inflammation, insulin resistance, and prolonged hyperglycemia, can be divided into microvascular and macrovascular problems. According to Ong (2022), microvascular complications including diabetic neuropathy, diabetic nephropathy, and diabetic retinopathy are developed in 45.6%, 33.7%, and 20.7% of patients respectively. Among the macrovascular complications, 75% percent of T2DM patients die from cardiovascular disease (Kumar S et al., 2018), 29.9% of individuals have diabetic foot, which might have serious consequences including amputation and, 27.8% of patients have coronary heart disease (Ong, 2022).

Hypertension and Dyslipidemia: According to "Long-Term Complications in Youth-Onset Type 2 Diabetes" (2021), the cumulative incidence of hypertension in young people with type 2 diabetes is 67.5% whereas 51.6% of impacted kids have dyslipidemia, which increases cardiovascular risks.

Diabetic kidney disease: The incidence of diabetic kidney disease is 54.8% ("Long-Term Complications in Youth-Onset Type 2 Diabetes," 2021), which emphasizes the danger of problems related to the kidneys that can lead to renal failure (Ong, 2022).

Neuropathy and Retinopathy: 32.4% of children suffer from neuropathy, and over time, the prevalence of retinal illness rose from 13.7% to 51.0% ("Long-Term Complications in Youth-Onset Type 2 Diabetes," 2021) which causes discomfort and increases their risk of foot ulcers and visual loss (Ong, 2022).

The long-term effects of young T2DM, such as an increased risk of cardiovascular illnesses and other chronic disorders that may result from early-onset diabetes, must also be taken into account, even though immediate consequences are crucial (Kehler et al., 2015). These complications are largely caused by factors including obesity, hyperglycemia, and minority race (Lascar et al., 2018). Despite these results, others contend that lifestyle changes and early intervention can reduce these risks, highlighting the significance of proactive care in young people with diabetes (Martinez & Zahra, 2022).

1.9 Pilot Study: An Exploratory Phase

Pilot studies are exploratory analyses intended to evaluate a research project's viability, budget, schedule, and unfavorable outcomes. Unlike full-scale investigations, which require stabilized processes, pilot studies provide trial-and-error adjustments without compromising data integrity (Reynolds, 2023). On the basis of the outcomes of a pilot study, full-scale research with refined methodologies is conducted by entailing larger populations and thorough data collection (Ying & Ehrhardt, 2023).

Smaller sample sizes, feasibility testing, preliminary data collection, and project design modifications based on preliminary findings are all key characteristics of a pilot project (Ying & Ehrhardt, 2023). Because pilot studies concentrate on information density and operational factors rather than formal statistical power calculations, they usually have fewer participants (Kunselman, 2024; Reynolds, 2023). To guarantee comprehensive planning for larger trials, researchers may devote up to 20% of their resources on pilot studies (Reynolds, 2023).

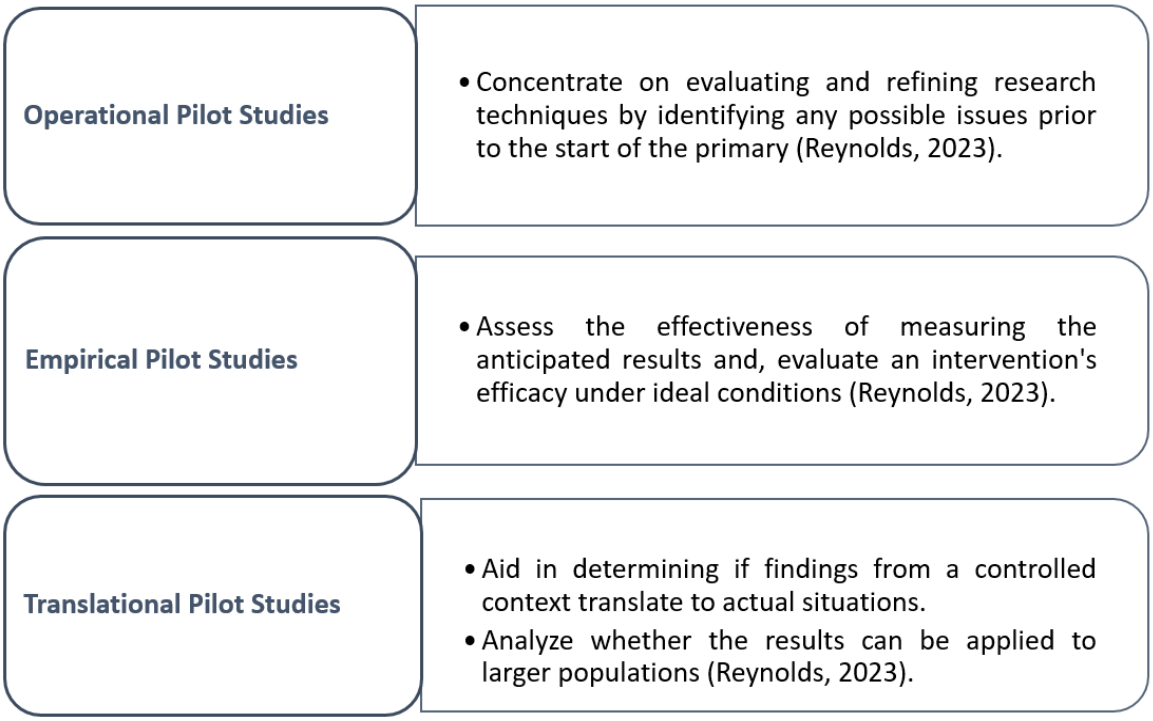

Figure 1.1: Types of pilot studies

This current study is an operational type of pilot study as it focuses on calculating the required sample size which is a critical operational aspect to ensure the feasibility of large-scale research as well as on optimizing and refining the study design and addressing potential issues prior to the main research.

Table 1.3: Advantages and Limitations of a Pilot Study

Category	Advantages / Limitations	References
Advantages of Pilot Study	**Feasibility Testing:** Pilot studies enable researchers to assess the viability of data collection techniques and recruiting tactics, guaranteeing the successful completion of the primary study.	(Silman et al., 2018)
	Methodological Refinement: They improve the quality of data obtained by standardizing methods and addressing potential problems throughout the primary study.	(In, 2017; Reynolds, 2023)

	Ethical Considerations: Before the main study starts, a pilot study can help identify ethical issues, especially in sensitive groups, and allow for necessary revisions.	(Sellers et al., 2023)
	Resource Optimization: Pilot studies can reduce resource waste and increase the chance of grant success by spotting possible issues early.	(Silman et al., 2018)
Limitations of Pilot Study	**Limited Generalizability:** Because of their limited sample numbers, pilot study results might not necessarily be generalizable to broader groups.	(Reynolds, 2023)
	Potential Bias: Due to the small scale, biases may arise that could influence the main study's findings.	(Hazzi & Maldaon, 2015)
	Underestimation of Challenges: Despite pilot testing, some problems could not become apparent until the entire study is underway.	(In, 2017)

Since pilot studies offer early insights that can improve the entire research framework, they are essential to the design of genetic association studies.

1.10 Addressing the knowledge gap

Although several studies have explored the connection between *LEPR* gene polymorphisms and Type 2 Diabetes Mellitus (T2DM) in various populations worldwide, there is limited research on the specific role of rs1137100 (Lys109Arg) and rs1137101 (Gln223Arg) in youth-onset T2DM. To the best of our knowledge, no research in Bangladesh has yet explored the association of the *LEPR* gene polymorphisms rs1137100 and rs1137101 with the risk of type 2 diabetes, particularly in younger age groups. The majority of the research that is currently accessible has been either on elderly people or on different ethnic groups, leaving a knowledge gap on how these polymorphisms contribute to early-onset type 2 diabetes in this population. Assessing this correlation may yield important information on the genetic variables affecting

the risk of type 2 diabetes in younger Bangladeshis, a population for which early detection and treatment are especially important. In light of this situation, the current observational cross-sectional pilot study has been designed to primarily investigate the role of two of the SNPs of the *LEPR* gene [rs1137100 (326A>G) & 1137101 (668A>G)] in T2DM in young Bangladeshi individuals, aged less than 30 years and to determine the minimum number of participants needed to conduct a full-scale genetic association study.

1.11 Objectives of the study

1.11.1 General Objectives

To assess the feasibility and generate primary data for larger-scale studies exploring the association of *LEPR* (rs1137100 & rs1137101) with T2DM in a sample of the young Bangladeshi population.

1.11.2 Specific Objectives

- To calculate the required sample sizes of both rs1137100 and rs1137101 for future full-scale research.
- To determine the frequency of *LEPR* gene variants (rs1137100 and rs1137101) in young Type 2 diabetic and non-diabetic Groups.
- To analyze the relationship between *LEPR* polymorphisms (rs1137100 and rs1137101) and clinical-demographic parameters in young individuals with T2DM.
- To explore *LEPR* gene variants (rs1137100 and rs1137101) as risk markers for early-onset Type 2 Diabetes Mellitus.

1.11.3 Additional Objectives

- To compare anthropometric and metabolic parameters between subgroups in both T2DM and NGT.
- To compare rs1137100 and rs1137101genotype between T2DM and NGT.

Chapter 2

Materials and Methods

The study aimed to investigate the association of the *LEPR* gene (rs1137100 and rs1137101) single nucleotide polymorphisms with type 2 Diabetes Mellitus (T2DM) in a sample of young Bangladeshi population. The following section details the methods and materials used in this research.

2.1 Place of study

The study was carried out at the Laboratory of Molecular Biotechnology Division, National Institute of Biotechnology (NIB), Dhaka -1349, a specialized institute under the Ministry of Science and Technology, Bangladesh.

2.2 Study Design

This case-control pilot study was conducted from October 2023 to April 2024. In total, blood specimens from 56 young DM patients and normal glucose tolerance (NGT) individuals ranging from 18-29 years were collected which were later on used for molecular analysis purposes.

The two diagrams below represent the overall study procedure adopted for this research.

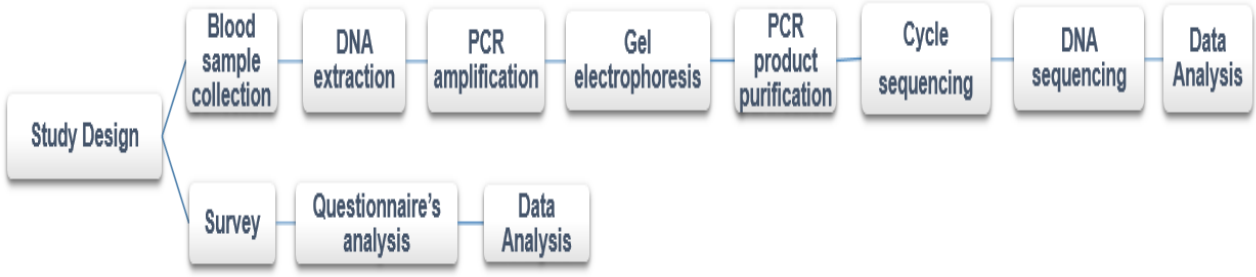

Figure 2.1: An overview of the steps followed to identify the association between the *LEPR* gene (rs1137100) SNP and young type-2 DM.

Figure 2.2**:** An overview of the steps followed to identify the association between the *LEPR* gene (rs1137101) SNP and young type-2 DM.

2.3 Population and sample

In this study, the sample collection type was purposive sampling and patients who gave their informed consent were included. The study's eligibility criteria required the participants to exhibit indications of T2DM. Glycemia, an essential measure for diagnosing and managing diabetes and other metabolic disorders, refers to the concentration of glucose in the bloodstream ("Tests of Glycemia in Diabetes," 2004; News-Medical, 2023). Based on glycemic status which is measured through the oral glucose tolerance test (OGTT), patients can be classified into 3 categories: NGT, prediabetes, and DM (Association, 2019). To create clear class distinctions in the research following ADA 2019 criteria, NGT and DM were included as controls and cases, respectively, while excluding prediabetes.

2.3.1 Criteria for Control Group (NGT)

The control group was selected when the following criteria were met: individuals with normal glucose tolerance as determined by the standard Oral Glucose Tolerance Test (OGTT), age range similar to the case group, and willingness to participate in the study.

2.3.2 Inclusion criteria for cases

Individuals diagnosed with type 2 diabetes mellitus according to the 2019 criteria set by the American Diabetes Association (ADA), including both new and existing cases, whether treated or untreated were recruited. Participants were both males and females aged 18 to under 30 years.

2.3.3 Exclusion criteria for cases

The study excluded patients with proven type 1 diabetes mellitus, gestational diabetes or diabetes in pregnancy, drug-induced diabetes, or diabetes secondary to other diseases. Patients with acute critical illness or infection within the past month, as well as, those with pre-existing chronic medical conditions such as cardiac, hepatic, renal, or thyroid disorders, chronic infections, or malignancy, and participants who declined to participate were not included.

2.3.4 Grouping

The research subjects were divided into two groups: **1. Case** (18 individuals with diabetes mellitus); **2. Control** (38 individuals with normal glucose tolerance).

2.4 Data Collection

Clinical and biochemical data along with general information about all subjects were meticulously gathered through a survey and physical examination conducted by the 'Young Diabetes Clinic' of the Department of Endocrinology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. Later on, the data were collected from the rightful authorities in order to use in this study.

2.4.1 Survey

A well-structured questionnaire was used to obtain some basic data, including name, age, gender, contact address, contact number, height, weight, drug history, family history of diabetes, prior history of T2DM, etc.

2.4.2 Clinical data

Clinical data recorded through physical examination included systolic blood pressure, diastolic blood pressure, body mass index, waist, hip, waist-hip ratio, plasma glucose level, and fasting insulin assessments.

2.4.3 Biochemical data

Biochemical data comprised of OGTT test results. The glucose tolerance test, sometimes referred to as the oral glucose tolerance test or OGTT, is a common screening tool for all types of diabetes including T2DM which measures the body's response to sugar or glucose (*Glucose Tolerance Test - Mayo Clinic*, n.d.).

2.4.3.1 75 g OGTT test procedure

The participant was instructed to measure their blood sugar level after fasting for at least eight hours to prepare for the test. The individual was then permitted to consume around 75 grams of sugar syrup solution. At last, two hours after drinking the solution, the glucose level was measured again (*Glucose Tolerance Test - Mayo Clinic*, n.d.).

In **Table 2.1**, the criteria for diagnosis of NGT and T2DM have been presented (Association, 2019; *Diabetes - Diagnosis and Treatment - Mayo Clinic*, n.d.). Based on the specific criteria during the OGTT test, study subjects were grouped into control and case.

Table 2.1: The criteria for diagnosis of NGT and T2DM

Plasma glucose criteria	Plasma glucose concentration (mmol/L)	
	NGT	T2DM
Fasting plasma glucose	< 5.6 mmol/L	≥ 7.0 mmol/L
2-hour plasma glucose	< 7.8 mmol/L	≥ 11.1 mmol/L

Here, NGT= Normal glucose tolerance and T2DM= Type 2 diabetes mellitus.

2.5 Sample Collection

Blood samples of the research subjects were collected at the Department of Endocrinology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka. After that, the samples were preserved and transported to the National Institute of Biotechnology for molecular analysis.

2.5.1 Blood collection

A total of 56 blood samples were collected for SNP genotyping. A 3 ml syringe was used to aseptically draw about 2 mL of blood from each participant, which was quickly transferred to K2EDTA tubes. The name, identification number, and collection date of each patient were written on the labels of the tubes.

2.5.2 Preservation

Soon after the specimen collection, tubes were kept in the vertical position at room temperature for 15-20 minutes, followed by preservation at -70◦C refrigerator in Dept. of Endocrinology, BSMMU. At a later time, specimens were transported to the molecular laboratory of NIB in a cold box with dry ice via an air-conditioned vehicle. For a further extended period of preservation before DNA extraction, blood samples were frozen at -20°C or lower temperature in the lab.

2.6 DNA extraction from blood samples using Monarch® Genomic DNA Purification Kit

At first, genomic DNA (gDNA) from the collected blood samples needed to be isolated to genotype SNPs. Commercial DNA extraction kit **Monarch® Genomic DNA Purification Kit (NEB #T3010S)** was used for this purpose. Lysis of the blood cells, removal of RNA and extraction of intact gDNA from the blood cells were performed following the protocols of the kit.

2.6.1 Kit components

The kit contained Monarch gDNA Purification Columns, Monarch Collection Tubes II, Monarch gDNA Tissue Lysis Buffer, Cell Lysis Buffer, Blood Lysis Buffer, Binding Buffer, Wash Buffer, Elution Buffer, Monarch RNase A, Proteinase K (Molecular Biology Grade).

2.6.2 Preparation of working buffers

Prior to first-time use, ethanol (≥ 95%) was added as indicated on the bottle of gDNA Wash Buffer to obtain a working solution and mixed well by inverting it ten times.

2.6.3 Protocol

By following the standard protocol of **Monarch® Genomic DNA Purification Kit**, gDNA from blood samples were isolated. The step-by-step procedure of gDNA purification process from mammalian whole blood consisting of two stages is described below:

Part 1: Sample Lysis

1) 100µl of blood was transferred to a 1.5ml microfuge tube.
2) 10µl Proteinase K, 3µl RNase, and 100µl Blood Lysis buffer were added and mixed immediately by vortexing.
3) Then, incubated for 5 minutes at 56°C in a thermal mixer with agitation at full speed (~1400 rpm).

Part 2: gDNA Binding and Elution

4) 400 µl gDNA Binding Buffer was added to the sample and mixed thoroughly by pulse-vortexing for 5-10 seconds.
5) The lysate/binding buffer mix (~600 µl) was transferred to a gDNA Purification Column which was pre-inserted into a collection tube, without touching the upper column area.

6) After that, the cap was closed and centrifugation for 2times was done: first for 3 minutes at 1,000 x g to bind gDNA (did not need to empty the collection tubes or remove from centrifuge) and then for 1 minute at maximum speed (> 12,000 x g) to clear the membrane.
7) The flow-through and the collection tube were discarded.
8) The column was then transferred to a new collection tube.
9) 500 µl gDNA Wash Buffer was added and the cap was closed and the collection tube was inverted a few times so that the wash buffer reached the cap.
10) Immediate centrifugation for 1 minute at maximum speed (16000 x g) was done and the flow through was discarded.
11) Then, the column was reinserted into the collection tube,
12) After which, 500 µl gDNA Wash Buffer was added again and the cap was closed.
13) Centrifugation for 1 minute at maximum speed (16000 x g) was done immediately and afterward the collection tube and flow through were discarded.
14) Subsequently, the gDNA Purification Column was placed in a DNase-free 1.5 ml microfuge tube.
15) 100µl preheated (60°C) gDNA Elution Buffer was added, the cap was closed and was incubated at room temperature for 1 minute.
16) The tube was centrifuged for 1 minute at maximum speed (16,000 x g) to elute the gDNA.
17) Finally, the supernatant was discarded, the tube with pellet was kept and the purified gDNA was stored at -20°C.

2.6.4 Precautions

Throughout the whole process of extracting genomic DNA, all the directions were followed carefully as indicated, yet certain crucial precautions and maintenance were taken.

1) All the kit components were kept at room temperature before use.
2) Based on the starting material (either tissue/cultured cell/blood) the specific lysis buffer was used.
3) To avoid any contamination, a sterile environment including pipette tips and micro-centrifuge tubes was maintained and ensured to be DNase/RNase-free during the handling of DNA.
4) While handling all components, a laboratory coat and a pair of gloves were worn to protect us from the reagents and kit components, as well as to prevent nucleic acid degradation by nuclease that might be present on human skin.
5) To minimize DNA degradation, repeated freezing and thawing of DNA samples were avoided.
6) Precisely maintained the recommended volumes of enzymes and buffers that were used.
7) During pipetting, air bubble formation was avoided cautiously.
8) All vortex steps were maintained at maximum speed to ensure thorough mixing.
9) Other kit components were kept at room temperature (15-25°C) while Proteinase K and RNase A solutions were stored at -20°C.
10) The Lysis buffer and Wash Buffer were checked and slightly shaken before each use,
11) Reuse of "used" tips was strictly avoided.

2.7 DNA Concentration and Purification Determination using NanoDrop Spectrophotometer™

To increase the chances of successful downstream applications, it is crucial to determine the concentration and purity of DNA (Boesenberg-Smith et al., 2012) . Thus, the concentration and purity of each isolated gDNA sample were determined according to the **Thermo Scientific NanoDrop™ 2000/2000c Spectrophotometer manual** after the isolation process and before proceeding with the PCR.

Process:

1st step: The computer is linked to the Thermo Fisher NanoDrop SpectrophotometerTM was turned on, the Nanodrop 2000/2000C software was launched and the 'nucleic acid' option was selected.

2nd step: To make sure there were no impurities left, the spectrophotometer's upper and lower pedestal sections were carefully cleaned using lint-free laboratory tissues and ultra-pure water.

3rd step: Initially, a blank cycle with an aliquot of the blanking buffer was run as though it were a sample to make sure the instrument was operating properly and that the dried-down sample on the pedestal was not a problem. For the blanking cycle,

- A 2 µl aliquot of elution buffer devoid of DNA samples was thereafter placed on the spectrophotometer's bottom pedestal.
- Following the placement of the blank solution, the sampling arm was cautiously lowered.
- **Blank** was clicked to measure and the reference spectrum was stored.
- After that, a dry lab wipe was used to clean the blank from both measuring pedestal surfaces.

4th step: Following blanking with elution buffer, 2 µl of DNA sample was deposited on the Nanodrop's bottom pedestal and the sampling arm was lowered carefully. Then, the 'Measure' option on the left side panel was clicked to measure DNA concentration (ng/µl) and purity (at absorbance ratio 260 nm/280 nm). Additionally, a graph displaying the concentration and purity of DNA was displayed.

5th step: The top and lower pedestals were thoroughly cleaned with ultra-pure water after measuring all the samples, the program was closed and, the computer was shut down.

2.8 Polymerase Chain Reaction (PCR) Amplification

In this study, the targeted regions for the *LEPR* gene (rs1137100 and rs1137101) of each extracted DNA sample were amplified through the conventional PCR process using the Applied Biosystems ProFlex PCR System.

2.8.1 Primer properties for *LEPR* gene

In this study, one pair of primers (forward and reverse) for each SNP of *LEPR* gene were used (**Table 2.2**).

Table 2.2: Primers used for PCR amplification

SNP	Primer	Primer Sequence (5'→ 3')	PCR product	Annealing Tm (°C)
rs 1137100 (+326 A>G)	Forward L326F	5`CTTTTGCCTGCTGGACTCTC 3`	217 bp	58
	Reverse L326R	5`AAACTAAAGAATTTACTGTTGAAACAAATGTC 3`		
rs 1137101 (+668 A>G)	Forward L668F	5`AAACTAAAGAATTTACTGTTGAAACAAATGTC 3`	330 bp	57
	Reverse L668R	5`CAATATTTATGGGCTGAACTGACATT 3`		

Here, SNP = Single nucleotide polymorphism; bp = base pairs; Tm = Temperature.

2.8.2 Primer Dilution

The necessary volume of nuclease-free water was added to the concentrated forward and reverse primers to dilute them and make a working solution to use during PCR. For each 200µl working primer solution, 180 µl nuclease-free water and 20 µl stock primer were mixed. Similarly, 90µl nuclease-free water and 10µl stock primer were combined to create each 100µl working primer solution.

Steps:

1) Primers, nuclease-free water, eppendorf tubes, and pipets were taken in the laminar hood.

2) The workbench and equipment were cleaned with 70% ethanol prior to starting dilution.
3) Eppendorf tubes were labeled.
4) Nuclease-free water (either 180µl or, 90µl) was transferred into each eppendorf tube.
5) Each primer was transferred into each eppendorf tube in either 20µl or 10µl amount.
6) Then, mixed through pipetting after transferring every time.
7) Vortex and spin was done.
8) Lastly, the diluted working primer solution is stored at -20°C.

Precautions:

1) The UV (Ultraviolet) light of the laminar hood was turned on 15 minutes before working.
2) Pipetting was done 2 times after each transfer to ensure better mixing.

2.8.3 Protocol of PCR

Using two different primer pairs (one pair for each SNP), two targeted sequences were amplified employing different annealing temperatures. Single amplicon reactions were carried out in 12.5µL or 25µL volumes where for each reaction 11µL or 23.5µL of PCR cocktail was prepared as demonstrated in table 2.3 below with 1.5 µL of DNA. DNA amplification conditions were: initial denaturation at 95 °C for 5 minutes as a hot start, followed by 35 cycles of denaturation at 95 °C for 60 seconds, annealing at suitable annealing temperature for 60 seconds, extension at 72 °C for 60 seconds, and the final extension stage was at 72 °C for 8 minutes (Figure 2.3 and Figure 2.4).

Step 1: Preparation of PCR reaction mix

a) At first, the isolated DNA samples were taken out from the refrigerator and kept on ice for thawing. Meanwhile, the PCR working mix was prepared.

- In a 1.5ml Eppendorf tube, the total required volume of GoTaq® Green Master Mix,2X (ready-to-use premixed solution containing *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers); Forward primer; Reverse primer; Nuclease-free water was taken according to the calculations based on the DNA samples no. to be copied by PCR. A gentle spin was done.
- Then, either 11µL or 23.5µL was taken into each PCR tube (labeled with each sample no).

b) 1.5µL DNA was added into each respective tube and mixed through pipetting, followed by tapping and spinning.

Table 2.3: Preparation of PCR reaction mix

Components	Measurement for 1 sample (12.5µL)	Measurement for 1 sample (25µL)
GoTaq® Green Master Mix, 2X	6.25	12.5
Primer (forward)	1	2
Primer (reverse)	1	2
Nuclease free H2O	2.75	7
DNA	1.5	1.5

Step 2: PCR cycling conditions

a) The PCR tubes were assembled in the rows of the **ProFlex PCR System**.

b) The PCR conditions for specific sequence amplification was set and run. It took about 1 hour 38 minutes to complete the amplification.

Figure 2.3: PCR cycling program to amplify *LEPR* rs1137100 sequence.

Figure 2.4: PCR cycling program to amplify *LEPR* rs1137101 sequence.

2.8.4 Precautions

1) All the laboratory glassware and equipment for conducting this work were autoclaved at 121°C at 15 psi for 15 minutes before using it.
2) The sterile surface was maintained, and hand gloves and the laboratory coat were worn.
3) Pipetting was done cautiously to avoid bubble formation.

2.9 Agarose Gel Electrophoresis

To check whether the desired bands were successfully achieved during PCR or not, the resultant PCR product was run on a 2% agarose gel, and the length of the PCR product was determined using a 50 bp DNA Ladder (NEB # N3236L).

2.9.1 2% Agarose gel preparation

For agarose gel electrophoresis, 2% agarose gel was prepared by mixing agarose with 1X TBE (Tris-borate EDTA) buffer. To visualize the amplified DNA band under ultraviolet light, 5% SYBRT™ Safe was added to the solution. Depending on the size of the tray, the amounts of the components varied as shown in table 2.3.

Table 2.4: Preparation of Agarose gel for visualization

Components	Measurement for small tray (5 x 8.3 cm)	Measurement for large tray (10.5 x 8.3 cm)
TBE buffer	20 ml	35 ml
Agarose	0.4 gm	0.7 gm
SYBRT™ Safe	2 µL	3.5 µL

For casting the gel the following steps were followed:

1) Initially, electrophoresis buffer (1x TBE buffer) was added to the flask.
2) Then, agarose powder was weighed out and placed into a 50ml conical flask.
3) The flask was then placed into a microwave oven and the timer was set for 1 min.
4) The flask was taken out from the microwave oven by holding it carefully with tissue or cotton.
5) When the agarose solution was cooled a bit, SYBRT™ Safe was added and mixed by gently whirling the flask.

2.9.2 Gel electrophoresis method

1) At first, the comb was placed into the appropriate groove or slot of the tray.
2) Then the melted agarose was poured onto the tray.
3) The gel was allowed to solidify at room temperature for 20-30 min.

4) The comb was removed carefully from the solidified gel.
5) The casting dams were removed from the edges of the gel tray.
6) The tray was placed by keeping the gel horizontal onto the base of the electrophoresis chamber so that the sample wells were near the cathode (negative end generally marked as black).
7) About 3 µL samples were loaded carefully in each well of the gel.
8) A molecular weight marker DNA (50bp DNA ladder) was loaded on the other well of the gel.
9) A sufficient amount of 1xTBE buffer (about 600 ml) was added to cover the depth of the chamber.
10) Electrophoresis was carried out at 80 Volts for about 40 minutes.
11) DNA sample would migrate towards the anode (positive end generally marked as red during electrophoresis).
12) The separation process was monitored by the migration of the dye in the loading buffer.
13) When the SYBRT™ Safe dye had reached about three-fourths (3/4) of the gel length, the electrophoresis was completed and stopped.

2.9.3 Documentation of DNA sample

For clearer visualization and storage to use in future analysis, the gel image was checked in a gel documentation machine.

1) Upon completion of the electrophoresis, the gel was taken out carefully from the electrophoresis chamber.
2) After that, the gel was placed *on the* gel *documentation* imaging system **Azure** 200 (Azure Biosystems).
3) The gel picture where the DNA was observed as bands was photographed and saved.

2.9.4 Precautions

1) Casting dams were removed carefully from the gel casting tray so that the gel would not slide off.
2) The volume of the electrophoresis buffer maintained precisely below the maximum buffer mark on the electrophoresis system.

2.10 Restriction Fragment Length Polymorphism (RFLP)

For the SNP Genotyping of the *LEPR* (rs1137101) gene, restriction digestion was performed according to the standard RFLP method. 7µl PCR products of this specific gene were treated with 0.5µl restriction enzyme **MspI** (New England Biolabs, USA, Cat no: R0106S**)** to carry out the digestion.

2.10.1 Prior restriction reaction mixture preparation

All the components were taken out of the refrigerator and thawed on ice. Meanwhile, 0.5ml microfuge tubes were labeled.

2.10.2 Preparation of restriction digestion reaction mixture

Restriction digestion reaction mixture was prepared as follows to a final volume of 10.0µl each.

Table 2.5: Preparation of restriction digestion reaction mixture

Components	Measurement for 1 sample (10.0µl)
PCR product	7.0 µl
Reaction buffer	1.0 µl
Restriction Enzyme (MspI)	0.5 µl
Nuclease free H_2O	1.5 µl
Total Volume	10.0 µl

2.10.3 Incubation in water bath

After the reaction mixtures were prepared, they were kept for overnight (16 hours) incubation at 37°C in the Stuart SBS40 Shaking Water Bath.

2.10.4 Gel run and documentation

The next day when the incubation period was over, the tubes were taken out of the water bath and the whole 10µl digestion reaction mixture was run on 2% agarose gel, followed by capturing the image of the gel in the gel documentation machine for result confirmation.

2.10.5 Precautions

1) The restriction enzyme was thawed on ice for a minimal period.
2) The components altogether were mildly spinned down for a better mixture.
3) The water bath was turned on a while ago so that the temperature would reach the required degree by the time the reaction mixture was prepared.

2.11 DNA sequencing

In this study, to genotype the SNP of the *LEPR* (rs1137100) gene and to re-confirm the results of the *LEPR* (rs1137101) gene obtained previously via the RFLP method, cycle sequencing of PCR product, followed by DNA sequencing was conducted.

2.11.1 PCR Product Purification

Using the working manual of the ExoSAP-IT™ Express PCR Product Cleanup (as demonstrated in Figure 2.6), the PCR product for the specific gene was purified before sequencing procedures.

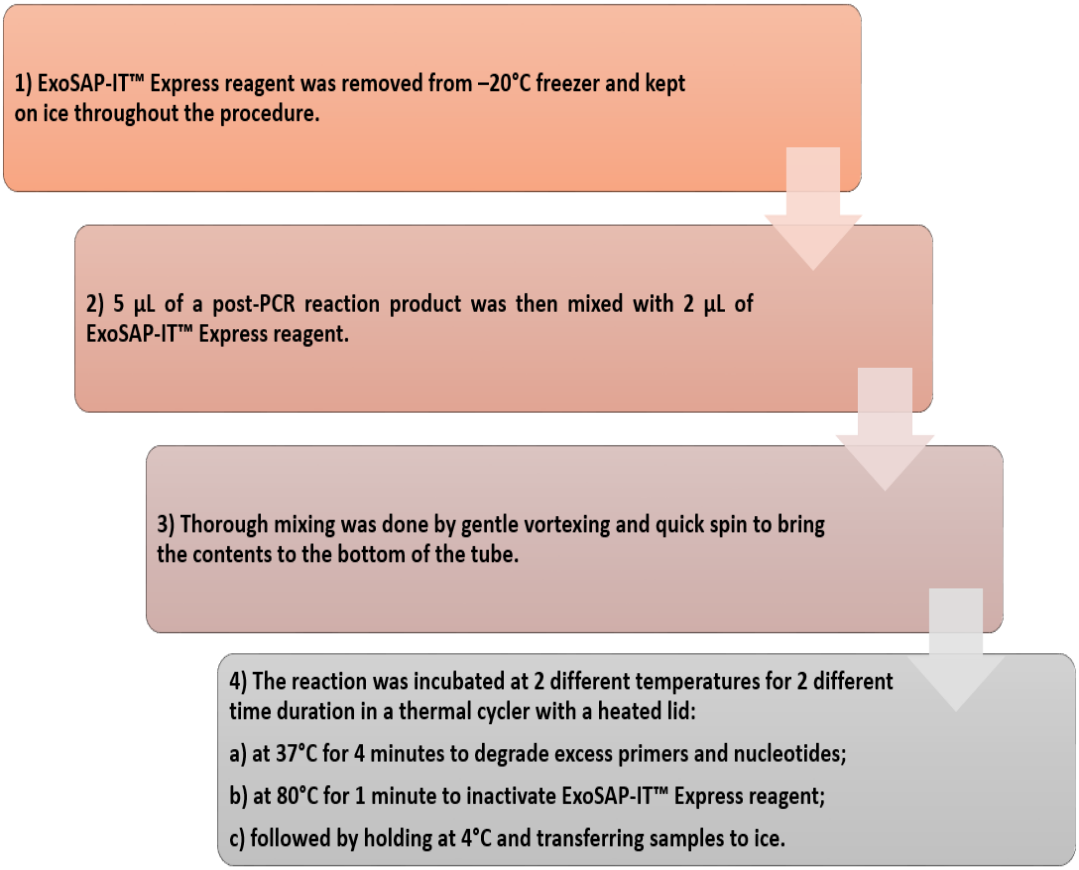

Figure 2.5: Diagram of ExoSAP-IT™ Express PCR Product Cleanup protocol

This purified PCR product was either stored at –20°C or used in cycle sequencing after measuring its concentration and purity.

2.11.2 Measurement of purified PCR products using Thermo Scientific NanoDrop™ 2000/2000c Spectrophotometer

A spectrophotometer™ was used to determine the DNA concentration present in each tube after purification. The standard DNA concentration for the next step of the molecular characterization was considered to be 9-10 nano-grams per µl (ng/ µl). In the Nanodrop, 2 µl elution buffer was used as blank. The blank was removed and 1 µl of each purified PCR product was loaded. The DNA concentration was measured in ng/µl units. Along with that, the OD of 260/280 ratio which indicated the purity of the sample, was measured with the integrated

software. After being used each time, the sample holder was sterilized before loading the next sample.

2.11.3 Cycle Sequencing

2.11.3.1 Preparation for Cycle Sequencing

Before performing cycle sequencing, the purified samples were diluted for them to be in the exact concentration. After diluting, the cycle sequencing reaction mixture was prepared using the BigDye Terminator v3.1 Cycle Sequencing Kit.

Table 2.6: Preparation of cycle sequencing reaction mixture

Components	Measurement for 1 sample (20.0µl)
BigDye Terminator v3.1 Ready reaction premix (RRP)	4.00 µl
BigDye Sequencing Buffer (5X)	2.00 µl
Template	2.00 µl
Primer (Forward/Reverse)	0.32 µl
Nuclease free H_2O	11.68 µl
Total Volume	20.00 µl

2.11.3.2 Cycle Sequencing Program

After reagent preparation for each sample, each tube was rotated to eliminate bubbles by using a rotator and finally, the samples were placed in the Applied Biosystems Veriti™ 96-Well Thermal Cycler for cycle sequencing. The thermal cycling profile included: (a) initial denaturation at 96°C for 1 minute (b) 25 cycles of cyclic denaturation at 94°C for 10 seconds, annealing at 58°C (for LEPR rs1137100) for 5 seconds, and extension at 60°C for 4 minutes, and (c) Holding at 4°C until ready for purification (Table 2.7).

Table 2.7: Cycle sequencing program for *LEPR* rs1137100

Steps	Temperature	Duration	Cycles
1	**96℃** (Initial denaturation)	1 min	1 cycle
2	**96℃** (Denaturation)	10 sec	25 cycles
	58℃ (Annealing)	5 sec	
	60℃ (Extension)	4 min	
3	**4℃** (Hold)	∞	∞

2.11.3.3 Post-Cycle Sequencing Purification

After cycle sequencing, the individual cycle sequencing PCR product was purified as per the purifying extension product protocol of **the BigDye® Terminator v3.1 Cycle Sequencing** Kit. Precipitation of each 20-µL cycle sequencing reaction mixture was performed through the Ethanol/EDTA/sodium acetate precipitation method in the following steps:

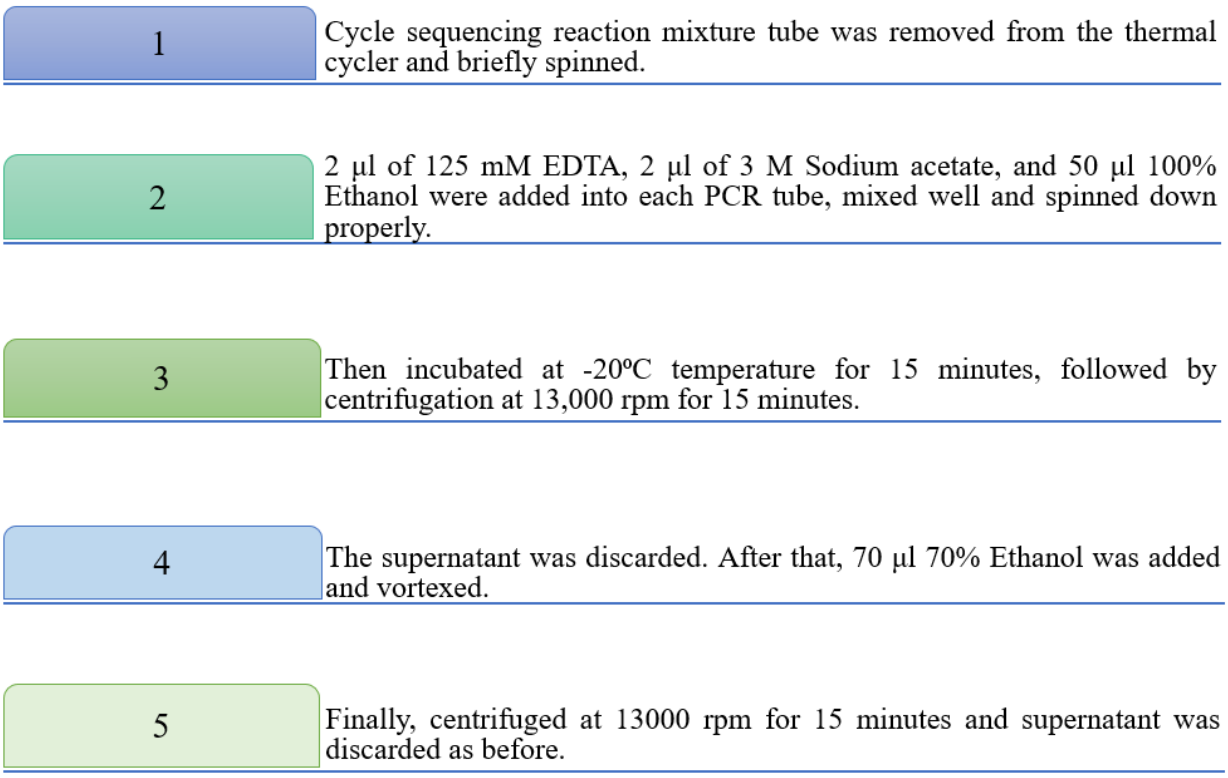

Figure 2.6**:** Post-cycle sequencing purification process.

2.11.4 Sequencing DNA sample using Applied Biosystems™ 3500 Genetic Analyzer

Before loading into the genetic analyzer, the purified samples of cycle sequencing went through a few preparatory steps mentioned below–

i. Samples were incubated at 95ºC for 5 minutes inside a thermocycler.
ii. Then, 10 µl Hi-Di™ Formamide was added to each tube containing individual samples.
iii. Denatured at 95°c for 5 minutes.
iv. Immediately after incubation, the samples were put into ice.

Subsequently, the samples were loaded into Applied Biosystems™ 3500 Genetic Analyzer following the manufacturer's instructions.

2.11.5 Sequence Analysis

It took up to 4 hours for each sample to get a sequence with the help of the 3500 Series Data Collection Software system of the machine.

2.11.5.1 Sequence correction

To begin with, the sequencing results were transferred to the personal laptop via pen drive. Raw sequence data was then converted to FASTA format by using the **UGENE software.** *This* software analyzed the quality of the raw sequence data. While checking, the base was selected and positioned in the upper direction in the UGENE **chromatogram** image to find out the more clarified result.

2.11.5.2 SNP Sequence Identification

1) The reference sequence of specific SNP (*LEPR*-rs1137100 gene) was selected from the NCBI (National Centre for Biotechnology Information) website by searching in the SNP category.

2) The reference sequence showed the flanking region. A few bases before this region were selected and copied.

3) The UGENE chromatogram image of each sample was opened. Ctrl+F was pressed which opened the search box and then the copied few base sequence was pasted in it.

4) If successful sequencing, the copied region was found out and marked.

5) The exact next base after the region was the targeted SNP base. Depending on the base, the genotype of the sample was decided.

2.12 Statistical Analysis

For qualitative values such as gender, allele, genotype, and haplotype, data were expressed in numbers (n) and percentages (%) whereas for quantitative values with normal distribution, data were expressed in mean (±SD). Numerical variables for instance age, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting plasma glucose, plasma glucose levels 1-h and 2-h after 75 gm glucose loads between T2DM and the control group were compared by using **Student's t-test** and, **Mann-Whitney U Test** when applicable. On the other hand, the frequency of SNPs between the DM and control groups was compared using the **Chi-square** (χ^2) test within different genetic models (dominant, recessive, co-dominant, and allelic). The independent segregation of alleles was tested for Hardy-Weinberg equilibrium comparing the observed vs expected genotype frequency using **Pearson's Chi-square (x^2) test** with a threshold of $P \geq 0.05$ in the case and controls separately. Risk factors of T2DM were

detected by **multivariate logistic regression**. The general association of genotypes with T2DM was assessed with multivariate logistic regression analysis under codominant, dominant, recessive, overdominant, and log additive models by using **SNPStats.** Risk estimation of SNPs was expressed by means of **OR** (odds ratio) with a 95% confidence interval.

2.13 Sample Size Calculation

Based on the preliminary result of this pilot study using 56 samples, the sample size of each group (case and control) for the full-scale study was calculated with an estimated *P* value of <0.05, 95% confidence interval (CI), and a power of 80 percent. The formula used for calculating the sample size is as follows (Charan & Biswas, 2013).

$$\boldsymbol{N = \frac{r+1}{r} \frac{(p^*)(1-p^*)\left(Z_\beta + Z_{\alpha/2}\right)^2}{(p_1 - p_2)^2}}$$

In this equation, Z_β = z-value of standard normal distribution for 80% power = 0.84; $Z_{\alpha/2}$ = z-value of standard normal distribution for 5% level of significance (P<0.05) = 1.96; r = Ratio of control to cases; p_1 = Proportion of risk allele in cases; p_2 = Proportion of risk allele in controls; $p_1 - p_2$ = The effect size or difference in risk allele frequencies between cases and controls; p^* = Average proportion of risk allele = $\frac{p_1 + p_2}{2}$.

CHAPTER 3

Results

3.1 Characteristics of the young T2DM and control subjects

Clinical and demographic characteristics of the 56 study subjects categorized into a control group having 38 individuals and a case group having 18 individuals are depicted in **Table 3.1**.

The mean age (T2DM vs. NGT: 26.44 ± 4.15 vs. 24.45 ± 3.68 years, *P*= 0.0248; mean±SD) and gender (T2DM vs. NGT: M:F = 37.5:62.5 vs. 50:50; *P* = 0.6009 was similar between the two groups). Subjects with T2DM showed higher height (157.47 ± 9.18 cm. vs. 156.67 ± 23.19 cm; *P*=0.2862), weight (68.22 ± 15.09 kg vs. 66.39 ± 23.39 kg; *P*=0.6149), body mass index (27.80 ± 5.94 kg/m^2 vs. 25.62 ± 5.31 kg/m^2; *P*=0.2163), hip circumference (99.82 ± 12.86 cm. vs. 98.54 ± 12.28 cm; *P*=0.9554), waist circumference (93.47±12.59 cm. vs. 86.32±16.41 cm; *P*=0.085) and, with statistically significant waist-hip ratio (0.91±0.15 vs. 0.89±0.08; *P*=0.0335) than NGT. Furthermore, systolic blood pressure (118.75±14.55 mmHg vs. 118.29±16.45 mm Hg; *P*=0.8692), and diastolic blood pressure (79.75±13.36 mmHg vs. 79.47±9.85 mmHg; *P*=0.9358) were marginally higher in T2DM group than NGT group.

According to **Table 3.1** lipid profile**,** all the lipid components were higher in T2DM than those of NGT (T2DM vs NGT; TC: 189.12± 52.61 vs. 174.29 ± 33.13 mg/dl, *P*=0.0276; TG: 205.39 ± 99.19 vs. 131.37 ± 64.58, *P*=0.0036; LDL: 123.53 ± 44.31 vs. 106.74 ± 27.52 mg/dl; *P*=0.0937) except HDL-C (36.78 ± 6.84 vs. 40.61 ± 7.78 mg/dl; *P*=0.0164) with significant difference between the two groups other than LDL-C. Similarly, the TG/HDL ratio was significantly higher in T2DM than in NGT (5.93 ± 2.95 vs 3.59 ± 2.16; *P*=0.0028).

The glycemic profile of the study subjects showed that subjects with T2DM had statistically significant higher fasting plasma glucose (10.34 ± 3.94 vs. 6.01±7.00 mmol/l; *P*<0.001), 2-

hour plasma glucose (16.57 ± 5.95 vs. 5.96±0.89 mmol/l) (mean±SD, *P*<0.001) following 75-gram oral glucose tolerance test (OGTT).

Table 3.1: Clinical-demographic characteristics of T2DM and control subjects

Variables	T2DM (n=18)	NGT (n=38)	*P* value
Age (years ±SD)	26.44 ± 4.15	24.45 ± 3.68	0.0248
Gender			
Male	6 (37.50%)	17 (50.00%)	0.6009
Female	10 (62.50%)	17 (50.00%)	
Family History of T2DM			
Present	9 (50.00%)	21 (55.26%)	0.9347
Absent	9 (50.00%)	17 (44.74%)	
Height (cm ±SD)	157.47 ± 9.18	156.67 ± 23.19	0.2862
Weight (Kg ±SD)	68.22 ± 15.09	66.39 ± 23.39	0.6149
BMI (Kg/m^2)	27.80 ± 5.94	25.62 ± 5.31	0.2163
Waist (cm)	93.47 ± 12.59	86.32 ± 16.41	0.0850
Hip (cm)	99.82 ± 12.86	98.54 ± 12.28	0.9554
Waist-hip ratio	0.91 ± 0.15	0.89 ± 0.08	0.0335
SBP (mmHg)	118.75 ± 14.55	118.29 ± 16.45	0.8692
DBP (mmHg)	79.75 ± 13.36	79.47 ± 9.85	0.9358
Lipid profile			
Total Cholesterol (mg/dl)	189.12 ± 52.61	174.29 ± 33.13	0.0276
HDL-C (mg/dl)	36.78 ± 6.84	40.61 ± 7.78	0.0164
LDL-C (mg/dl)	123.53 ± 44.31	106.74 ± 27.52	0.0937
Triglyceride (mg/dl)	205.39 ± 99.19	131.37 ± 64.58	0.0036
Triglyceride/HDL-cholesterol ratio	5.93 ± 2.95	3.59 ± 2.16	0.0028
Plasma Glucose level			
FPG (mmol/L)	10.34 ± 3.94	6.01 ± 7.00	0.0000
2hrPG (mmol/L)	16.57 ± 5.95	5.96 ± 0.89	0.0000

(Within parentheses are percentages over column total); *P* values stand for comparison between T2DM and NGT; Significance values stand for comparison between T2DM and NGT groups by independent sample t-test, Chi-square, and Mann-Whitney U Test, test as applicable. BMI: Body mass index, SBP: Systolic blood pressure, DBP diastolic Blood pressure, FPG: Fasting Plasma Glucose, 2-hr PG: 2-hour plasma glucose during oral glucose tolerance test; DM: diabetes mellitus of young.

As shown in **Table 3.2**, about 59% of T2DM were obese, followed by 29.4% overweight and around 12% were normal weight, while almost 28% were normal and 6% were underweight in the NGT group. Frequencies in the two groups were significantly different only in the normal weight range (*P*=0. 0283). **Figure 3.1** represents all the comparisons under the BMI Category (T2DM vs. NGT).

Table 3.2: Distribution of the study population according to BMI (N=56)

BMI Category (kg/m^2)	T2DM (n=18)	NGT (n=38)	*P* value
	n(%)	n(%)	
Normal (18.0-22.9)	2 (11.8)	10 (27.8)	0.0283
Obese (≥25.0)	10 (58.8)	19 (52.8)	0.0804
Overweight (23.0-24.9)	5 (29.4)	5 (13.9)	1.0000
Underweight (<18.0)	0 (0)	2 (5.6)	0.4952

(Within parentheses are percentages over column total); Data were expressed as a chi-square test.

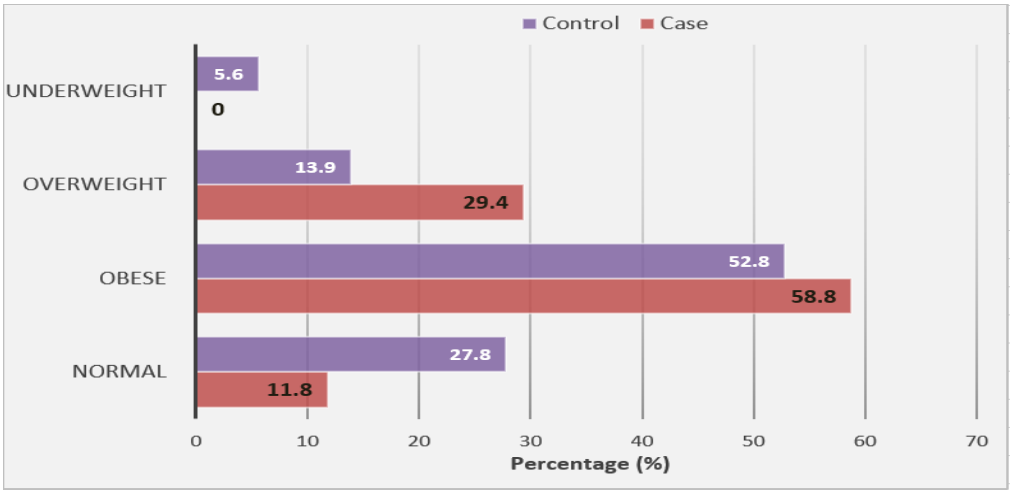

Figure 3.1: Distribution of the study population according to BMI

In this study, a positive family history of T2DM was more prevalent in the control group than in the case group whereas a negative family history of T2DM was higher in the case group with no significant difference (T2DM vs. NGT: Yes : No = 50:50 vs. 55.26 : 44.74; *P*=0.9347) (**Table**

3.1). There was a significant difference in terms of having parents with T2DM (T2DM vs. NGT: 44.4 vs. 56.8; *P*=0.0087) between cases and controls.

Table 3.3: Previous family history of type 2 diabetes mellitus (Case vs. Control)

Family History	Case n (%)	Control n (%)	*P* value
None	9 (50%)	16 (43.2%)	0.1715
Parents	8 (44.4%)	21 (56.8%)	0.0087
Siblings	1 (5.6%)	0 (0%)	1.0000

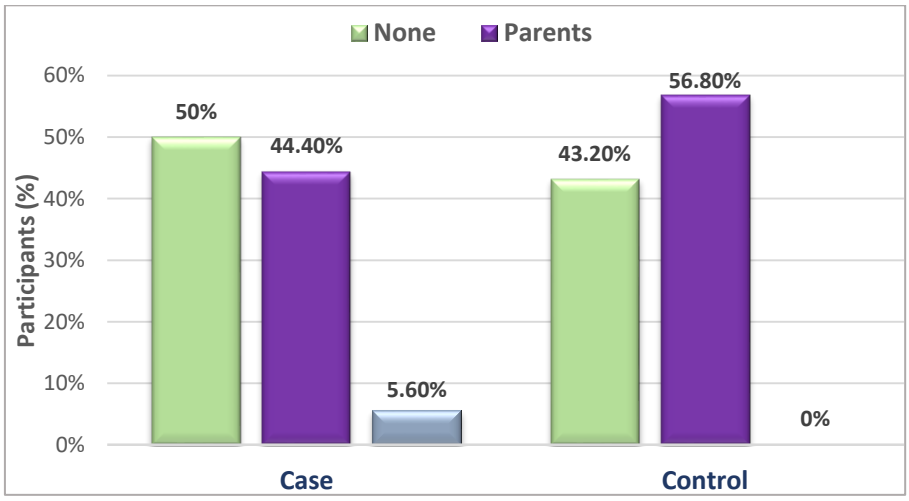

Figure 3.2**:** Previous family history of type 2 diabetes mellitus

3.2 Molecular Analysis

3.2.1 Genotype and Allele frequencies of *LEPR* rs1137100 (+326A>G)

The results of molecular analysis processes targeting rs1137100 are represented in **Figure 3.3**.

The presence of sharp and clear PCR product bands at 217 bp position on 2% agarose gel electrophoresis indicated that the chosen annealing temperature was optimal, resulting in the successful PCR amplification with the designed primer specific for the region spanning the

target SNP rs1137100 (Figure 3.3:A). Subsequent Sanger sequencing output chromatogram confirmed the following genotypes: the presence of 'A' nucleotide indicated wild type AA genotype; 'R' denoting A/G and overlapped peak suggested heterozygous AG genotype; 'G' nucleotide pointed towards altered GG genotype (Figure 3.3:B, C and D respectively).

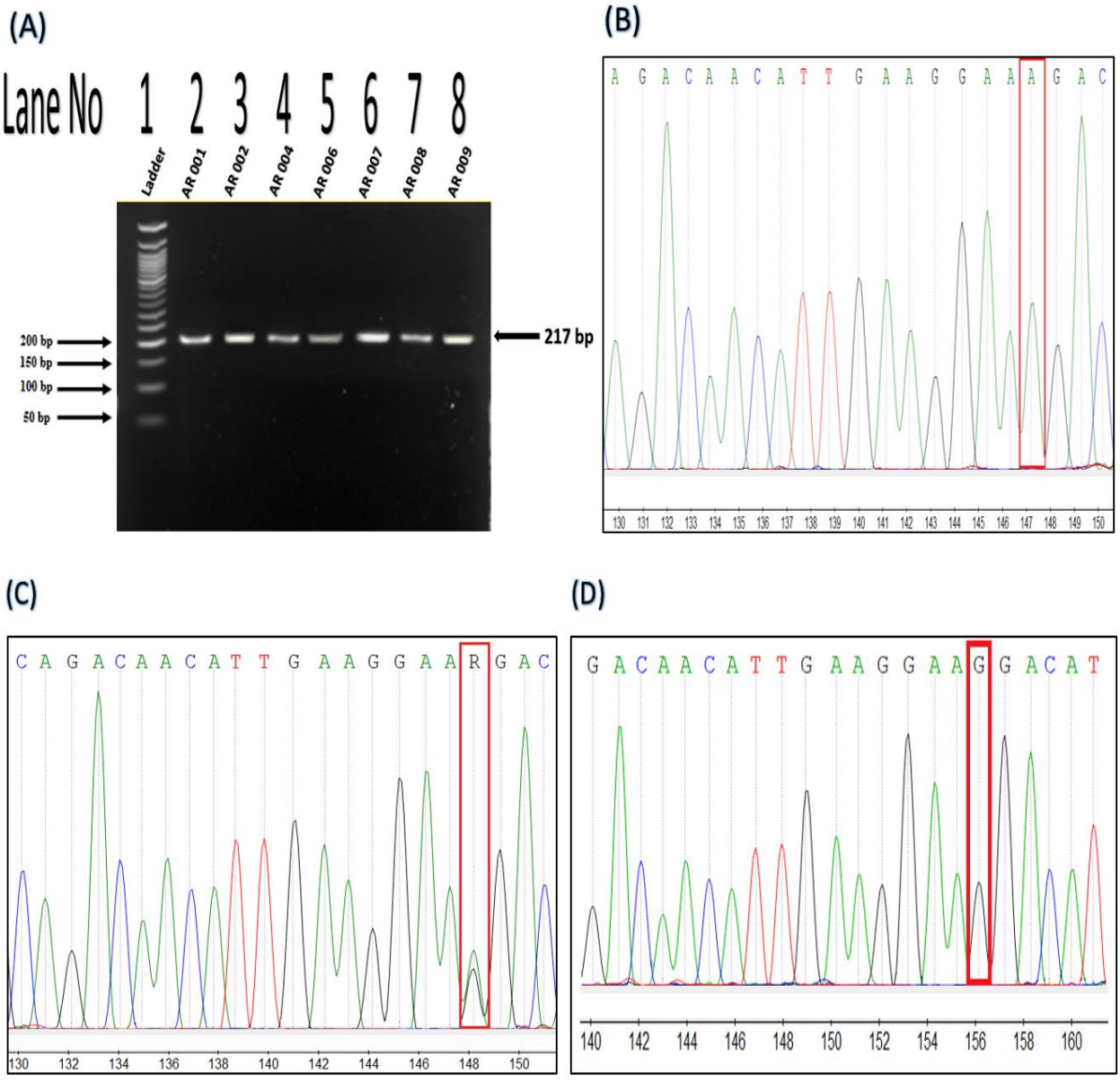

Figure 3.3: Molecular analysis of study subjects for *LEPR* rs1137100 (+326A>G). (A) PCR products of *LEPR* rs1137100 at 217 bp position on 2%TBE agarose gel; Chromatogram results of PCR direct sequence showed (B) wild type AA genotype (C) heterozygous AG genotype and, (D) altered GG genotype.

As **Table 3.4** shows genotyping for *LEPR* rs1137100 polymorphism revealing that AA, AG, and GG genotype frequencies of the *LEPR* rs1137100 variant differed between T2DM and NGT (T2DM vs. NGT: AA-AG-GG: 78-22-0 % vs 61-34-5 % respectively). The results indicated that the AA genotype was more common in the T2DM group (78%) compared to the control group (61%). On the other hand, the AG genotype (34% vs. 22 %) and GG genotype (5% vs. 0%) were more prevalent in the control group. Combinedly, all study subjects had the highest prevalence of the AA genotype and the lowest prevalence of the GG genotype (AA vs. AG vs. GG: 66% vs. 30% vs. 4%) according to Table 3.4 and Figure 3.4.

Table 3.4: Genotype frequencies of *LEPR* rs1137100 gene variant in the study subject

SNP	Genotype	All subjects (N=56) n (%)	T2DM (N=18) n (%)	NGT (N=38) n (%)
***LEPR* rs1137100 (+326A>G)**	A/A	37 (66)	14 (78)	23 (61)
	A/G	17 (30)	4 (22)	13 (34)
	G/G	2 (4)	0 (0)	2 (5)

Here, N = total number; n = count of the specific genotype (Within parentheses are percentages over column total); T2DM = Type 2 diabetes mellitus; NGT= Normal glucose tolerance.

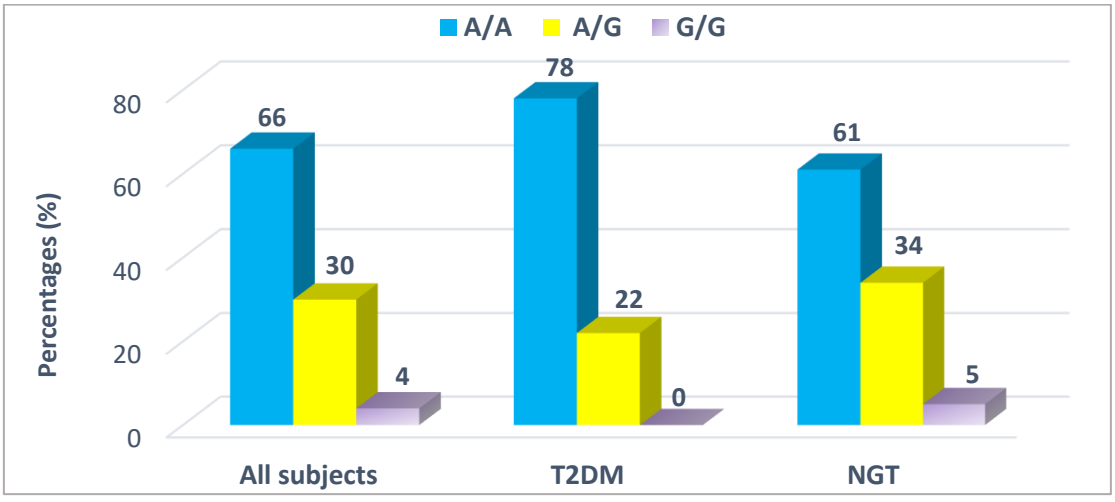

Figure 3.4: Genotype frequencies of *LEPR* rs1137100 (+326A>G)

Table 3.5 and **Figure 3.5** show that the A allele frequency was higher in T2DM than in NGT whereas the G allele frequency was higher in NGT than in T2DM for *LEPR* rs1137100 (A allele: 89% vs. 78%; G allele: 22% vs. 11%). All study subjects had a higher prevalence of the A allele compared to the G allele (A allele vs. G allele: 81% vs. 19%).

Table 3.5: Allele frequencies of *LEPR* rs1137100 gene variant in the study subject

SNP	Allele	All subjects (N=56) n (%)	T2DM (N=18) n (%)	NGT (N=38) n (%)
***LEPR* rs1137100 (+326A>G)**	A	91 (81)	32 (89)	59 (78)
	G	21 (19)	4 (11)	17 (22)

Here, N = total number; n = count of the specific allele (Within parentheses are percentages over column total); T2DM = Type 2 diabetes mellitus; NGT= Normal glucose tolerance.

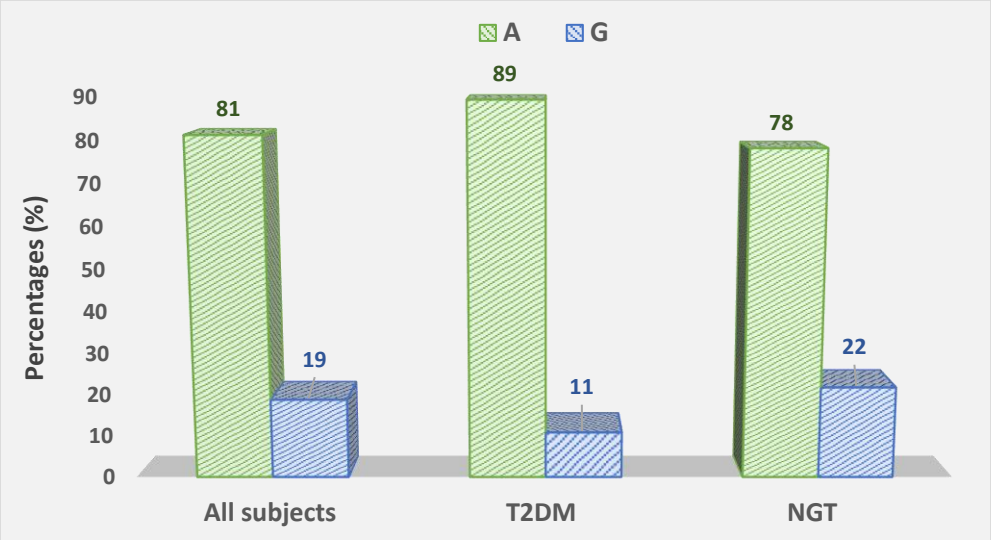

Figure 3.5: Allele frequencies of *LEPR* rs1137100 (+326A>G)

3.2.2 Genotype and Allele frequency of *LEPR* rs1137101 (+668A>G):

In **Figure 3.3,** the results of molecular analysis processes targeting rs1137101 are represented.

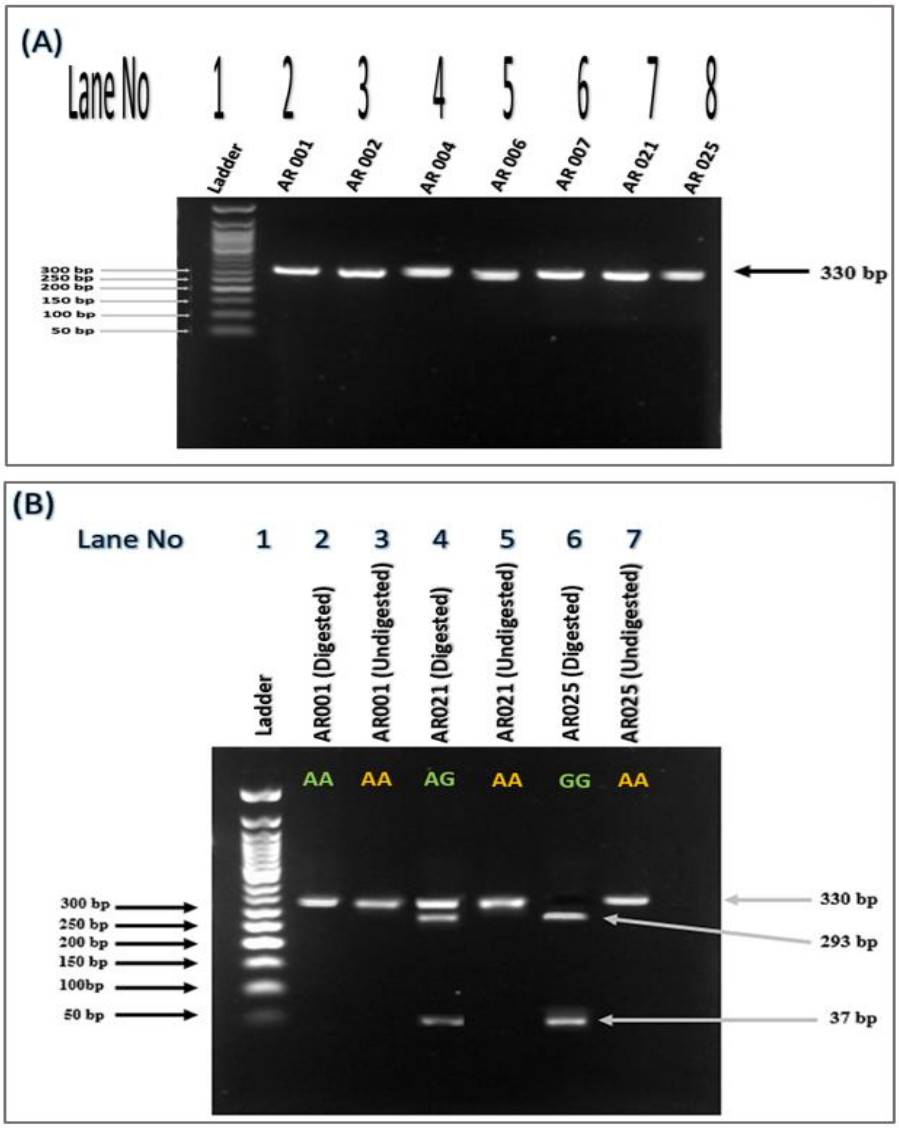

Figure 3.6**:** Molecular analysis of study subjects for *LEPR* rs1137101(+668A>G). (A) PCR products of *LEPR* rs1137101 at 330bp position on 2%TBE agarose gel; (B)MspI restriction map of PCR products in the 2% agarose gel electrophorese, which stained by SYBRT™ Safe, AA, AG, and GG lanes indicate genotypes: Lane 2 homozygous wild type AA with 330bp; Lane 4 heterozygous AG with 330bp, 293bp, 37 bp; Lane 6 homozygous mutant GG with 293 bp, 37 bp; Lane 1 with 50bp ladder; Lane 3,5,7 undigested reference wild type AA with 330bp.

The successful PCR amplification of the target sequence on 2% agarose gel electrophoresis was confirmed by the clarity and sharpness of the PCR product bands at 330 bp position and was an indication that the designed primer was specific for the region spanning the rs1137101 and the selected annealing temperature was optimal for producing the desired 330 bp PCR

product (Figure 3.6:A). Consequently, the successful restriction digestion with MspI was observed by the digested bands at the desired position according to the genotype (homozygous wild type AA=330bp; heterozygous AG=330bp, 293bp, 37bp and, homozygous altered GG=293bp, 37bp) when compared to the reference homozygous wild type AA (330bp).

Table 3.6: Genotype frequencies of *LEPR* rs1137101 gene variant in the study subject

SNP	Genotype	All subjects (N=56) n (%)	T2DM (N=18) n (%)	NGT (N=38) n (%)
***LEPR* rs1137101 (+668A>G)**	A/A	17 (30)	5 (28)	12 (32)
	A/G	37 (66)	4 (72)	24 (63)
	G/G	2 (4)	0 (0)	2 (5)

Here, N= total number; n= count of the specific genotype (Within parentheses are percentages over column total); T2DM= Type 2 diabetes mellitus; NGT= Normal glucose tolerance.

Table 3.6 presents genotyping for *LEPR* rs1137101 polymorphism which revealed that AA, AG, and GG genotype frequencies of the *LEPR* rs1137101 variant differed between T2DM and NGT (T2DM vs. NGT: AA-AG-GG: 28-72-0 % vs 32-63-5% respectively). The results showed that the AA genotype (32% vs. 28%) and GG genotype (5% vs. 0%) were more common in the NGT group compared to the T2DM group. On the contrary, the AG genotype (72 % vs. 63 %) was more prevalent in the T2DM group. As Table 3.6 and Figure 3.7 show, all study subjects collectively had the highest prevalence of the AG genotype and the lowest prevalence of the GG genotype (AA vs. AG vs. GG: 30% vs. 66% vs. 4%).

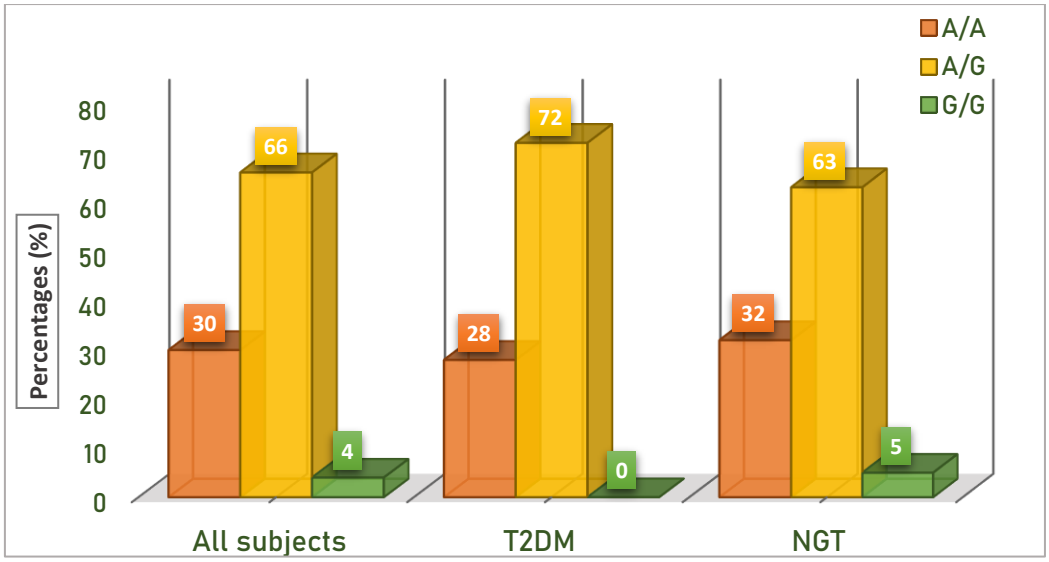

Figure 3.7**:** Genotype frequencies of *LEPR* rs1137101 (+668A>G)

Table 3.7 and **Figure 3.8** show that the A allele frequency was higher in T2DM than in NGT whereas the G allele frequency was higher in NGT than in T2DM for *LEPR* rs1137101 (A allele: 64% vs. 63%; G allele: 36% vs. 37%). All study subjects had a higher prevalence of the A allele in comparison with the G allele (A allele vs. G allele: 63% vs. 37%).

Table 3.7: Allele frequencies of *LEPR* rs1137101 gene variant in the study subject

SNP	Allele	All subjects (N=56) n (%)	T2DM (N=18) n (%)	NGT (N=38) n (%)
***LEPR* rs1137101 (+668A>G)**	A	71 (63)	23 (64)	48 (63)
	G	41 (37)	13 (36)	28 (37)

Here, N= total number; n= count of the specific genotype (Within parentheses are percentages over column total); T2DM= Type 2 diabetes mellitus; NGT= Normal glucose tolerance.

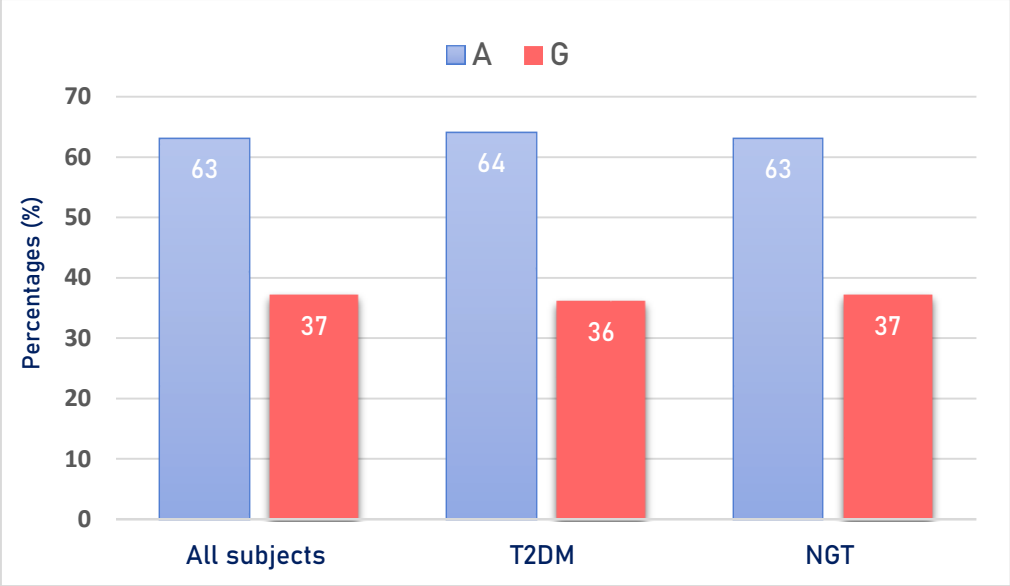

Figure 3.8: Allele frequencies of *LEPR* rs1137101 (+668A>G)

3.2.3 Hardy–Weinberg equilibrium for *LEPR* rs1137100 (+326A>G) and *LEPR* rs1137101 (+668A>G)

The Hardy-Weinberg Equilibrium (HWE) *P* value for the *LEPR* rs1137100 SNP, both in the control and case group was exactly 1 (more than 0.05) which indicated that the sample population was consistent with the equilibrium, with no significant deviation (Table 3.8).

Table 3.8: Genotype exact test for Hardy-Weinberg Equilibrium

SNP	*P* value		
	All subjects (N=56)	T2DM (N=18)	NGT (N=38)
***LEPR* rs1137100 (+326A>G)**	**1**	**1**	**1**
***LEPR* rs1137101 (+668A>G)**	**0.0031**	**0.039**	**0.042**

P<0.05 - not consistent with Hardy Weinberg Equilibrium (HWE), *P*> 0.05- consistent with Hardy Weinberg Equilibrium (HWE).

However, the Hardy-Weinberg Equilibrium (HWE) *P value* for the *LEPR* rs1137101 SNP, in both the T2DM and NGT group was less than 0.05 indicating that the sample population was

not consistent with the equilibrium. In the control group, the *P value* of 0.042 signifies a significant deviation from the equilibrium. Similarly, in the T2DM group, the *P value* is 0.039, suggesting a significant deviation from the equilibrium as well (Table 3.8).

3.2.4 Analysis of Genotype Alteration in T2DM vs. Control Groups

3.2.4.1 Analysis of Genotype *LEPR* rs1137100 Alteration in T2DM vs. Control Groups

Table 3.9 and Table 3.10 demonstrate the analysis of the likelihood of having an altered allele between two groups: those exposed (T2DM, case group) and those non-exposed (NGT, control group). In the T2DM (Exposed) group, there are 4 altered alleles and 32 reference alleles out of the total 36 alleles. In the Control (Non-exposed) group, out of 76 alleles, 17 are the altered allele and 59 are the reference allele. This data indicates a higher prevalence of the altered allele in the NGT group compared to the T2DM group.

Table 3.9: Data summary (*LEPR* rs1137100)

Group	Altered Allele	Reference Allele	Total
T2DM(Exposed)	4	32	36
NGT (Non-exposed)	17	59	76
Total	21	91	112

Table 3.10: Statistical Result (*LEPR* rs1137100)

Measure	Value
Odds Ratio (OR)	0.4338
95% Confidence Interval (CI)	0.1345 to 1.3994
Z Statistic	1.398
***P* value**	0.1622

Odds Ratio (OR) = 0.4338: indicates that the odds of having the altered allele is less likely in the T2DM (Exposed) group compared to the control group.

95% Confidence Interval (CI) = 0.1345 to 1.3994, this confidence interval includes 1 which suggests that the observed result of association might not be statistically significant.

Z Statistic = 1.398: at a standard threshold, this Z value falls short of the critical value needed for statistical significance.

***P* value =** 0.1622**:** Because the *P* value is higher than the generally accepted significance level of 0.05, it may be concluded that there is no statistically significant difference in the allele distribution between the T2DM and control groups.

Based on the odds ratio, confidence interval, and *P* value, we conclude that there is no significant association between T2DM and the likelihood of having an altered genotype in the case of *LEPR* rs1137100.

3.2.4.2 Analysis of Genotype *LEPR* rs1137101 Alteration in T2DM vs. Control Groups

Table 3.11 and Table 3.12 demonstrate the analysis of the likelihood of having an altered allele between two groups: those exposed (T2DM, case group) and those non-exposed (NGT, control group). In the T2DM (Exposed) group, there are 13 altered alleles and 23 reference alleles out of the total 36 alleles. In the Control (Non-exposed) group, out of 76 alleles, 28 are the altered allele and 48 are the reference allele. This data indicates a higher prevalence of the altered allele in the NGT group compared to the T2DM group.

Table 3.11: Data summary (*LEPR* rs1137101)

Group	Altered Allele	Reference Allele	Total
T2DM(Exposed)	13	23	36
NGT (Non-exposed)	28	48	76
Total	41	71	112

Table 3.12: Statistical Result (*LEPR* rs1137101)

Measure	Value
Odds Ratio (OR)	0.9689
95% Confidence Interval (CI)	0.4248 to 2.2099
Z Statistic	0.075
***P* value**	0.9402

Odds Ratio (OR) = 0.9689: indicates that the odds of having the altered allele is nearly same in the control group compared to the T2DM (Exposed /case) group.

95% Confidence Interval (CI) = 0.4248 to 2.2099, this confidence interval range includes 1 which suggests that the observed result of association might not be statistically significant.

Z Statistic = 0.075: at a standard threshold, this Z value falls short of the critical value needed for statistical significance.

***P* value =** 0.9402**:** Because the *P* value is much greater than the generally accepted significance level of 0.05, it may be concluded that there is no statistically significant difference in the allele distribution between the T2DM and control groups.

Based on the odds ratio, confidence interval, and *P* value, we conclude that there is no significant association between T2DM and the likelihood of having an altered genotype in the case of *LEPR* rs1137101 as well.

3.2.5 Association of targeted SNPs and the risk of young T2DM

3.2.5.1 Association between *LEPR* rs1137100 polymorphism and risk of young T2DM

In this analysis, the association of the genetic variant of *LEPR* rs1137100 with the risk of T2DM was tested under dominant, overdominant, and log additive models (Table 3.11).

Table 3.13: Association of *LEPR* rs1137100 under different genetic models

Model	Genotype	Case	Control	OR (95% CI)	*P* value
Dominant	A/A	14 (77.8%)	23 (60.5%)	1.00	0.19
	A/G-G/G	4 (22.2%)	15 (39.5%)	2.28 (0.63-8.27)	
Overdominant	A/A-G/G	14 (77.8%)	25 (65.8%)	1.00	0.35
	A/G	4 (22.2%)	13 (34.2%)	1.82 (0.50-6.66)	
Log-additive	---	---	---	2.33 (0.71-7.68)	0.14

OR: odds ratio; CI: confidence interval; $P < 0.05$ was considered statistically significant.

Under the dominant model, the A/A genotype: T2Dm vs. NGT (77.8% vs. 60.5%; OR = 1.00). The A/G and G/G combined: T2Dm vs. NGT (22.2% vs. 39.5%); yielding an OR (95% CI) = 1.98 (0.54-7.28). A *P* value of 0.19 for this genetic model is not statistically significant. In the overdominant model, T2DM vs. NGT: A/A-A/G: G/G = 77.8%: 22.2% vs. 65.8%: 34.2%; *P value* = 0.35(>0.05) and, OR (95% CI): T2DM vs. NGT: 1.00 vs. 1.82 (0.50–6.66). Moreover, in the log-additive model, OR (95% CI): 2.33 (0.71–7.68) with a *P value* of 0.14 (>0.05). As

indicated by the *P* value, none of the models in Table 3.13 could reach statistical significance, no association of the genetic variant of *LEPR* rs1137100 with the risk of T2DM could be established.

3.2.5.2 Association between *LEPR* rs1137101 polymorphism and risk of young T2DM

Any association analysis between *LEPR* rs1137101 polymorphism and risk of young T2 diabetes mellitus could not be carried out because the Hardy-Weinberg Equilibrium (HWE) *P* value for *LEPR* rs1137101 (Table 3.8) did not follow the equilibrium concept making rs1137101 SNP not suitable for further analysis.

3.3 Sample Size Calculation for Association Study

Using the resulting allele frequency data from this pilot study, the required sample size for a large-scale genetic association study was calculated.

3.3.1 For *LEPR* rs1137100

To compute the sample size for each group in the case of the association study of *LEPR* rs1137100 polymorphism with T2DM following calculations were done.

Step-by-step calculation:

Here,

Participants no. in 'Case' group = 38

Participants no. in 'Control' group = 18

Z_{β} = z-value of standard normal distribution for 80% power = 0.84

$Z_{\alpha/2}$ = z-value of standard normal distribution for 5% level of significance (P<0.05) = 1.96

r = Ratio of control to cases = $\frac{38}{18}$ = 2.1

p_1 = Proportion of risk allele (G) in cases [from Table 3.5] = $\frac{4}{36}$ = 0.11

p_2 = Proportion of risk allele (G) in controls [from Table 3.5] = $\frac{17}{76}$ = 0.22

$p_1 - p_2$ = (0.11 – 0.22)

p^* = Average proportion of risk allele (G) = $\frac{p_1 + p_2}{2}$ = $\frac{0.11 + 0.22}{2}$ = 0.17

Now, Sample size, $\boldsymbol{N = \frac{r+1}{r} \frac{(p^*)(1-p^*)\left(Z_\beta + Z_{\alpha/2}\right)^2}{(p_1 - p_2)^2}}$

$$= \frac{2.1 + 1}{2.1} \frac{(0.17)\,(1 - 0.17)\,(0.84 + 1.96)^2}{(0.11 - 0.22)^2}$$

$$= 1.5 \times \frac{(0.17)\,(0.83)\,(7.84)}{0.0121}$$

$$= 1.5 \times \frac{1.11}{0.0121}$$

$$= 1.5 \times 91.7 = 138 \text{ (approx.)}$$

Approximately **138** participants per group (cases and controls) are required to detect an association with 80% power and a significance level of 0.05 based on our pilot study data.

3.3.2 For *LEPR* rs1137101

To compute the sample size for each group in the case of the association study of *LEPR* rs1137101 polymorphism with T2DM following calculations were done.

Step-by-step calculation:

Here,

Participants no. in 'Case' group = 38

Participants no. in the 'Control' group = 18

Z_β = z-value of standard normal distribution for 80% power = 0.84

$Z_{\alpha/2}$ = z-value of standard normal distribution for 5% level of significance (P<0.05) = 1.96

r = Ratio of control to cases = $\frac{38}{18}$ = 2.1

p_1= Proportion of risk allele (G) in cases [from Table 3.7] = $\frac{13}{36}$ = 0.36

p_2 = Proportion of risk allele (G) in controls [from Table 3.7] = $\frac{28}{76}$ = 0.37

$p_1 - p_2$ = (0.36 – 0.37)

p^*= Average proportion of risk allele (G) = $\frac{p_1 + p_2}{2} = \frac{0.36 + 0.37}{2}$ = 0.37

Now, Sample size, $\boldsymbol{N} = \frac{2.1 + 1}{2 .1} \frac{(0.37)\,(1 - 0.37)\,(0.84 + 1.96)^2}{(0.36 - 0.37)^2}$

$$= 1.5 \times \frac{(0.37)\,(0.63)\,(7.84)}{0.0001}$$

$$= 1.5 \times \frac{1.83}{0.0001}$$

$$= 1.5 \times 18{,}300 = 27{,}450 \text{ (approx.)}$$

Approximately **27,450** participants per group (cases and controls) are required to detect an association with 80% power and a significance level of 0.05 based on our pilot study data.

CHAPTER 4

Discussion

The increased incidence of youth-onset Type 2 Diabetes Mellitus (T2DM) worldwide as mentioned in the introduction, is a serious public health problem, particularly in areas with few resources for early identification and treatment. This study aimed to investigate the clinical, demographic, and genetic factors, with an emphasis on the role of *LEPR* gene polymorphisms rs1137100 and rs1137101 linked to young-onset T2DM in a Bangladeshi population. Another objective of this study was to conduct it as a pilot project to check the feasibility and to determine the minimum number of participants required for large-scale research. Although, according to our study's findings, the connection with the *LEPR* polymorphisms was not statistically significant, results showed strong correlations between metabolic variables and type 2 diabetes, conforming to global trends.

In the case of demographic characteristics analysis of our study, a statistically significant (P =0.0248) difference in mean age between the case and control group was observed where the mean age in the case group was higher than in the control group. Although this result could suggest age as a risk factor for T2DM in young, it might be contradictory to our expectations of younger individuals more likely to have type 2 diabetes. This contradiction could be due to the small sample size (only 18 cases and 38 controls), delayed diagnosis, and a combination of other biological, behavioral, and methodological factors. However, it could be interpreted that at this age type 2 diabetes usually becomes diagnosed and also that over time as individuals age, the risk of T2DM may increase, even including those over 20 years (Colagiuri et al., 2005). A study by Cowie et al. (2018) stated that diagnosed T2DMis more common as people age, especially after the age of 20, and peaks between the ages of 65 and 74. On the contrary, a SEARCH study in the US from 2002 to 2018 by SAUDER et al. (2022) highlighted an earlier

peak age for diagnosis with notable variations in which the lowest age for non-Hispanic Black kids was 13 years, whereas the highest age for American Indian, Asian and Pacific Islander, Hispanic, and non-Hispanic White adolescents was 16 years. A further comparison of gender between the T2DM and NGT groups showed no statistically significant difference since the *P* value (*P*=0.6009) is quite greater than the significance threshold of 0.05. In the case group, there is a higher proportion of females (62.5%) than males (37.5%) whilst there is equal distribution of males (50%) and females (50%) in the control group. Despite this, based on our analysis, there is not any strong evidence to suggest gender as an important risk factor for developing T2DM, and cannot even conclusively claim that females are at higher risk of developing T2DM in this sample of young people, rather this male-to female ratio variation could be implied as the result of chance. Female gender has been identified in several studies as a risk factor for T2DM that develops in youth (Hernandez et al., 2024; Marcovecchio et al., 2005). For example, Pinhas-Hamiel et al. (2018) found that among younger generations, the female-to-male ratio for T2DM is about 2.0, making gender a risk factor. A female sex preponderance in youth-onset type 2 diabetes was evidenced by studies like SEARCH and TODAY where a greater frequency with ratios of 4-6:1 was discovered especially among American Indian tribe females (Pinhas-Hamiel et al., 2018) and, by another study where females constituted 63% of the 30 cases of the young T2DM (SRINIVASAN et al., 2018). However, research by Garbuzova et al. (2023) found that young men were more likely than young women to haveT2DM, as shown by the fact that young men had a greater prevalence of the disease (0.96%) than women (0.69%). Similarly, in another research on Korean children, adolescents, and adults under 30, the prevalence of T2DM increased from 1.96 to 8.63 per 10,000 in girls and from 2.57 per 10,000 in men from 2002 to 11.41 in 2016 (Hong et al., 2022). Hence, further analysis is required to draw a clear and non-debatable conclusion.

As per the analysis of the clinical parameters of our study, even though parameters such as height, systolic blood pressure (SBP), and, diastolic blood pressure (DBP) were marginally higher in the T2DM group compared to the NGT group, no strong statistical correlation could be established as *P* values for each variable comparison were more than 0.05. Conversely, some studies evaluating the association between height and increased risk of T2DMrevealed that shorter height during late adolescence and young adulthood was linked to a higher risk of developing T2DM indicating an inverse and positive relation (Bjerregaard et al., 2017, 2020; Shrestha et al., 2019). In a Mendelian randomization study, authors reported a 2% increase in the risk of T2DM for every 1 mmHg increase in SBP, indicating a causal association (Aikens et al., 2017). According to another study by Wise (2015), there is a strong correlation between hypertension and the onset ofT2DM, as the study shows that those with high blood pressure, especially systolic and diastolic blood pressure levels, have approximately 60% higher chance of getting the disease.

Increased risks of T2DM are noticeably linked to high levels of triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and a high triglyceride-to-high-density lipoprotein cholesterol (TG/HDL-C) ratio (T. Yang et al., 2022), as well as low HDL-C levels by causing insulin resistance and pancreatic β-cell dysfunction (Sun et al., 2022). A study with over 120,000 individuals found that a TG/HDL-C ratio of more than 2.1 was a better predictor of the onset of type 2 diabetes (Yuge et al., 2023) despite the fact that, total cholesterol (TC) and LDL cholesterol levels are equally significant (T. Yang et al., 2022; Yuge et al., 2023). The lipid profile **table 3.1** in chapter 3 of our study showed statistically significant higher levels of TC (P=0.0276), HDL-C (P=0.0164), TG (P=0.0036), and TG/HDL-C (P=0.0028) in the case group compared to the control group, indicating a positive correlation of them with the development of T2DM in the sample population. We found higher levels of LDL-C in the case group than the control group but because of an insignificant *P* value , this finding could not be

considered to suggest any correlation. Another fact to notice here was the TG/HDL-C level (3.59) more than 2.1 in the control group, a typical indicator of T2DM onset. Factors like body composition, insulin resistance, and metabolic health of the non-diabetic study subjects might be responsible for this. According to this dataset, those with T2DM had lower HDL-C levels, higher triglyceride levels, and larger waist-hip ratios than those in the control group with statistically significant difference between two groups. These results are in line with previous research that shows a substantial correlation between the pathophysiology of type 2 diabetes and insulin resistance, central obesity, and dyslipidemia (Maki et al., 2021; Twig et al., 2020; Wang & Ahmadizar, 2021).

As per ADA-2019 criteria, to be characterized as a type 2 diabetes patient, an individual's fasting plasma glucose (FPG) and 2-hour plasma glucose (2hrPG) should be more than 7.0 mmol/L and 11.0 mmol/L respectively (Association, 2019). In our analysis, the FPG and 2hr PG levels were 10.34 mmol/L and 16.57 mmol/L respectively in the case group where the control group had an FPG level of 6.01 mmol/L and 2hr PG level of 5.96 mmol/L, aligning with the expected result. The T2DM group's higher fasting and 2-hour plasma glucose levels support the glycemic dysregulation that is typical of diabetic patients and point to the necessity of focused early therapy in the younger population.

An individual's BMI, weight, hip circumference, waist circumference, and waist-hip ratio have always been important parameters linked to obesity which is widely discussed as a risk factor for developing T2DM. Individuals with unusual waist circumference (WC), waist-to-height ratio (WHtR), and BMI had considerably greater risks of T2DM, according to a cohort study by X. Y. Yang et al. (2016). In our study, the weight, waist circumference, hip circumference, and overall BMI in the T2DM group were higher when compared to the NGT group still, no correlation could be drawn since the *P* values were statistically insignificant. Only the *P* value of waist-hip ratio was 0.0335 (<0.05), leading to an assumption that the greater waist-hip ratio

in the case group might be associated with the onset of T2DM in youth in our sample. Twig et al. (2020) in a national survey stated that teenagers who were severely obese had a much higher risk of type 2 diabetes, likewise, Al Mamun et al. (2009) in a long-term study found that those who were overweight at age 5 had an odds ratio of 2.60, highlighting a significant correlation between childhood overweight status and developing diabetes by the age of 21. The comparison between the NGT and T2DM groups of this study under different BMI categories was presented in **Table 3.2** (chapter 3). In that table, a statistically significant *P* value of 0.0283 (<0.05) was found for the normal BMI category, implying that those with youth-onset T2DM to far less likely to have a normal BMI than those with normal glucose tolerance. A lower risk of youth-onset T2DM may be linked to a normal BMI, as seen by the lower proportion of T2DM patients in the normal BMI category (11.8% vs. 27.8%). Compared to those with less severe obesity, youth with severe obesity have a 2.3–5.1 times greater incidence of youth-onset diabetes, according to research on American Indian tribes (Tanamas et al., 2018). Given that the obtained *P* value for the obese category was 0.0804 (>0.05), it was not statistically significant. Still, the higher proportion of obese individuals in the T2DM group (58.8%) than those with NGT (52.8%) may indicate a trend of increased obesity rates associated with T2DM that emerges in youth. In the overweight BMI category, the *P* value of 1 indicates that there is no statistically significant difference between the T2DM and NGT group's status because the number of individuals in this category is the same in both groups (29.4% for T2DM and 13.9% for NGT). Furthermore, in the underweight category, the *P* value of 0.4952, which is much greater than 0.05, indicates no statistical significance. The findings might show that underweight does not seem to be associated with an increased incidence of T2DM in young individuals as just 5.6% of those in the NGT group are underweight, and none of the individuals with T2DM are underweight. The findings suggest that those with normal BMI seem to be at a decreased risk of acquiring youth-onset T2DM, though obesity and overweight do not

statistically significantly affect this dataset. Therefore, maintaining a normal body mass index (BMI) may be essential for reducing or preventing young people's risk of type 2 diabetes.

A person's risk of developing T2DM is considerably increased if they have two or more first-degree relatives with T2DM (Smith et al., 2024). To evaluate the association of family history of T2DM with an increased risk of youth-onset T2DM in this sample population, data comparing the presence of family history of type 2 diabetes between the T2DM and NGT group was presented in **Table 3.3** (chapter 3). The absence of family history and a family history of T2DM through siblings showed no statistically significant difference between the case and control groups, as per the *P* value of 0.171 and 1.000 (higher than 0.05) respectively. Even so, it is challenging to draw any significant conclusions because there are so few people in both groups with a sibling history (1 case vs. 0 control). According to Middleton et al. (2019), the incidence of parental diabetes history is higher in young-onset patients where the difference between maternal and paternal history is less pronounced. In the case of family history through parents, with a statistically significant *P* value of 0.0087 (<0.05 cut-off), a greater percentage of T2DM individuals (44.4%) had a parental history of T2DM than NGT individuals (56.8%). This suggests that not having a family history and a family history through siblings do not seem to significantly influence the likelihood of developing youth-onset T2DM whereas a parental history of T2DM may be a risk factor for the development of youth-onset T2DM in our data set, supporting the notion of genetic and family factors, probably inherited from parents having a role in the development of T2DM in young people.

The assessment of *LEPR* gene polymorphisms rs1137100 and rs1137101, which have both been linked to obesity and the tendency to T2DM, was a noteworthy component of this investigation. In this study, **Table 3.4** in Chapter 3 highlighted the distribution of *LEPR* rs1137100 genotypes among the study subjects and the comparison between T2DM and NGT groups. Upon overall study subjects analysis, a minority of participants had the homozygous

altered genotype GG (4%) whereas the majority were homozygous for the wild-type allele AA (66%) followed by heterozygous carriers AG (30%). In the T2DM group, the AA genotype was found to be more common (78%) than the AG genotype (22%) and this subgroup did not have the GG genotype. In the NGT group, although 61% of people still had the AA genotype, 34% more people had the AG genotype, and 5% of people had the GG genotype. These results point to a possible link between T2DM and the AA genotype. On the other hand, the GG genotype seems to be exclusive to the NGT group, suggesting either a lack of association or a potential protective function in the development of T2DM. According to **Table 3.5** analysis, the frequency of the A allele is higher (81%) than that of the G allele (19%) within the overall study participants. The dominant presence of the A allele in diabetic people is further supported by the increased A allele frequency of 89% and decreased G allele frequency of 11% in the T2DM group. In the NGT group, while the G allele is comparatively more common (22%) than in the T2DM group, the A allele is still present (78%). The observed results, particularly in the T2DM subgroup, the prevalence of the AA genotype and A allele may suggest a genetic susceptibility associated with this variant which is opposite to the expected outcome since G is the risk allele here. The disproportion or imbalance in the case and control number where the control is almost 2.1 times higher than the case number may have played an important role in this unexpected result. Almost similar trend is seen in **Table 3.6** and **Table 3.7** for the rs 1137101. In **Table 3.6** increased prevalence of heterozygote AG genotype is observed in the T2DM group than the other two homozygous genotypes, suggesting a potential role in the studied population that may need further investigation. To fully comprehend the clinical relevance of the G allele frequency in the T2DM and NGT group, full-scale research is necessary. The GG genotype may play a complex function, possibly altering metabolic pathways in a way that calls for more research, as evidenced by the lack of this genotype in T2DM participants and its generally low representation.

In spite of its potential role in obesity, the association of rs1137100 polymorphism with T2DM particularly in youth appears to be less influential based on the minimal current research targeting this population. A study by He et al. (2015) showed that the rs1137100 polymorphism increases the incidence of type 2 diabetes (OR = 1.46, 95% CI: 1.15-1.85). While examining the association between T2DM and the likelihood of having an altered *LEPR* rs1137100 genotype, a summary of data in **Table 3.9** in this updated analysis highlighted that there were more negative outcomes than positive outcomes. The positive outcome represents the presence of an altered allele, while the negative outcome indicates the presence of a reference allele. This discrepancy of the lower presence of altered allele (G) frequency in the T2DM group than the control group calls into doubt the potential protective or neutral effect of *LEPR* rs1137100 in the susceptibility to T2DM. The statistical analysis data in **Table 3.10** (OR = 0.4338; CI (95%) = 0.1345-1.3994; Z statistic = 1.398; *P* value = 0.1622) indicated no significant association between the likelihood of having an altered allele of *LEPR* rs1137100 genotype and T2DM within the studied population. In a similar manner, research has not shown a significant correlation between this SNP and the incidence of obesity or T2DM, despite its possible influence (Grzeszczak et al., 2018; Y. Yang & Niu, 2018).

With an OR of 1.43 and a substantial correlation with T2DM in the Chinese population, the Gln223Arg variant (rs1137101) suggested heightened vulnerability among carriers (Li et al., 2017). Conversely, the Q223R polymorphism and T2D risk did not significantly correlate, according to a meta-analysis of 13 studies, with an odds ratio (OR) of 1.09 (95% CI: 0.80–1.48) (Y. Yang & Niu, 2018). In **Table 3.11**, the T2DM group has a lower frequency of the altered allele (G) than the control group for *LEPR* rs1137101 meaning there is more negative outcome exceeding the positive outcome. According to the statistical analysis data in **Table 3.12,** (OR = 0.9689; CI (95%) = 0.4248-2.2099; Z statistic = 0.075; *P* value = 0.9402) there is a lack of evidence suggesting any association of altered allele of *LEPR* rs1137101 with T2DM

in this dataset. The fact that the *P* values and confidence ranges neither of the *LEPR* rs1137100 nor rs1137101 variations showed statistical significance implying that these particular genotypes within this studied population might not have a key role in susceptibility to type 2 diabetes. Even if the OR below 1 suggests that the odds of having the disease are decreased, the OR for rs1137100 suggests a potential protective impact and rs1137101 indicates a neutral impact, the absence of statistical evidence makes the results inconclusive. Smaller sample sizes and broader CI highlight the need to further study with large datasets to support or contradict these findings.

The Hardy-Weinberg equilibrium (HWE) is a principle in population genetics study that enables scientists to evaluate variations from anticipated genotypic proportions and estimate allele frequencies, which might reveal evolutionary processes at play (Allendorf et al., 2022). According to HWE, if a population is not influenced by any evolutionary consequences, the frequency of a specific allele or genotype in that population will remain the same. Under the assumptions of random mating and the absence of evolutionary forces like selection or mutation, the HWE creates a baseline for genetic equilibrium (Allendorf et al., 2022). The HWE is a useful tool for finding alleles linked to disease since deviations from HWE (typically $P<0.05$) might indicate the presence of rare genetic variations or genotyping mistakes (Neamatzadeh et al., 2024). In this study, *LEPR* rs1137101 (+668A>G) exhibits inconsistency whereas *LEPR* rs1137100 (+326A>G) maintains equilibrium which may indicate that rs1137101 is more relevant to the phenotypic features being studied. The significant disagreement between the two SNPs' HWE results suggests that their evolutionary, biological, or population-level traits may differ. In the overall study population, T2DM group, and NGT group, all of the *P* values for the *LEPR* rs1137100 SNP were equal to 1 (Table 3.8). These findings show that in every group, the genotype distribution for *LEPR* rs1137100 is in agreement with HWE where perfect equilibrium is indicated by a *P* value of 1. This implies

that the genetic distribution of rs1137100 across all groups of this study is stable and unaffected by T2DM status and there are no visible deviations brought on by selection bias, non-random mating, or population stratification. Hence, we can suggest based on this stability that the genetic susceptibility to T2DM in this study subjects may not be significantly influenced by *LEPR* rs1137100. Nonetheless, *LEPR* rs1137101 SNP had *P* values of 0.0031 for the overall population, 0.039 for the T2DM group, and 0.042 for the NGT groups (Table 3.8), showing that in all groups, the genotype distribution for *LEPR* rs1137101 is not consistent with HWE ($P < 0.05$). These statistically significant deviations in both subgroups and the whole population may be an indication of potential genetic influences such as selection pressure, population stratification, genotyping errors, and association with disease (Grover et al., 2010; Sen & Burmeister, 2008).

In genetic association studies, the link between an allele or genotype and the risk of a certain disease or trait is described by several genetic models different genetic models (dominant, co-dominant, recessive, over-dominant, and log-additive). Based on allele frequencies, these models have an impact on the computation and interpretation of odds ratios (OR) and confidence intervals (CI). In this study, dominant, overdominant, and log-additive genetic models were used to assess the relationship between the *LEPR* rs1137100 polymorphism and the likelihood of developing youth-onset T2DM. Under a dominant model, a true association is shown by a significant OR with a CI that excludes 1, and people who have one or two copies of the allele are at a higher risk if the OR is greater than 1. According to the findings of this study, the A/G-G/G group outperformed the A/A group with an odds ratio (OR) of 2.28 (95% CI: 0.63–8.27) in the dominant model (Table 3.13). The link was not statistically significant ($P = 0.19$), even though it indicates a more than twofold increase in risk for T2DM. This can be assumed that the minor allele (G) only needs one copy to influence the phenotype. Additionally, variability and the potential for insufficient power to identify a true relationship

are indicated by the wide confidence range. In the overdominant model, the risk of heterozygotes differs from that of both homozygotes, frequently indicating a distinct protective or dangerous impact. By determining whether the CI excludes 1, the significance is deduced in which heterozygotes are protective if their OR is less than 1, and they are at increased risk if their OR is greater than 1. In our analysis, heterozygotes (A/G) are contrasted with the combined homozygous genotypes (A/A and G/G) in the overdominant model where OR was 1.82 (95% CI: 0.50–6.66), with a statistically insignificant *P* value of 0.35. Although the data suggested a higher risk for heterozygotes, the findings were not definitive, highlighting the need for more studies to confirm this finding. The log-additive model is frequently regarded as robust and is useful for identifying linear trends across genotypes. To evaluate how each extra allele raises the risk, ORs are computed on a log scale in this model and the genotype impact is treated as cumulative. We found a *P* value of 0.14 and an OR of 2.33 (95% CI: 0.71–7.68) in this model. Although not statistically significant, this finding raises the possibility of a dose-dependent risk increase for every extra G allele by a significant trend in OR with CI excluding 1. The findings consistently indicate a tendency toward a higher risk of young-onset T2DM linked to the presence of the G allele in all three models. Nevertheless, none of the correlations were statistically significant, suggesting that the patterns might be random or constrained by statistical power and sample size. This study's lack of strong associations emphasizes how crucial it is to take into account additional genetic, epigenetic, and environmental factors that might interact with the *LEPR* rs1137100 polymorphism. In a meta-analysis involving 17 case-control studies, a significant association between *LEPR* rs1137100 polymorphism and T2DM was found under the recessive genetic model (OR=0.67, 95%CI 0.52-0.88, *P*=0.00) and allele contrast genetic model: OR=1.46, 95%CI 1.15-1.85, *P*=0.00) (He et al.; 2015), though the age group was not clearly mentioned. Besides, to determine whether a polymorphism is associated with an increased risk of disease, unbiased allele distributions are used in association studies.

The association analysis of the *LEPR* rs1137101 polymorphism with the risk of youth-onset T2DM in our study was limited due to the deviation of this genotype distribution from Hardy-Weinberg Equilibrium (HWE), because the deviation might amplify or lower the true relationship between the SNP and the disease phenotype, compromising the validity of subsequent association studies. When a particular SNP deviates from HWE, the possibility of the data not fairly representing the population's inherent genetic variation is raised. Therefore, it is difficult to detect genuine correlations when HWE is deviated from (Mao et al., 2010; Zhang & Sun, 2020).

The investigations of the genetic and clinical factors of this pilot study revealed areas that require more research, even if statistical significance was not found for several correlations. It is important to recognize the limitations of this pilot research. Both the statistical strength of the genetic association tests and the generalizability of these results are limited by the small sample size which may have diminished the ability to identify modest genetic effects, as seen by this lack of relationship. Furthermore, stratified analyses that further clarify the interplay of genetic, metabolic, and environmental variables would be made possible by a larger sample size. Hence, the minimal number of participants needed for large-scale case-control research in the context of the study on youth-onset T2DM in Bangladesh was determined using the results of this pilot study. For *LEPR* rs1137100, the required sample size is approximately **138** participants per group whereas for *LEPR* rs1137101, it is approximately **27,450** participants per group. The different effect sizes and allele frequencies between the SNPs results in this difference in sample sizes. Since, the allele frequency difference $(p_1 - p_2)$ between cases and controls is significantly less and squared difference between the case and control allele frequencies $(p_1 - p_2)^2$ is very small, a larger sample size for rs1137101 is needed to find a statistically significant link. For rs1137100, a much smaller sample size is required for the same power and significance level since the difference in allele frequencies is greater and the squared

difference is larger. Finally, we can hope to achieve noteworthy results through future research with this estimated sample size.

CHAPTER 5

Conclusion

In conclusion, this pilot study does not link *LEPR* polymorphisms (rs1137100 and rs1137101) to the risk of T2DM in young Bangladeshis, even though it does demonstrate major metabolic differences between T2DM and control groups. The research highlighted the significance of these indicators in detecting early-onset T2DM risks by revealing important metabolic differences, such as higher fasting plasma glucose, 2-hour plasma glucose, and particular lipid profile markers including triglycerides and TG/HDL-C ratios. While gender and BMI showed fewer clear correlations in this group, demographic analysis identified age and parental history as significant risk factors. Significantly, the deviation of rs1137101 from the Hardy-Weinberg Equilibrium raises the possibility of selection bias or genotyping mistakes, underscoring the necessity for bigger, more thorough research to fully understand its function. Additionally, the patterns in genotype distributions that have been identified highlight the intricate interactions between metabolic, environmental, and genetic variables in the pathophysiology of type 2 diabetes in young people.

The feasibility of carrying out genetic research on type 2 diabetes in environments with limited resources is also demonstrated by this work, which offers important insights into how to choose sample size and the methodological difficulties that may arise in subsequent large-scale investigations. These results highlight the significance of focused treatments, such as metabolic health monitoring and genetic screening, to combat the growing incidence of youth-onset type 2 diabetes, especially in susceptible groups. Further research with bigger cohorts, varied groups, and additional genetic markers is required to validate these findings and provide individualized preventative efforts, even though the current study offers fundamental evidence.

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