ASSOCIATION OF *EGF* GENE POLYMORPHISM rs4444903 WITH DIABETIC FOOT ULCER

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PAKISTAN

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"Association of *EGF* Gene Polymorphism rs4444903 with Diabetic Foot Ulcer"

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Dedicated to My Beloved Mother

&

My mentor Syed Sarfraz A.Shah

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ABSTRACT

The most common and debilitating complication associated with type 2 diabetes mellitus (T2D) is diabetic foot ulcer (DFU), which can increase morbidity, amputation, and the burden on the health system. It begins with a loss of sensory, motor, and autonomic neural functions, leading to amputation and gangrene. Recent advancements suggest that growth factors significantly contribute to the development of diabetic foot ulcers in diabetic patients. Epidermal growth factor (EGF) is a highly dynamic cytokine that is primarily involved in wound healing as well as tissue reconstruction after injury. *EGF* gene polymorphism may provide a vision for the molecular mechanism of DFU onset. The goal of this study was to find out the role of *EGF* gene polymorphism rs4444903 (+61 A>G) with the pathogenesis of diabetic foot ulcer (DFU). This study may enhance treatment strategies, potentially reducing DFU morbidity and recurrence. The case-control-based study divided the subjects into two groups. Cases with DFU (n = 58) and controls with T2D (n = 116). The subjects were diagnosed cases DM, and DFU, were assessed using the SINBAD classification. The Tetra Primers Amplification Refractory Mutation System-PCR technique was applied for the amplification and detection of different alleles of *EGF* gene polymorphism. The Chi square test was used for analysis, which showed *EGF* SNP rs4444903 was not statistically significant with progression of DFU (p = 0.426). The odds ratio values at p 0.426 suggest that *EGF* polymorphism is not significantly associated with the DFU but may still increase a risk. The association of genotypes were analyzed by applying genotype models which confirms that the heterozygous variant AG genotype in over-dominant model is statistically significant confirming the risk factor for diagnosis of disease, AG+GG in

dominant models is not statistically significant, GG in recessive model may play a protective role and it's the mutant genotype, whereas AG and GG in codominant model are statistically significant and is associated with diagnosing DFU.

Keywords: Diabetic Foot Ulcer, Epidermal growth factors, rs4444903, single nucleotide polymorphism, Tetra Primers Amplification refractory mutation system-PCR.

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AGEs	Advanced glycation end-products
AIDS	Acquired immunodeficiency syndrome
Akt	AK strain transforming
ANS-Me	Anterior Nasal Spine-Menton
Cbl	Casitas B-lineage Lymphoma
c-Myc	Multifunctional transcription factor
CTLA4	The cytotoxic T lymphocyte antigen-4 gene
BMI	Body Mass Index
DFU	Diabetic foot ulcer
DKA	Diabetic ketoacidosis
DM	Diabetes mellitus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ELK-1	Ets LiKe gene 1
ErbB	Erythroblastic leukemia viral oncogene
ESRD	End-stage renal disease

ETS	E26 transformation-specific
FGF	Fibroblast growth factor
GO	Glyoxal
HbA1c	Glycated hemoglobin
HDL	High density lipoprotein
HGF	Hepatocyte growth factor
HIF-1	Hypoxia-inducible factors
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSP-70	Heat shock proteins
IFIH1	Interferon induced with helicase C domain 1 [(human)]
ITLN1	Intelectin 1
IWGDF	International Working Group on the Diabetic Foot Risk Classification System
JAK	Janus kinases
LADA	Latent autoimmune diabetes in the adult
LDL	Low density lipoprotein
LEA	Lower extremity amputation
LOX	Lipo-oxygenase
MAPK/MEK	Mitogenic activating protein kinase
MCP	Monocyte Chemotactic Protein-1
MDA	Malondialdehyde
MDRL	Multi-Disciplinary Research Laboratory
MGO	Methylglyoxal

mRNA	messenger RNA
MRSA	Methicillin-resistant S. aureus
MODY	Maturity-onset diabetes of the young
NADPH	Nicotinamide adenine dinucleotide phosphate oxidases
NCBI	National Center for Biotechnology Information
NETs	Pancreatic neuroendocrine tumors
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next-generation sequencing
NRF2	Nuclear transcription factor
OPG	Osteoprotegerin
PAD	Peripheral artery disease
PDGF	Platelet derived growth factor
PEDIS	Measures perfusion, extent/size, depth/tissue loss, infection and sensation
PPARγ	Peroxisome proliferator-activated receptor γ.
PLC	Phosphoinositide-specific phospholipase C
PI3K	Phosphatidylinositol-3 kinase
PVD	Peripheral vascular disease
qPCR	quantitative Polymerase chain reaction
RAF	Rapidly Accelerated Fibrosarcoma
RAGE1	Receptor advanced glycation end product 1
Ras	Rat sarcoma virus
rhEGF	Recombinant human epidermal growth factor
ROS	Reactive oxygen species

RTKs	Receptor tyrosine kinase
SDF	Stromal cell-derived factor
SITR1	Short tau inversion recovery
SINBAD	Site, Ischemia, Neuropathy, Bacterial infection, Area, and Depth
STAT	Signal transducer and activator of transcription proteins
T-ARMS-PCR	Tetra Primers Amplification refractory mutation system-PCR
TC	Total cholesterol
TGF	Transforming growth factor
TG	Triglycerides
TLRs	Toll-like receptors
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
WAKMAR1	wound and keratinocyte migration-associated lncRNA 1
WHO	World Health Organization
WIfI	Wound, Ischemia and foot infection

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CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is a rapidly growing global public health epidemic affecting a large number of people worldwide because of its complex interplay of familial, environmental, and lifestyle factors. DM is characterized by a heterogeneous metabolic disorder of carbohydrates, proteins, or lipids, resulting in excessively high blood sugar levels. This may arise from insufficient insulin synthesis or a combination of insulin resistance and poor insulin secretion. There is expected to be a rise in the occurrence of diabetes among individuals aged 20 to 79, with the prevalence increasing from 10.5% in 2021 to 12.2% in 2045. Among this age group, persons aged 75 to 79 are projected to have the greatest prevalence. High-income countries and urban areas have a higher prevalence. Healthcare expenditures will reach a total of USD 1,054 billion by 2045, as predicted (Sun *et al*., 2022). In Pakistan, the prevalence of DM has reached alarming levels of 26.7%, posing significant challenges to healthcare systems and public health initiatives. Pakistan ranks third globally in terms of diabetes prevalence, following China and India. Furthermore, it is worrisome that a significant number of individuals may go unnoticed, leading to a higher prevalence of issues and an increased risk of harm due to a lack of treatment (Azeem *et al*., 2022).

Although traditionally, diabetes has been classified as either a form that develops early (known as Type 1 diabetes mellitus or T1D due to autoimmune disorder) or a non-autoimmune form that develops later in life (known as type 2 diabetes mellitus or T2D), other subtypes can be identified clinically, such as monogenic *diabetes* (e.g. maturity-onset diabetes of the young (MODY or neonatal diabetes), diabetes in pregnancy appearing for the first time in the second or third trimester of pregnancy which never existed earlier (Li *et al*., 2021), drug-induced e.g., corticosteroids induced DM or during the treatment of HIV/AIDS, and perhaps a late-onset autoimmune form (latent autoimmune diabetes in the adult) or LADA. Numerous endocrinopathies exist, such as Cushing's syndrome or acromegaly, as well as exocrine pancreatic disorders, such as pancreatitis, pancreatic tumors, and hemochromatosis. There is also a new type of diabetes mellitus that happens after a transplant (Haney, 2020).

Environmental and genetic variables influence Type 1 diabetes (T1D), making it a complex condition. Risk of T1D increases markedly with HLA II gene region, INS, PTPN22, and CTLA4 genes. Besides HLA region other than loci holding the risk can raise the chances of development of DM due to viral infections (Krzewska & Ben-Skowronek, 2016). DM is a multifactorial genetic disorder which has been concluded in various studies, and multiple loci are involved to affect the autoimmune pathways in type1 diabetes patients (T1D). (Qu *et al*., 2021; Jerram, 2017).

Type 2 diabetes mellitus (T2D) is a common metabolic complication present in almost 90–95% of DM and is mainly affected by two main risk factors: impeded insulin production by beta cells of pancreas and low insulin sensitivity in the insulin sensitive receptors of the body. Insulin is the main hormone involved in regulation of glucose in blood, hence if any disruption occurs in synthesis, activation and or secretion may dysregulate the metabolism and T2D may occur. The T2D is affected by multiple factors such as food, physical activity, gastrointestinal health, and metabolic memory (Goyal, Jialal, & Singhal, 2023). T2D also increases the probability of progression of heart disease by ameliorating the process of arterial occlusion. A thorough understanding of molecular links between T2D, resistance developed on receptors of insulin, and cardiovascular complications is essential for properly managing this disease (Galicia et al., 2021).

Diabetes mellitus (DM) manifests in a variety of long-term and variable symptoms, including polydipsia, dry mouth, increased frequency of urine, tiredness, blurred vision, numbness of limbs, delayed healing of wound or cut, and skin or vaginal fungal infections (ElSayed et al., 2023). The chances of developing insulin independent diabetes depend on increasing age, reduced physical activity due to a sedentary and immobile lifestyle, and obesity or overweight due to overconsumption of unhealthy foods, according to standards of BMI (>23 kg/m2 for Asians) (Bae et al., 2021). Other risk factors may include a history of diabetes in pregnancy or polycystic ovarian syndrome in women, as well as lipid disorders or high blood pressure, along with some ethnic predispositions of Afro-Americans, Hawaiian Natives, Latins, and Asians. The genetic association and family history of 1st-degree relatives, who are more at risk to develop DM (Lo et al., 2017).

Diabetes is diagnosed when HbA1c values are almost 6.5% or higher, fasting blood glucose readings are 126 mg/dl or higher, and random blood glucose values are 200 mg/dl or higher. The HbA1c test is a highly effecti-e test in monitoring diabetic management and compliance. It offers a complete picture of diabetes control over the past two to three months, a unique feature not found in any other test (ElSayed et al., 2023).

Hyperglycemia has been found to have negative effects on cognitive function and neuronal health. One of the consequences is the glycation of tau proteins, which can lead to the formation of neurofibrillary tangles, a key factor in the development of Alzheimer's disease (Kumar et al., 2022). In DM, the presence of advanced glycation end products leads to neuron apoptosis and angiogenesis. Additionally, a particular A beta-receptor generates reactive oxygen species, which in turn impair mitochondrial function and deplete neuronal energy resources. Through the process of increasing tau protein phosphorylation and neurofibrillary tangle generation, insulin resistance is associated with dysregulation syndrome and can potentially raise the risk of Alzheimer's disease Insulin therapy has been shown to have a marked contribution on the behavior and memory of individuals with this neurodegenerative disorder. Additionally, maintaining excellent glycoregulation is crucial for preventing permanent loss of memory (Kubis-Kubiak et al., 2020). Gaining a deeper understanding of the connection between DM and brain functioning may open up alternatives for developing therapies to combat dementia. Various factors, including

decreased insulin production, amyloid protein accumulation, glucose absorption, oxidative stress, blood vessel damage, apoptotic pathway activation, aging, increased CHO-protein reaction, tau protein phosphorylation, brain atrophy, fat metabolism impairment, and mitochondrial damage, link Alzheimer's disease and T2D (Agarwal, 2022).

Renal insufficiency plays a significant role in the development of end-stage renal disease (ESRD), peripheral neuropathy leading to the risk of diabetic foot ulcer/gangrene, amputations, and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular or cerebrovascular (stroke) manifestations or neurodegenerative disorder, i.e., Alzheimer’s disease, as well as loss of sexual desire, are the next complications that result from long-term or chronic hyperglycemia or any inadequacy in controlling Type 2 diabetes after retinal degeneration being the prime (Gowthami, 2023; Petersmann *et al*., 2018).

Diabetes mellitus is a significant contributor to endothelial dysfunction, cellular defense abnormalities, and other diseases associated with diabetes. It is characterized by high blood sugar levels (hyperglycemia), resistance to insulin, high levels of lipids in the blood (hyperlipidemia), and elevated oxidative stress (Antar *et al*., 2023). Hyperglycemia inhibits the generation of nitric oxide by endothelial cells, resulting in elevated amounts of reactive oxygen species (ROS), specifically superoxide radicals, which cause damage at the cellular level. This leads to the production of peroxynitrite, which has an impact on the dilation of blood vessels in the endothelium and the oxidation of lipids (Dubsky *et al*., 2023). As a result, there is an elevation in levels of low-density lipoprotein, which can contribute to the development of atherosclerosis, inflammation, abnormal growth of the inner lining of blood vessels, clumping of platelets, and the formation of blood clots. Deterioration of the autonomic nervous system leads to the development of dry skin, thick plaques, and calluses, which contribute to the occurrence of diabetic neuropathy and diabetic foot ulcers (Rosen & Yosipovitch, 2022).

Inflammatory cytokines stimulate NF-κb activation, causing osteoclast maturation and bone abnormalities (Mussbacher *et al*., 2019). Impaired blood vessel function increases thromboxane A2, causing blood vessels to constrict, leading to excessive blood clotting,

reduced blood flow, tissue damage, ulcer formation, tissue death, and infection development (Antar *et al*., 2023).

Typically, these infections involve multiple types of microorganisms. In diabetic foot infections, methicillin-resistant S. aureus (MRSA) can be found in approximately 10% to 32% of cases (Kaimkhani *et al*., 2018). Staphylococcus aureus is the most common organism responsible for infections in DFUs, followed by Proteus species, Klebsiella species, and Pseudomonas aeruginosa and identification of Staphylococcus aureus as the prime bacteria responsible for infections in cases of DFU emphasizes its importance in the management of wounds (Kumar, 2022; Kaimkhani et al., 2018).

Besides the bacterial species mentioned earlier, E. coli, is also one of the important causing agent. In many researches it has been reported that infection is caused by a variety of organisms. Precise pointing of organisms are important for antibacterial treatment for DFU and avoid the risk of development of complication like gangrene and amputations (Vincent *et al.*, 2020; Hurlow, 2018).

Mostly long duration of DM and high HbA1c levels for prolonged duration besides treatment are important risk factor for DFU. Smoking, inefficient metabolism of lipids, as well as raised blood pressure are also important risk factors as discovered by the EURO-Diab group. Males are 6 times more prone to develop DFU in Western nations. Patients with microvasculature diseases like retinopathy and nephropathy have more likely chances of developing DFU (Abdulaziz *et al*., 2023). Atherosclerotic plaques in circulation leads to activation of inflammatory cascade and angiogenesis, which slows down the healing of wounds and may result in death of tissues and gangrene (Syafril, 2018).

The chronic wounds are developed mainly due to diseases of peripheral nerves and arteries (PN, PAD) and inefficient control glucose in blood. This interplay of different risk factors delays the wound healing and susceptibility of infection in diabetes patients. Breaking down the percentage of DFU, neuropathy covers around forty to sixty percent of total foot ulcer cases whereas PAD accounts for 45% of foot ulcers (Amin & Doupis, 2016). Diabetic retinopathy is the most frequent complication of DM, and diabetic peripheral neuropathy is also similar in occurrence and mainly dependent on long duration of diabetes.

Ischemia of the lower limb mainly occur due to peripheral arterial disease also adds the burden and accounts almost 10% of total DFUs (Ojalvo *et al*., 2017).

Individuals with diabetes develop an ulcer below the ankle extending all layers of skin, from superfiscial to deepest layers of skin and even involvement of bones as in osteomyelistis. DFU is the main contributing factor for amputations mainly without any major trauma, consquently leads to limitations of normal functions of life, morbidity as well as poor quality of life lacking the basic needs for human life. A study predicted that foot ulcers will increase around 125 million by 2025, and among these 20 million may require amputations of lower extremity (LEA). It was also seen that DFUs have higher mortalities compared to aggressive cancers within 5 years of duration (Armstrong, 2017).

It was reported in the World Health Organization (WHO) that most of the amputations were reported in diabtes patients in comparison to the non-diabteics and risk is 10 times more when compared according to the available prevalence in literature. The patients of DM have around 15-25% risk of developing DFU. DFU has no specificity with age of occurrence but mostly patients presented with 45 years of age or older (Oliver, 2023).

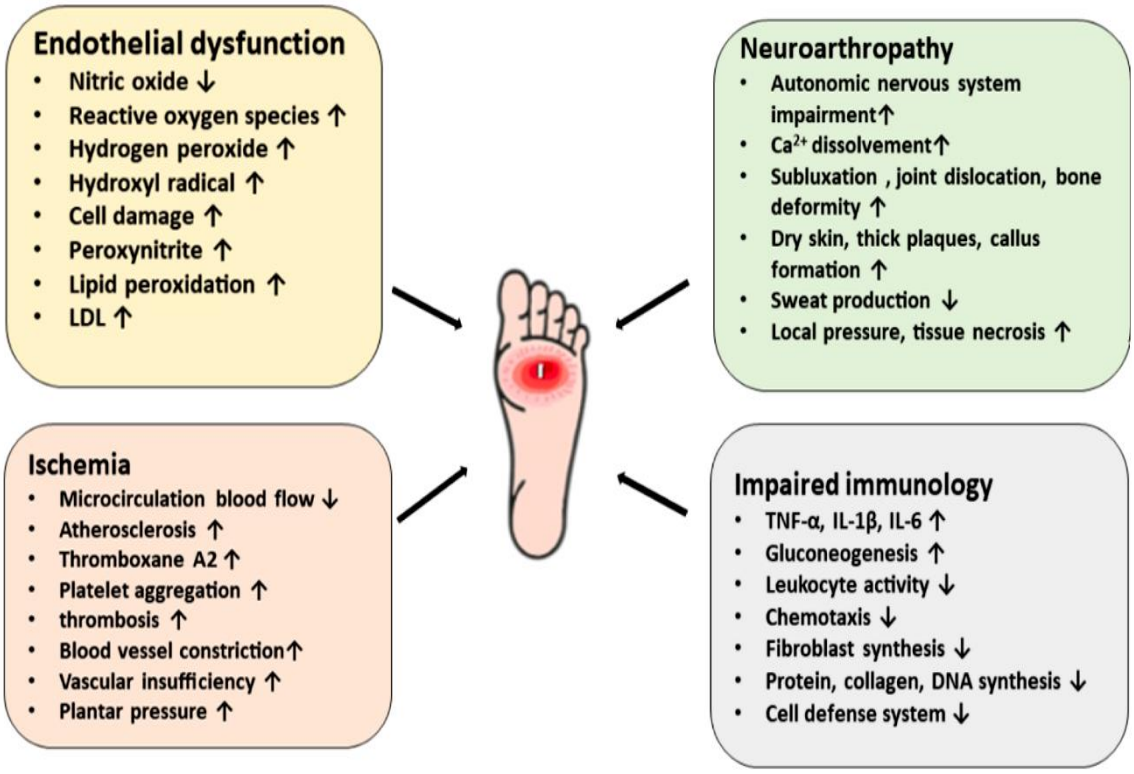


Figure 1.1: A schematic diagram representing the pathophysiology of diabetic foot ulcers via different mechanistic pathways. Endothelial dysfunction, ischemia, neuroarthropathy, and impaired immunology contribute to the pathophysiology of DFU (Ansari *et al*., 2022)

In Pakistan, 16.83% of the population suffers from diabetic foot ulcers. Even with improvements in diabetes care, DFU prevalence is still too high, especially in some demographic areas. Pakistan, a South Asian nation, is dealing with a concerning increase in the prevalence of diabetes and its related problems. DFU is one of the many problems that people with diabetes in Pakistan confront, and because of its chronic nature, frequent recurrences, and related risks of infection, gangrene, and lower limb amputation, it poses a significant challenge to healthcare practitioners and policymakers (Akhtar *et al*., 2022).

A DFU's process typically consists of three stages. Callus formation is the first step that occurs throughout this process. Calluses are a result of neuralpathy. The neuropathic foot often exhibits warmth, dryness, numbness, and an absence of pain sensations. It is associated with neuropathic ulcers, charcot joints, and neuropathic edema. In contrast, the ischemic foot typically exhibits a low temperature and shows specific areas of tissue death and gangrene, accompanied by the absence of pulses (Perez-Favila *et al*., 2019).

Sensory neuropathy leads to a loss of sensation, leading to ongoing limb injuries, while motor neuropathy changes the physical shape of the foot. Another factor to consider is autonomic neuropathy, which can lead to dry skin. Eventually, consistent pressure on the callus leads to bleeding beneath the skin, which gradually wears it down and forms an ulcer. If bacteria invade the area, the ulcer may become infected. The plantar metatarsal head, heel, and hammertoe tips are the most common locations of ulcers (Syafril, 2018). Hammertoes, calluses, fissures, and brittle nails are further physical characteristics (Oliver, 2023). Individuals with diabetic neuropathy usually do not experience any symptoms initially. However, a minority of them may eventually encounter neuropathic symptoms, such as numbness, paresthesia, or a burning sensation that tends to worsen during the night. The prime site of occurrence is foot (ankle joint or below) infection, ulcer, and/or deep tissue loss due to distal nerve anomalies in the lower limbs and varying degrees of peripheral vascular disease (Ojalvo *et al*., 2017).

The groundbreaking finding of insulin in 1921 completely transformed the way diabetes is managed, greatly increasing the life expectancy of those affected by the disease. Recent developments have increased the frequency of DFUs. In the second century AD,

the term "diabetes" was introduced by the Greek physician Aretaeus of Cappadocia. This highlights the immense danger that diabetes presented prior to the discovery of insulin (Haney, 2020). The Greeks and Romans made remarkable progress in the field of medicine, specifically in the domains of wound dressing, tissue removal, and amputation procedures. After discovery of insulin, diabetes with low life expectency, improved and changed it leading to onset of chronic complications. In the earlier era , foot ulcers were mainly linked with patients with diabetes. These challenges with longstanding diabetes mellitus opened up the new horizons of management of DM (Porta, 2020).

Besides increasing the rates of ulcers in diabetes, discovery of insuln, also increased the rate of developing more complications related to metabolic and vascular changes. For pathogenesis of DFU, ischemia of lower limb, diseases of peripheral nerves, poor compliance with medicinal treatments, poor wound healing and improper immunity hold responsibility (Aljohary *et al*., 2024).

Wound in diabetes may appear with multiple symptoms usually with open wound after minor trauma mainly located on the sole of foot, prominent changes in color of skin either red or darker and increase or decrease temperature perception of feet, thickness and indentation around the are of ulcer, although nerve damage may affect pain sensations but pain can be remarkable system along tenderness as cellulitis may be suspected, usually bacteria colonize on the site of open wound, pus is formed with foul smell and may or may not present with fever with or without chills (Dias *et al.,* 2022).

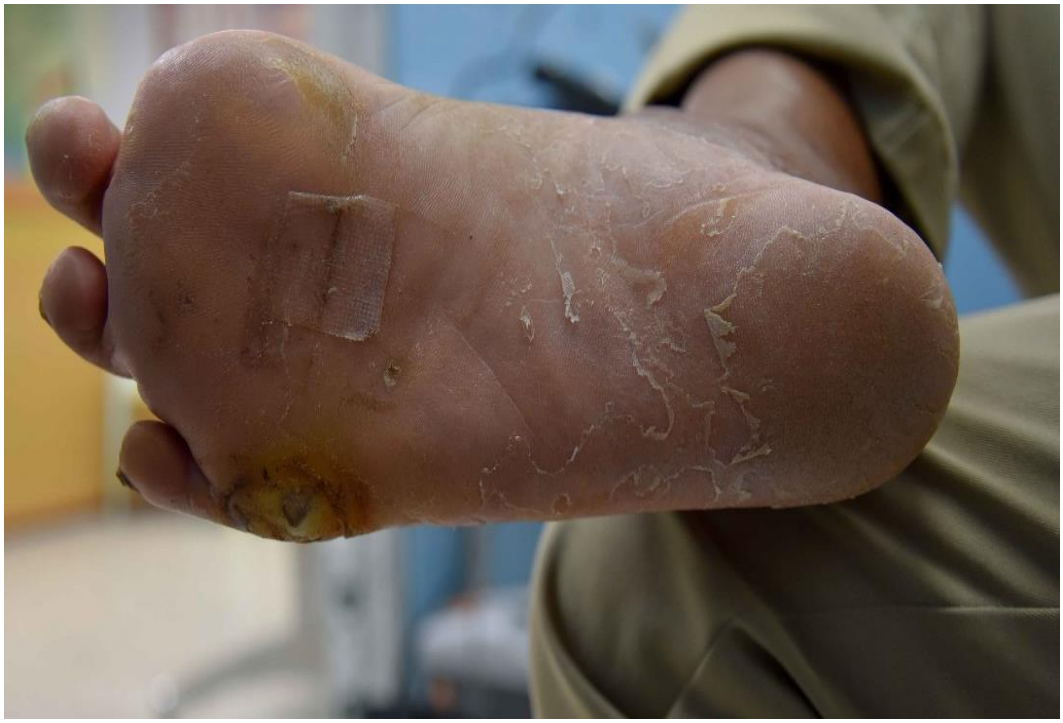


Figure 1.2: Right foot of a patient with diabetic foot infection wound

DFU is a multifaceted disorder may occur due to familial or environmental factors. The familial or genetic predispositions are mainly involved in resistence developed in islets of langerhans of pancreas for insulin as well as metabolism of macronutrients including glucose, proteins and lipids which are interlinked with each other. Whereas environmental reason may involve presence of pollutants in air as well as heavy metals used in factories, or viruses increasing the reactive oxygen species or free radicals in body (Pearson, 2019).

Pathogenesis of DFU mainly involve a term named as triad, which include nerve damage, occlusion in blood vessels and infections followed by any minor injury or trauma. The ulcer initially is the forms of scales or scabs, thus protecting the underneath skin, may heal or converted into dead tissues. Healing of wound may involve slowing of blood, activation of coagulation and inflammatory cascade, migration and multiplication of cells and restoration (Wang *et al*., 2021).

In the beginning of the 20th century, Elliott Joslin introduced a multidisciplinary approach for the treatment and managemnt of DFU. This approach completely changed the lens of managing DFU and broadened the vision to assess the complicated disease. As after discovery of insulin, increased the burden of DFU, fad diets, sedentary lifestyles, lack of movements had also raised the burden of DM globally and converted to an epidemic. Multiple RCT and meta-analyses were conducted in order to expand the detailed knowledge of modalities through which diabetic foot ulcer could be evaluated and controlled (Aljohary *et al*., 2024; Monteiro-Soares *et al*., 2020). Using multidisciplinary approach medicinal and surgical experts encompassed the effectiveness identification of ulcers, thorough checkup , controlling blood glucose levels, reporting and controlling co-morbidities and their associated symptoms, unloading the pressure on foot, cleaning of the wound debris and dead tissues, covering of wounds, antibiotics to present and control the spread of infections, revascularization as well as awareness about grievance of the disease are the prime targets of dealing diabetic wounds. Educating the diabetic patients about foot care is the crucial step of diabetes and it involves (Maria *et al*., 2022 ; Kaimkhani *et al*., 2018).

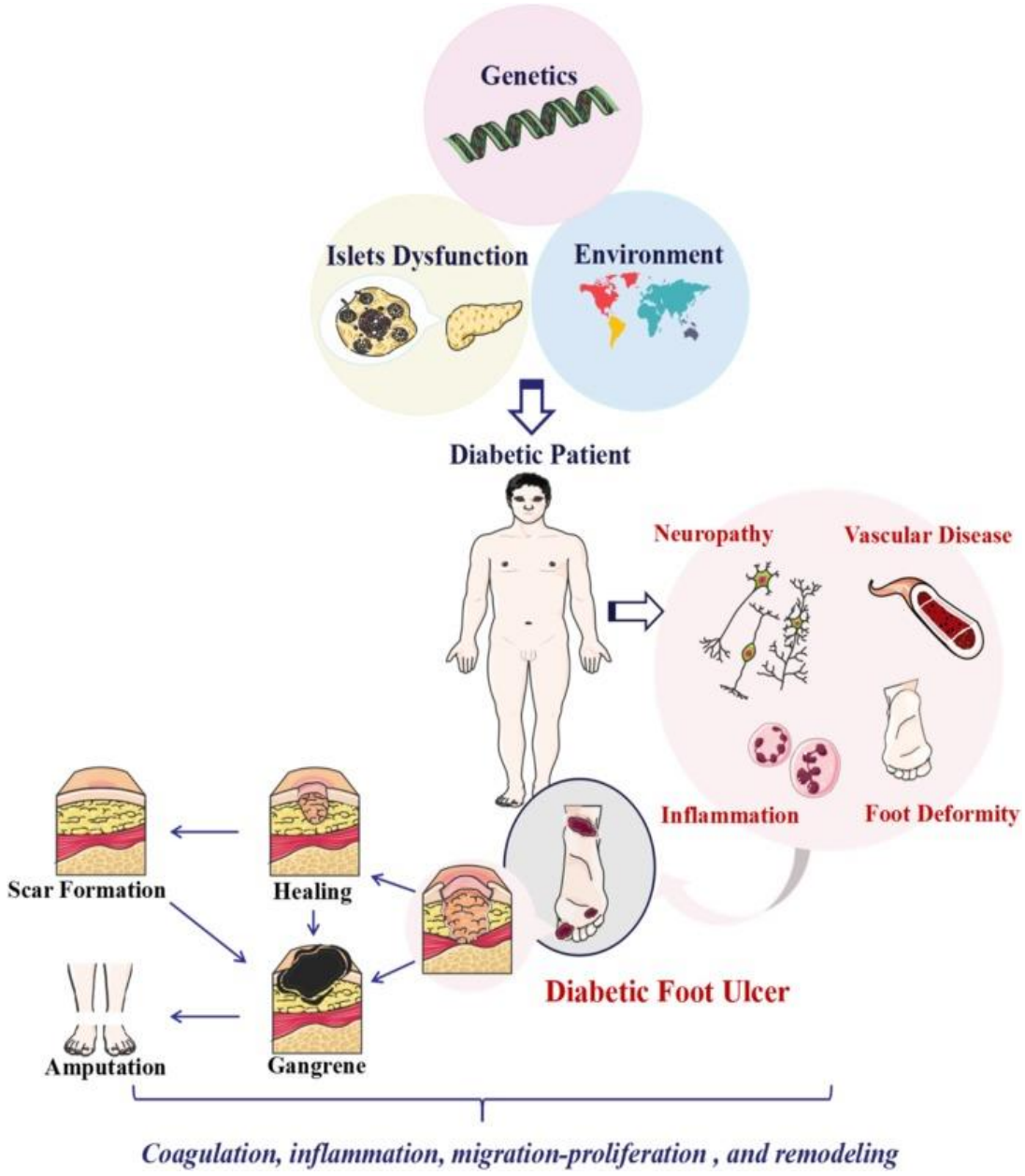


Figure 1.3: A schematic demonstration of factors contributing to diabetic foot ulcer (Wang *et al*., 2021)

While different foot ulcers reported it became necessary to devise a classification system to categorize detection, management and treatment options viz medical or surgical, or manifestation of ulcers whether smputation or healing. For this purpose Meggitt-Wagner presented with a system of classification of wounds in 1976 and was named as Wagner's classification. It introduced grading system from 0 to 5, involving depth wound, ocurrence of gangrene and affects on bone. It is helps in choice of management whether wound debridement is sufficient or surgical intervention can be introduced to the wound. (Monteiro-Soares *et al*., 2020). Although Wagner systm of classification is easy to use but it doesn't include the diagnosis of peripheral arterial disease and hence not reliable tool for assessment (triage tool) of DFU as assessed by Jeon *et al*. (2016).

The system of classification presented by Wagner was further modified in 1996 by the University of Texas, which identify DFU into two prominent groups one showing depth which is presented as grades 0 to 3 and an infection as stage B and ischemia as stage C or if co-exist then stage D. Thus the UT system became more explorative and involved in better assessment of prognosis (Wang *et al.*,2022). An International Working Group on the Diabetic Foot Risk Classification System (IWGDF) or PEDIS, focuses more on depth,sensation status, infection and extent of neuropathy and presence of ischemia (perfusion) (Bus, 2020; Monteiro-Soares et *al*., 2020).

Two quantitative classification systems are known for assessment of healing and amputations among DFUs. One is WIFi and other is Diabetic Ulcer Severity Score (DUSS) and important traiging tools in primary or community based setups. Where WIFi system covers the estimation of details of location, margins, size and depth of ulers besides the impede in blood flow of lower limbs as well as bacterial growth while DUSS include similar factors with detailed evaluation of perfusion of limbs by using ankle brachial index (ABI) (Jeon *et al*.,2016).

The SINBAD scoring system is the simplest, valid and reliable tool for screening of DFU. It assigns 0 or 1 points to six factors i.e., site, loss of blood flow or ischemia, nerve damage, bacterial infection, area of ulcer and depth of ulcer and a chance of maximum scoring of six points. Usually ulcers are divided into two groups <3 are ulcers having more

chances of healing and ≥3 are aggressive ulcers with poor prognosis and amputations (Oliver, 2023; Maria *et al*., 2022).

All the systems of classification e.g., Wagner, UT, WIFi, PEDIS, DUSS and SINBAD have been trialed and tested in multiple studies to estimate healing of DFU as well as amputation of lower extremity, morbidty and mortality. Hence the classification systems are very effective tool to manage the global, psychological, financial burden of DFU (Monteiro-Soares *et al*., 2020).

Growth factors play an integral part in poor wound healing, particularly in wounds caused by diabetes. GFs are essentially polypeptides with hormone-like properties that regulate the process of tissue repair. Various growth factors, including VEGF, PDGF, EGF, FGF, TGF-beta, HGF, and others, play important role in quick recovery and healing of wound (Zheng *et al*., 2023).

Table 1.1: SINBAD classification for diagnosis of DFU (Niță *et al*., 2023)

Category	Definition	Score
Site	Forefoot	0
	Midfoot and hindfoot	1
Ischemia	Pedal blood flow intact: at least one palpable pulse	0
	Clinical evidence of reduced pedal flow	1
Neuropathy	Protective sensation intact	0
	Protective sensation lost	1
Bacterial infection	None	0
	Present	1
Area	Ulcer <1 cm^2	0
	Ulcer ≥ 1 cm^2	1
Depth	Ulcer confined to skin and subcutaneous tissue	0
	Ulcer reaching muscle, tendon or deeper	1

Researchers are developing more interests in growth factors linked to nerve damage in the limbs, vascular diseases, inflammatory responses, and the extracellular matrix (ECM) components like collagen involved in healing because they can be helpful in treating diabetic foot ulcers (Qi *et al*., 2018).

The epidermal growth factor (EGF), a potent cytokine, is main contributor in the healing of wound. As it encourages proliferation of cells. EGF has been frequently researched in randomized control trials (RCT), animal studies and in vitro cell lines. It has been observed that EGF is lowered in patients of DM with poor glycemic controls (Mahmoud *et al.,* 2024; Trimal *et al.,* 2019). While DM lowering the EGF levels trigger the unfolding of both local and systematic complications (Berlanga *et al.,* 2020).

EGF is a prominent peptide that plays a critical part in various biological mechanisms. Earlier, a molecule was isolated from human urine was discovered having double properties of inhibiting the gastric acid secretion and proliferation of fibroblasts. Human derived EGF (hEGF) got the similarities in structure and function with urogastrone. EGF acts via its receptor, EGFR which activates intracellular pathways and promotes differentiation and multiplication of cells. The molecular weight of hEGF is 6 kilo Daltons, made up of 53 amino acids residues made after proteolytic processes. It possess three disulfide bonds within the molecule (Custo, 2022).

EGF is usually found in platelets, urine, saliva, milk, tears, and blood plasma. It may also present in submandibular and parotid glands. The male dominant hormone, testosterone has been discovered to enhance the formation of EGF (Mella, 2020). The epidermal growth factor receptor (EGFR), present on the surface of the cells, EGF bind onto receptor to perform its functions. This enables the binding of different mediators, leading to activation of receptor tyrosine kinase activity. This tyrosine kinase triggers multiple events of reactions within the cells thus enhancing the breakdown of glucose, anabolism of proteins and activation of EGFR gene on upstream of gene. These chains of reactions affect the DNA and cell proliferation (Patrizia, 2020).

EGFR is a single polypeptide chain with molecular weight of 170 kilo Daltons, is mainly located on the surfaces of human cells. The EGFR cluster is a mainly grouped under

receptor tyrosine kinases (RTKs), which consists of four members: ErbB1, ErbB2, ErbB3, and ErbB4 (Seebacher, 2019). Main pathway involved in EGF activation is JAK-STAT-MAPK which is induced by tyrosine kinase. Advanced glycation end products (AGEs) and their precursors have the ability to hamper the EGFR signaling via multiple mechanisms. This shows the complicated interplay of AGEs, RAGE and other signally mechanisms in cells, specifically for DFUs (Mengstie *et al.*, 2022). The signaling pathways are involved in the alteration at the level of transcription On the 5 prime untranslated region, modification occurs and thus EGF is either upregulated or downregulated (Ortega *et al.*, 2020).

Advanced glycation end-products (AGEs) are important and highly reactive molecules and are formed by non-enzymatic glycation of molecules with glucose. The receptor for AGEs is an important modulator involved in transduction of signaling pathways and are involved in initiation of inflammatory cascade and is responsible for aging and age related illnesses such as diabetes, plaques in arteries, and degeneration of neurons (Twarda-Clapa, 2022). Precursor of AGE, that is, glyoxal (GO) and methylglyoxal (MGO) interrupt the onset of signaling for epidermal growth factor receptor (EGFR). Auto-phosphorylation in tyrosine kinase and phospholipase Cgamma1 is interrupted (Zgutka, 2023).

Human cells have approximately sixty different kinds of receptor tyrosine kinases (RTKs), classified into 20 subfamilies based on their domain structure. Each RTK subfamily is different because it has an extracellular domain that binds to ligands, a transmembrane region, and an intracellular region that contains the tyrosine kinase domain and other regulatory and protein interaction domains. When a ligand binds to receptor tyrosine kinases (RTKs), they usually pair up and activate auto-phosphorylation on specific tyrosine residues inside the cell. Auto-phosphorylation enhances the catalytic effectiveness of the receptor and creates opportunities for the formation of downstream signaling. Many times, receptor tyrosine kinases (RTKs) set off signaling pathways like RAF/MAP kinase cascades, AKT signaling, and PLC-gamma-mediated signaling. Activation of these pathways eventually leads to alterations in gene expression and cellular metabolism (Babu *et al*., 2023).

Gene polymorphisms are a form of genetic variation that occurs often in humans, with a frequency that is more than one percent. Conversely, researchers often discover DNA mutations in a smaller number of individuals and at a lower frequency than other types of mutations. Variations in genes affect an individual's metabolism response as well as their risk of developing disease, and they may act as effective biomarkers in the study of disease diagnosis, drug development, boosting immunity against different environmental factors, and behavioral studies among different races (Giandalia *et al*., 2021). Cultural differences and risk factors can cause variations in genes across different ethnicities, which in turn can impact health-related instances. Personalized medicine places significant importance on understanding gene variations, as it designs drugs based on an individual's genetic architecture or genotype, medical history, and lifestyle to optimize effectiveness and minimize risks. Gene mutations primarily contribute to the pathogenesis of disease, while variations among genes may not have the same impact (Chiarella, 2023).

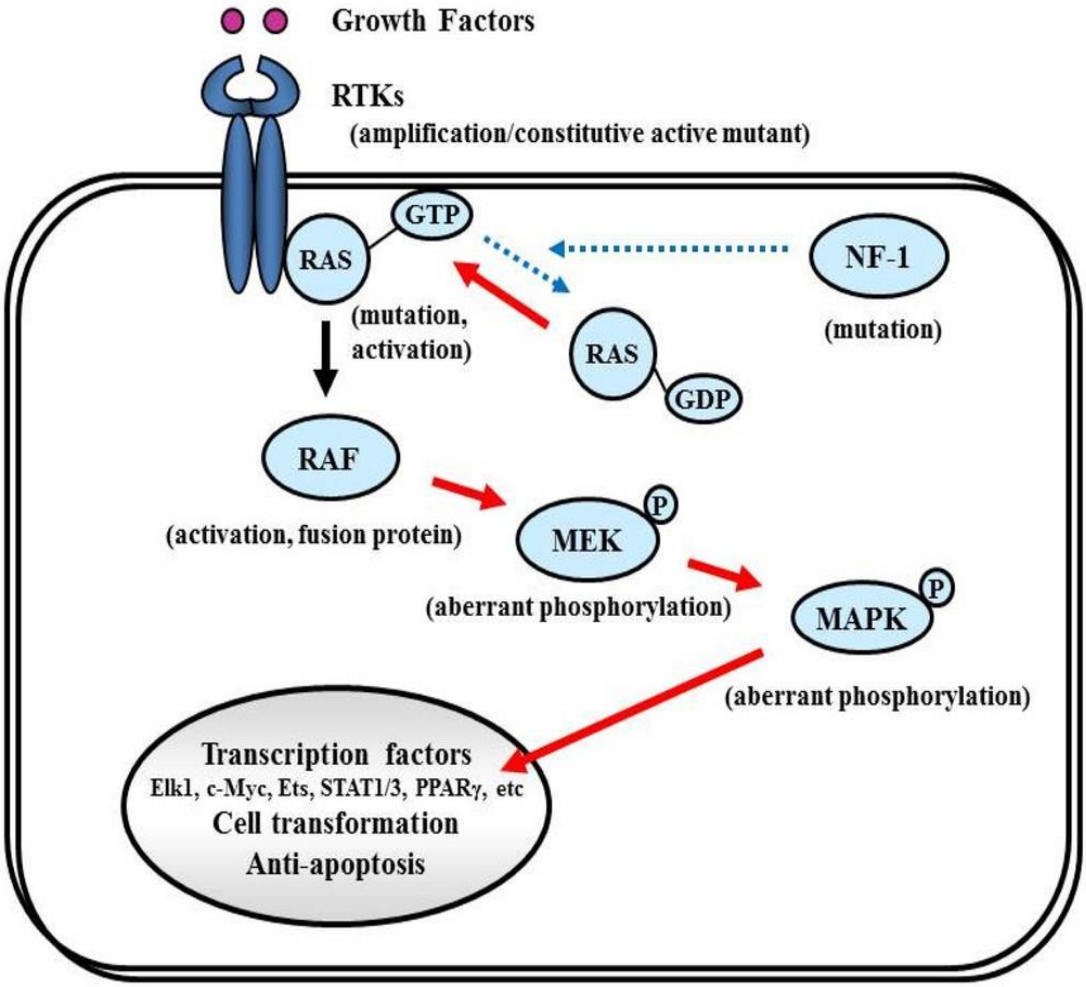


Figure 1.4: EGF signaling pathway (Zhang & Wang, 2019)

RAS signaling. RTKs and NF-1 regulate RAS proteins' on/off (RAS-GDP/RAS-GTP) switches. RAS-GTP stimulates RAF for serine/threonine kinase. MEK, triggered by RAF, triggers MAPK. MAPK activation activates transcription factors such Elk1, c-myc, Ets, STAT1/3, and PPARγ, leading to cell transformation and inhibiting apoptosis. Solid and dashed arrows show activation and suppression. STAT: signal transducers and activators of transcription; PPARγ: peroxisome proliferator-activated receptor γ (Zhang & Wang, 2019).

Epidermal growth factor (*EGF*) has a genetic variant named rs4444903, also known as +61 which shows A allele is replaced with G at position 61. There is a association between the *EGF* and a specific genetic variation in the 5 prime untranslated regions of the *EGF* gene (A>G rs4444903) (Ayman El Shayeb, 2022; Baghdadi *et al*., 2020). It is concluded from literature search that this particular genetic variation, when it is present on the promoter region of the *EGF* gene, is mainly accountable for the production of EGF. Different research data identify that this variation is a critical part in the EGF synthesis. It appears that the rs4444903 (G) allele results in higher levels of EGF compared to the (A) variant is evident more with cancers. Individuals with the GG genotype show higher levels of EGF formation and synthesis in comparison to those with the AA genotype. Hence this polymorphism increase the risk of developing cancers and fibrosis (Zhang *et al*., 2017). The *EGF* gene is located on chromosome 4, on long arm 4q25–q27 where 25 and 27 shows special bands present on the long arm of chromosome (Leal *et al*., 2020). Multiple clinical trials have established EGF as an adjunct therapy for DFU, but no study has examined explored gene polymorphism corresponding to DFU (Akkus *et al*., 2022).

The primary objective of this investigation is to determine the *EGF* gene polymorphism rs4444903 and its association with DFU for a better understanding of T2D complications as well as to design an effective strategy for the treatment of DFU. EGF over-stimulation, which is involved in many cancers, may play an important role in wound healing. Early identification of persons at risk and prompt diagnosis would aid in the recommendation of mid-adult lifestyle adjustments that will prevent or decrease the course of illness.

1.2 Rationale of the study/ Research Gap

The study aimed to understand the role of *EGF* gene polymorphism rs4444903 in the pathogenesis of DFU; however, *EGF* gene polymorphism association with DFU hasn't been established yet.

1.2.1 Theoretical Gap

So far, the *EGF* gene polymorphism rs4444903 has not been investigated in association with DFU in T2D patients, and there is limited knowledge in it. Although, DFU is a serious complication of T2D and has been explored for many other growth factors and genetic variations.

1.2.2 Contextual Gap

Before this research there was a gap for *EGF* gene polymorphism rs4444903 (+61 A>G) in the Pakistani population, henceforth susceptibility to DFU, healing processes, and correspondence with other risk factors can be targeted to explore.

1.2.3 Methodological Gap

Despite its widespread usage in genotyping and gene variation investigations, the amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) has not yet been examined with the *EGF* gene polymorphism rs4444903 (+61 A>G) and diabetic foot ulcers (DFU).

1.3 Problem statement

Pakistan ranked 3rd globally for DM and DFU is the main complication of DM and burden of DFU is 16.83% in Pakistan. EGF has been linked with pathogenesis of DFU but how *EGF* gene polymorphism associate with DFU is not explored that's why the role of *EGF* gene polymorphism with susceptibility is explored in this study.

1.4 Research Question/ Hypothesis

(A) Null Hypothesis

There is no association between *EGF* gene polymorphism rs4444903 (+61 A>G) in the occurrence of diabetic foot ulcers (DFUs).

(B) Alternative Hypothesis

There is an association between *EGF* gene polymorphism rs4444903 (+61 A>G) in the occurrence of diabetic foot ulcers (DFUs).

1.5 Objective

To determine the association of *EGF* gene polymorphism rs4444903 (+61 A>G) in T2D patients with DFU as compared with T2D patients without DFU.

1.6 Significance of study

This study sought to investigate the link between *EGF* gene polymorphism rs4444903 (+61 A>G) and the susceptibility to diabetic foot ulcers. The goal was to shed light on the genetic factors that play a role in the development of DFUs. A polymorphism may be associated with disease if the targeted allele is found more frequently in cases compared to controls. These findings hold great promise in enhancing risk assessment, treatment strategies, and clinical management of DFUs, ultimately leading to improved outcomes and quality of life for those affected. This could lead to the development of genetic screening tools.

CHAPTER 2

LITERATURE REVIEW

Diabetes mellitus of type 2 (T2D) is a progressive disease with gradual onset, mainly characterized by metabolic abnormalities linked to insulin resistance, inadequate insulin synthesis, and a raised level of glucose in the blood chronically. Obesity, waking up late at night, and minimal activity during the day all contribute to the rise in diabetes mellitus (DM). It exhibits a strong correlation with both micro- and macroangiopathic abnormalities, leading to significant physical and psychological distress. This, in turn, impacts personal and governmental finances, which are crucial for maintaining economic equilibrium (Carmichael, 2021). So far, researchers have identified a total of 76 genes, with the development of T2D primarily impacting pathways related to cellular signaling by p53 and necrobiosis. As DM gets worse, oxidative stress from radicals, apoptotic pathways, and cell signaling pathways like p53 and MAPK can cause the loss of islet cells in the pancreas, which hurts insulin production (Luo *et al*., 2024) using animal studies. Over the last few decades, the prevalence of diabetes has sharply increased worldwide. According to the International Diabetes Federation (IDF), there were around 463 million diabetics worldwide in 2019. By 2045, the International Diabetes Federation (IDF) projects a 700 million-dollar increase in this number. According to Azeem *et al.* (2022), there will be a 69% rise in diabetes in underdeveloped countries and a 20% rise in developed ones between 2010 and 2030.

Diabetes-related problems of the lower extremities rank tenth among the leading causes of disease burden and disability worldwide, placing a heavy burden on patients and society (Lazzarini *et al*., 2018). DFU is one of diabetes's most complicated and expensive co-morbidities. Each year, an estimated 9.1-26.1 million diabetics around the world develop a foot ulcer. A foot ulcer will appear in 19–34% of diabetics at some point in their lives (Azeem *et al.,* 2022). Burning sensations, loss of senses, prickling, and pain are all symptoms of peripheral neuropathy. Weakness in the lower limbs, including the foot, follows these symptoms, potentially exacerbating the neuropathic pains experienced by approximately one-third of those with peripheral neuropathy. Later on, this neuropathy may develop into calluses and ulcers, or it can worsen ulcers that are already present by producing a lack of sensation, which can conceal the ulcers from detection for lengthy periods of time (Yang *et al*., 2022). Therefore, early identification is crucial to preventing ulcers. In certain cases, the very first indication that manifests itself in peripheral neuropathy is DFU. This symptom may progress to include irreversible tissue damage, LEA, and severe suffering (Carmichael, 2021).

DFU not only exerts a significant impact on the physical well-being of patients, but it also gives rise to psychological issues such as anxiety, depression, and other detrimental emotions. Moreover, DFU places a substantial economic burden on both families and society. A patient with a diabetic foot ulcer has a 2.5-fold increased likelihood of dying at 5 years than a patient without a foot ulcer (Polikandrioti *et al*., 2020).

Estimates provided by the IDF, the global burden associated with diabetes will be reaching USD 760 billion in 2019 (Top, 2020) and Pakistan's IDF survey estimated, the prevalence of DM is 26.7% (Azeem *et al*., 2022), while the prevalence of DFU is 16.83% (Akhtar *et al*., 2022). Diabetes patients in Pakistan regularly experience foot ulcers and amputations. This problem is caused by a lack of adequate medical care, low economic status, and worsening wound care conditions. Due to a dearth of healthcare resources for diabetes and its related co-morbidities, Pakistan is poorly equipped to tackle this burden (Aslam *et al*., 2023).

Humans have gene polymorphisms, a kind of genetic diversity, with a frequency greater than 1%. On the other side, DNA mutations are less common and often found in a smaller sample size than other forms of mutations. Genetic variations influence metabolic responses and disease risk; these variations may serve as useful biomarkers for research into disease diagnosis, medication development, enhancing immunity to various environmental factors, and racial differences in behavior. Ethnic variations in genes may have an effect on health-related cases, and this effect can be amplified by cultural differences and risk factors. Knowledge of gene variants is crucial in personalized medicine since each patient's medication is tailor-made according to their unique genetic makeup, medical history, and lifestyle choices in order to maximize efficacy and reduce side effects. While gene changes may not have a significant role in illness development, mutations in genes are the main culprits (Chiarella, 2023; Goetz & Schork, 2018). One of the most recent developments in the area of medicine is personalized medicine, which encompasses a wide range of health disciplines and seeks to target genetic, transcriptional, and translational alterations that occur as a disease advances (Luo *et al*., 2024).

EGF is important in healing wounds as well as tissue repair, it has been explored in diabetes mellitus by doing RFLP-PCR and expression studies via real-time qPCR. They also measured the EGF levels by ELISA and found that the GA heterozygous variant genotype may have an association with glycemic control as in poor glycemic control GA genotype was frequent and also found that higher levels of EGF are linked with good control of diabetes mellitus (Mahmoud Alfaqih, Jannat Maraqah, & Ebaa Ababneh, 2024). Another study was done in the Indian population by using the same methodology with the difference of real-time PCR for expression of *EGF* gene analysis and it was concluded that the AG genotype is statistically significant and possesses strong susceptibility to T2D (Trimal *et al*., 2019).

DFU can arise from various causes, with the primary factor being infection that advances into gangrene accompanied by sepsis, posing a risk of amputation to the affected area (Viswanathan *et al*., 2019). Researchers conducted a study on the ethnicity of the South Indian people, specifically focusing on SNPs of chemokines and cytokines. The study also examined the progression and treatment strategy for DFU (Viswanathan, 2018).

A research explored the *VEGF* +405 G/C SNP its relationship to levels, infection severity, and amputation in the south Indian DFU population. The CC genotype of SNP was found to be a major risk factor for both T2D and DFU. Researchers analyzed and concluded results by using Restriction Fragment Length Polymophism-PCR and sandwiched ELISA. The CC genotype may also be associated with reduced levels in the serum as observed by ELISA. As a result, the *VEGF* +405 G/C SNP may have a genetic prediction and association for DFU and may help to avoid limb amputations (Ganapathy *et al*., 2023).

Recent research has identified several single nucleotide variants (SNVs) in several genes as potentially important in DFU progression and onset. They participate in a variety of biological processes, such as inflammatory reactions, the development of vessels, the oxidative damage response, extracellular matrix remodeling, and immune-mediated adjustment. Numerous SNPs within the respective genes may contribute to the ongoing severity of DFUs. Several studies have looked at how SNPs in genes related to *C-reactive proteins, IL-6, TNF-, SDF-1, VEGF, NRF2, SITR1, HSP-70, HIF-1, LOX, ITLN1, MAPK14, TLRs, OPG, VDR,* and *FIB* may impact how each DFU patient is diagnosed, their prognosis, and possible targeted therapies. (Hu, 2022).

Another study looked at the effects of four single nucleotide polymorphisms (SNPs): *TNF-α* (−308G > A), *SDF-1* (+801 G > A), IL-6 (−174G > C), and another SNP. People with DFUs were more likely to get a severe podal microbial infection, a higher grade of ulcer, or have to have a limb amputated if they had these SNPs: TNF-α (−308G > A) and SDF-1 (+801G > A). Furthermore, studies have linked *IL-6* (174G > C) to an increased risk, and this study's findings suggest that at least three of the four SNPs could be definitive risk factors for the development of DFUs (Viswanathan, 2018).

A study found that people with diabetes who have at least one T allele in their genotype of the *eNOS* Glu2983Asp polymorphism are less likely to get diabetic foot ulcers (DFUs). The T allele in this variation can be regarded as a risk for DFU pathogenesis. Therefore, ARMS-PCR amplification of genes may have beneficial effect for screening of DFUs in patients of DM. This helps an early diagnosis, management and treatment (Sadati *et al.,* 2017).

The EGF- receptor activates main intracellular pathways, resulting in the two most needed activities of EGF in tissue repair: cell proliferation and cell remodeling. The RAS-RAF-MEK-MAPK network controls the initial stages of cell cycle from the G1 phase to the S phase and is mandatory for the induction of mitosis. The PI3K-Akt route starts a cascade of apoptotic opposing mediators and cell protective mediators which help recovery of damaged tissues. One of the potential anti-aging effect of EGF is part of its natural structure. EGF hinders proliferation, programmed cell death, and premature senescence. EGF decreases at a certain level in saliva and circulation in diabetes. There are multiple studies to ensure that EGF and other GFs, are needed for cell cycle proliferation, are markedly reduced in long standing wounds e.g. diabetic foot ulcers (Berlanga-Acosta, 2019).

Growth factors (GFs) play important role in pathogenesis and healing of DFU. A study was explored in Alzheimer's disease by using gene expression studies was done to identify wild and variant alleles. Where mutant allele with missense type of mutation was identified in *EGF* gene linked to AD. A variant, rs4698800 of *EGF*, has shown notable association of risk with AD. The allele linked to increased risk, rs4698800, has shown an association with elevated *EGF* gene expression. This finding suggests that *EGF* gene may be associated with an increased risk of AD (Li *et al*., 2023).

Multiple studies had explored the single nucleotide polymorphism (SNP), rs4444903. The tumors in glial cells are initiated following complex pathways and are affected by multiple factors. One related to our interest is *EGF* gene polymorphism, where EGF proliferates the cells hence enhance the growth of tumor cells. *EGF* polymorphism mainly influence the expression of genes on promoter region and hence increases the synthesis of EGF. Glial cell tumors are most common neuronal tumors with poor prognosis although new and advanced treatments are available. Although the etiology of tumors is still debatable, familial and hereditary involvement is well known. Extensive research on glioma has yielded variable results. To sort out this ambiguity, all the case-control studies of glioma were gathered into this meta-analysis. Almost ten case and control studies from Asians and Caucasians were gathered from different databases and depicted that there was

a significant link between *EGF* rs4444903 polymorphism and glioma risk (Dugăeșescu *et al*., 2021).

Another meta-analysis of Caucasian ethnicity on multiple case control studies on melanoma and association with *EGF* gene polymorphism was conducted due to controversial findings in individual studies. The study did not find any connection between *EGF* polymorphism and the start and spread of melanoma, even though EGF is a strong mitogen and activates many cell signaling pathways (Wu, 2013).

The *EGF* gene polymorphism 61*G (rs4444903 G) was associated with a higher risk of hepatocellular carcinoma (HCC) in the Egyptian population study (patients with the G/G genotype are at a higher risk than those with the A/G and AA genotypes). These results suggest that this particular gene variation may have a significant impact on the emergence of HCC. The G/G genotype of tumor tissue had higher *EGF* expression, explaining the correlation. Consequently, the risk of developing HCC in Egyptian cirrhotic HCV patients could rise. Therefore, it is important to regularly monitor individuals who contain the risk alleles to enable early identification and better treatment outcomes (Baghdadi *et al*., 2020). Also in the Iranian population by Gholizadeh (2017), *EGF* gene polymorphism rs4444903 was studied for hepatocellular carcinoma; both followed RFLP-PCR for analysis, except Baghdadi *et al*. (2020), which included cirrhotic subjects along with HCC and healthy subjects, as well as analyzed the expression of *EGF* with similar findings.

Since EGF plays a crucial role in mitogenesis, DNA synthesis, and tumor progression, researchers evaluated the SNP G61A in RCC, revealing a link between variant genotype AA and an increased risk of RCC. This study also measured serum levels of EGF, finding lower levels associated with RCC. Here, the mutation occurs at the 5' untranslated region of the respective gene, replacing G with A. The variant AA leads to decreased EGF synthesis in peripheral circular blood cells. Renal cell carcinoma (RCC), the third most common cause of genitourinary tumors, accounts for 2–3% of all cancers in developed countries. Half of the Algerian populace is at risk of developing RCC, with a higher prevalence in males (Bensouilah *et al*., 2020).

Growth factors, such as epidermal growth factor (EGF), play an important role in proper spermatogenesis and are largely generated by leydig cells in the testis. EGF and its receptor (EGFR) control spermatogenesis and gap junction development in spermatogenic cells. Infertility is defined as the inability to get pregnant after one year of unprotected intercourse. In approximately half of instances, men have poor sperm parameters. EGF increases cell proliferation, regulates other biological processes, and stimulates human sperm capacitation. EGF/HER2 signaling is most likely involved in spermatogenesis and Leydig cell steroidogenesis. A connection between the *EGF* +61A/G (rs4444903) SNP and serum levels has been proposed for idiopathic male infertility. Researchers investigated the AG and GG genotypes of the *EGF* SNP (rs4444903) and male infertility, discovering a strong relationship (Aminmalek, 2020). Leal (2020) proposed the effect of first-generation tyrosine kinase inhibitors on patient prognosis via the *EGF* rs4444903 polymorphism (AG and AG + GG), which was independently associated with stable illness in lung cancer patients. Researchers have linked this polymorphism to higher levels of EGF, which in turn poses a risk for the growth of specific tumors in cancer patients. This study looked at how the *EGF*+61A>G SNP can be used to spot and track lung cancer in Brazilian patients who had been treated with *EGF*R-mutated TKIs. There were 31.5%, 49.6%, and 18.9% of people with the *EGF* gene, as well as 56.3% and 43.7% of people with the allelic types. There was no link between genes and total or progression-free mortality. The *EGF*+61 allele was linked to steady disease in lung cancer patients, but not to the total response rate to first-generation TKIs or the result of the patients.

EGF is a protein found in the human epidermis, is involved in the multiplication and divergence of cells, and may promote the healing of epidermal wounds. Studies in periodontitis have explored and reported abnormal expression of the *EGF* gene. A study was done in dental clinics to evaluate inflammation in the tissues around the dental implant. It is a usual complication that occurs after the repair of implants and may cause the failure of dental implants too. This area of the implant holds a deficient blood supply and more room for inflammation, and after the initiation of the peri-implantitis, inflammation spreads faster and more aggressively, thus leading to implant failures. Recently, the pattern of peri-implantitis has not been found to be uniform in distribution and is hypothesized to be linked with genetic susceptibility. The two most common SNPs with a familial outcome of

inflammation are rs2237051 and rs4444903 (Wang *et al*., 2021), and the 5'UTR is involved in the upregulation of gene levels (Chen, 2018). This study used AS-PCR and DNA sequencing and found no evidence of any association with perimplantitis (Chang *et al*., 2021; Scarano *et al*., 2020).

A study was done to find out if there was a link between genetic variations in *EGF* rs4444903 and transforming growth factor (TGF) β1 (rs1800470) and facial measurements in Brazilian patients with dentine and facial distortion. The results revealed that heterozygous patients exhibited a decrease in mandibular length and mandibular base length, whereas homozygous A patients experienced a reduction in mandibular base length only. When compared to individuals with the homozygous A genotype, patients with genotype AG showed an increase in anterior facial height and ANS-Me distance measurements. The mandibular base length was found to be increased in heterozygous AG patients when compared to homozygous A individuals. The presence of heterozygous AG patients was associated with an increase in angular measurements in the TGF-β1 polymorphism, specifically related to the upper angle of the jaw. The study examines the potential correlation between genetic variations in EGF and TGF-β1 and facial measurements in patients diagnosed with dentine and face-related deformities (Cavalcante *et al*., 2020).

Mohamed *et al*. (2021) conducted a study on chronic hepatitis B liver disease and its progression in Egyptian children, which suggested that SNP variation A>G worsened the development of HBV. They found a higher frequency of the GG genotype, while AA genotypes may provide a protective role. Subjects with advanced liver disease and potent liver transplant candidates had the maximum number of GG genotypes; researchers used allele-specific PCR to detect polymorphisms.

Collectively, with earlier findings linking low urine EGF to the onset of CKD in people and exogenously administered EGF accelerating tubule repair in animal models, it suggests that physiological levels of EGF provide a protective effect on the adult kidney. Instead, the fact that some EGF-deficient mice developed glomerulonephritis, renal fibrosis, and inflammation shows that EGF helps protect and repair functions in the

nephron along with other modifier genes. These genes may have different activities in different mice, which can be explained by their varied genetic background (Zeid, 2022).

Pre-eclampsia, a dangerous syndrome in pregnancy consisting of high blood pressure and protein-containing urine, is hazardous for both mother and fetus, worsening both morbidity and the risk of death. The major outcome is babies with low birth weight. Pre-eclampsia is defined as high BP ≥140/90 mmHg, 2 readings with a six-hour gap, appearing after 20 weeks of pregnancy, and being diagnosed for the first time with a protein ≥1+. It was a case-control study in Srilankan people and was experimented with RFLP-PCR and massAssey. It was assumed that all the haplotypes of genotypes of *EGF* were associated with low birthweight in mothers of pre-eclampsia, except rs3756261G, which was associated with a relatively higher weight at the time of birth (Clemente & Bird, 2022).

Rare pancreatic neuroendocrine tumors, commonly known as NETs, account for approximately seven percent of all pancreatic malignancies. The purpose of this research was to determine whether or how much there is a connection between certain genetic polymorphisms in the *EGF, EGFR, and HER2* genes and the likelihood of developing PNETs. The researchers' genotyped 68 patients with incurable PNETs and 300 healthy people as controls to look into how genetic changes affect the chance of having PNETs and the development of metastases. People with the *EGF*R +1562 AG genotype and the AA/AG *EGF* +61/HER2 +1963 genotype were more likely to get personal network tumors (PNETs), according to the results. Furthermore, the *EGFR* +1562 AA genotype may be associated with an increased risk of developing insulinoma. There was an application of Weinberg equilibrium for both the controls and the cases, Weinberg equilibrium was applied (Marinović *et al*., 2022).

In a study, researchers in Central Europe aimed to explore the relationship between *EGF/EGFR* gene polymorphisms, mRNA expression, and plasma levels of EGF in relation to GERD and its complications. The research did not link the development of these gene variations or their expression levels to reflux esophagitis (RE), Barrett's esophagus (BE), and esophageal adenocarcinoma (EAC). Subjects were observed by endoscopic changes, expression was observed via reverse transcription by qPCR, and EGF levels were measured

using ELISA. Nevertheless, some gene variant carriers exhibited reduced *EGF* mRNA expression, which may indicate an impact on *EGF* gene expression. In this particular group of people, the research found no association between GERD, its consequences, and variations in the *EGF or EGFR* genes (Deissova *et al*., 2022).

The biliary tree is the most targeted site for a vast group of tumors called cholangiocarcinoma (CCA). It comes second to hepatocellular carcinoma-causing hepatomas. Henceforth, prompt identification is a must for cases at risk (Saffioti & Mavroeidis, 2021). CCA is a very fast-growing tumor with the worst outcomes and high mortality rates (Blechacz, 2017). Long-term inflammation may begin in the presence of molecular dysregularities caused by stimulation of various growth factors that enhance the epithelial lining of cells in the bile duct (Mavila, 2024). Metastasis directly correlates with the EGF/EGF receptor and its expression. When A swipes G, it results in a mutation that modifies the mRNA after transcription, thereby extending its half-life and increasing the EGF yield through elevated serum EGF levels (Jin *et al*., 2020). An Egyptian nation conducted a case-control study and experimented with genotyping using real-time PCR, finding that the G allele mutation may play a role in disease progression but not in tumor aggression (Gawish, 2023).

The research investigated the correlation between gene polymorphisms of *EGF/EGFR* in patients with oral squamous cell carcinoma (OSCC) and those without the disease. The research used a case-control design and included a total of 89 patients diagnosed with OSCC (oral squamous cell carcinoma) and 107 individuals who were deemed healthy controls. The conclusions suggested higher association of GG genotypes with oral squamous cell carcinoma, while wild genotype AA or A allele was more prevalent in healthy controls. The G allele was 2.3 times more associated in patients with oral squamous cell carcinoma (OSCC). This study suggests more investigations with bigger population size to explore better insight of correlation *EGF* gene polymorphism and OSCC. (Ertugrul *et al*., 2023).

Diabetic foot ulcers represent a prominent and deadly complication of diabetes. The low quality of life due to its negative influence on patients' is quite evident. To target

multidisciplinary approach in treatment of DFU, a study was conducted to assess the effects of intralesional epidermal growth factor in the treatment of foot ulcers in patients with diabetic foot ulcer. The study was conducted retrospectively on patients who met the study criteria and did not have wound infection or osteomyelitis. These patients underwent treatment with epidermal growth factor. The study involved the application of epidermal growth factor at a dosage of 75µg/day, three times a week. The administration of medication directly into diabetic ulcers, known as intralesional treatment, typically lasts for a duration of 4-8 weeks. The study yielded three significant findings. The observation of the efficacy of epidermal growth factor in the treatment of patients with diabetic foot ulcers has been documented. The second significant discovery was the importance of ensuring the safe epithelialization of the standing ulcers while maintaining the patients' quality of life. Additionally, following the debridement process, it was observed that treatment with epidermal growth factor resulted in a notable enhancement in wound healing (Baykan & Kara, 2022). Another research done by same mode and duration of treatment with intralesional EGF application, positive results were obtained in addition to good foot care in diabetic foot ulcers (DFUs). Furthermore, promising results were obtained by protecting the extremity from amputation by using it in patients whose vascular intervention methods are not appropriate and who have diabetic foot ulcers that do not heal with conventional wound care treatments (Çetinkaya *et al*., 2020).

. The rhVEGF and FGF-2, if used in combination of other growth factors may enhance the therapeutic efficacy. Further studies with big population sample are required to test these growth factors in randomized control studies. Extensive research in this domain may ease diagnosis and management strategies for DFU and its burden (Qi, 2018). Recombinant human epidermal growth factor (rhEGF) is a polypeptide made of 53 amino acids. It was picked up from the submaxillary glands of rats and later modified by biotechnology. It causes angiogenesis, cell differentiation as well as fibroblast and mesenchyme proliferation. These cascades are activated by synchronized activity of receptors tyrosine kinase and EGF (Çetinkaya *et al*., 2020).

Jeong *et al*. (2020) explored the significance of EGF coenzyme A, a novel EGF role, hastens healing of wound in longstanding wounds like DFU. If FGF or PDGF factors are modified similarly both may work synergistically and improve the ulcer of diabetes. EGF was infiltrated locally into the high graded DFU. EGF injections improves survival rate of fibroblasts and enables multiplication and hence important for healing of DFU (Berlanga *et al*., 2020).

These conclusion may suggest that healing of wound by using EGF infiltration is successful because of pharmacogenomics consequences. These advantages aid in a comprehensive healing process, resulting in a significant reduction in amputation risks and low long-term recurrence rates. EGF injection not only helps to correct soluble mediator imbalances, but it also has the potential to reverse the premature senescence associated with diabetes. This means that EGF infiltration works on more than one level, blocking the start of molecular events that, when put together, allow the body to heal normally again (Ojalvo *et al*., 2019).

Monoclonal antibodies targeting the EGFR protein have recently received approval for the treatment of advanced colorectal and head and neck tumors. Additionally, EGFR TKIs have been given the green light for the treatment of advanced non–small cell lung cancer, pancreatic carcinoma, head and neck tumors, and glioblastoma (Cai *et al*., 2020).

DFU lacks sufficient treatment to prevent ulcer spread. Researchers have tested a new drug, alagebrium chloride (ALT-711), in Wistar rats. This drug chelates the cross-links that are present in AGEs (Çiğdem & Semra, 2019). Firstly, DM was induced in rats by using the cytotoxic drug "Streptozotocin," which destroys beta cells of the pancreas. After a month, wounds were induced on the foot. Alagebrium (10 mg/kg) was dispensed by mouth for 2 weeks, measuring the wound size every third day. Sensations and gait were monitored. Blood was drawn to evaluate HbA1c and serum levels of AGEs, MDA, and NOX1. EGF and other cytokines were assessed by histological assessment and expression of respective cytokines. Alagebrium augmented the healing of diabetic wounds, improved sensory loss and disturbed gait, and mitigated the histological variations. It reduced AGEs

and improved EGF and other chemokines in wounded rats. Oral and topical alagebrium both improve diabetic wound healing (Harb *et al*., 2023).

Diabetic Foot Ulcers (DFUs) have a genetic code that reveals transcriptional alterations, reduced keratinocyte development, fibroblast line function, macrophage metabolism, inflammation, and extracellular matrix creation compared to non-DFUs. Recovered DFU patients have significant alterations in signaling pathways, elevated inflammatory fibroblasts, and M1-polarizing macrophages. DFU has a distinct inflammatory transcription specific to oral and skin wounds, enhancing cell proliferation and survival. The immune cell profile shows a compromised immune response. The "Healer" group has increased genes associated with inflammation and extracellular matrix, while the "Non-Healer" group has increased genes linked to metabolism. Hyperglycemia and hypoxia have little impact on wound healing molecules like TGF-β and WAKMAR1 (Putri & Azminah, 2023; Li *et al*., 2019).

Operational definitions

Type 2 Diabetes Mellitus: T2D is a metabolic disease mainly characterized by reduced sensitivity of insulin receptors, diagnosed clinically as blood fasting glucose levels >126 mg/dl (7 mmol/l) and/or random blood glucose level >200 mg/dl (11.1 mmol/l) for more than one episode), duration of DM for equal to or more than 5 years with age more than 35 years and use of oral hypoglycemic drugs or insulin.

SINBAD classification: Site**,** area of ulcer, depth, bacterial infection, ischemia, and neuropathy are the five factors that make up the SINBAD system. Each element is graded as 0 or 1 point, resulting in an assessment system with values ranging from 0 to 6 that describe increasing severity. Ulcers with SINBAD scoring <3 usually heal but equal to or more than 3 are more susceptible for lower extremity amputation (LEA).

Diabetic Neuropathy: Nerve injury or inappropriate functioning may exhibit clinical symptoms in a patient with an extended history of diabetes mellitus. It present with pain, loss of sensation mainly in feet and hands, pricking sensation confirmed by reduced senses for vibration detected by tuning fork examination, monofilament tests as well as minimized Achilles reflex on tapping by hammer.

Ischemia: Ischemia of the vessels of lower extremities leading to reduced pedal blood flow due to atherosclerotic plaques in DM may present as ulcers, gangrene, and weakness of limbs or pain in the limb and was measured by ankle brachial index.

Neuro-Ischemic Ulcers: Any ulcer holding both neuropathy as well as ischemia in the lower limb area making the healing of ulcer more difficult.

Infection: When dried and fissured foot is invaded by bacterial growth, infection may pursue initiating the inflammatory cascade. Skin becomes red, warm or swollen with pus. Culture of bacteria in deep tissue of foot was done to identify infection in ulcers.

Healed foot ulcer: A previously diagnosed ulcer replaced with complete epithelization and skin became intact again. Diagnosed on the basis of presence of granulation tissue.

Classification of BMI: Body mass index (BMI) is a basic weight-for-height metric frequently used to categorize persons who are overweight or obese. It may be calculated by dividing the square of an individual's height in meters by their weight in kilograms.

The Body Mass Index (BMI) was classified according to the Asia-Pacific categorization into the following categories: underweight (< 18.5 kg/m2), normal weight (18.5-22.9 kg/m2), overweight (23.0-24.9 kg/m2), obesity class I (25.0-29.9 kg/m2), or obesity class II (≥ 30.0 kg/m2) (Bae *et al*., 2021).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Study design

Case control study

3.2 Subjects

The study was conducted on Male and Female participants with age >35 years with duration of T2D fulfilling the inclusion criteria were included in the study.

3.3 Study Setting

The study was conducted in Multi-Disciplinary Research Laboratory (MDRL) in Bahria University Health Sciences, in collaboration with PNS, Shifa Hospital, Karachi.

3.4 Inclusion criteria

One hundred and seventy-four subjects with 58 cases of DFU and 116 controls of T2D of age >35 years with duration of diabetes more than five years.

Patients of diabetic foot ulcer were selected on the basis of SINBAD classification; PAD was confirmed by ABI, whereas neuropathy was detected on the basis of (i) history and symptoms; (ii) a monofilament test by applying light pressure to different areas of the foot; (iii) a vibration perception test by applying a tuning fork to the prominence of the foot bone; (iv) a pinprick sensation; (v) a foot response towards temperature changes; and (vi) the Achilles tendon reflex.

3.5 Exclusion criteria

a) Gestational diabetes

b) Type 1 diabetes

c) Chronic liver disease

d) Other endocrine disorders

3.6 Duration of study

Total duration of study was 6 months after approval from Bahria University Health Sciences Campus and Dental College, Institutional Review Board (IRB) BUHS-IRB 036/23, 01/12/24 and Faculty Review Committee (FRC) FRC-BUHS-09- 2024-508, 8/1/2024).

3.7 Sample size estimation

The sample size was determined using OpenEpi, Version 3.0, with a confidence interval of 95% and a 5% margin of error. The characteristics used in the article were taken into consideration when determining the sample size (Akhtar *et al*., 2022) with prevalence of DFU in Pakistan is estimated to be 16.83%. Sample size justified a 1:2 ratio of cases with DFU and controls with T2D.

Sample Size for Unmatched Case-Control Study

For:

Two-sided confidence level(1-alpha)	95
Power(% chance of detecting)	90
Ratio of Controls to Cases	2
Hypothetical proportion of controls with exposure	40
Hypothetical proportion of cases with exposure:	16.83
Least extreme Odds Ratio to be detected:	0.30

	Kelsey	Fleiss	Fleiss with CC
Sample Size - Cases	64	58	65
Sample Size - Controls	127	116	129
Total sample size:	191	174	194

3.8 Sampling technique

Non Probability Consecutive Sampling

3.9 Human subjects and consent

This study received approval from the Institutional Review Board & Research ethics Committee of Bahria University Health Science Campus, Karachi. Permission was acquired from the director and head of the medical institute to carry out the research at the

respective hospital and institute. Participants who were able to accept the study procedures were enrolled upon signing informed consent forms in Urdu and English as attached in the annex. Blood samples of cases with DFU (n=58) and controls with T2D (n=116) were collected through venipuncture after taking their informed consent. Blood samples were collected in vacutainers containing acid citrate dextrose (ACD) being the strong anticoagulant and keep the cells stable for longer periods of time. Specific codes were given to the samples to maintain the confidentiality of the patient's data. All the samples were stored at -20°C for the molecular analysis.

3.10 Materials

3.10.1 Subject Evaluation Form

Attached as annex.

3.10.2 Drugs

N/A

3.10.3 Equipment and Reagents

- Agarose powder
- Alcohol swabs
- Autoclave machine
- BMI chart

- Citric acid (anhydrous)
- Dextrose (monohydrate)
- Disposable syringes & gloves
- DNA Ladder 100bp
- DNA extraction Molequle-on kit from whole blood
- DNase and RNase- -free Micropipette tips (1000µl)(200µl)(10µl)
- Height scale
- Electrical weighing balance
- Eppendorf tubes 1.5ml
- Freezer -20°C for sample storage
- Ice box and ice pack
- Laboratory drying oven
- Mini gel electrophoresis tank and Gel documentation system
- Micropipette (1000µl)(200µl)(100µl)(20µl)
- Mini spinning vortex
- NanoDrop Spectrophotometer
- Red top vacutainers
- Refrigerated centrifugation machine
- Sodium Citrate (dihydrate)
- SYBR™ Safe DNA gel stain
- Thermal cycler Machine
- Tourniquet
- Ultra low temperature freezer
- Vortex Mixer
- Water bath

3.11 Parameters of Study

Demographic Parameters

Age, gender and smoking History

Anthropometric Parameters and Physical measurements

The patients underwent a baseline examination to measure their weight and height, utilizing standardized techniques. The BMI was calculated using the Quetelets index formula weight (kg)/height (m2). Arterial blood pressure was measured by using mercury Sphygmomanometer and rounded off for nearest 2mm of Hg before blood collection. The blood pressure was measured while the individual was seated, using a mercury sphygmomanometer on the right arm. The blood pressure was measured in millimeter of mercury (mmHg).

Biochemical Parameters

Serum samples of both cases and controls were collected and sent to laboratory for investigation of following parameters:

Total cholesterol, triglyceride, HDL, LDL and reports were collected.

Genetic Marker

This study evaluated the amplification of *EGF* gene polymorphism rs4444903 (+61 A>G) by using T-ARMS-PCR technique modified with tetra primers.

3.12 Protocol / Procedure of Study

3.12.1 Sample Collection

A sample of 3 ml of whole blood anti-coagulated with acid citrate dextrose (ACD) was drawn from the cases with DFU and controls with T2D maintaining the aseptic conditions and centrifuged within two hours at 3000 rpm for 10 minutes by using Beckman Coulter refrigerated centrifuge machine.

Preparation of ACD

Acid citrate dextrose (ACD) was made from sodium citrate (0.66g), citric acid (0.24g), and dextrose (0.735g) dissolved with autoclaved distilled water 40ml in a sterilized falcon tube of 50ml, and a volume of 50ml was made, shook well until dissolved completely. Later the mixture was filtered by using autoclaved filter paper in a separate falcon tube and an ACD mixture of 0.5ml was filled in each 3ml of red top vacutainer (Xinel, China).

3.12.2 Extraction of DNA

The Genomic DNA was isolated from whole blood using MQ Blood Genomic DNA Extraction Kit shown in Figure 3.1 and performance of kit extraction is shown in 3.2.

Principle of kit

With the use of specific binding buffers, the MQ Blood DNA Isolation Kit's silica-gel membrane absorb DNA fragments. Isolated DNA was analyzed quantitatively by

NanoDrop Spectrophotometer as well as also detected qualitatively by performing gel electrophoresis. Table 3.1 shows components of DNA extraction Kit.

Preparation of Solutions

- In order to prepare a 30 ml solution of diluted CW1, 17 ml of absolute ethanol was combined with 13 ml of concentrated CW1 solution. Prior to using the DNA extraction kit, 21 milliliters of absolute ethanol were combined with nine milliliters of concentrated CW2 solution.
- TE (Tris-EDTA) Buffer used in first step was prepared from stock solution. A beaker with autoclaved distilled water of 100ml was taken, then added 3ml of Tris Base (1M, pH 8.0) and 0.6ml/600µl (0.5 EDTA, pH 8.1), pH was adjusted with pH meter 7.6-7.9 (NaOH, HCl), final volume of 300ml was made in the cylinder and stored in small glass bottle of 500ml for further use.
- Ribo Nuclease A (Bioshop Cat# RNA 888.100) obtained from bovine pancreas (RNase A) was reconstituted by dividing the total mass of RNase (100 mg) by the desired final concentration (10 mg/ml):

$$\text{Volume of water (ml)} = \frac{\text{Total mass of RNase (mg)}}{\text{Final concentration (mg/ml)}}$$

$$\text{Volume of water} \quad \frac{100\text{mg}}{10\text{mg/ml}} \quad = 10\text{ml}$$

Table 3.1 Components of DNA extraction Kit

MQ Blood Genomic DNA Extraction Kit	50 Preps
PBS Solution	8 ml
Buffer CL Solution	12 ml
CW1 Solution (Concentrated)	13 ml
CW2 Solution (Concentrated)	9 ml
CE Buffer Solution (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0)	15 ml
Buffer TBP Solution	20 ml
Proteinase K Solution	1.2 ml
MQ purification Columns with 2ml Collection Tube	50

Then added the calculated volume of RNase-free water (10 ml) to the vial containing the 100 mg of RNase powder. Swirled carefully the vial to ensure thorough mixing until the RNase A powder is completely dissolved in the RNase A free water. In order to avoid having to go through numerous freeze-thaw cycles, the reconstituted RNase solution was later split into smaller aliquots of one milliliter each. The aliquots were kept at a temperature of -20 degrees Celsius. Labelled the tubes clearly containing the reconstituted RNase solution with important information such as concentration, preparation date, and initials of researcher's name. Strict sterilization was maintained to avoid any contamination.

Procedure

- The sterilization of the workbench was ensured by cleaning it with alcohol, removing contamination, gathering all reagents, and following all SOPs. Before pouring blood into 1.5 ml Eppendorf tubes for DNA isolation, vacutainers containing the blood sample were vortexed. 400µl of the TBP Buffer was combined with 200µl of blood. After mixing gently, tubes were stood upright for one minute to allow the red cells to lyse fully. Spinned the Eppendorf tubes in the centrifuge machine **(**Beckman Coulter refrigerated centrifuge machine**)** for one minute at 8,000 rpm (rotation per minute). Carefully, the supernatant was disposed of by holding the tube with the hinge facing above. Rinsed the precipitate twice with 500µl of TE Buffer and the supernatant was discarded twice too. Before every wash, spinned at 8,000 rpm for one minute. The last precipitate ought to have a clear appearance.

- After that, 20 microliters of proteinase K was added. For a gentle mixing, a micropipette tip was used to dispense the mixture. A volume of 200 microliters of buffer CL (Lysis buffer) was added. Once the samples had been gently vortexed, they were placed in a water bath and incubated for twenty minutes at a temperature of 56 degrees Celsius.

- Following the addition of 20µl of RNase A (10 mg/ml), the mixture was vortexed and allowed to remain at room temperature for a duration of five minutes. Proceeded to the subsequent stage in order to get genomic DNA that was free of RNA.
- 200µl of chilled absolute alcohol was added to the mixture and mixed well.
- MQ column was filled with the mixture from previous step (including precipitates), using a 2ml Collection Tube obtained from the kit. Gave it a minute or two to stand at room temperature. After that, it was spinned for two minutes at a speed of 10,000 rotations per minute. The flow-through was removed from the collecting tube and disposed of it in bleach containing beaker.
- Then discarded the flow through after adding 500µl of CW1 solution and spinning it for one minute at a speed of 10,000 rotations per minute.
- After adding 500µl of CW2 Solution, columns was spinned for one minute at 10,000 rpm.
- Flow-through was thrown away in the discarder. For one more minute, flow-through was spinned at 10,000 rpm to get rid of any remaining CW2 Solution.
- In a sterilized 1.5 ml Eppendorf tube, the column was inserted. 50µl of CE Buffer was poured into the membrane's center in the column. For two minutes, incubated at room temperature. The water bath was set at 50°C and tubes were incubated for 5 minutes.
- For one minute, the Eppendorf tube with the column was spinned at 10,000 rpm to extract DNA from the column.
- The isolated genomic DNA aliquots were stored at -20°C for PCR.

3.12.3 Quantitative Analysis of DNA

Using a NanoDrop spectrophotometer, DNA was quantified. This spectrophotometer analyzes the amount (concentration) of the test sample by measuring

the amount of light absorbed by the sample (Figure 3.3). In this process, the concentration of DNA in a Nano gram was determined using a 0.5-2µl of the sample. The dual-beam technique used by the NanoDrop allows light to pass through both the reference and test sample mixtures. The reference is regarded as the sample that has been adjusted to reduce background noise, including chemical and protein contaminants. Additionally, the device measures concentration in the UV (ultraviolet) band of 220 to 320 nm. On the other hand, the DNA was detected at 260 nm, and the sample concentration was determined using the Beer-Lambert law formula. This law states that the sample concentration and the total distance the light covers are directly correlated with the amount of light absorbed by the sample. The amount of sample that the device provides is determined by comparing the reference sample's concentration to the test sample in the ratio of A260/A280, where 1.8 is considered pure, >1.8 denotes RNA contamination, and <1.8 denotes protein contamination. Samples were pure with concentrations up to 1.8 (Figure 4.3.1(a)).

3.12.4 Qualitative Analysis of DNA

The DNA integrity was examined using agarose gel electrophoresis. The process of agarose gel electrophoresis is based on the idea of separating DNA bands according to their sizes and charges. This process allows modified molecules to pass through a gel matrix by applying an electric field. Using a 0.8% gel, genomic DNA quality was assessed. 100 ml of TBE (Tris-borate EDTA) 1X buffer was measured in a measuring cylinder of 100 ml, and 0.8 g of agarose powder (Molecular Biology Grade) was poured into a conical flask after weighing in the weighing balance. After combining 0.8 g of agarose powder with 100 ml of TBE, the mixture was boiled and mixed gently for 45 seconds in a microwave oven with intervals of 10 seconds to eliminate any remaining agarose until the powder was dissolved completely and the solution was clear. When the solution became warm, 5µl of SYBRsafe (Invitrogen) In order to observe the DNA bands under UV light, DNA gel stain was incorporated into the solution. Later, the solution with dye was poured into the already-set mini gel electrophoresis tank (COPE), a comb and two stoppers were applied, and it was left to set at room temperature. The conical flask containing the solution

with SYBRsafe dye was rinsed abruptly with tap water. Let the gel cool for 25 minutes. Once the gel hardened, the comb and stoppers were taken out, and TBE 1X buffer was poured into the electrophoresis chamber. 5µl of DNA ladder (Molequle-on) of 1kilo base pairs (kbp) was also used, which aided in detection of genomic DNA. Samples of DNA (4µl) were loaded into the wells after being combined with loading dye in the ratio 4:4 in which 4 µl of DNA was mixed with 4µl of loading dye (Thermoscientific) to visualize the colorless DNA sample under UV light. For 30 minutes, the setup was run at 200 mA of current and 100 volts of voltage. The gel underwent observation under the gel documentation system (Axygen®) as shown in figure 4.3.1(b).

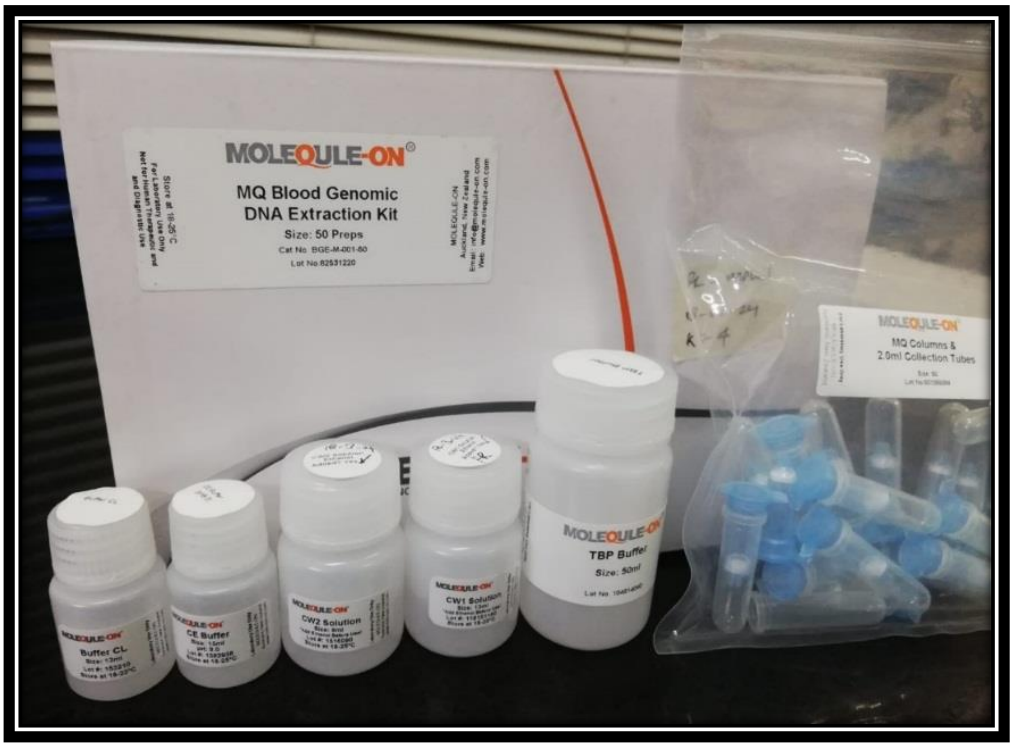


Figure 3.1: Molequle-on DNA extraction kit

Figure 3.2: Performance of extraction of DNA from Kit

Figure 3.3: Quantifying the optic density of DNA on NanoDrop

3.12.5 Selection of Targeted Single Nucleotide Polymorphism (SNP)

DNA samples collected were used to determine the frequency of SNP rs44444903 in the *EGF* gene among both the patients and controls. The SNP with a minor allelic frequency (MAF) greater than 0.05 was chosen, and information on this SNP was collected from Ensembl genome browser 92 (https://asia.ensembl.org/index.html) and NCBI dbSNP (National Center for Biotechnology Information, data base SNP).

3.12.6 Designing of Primers for the *EGF* gene

Sequence of *EGF* gene was retrieved from ensemble genome browser. Primer1 software (https://primer1.soton.ac.uk/primer1.html) was used to design primers for the T-ARMS-PCR. Information regarding single nucleotide polymorphism (SNP) rs4444403 of the *EGF* gene was checked in NCBI's SNPdb. Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to evaluate specificity of primers, primer length, melting temperature(Tm), and GC content.

- Forward outer (FO)/ Reverse outer (RO)
- Forward inner(FI)/ Reverse inner (RI)

Primer sequence is shown in Table 3.2.

3.12.7 Constitution of Primers

Lyophilized dry DNA oligo powder of 25nm synthesis scale was obtained from Synbio technologies reconstituted. The powder was first gathered to the bottom of the tubes by centrifuging them at 5,000 rpm for 15 seconds. Then opened the tubes gently and added nuclease free water, capped and shaken for dissolving it completely. 100 µM of solution was prepared by multiplying the total nmol by 10 and added that volume of NFW in it. Table 3.3 shows the calculations of primers. To avoid frequent thawing and freezing, small aliquots for 100 µl were further divided into 50 µl and 50 µl, labelled and stored at −20°C.

Table 3.2 List of Primers, length of amplified sequence, Tm, GC content and product size of *EGF* gene

Primers	Sequence	Primer Length	Tm	GC (%)	Product Size
***EGF*(FO)**	CCTCTCTCCCAGTTCTGCC CAGGATATT	28	66	54	507bp
***EGF*(RO)**	TGCTGTTGTCCTCCAAGCT CTTTCTTCA	28	63	46	
***EGF* (FI)**	GAAAGGAAGAACTGATGG AAAGTTCCATCC	30	63	43	296bp
***EGF* (RI)**	TTCTTTCAGCCCCAATCCA AGGGTTTTA	28	61	43	268bp

Table 3.3 Oligonucleotide or primers information

Primer names	nmol/Tube	Volume for 100µM /Tube	Working Solution **20** µM
1FO-EGF	20.8	208 µl	20 µl + 80 µl NFW
1RO-EGF	21.0	210 µl	20 µl + 80 µl NFW
1RI-EGF (G-V)	15.5	155 µl	20 µl + 80 µl NFW
1FI-EGF (A-W)	19.5	195 µl	20 µl + 80 µl NFW

3.12.8 Tetra-primer Amplification Refractory Mutation System Polymerase Chain Reaction

The *EGF* rs4444903 single nucleotide polymorphism (SNP) was amplified using T-ARMS PCR. The first step in performing PCR on samples was preparing the reaction mixture by sequentially adding all necessary reagents according to the order specified in the table 3.4. A single reaction tube was used to conduct T-ARMS PCR, which included two sets of outer and inner primers. The reagents were maintained at a low temperature by placing them on ice during the experiment. The solution was divided into individual sterilized PCR tubes, which were then labeled and kept on ice as shown in figure 3.4. The PCR reaction mixture was made by combining, 0.3µL of primers (20µM), and 10µL of Dream greenTM master mix (Thermoscientific) as shown in figure 3.5. The mixture was diluted to a level of 18µL by adding nuclease-free water. DNA sample 2µL (50ng/µL) was added in the end to avoid any contamination and made up the volume to 20 µl. After the addition of the reaction mixture and DNA, the contents were mixed on mini spinner briefly to ensure that all the materials settled at the bottom of the tube.

The optimization of T-ARMS PCR was done initially by applying temperature gradient in thermal cycler machine (Multigene Labnet Int. USA) from temperature 58 °C to 68°C to identify best temperature where maximum annealing of targeted primers was achieved. Electrophoresis on a 3% agarose gel allowed to see the PCR result that were produced (Figure 4.3.2).

Denaturation at 94°C for 5 minutes was the first step in the reaction, and denaturation at same temperature for 30 seconds was the second step. Primers were coupled to the required area of the target for polymorphism amplification by subjecting it to an annealing temperature of 64°C for 30 seconds. The sequence was extended further by providing an extension temperature of 72°C for 45 seconds. After 39 repetitions of this cycle, the sample was elongated for an additional 7 minutes at 72°C. Keep the amplified product at 4°C for storage and product was observed by agarose gel (3%) electrophoresis followed by gel visualization under the Gel document system. Figure 3.6 shows the cycles of PCR.

Table 3.4 Quantification of Reaction Mixture (20µL)

S. No.	Reagent	Initial Concentration	Volume/ Reaction
1	**Nuclease free Water**	___	6.8 µl
2	**OF-Primer**	20 µM	0.3 µl
3	**OR-Primer**	20 µM	0.3 µl
4	**IF-Primer**	20 µM	0.3 µl
5	**IR-Primer**	20 µM	0.3 µl
6	**DreamTaq Green PCR Master Mix**	2 X	10 µl
7	**DNA sample**	50 ng/µl	2 µl
	Total Volume Per Reaction		20 µl

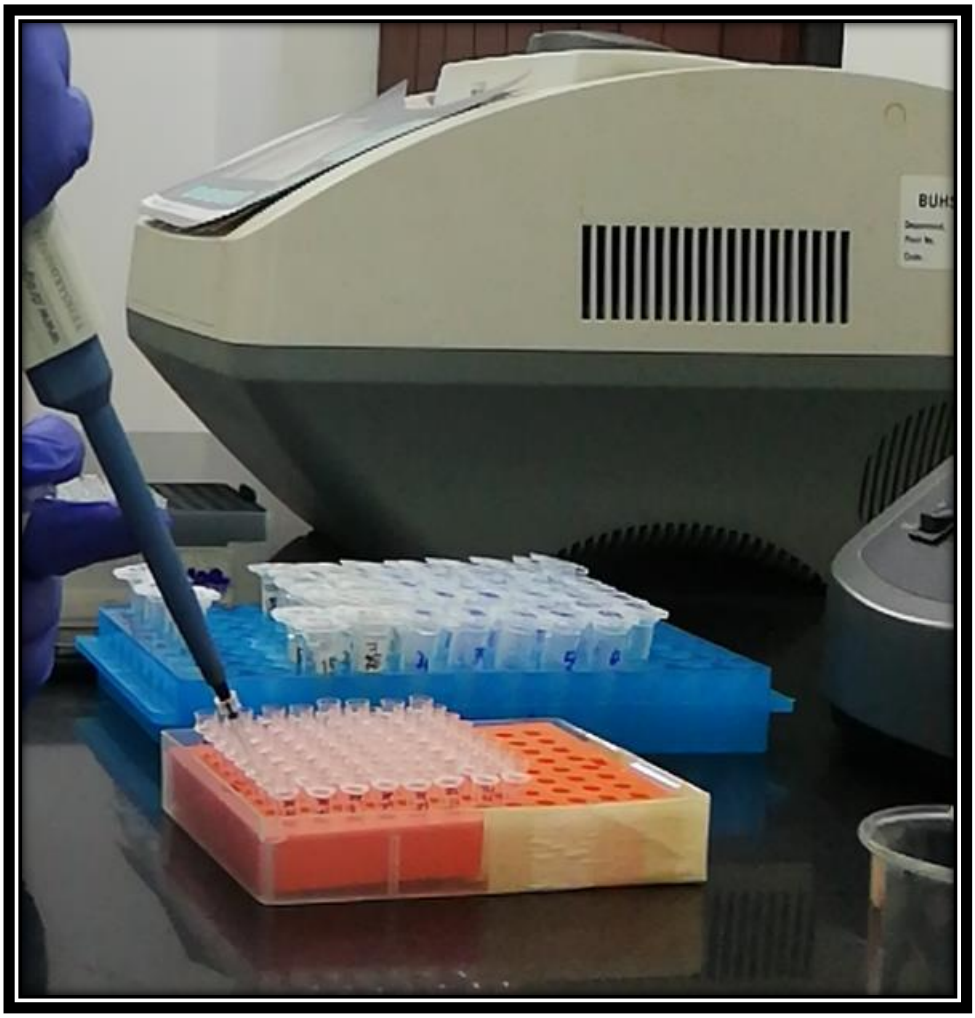


Figure 3.4: PCR workbench

Figure 3.5: PCR Master-mix

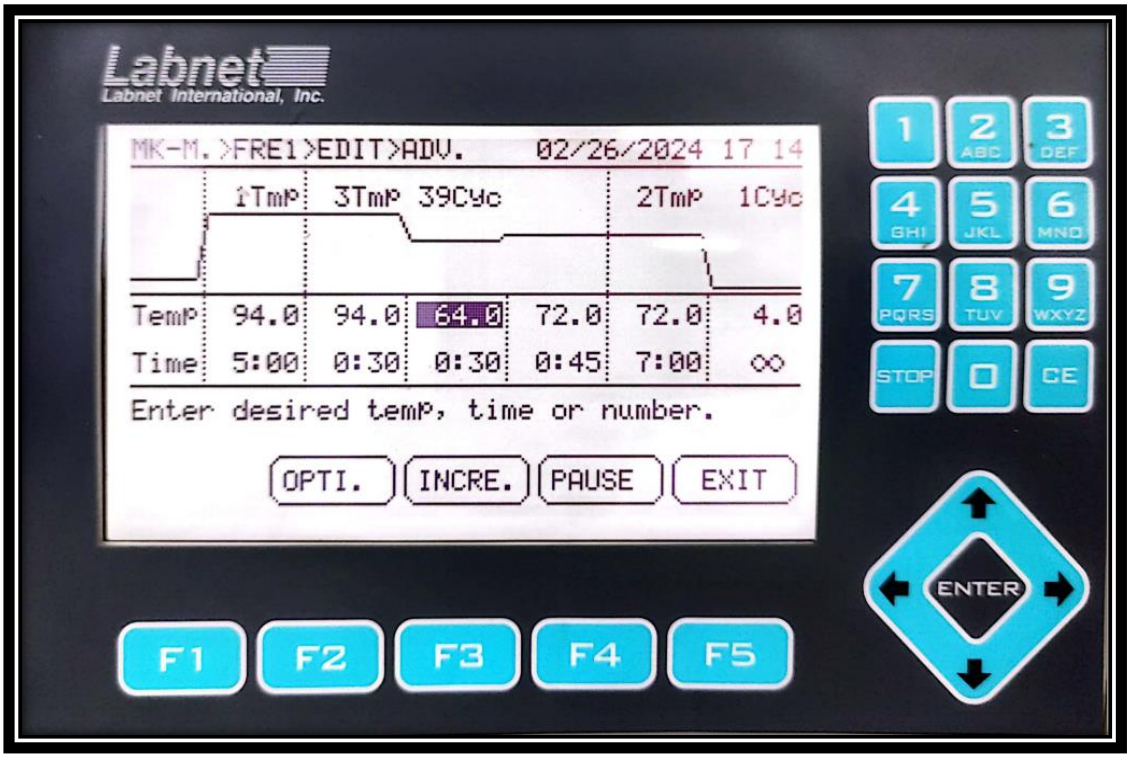


Figure 3.6: Cyclic conditions at 64°C for *EGF* gene polymorphism

3.12.9 Qualitative Analysis of PCR products

PCR result was analyzed by agarose gel electrophoresis. Applying an electric field, modified molecules move through a gel matrix. Quality pf PCR genotypes of different bands was tested using 3% gel. A 100-ml measuring cylinder measured 50 ml of TBE (Tris-borate EDTA) 1X buffer, and a weighing balance weighed 1 g of agarose powder into a conical flask. A combination of 1 g agarose powder and 70 ml TBE was heated and gently stirred for 45 seconds in a microwave oven with 10-second intervals to dissolve any leftover agarose and clarify the solution. To observe PCR product bands under UV light, 5µl of SYBRsafe (Invitrogen) DNA gel dye as shown in figure 3.7 was added to the heated solution. After pouring the dyed solution into the small gel electrophoresis tank (COPE) shown in figure 3.8, a comb and two stoppers were added, and it was permitted to set at room temperature. The SYBRsafe dye solution in a conical flask was quickly washed with tap water. Cool the gel for 25 minutes. Once the gel formed, the comb and stoppers were removed and TBE 1X buffer was added to the electrophoresis chamber. Use of 8 µl of 100 bp DNA ladder (Molequle-on) helped identify genotypes in PCR generated products. PCR samples (20µl) were placed in wells. The arrangement ran at 200 mA and 100 volts for 30 minutes as shown in figure 3.9. The gel documentation system (Axygen®) took and archived photos of each gel as shown in figure 4.3.2. Gel was zip-locked and dumped in biomedical waste collection as shown in figure 3.10.

Figure 3.7: SYBR®safe gel stain

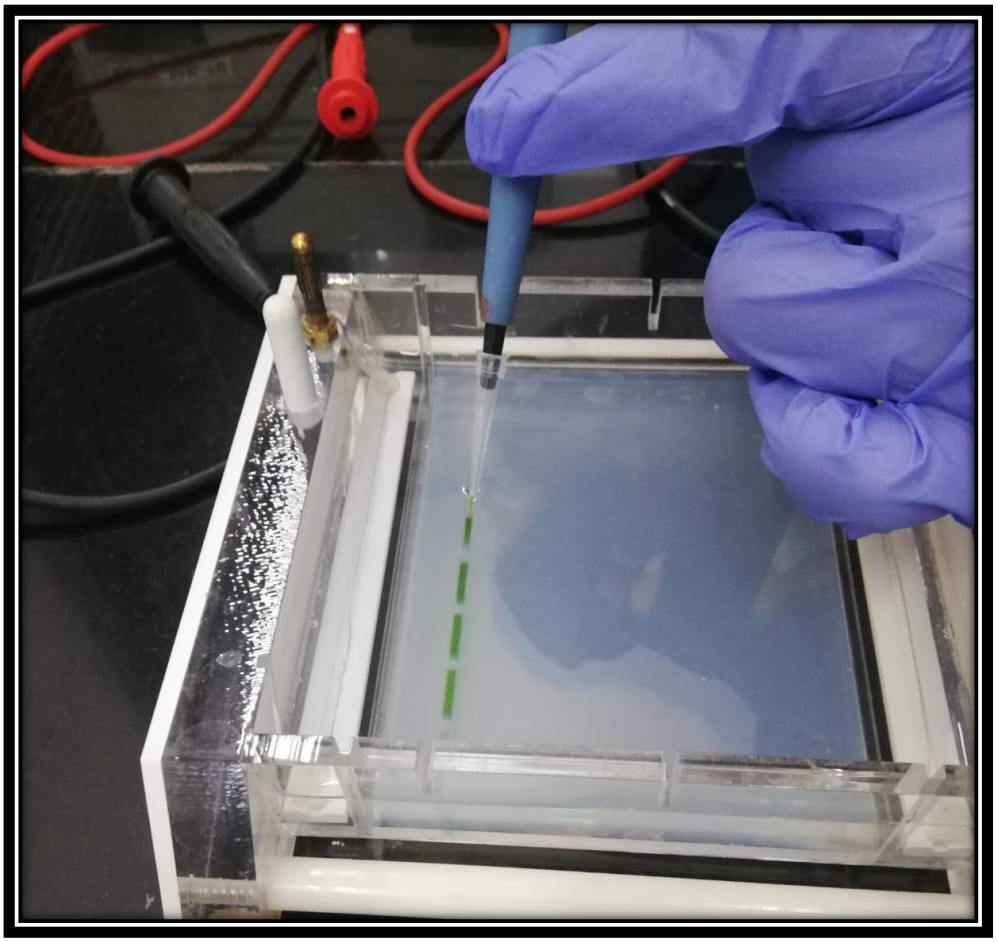


Figure 3.8: Loading samples into gel wells

Figure 3.9: Running gel electrophoresis

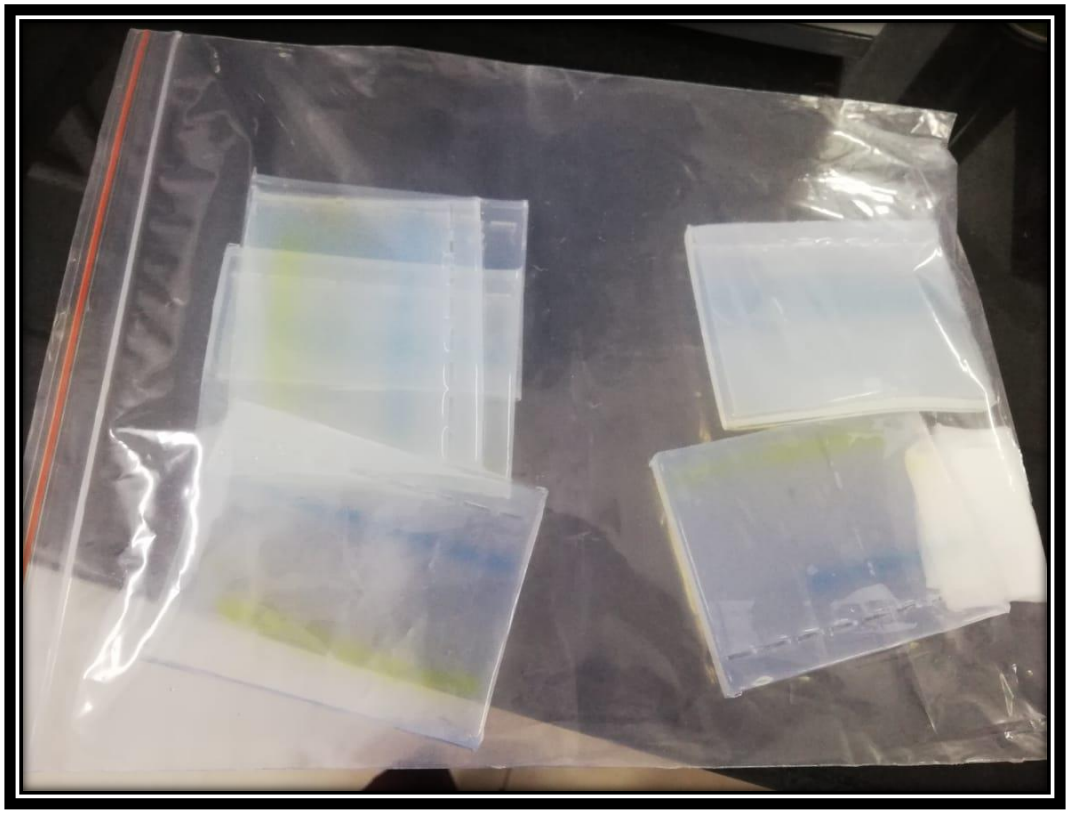
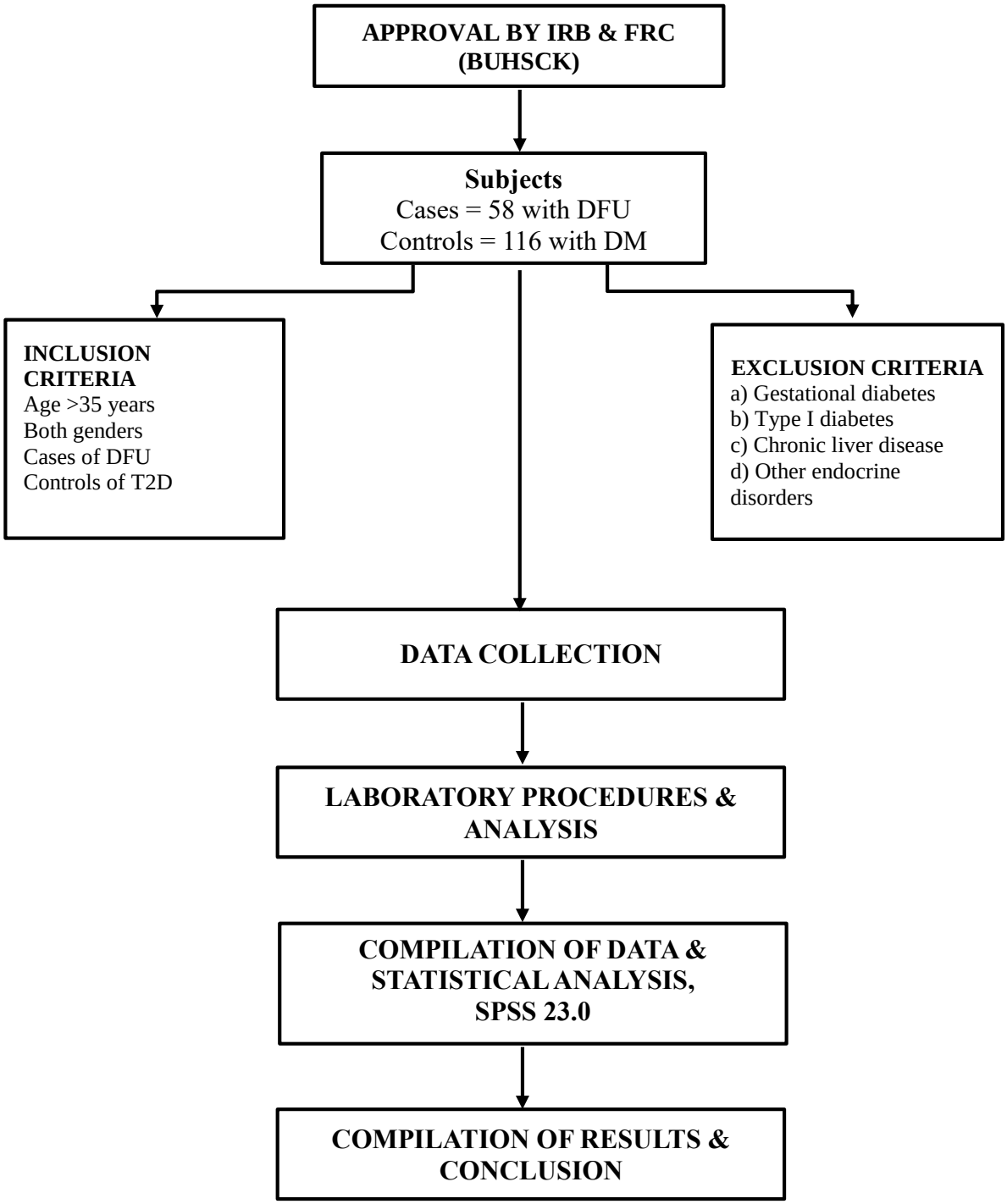


Figure 3.10: Zip-locks for used gels

3.13 Flow Chart/ Algorithm of the Study



3.14 Statistical Analysis

The analysis was carried out using SPSS version 23.0, developed in Chicago, IL, USA. The mean ± standard deviation was used to represent continuous variables (age, BMI, lipid profile), and statistical significance was determined at the standard levels of 5% ($p<0.05$), 1% ($p<0.01$), and 0.1% ($p<0.001$). The categorical data was collected and represented as frequencies and percentages. Descriptive statistics were then analyzed using Microsoft Excel 365 to compare the cases with DFU and controls with T2D. The Shapiro-Wilk test assessed the normality of the data distribution. The Mann-Whitney U-test compares two independent groups non-parametrically. Rather than comparing means, it compares distributions by significance. A suitable test statistic, such as the Pearson's Chi-square (X^2) test, was used to examine the association between the *EGF* gene SNP rs4444903 (+61 A>G) and several clinic-pathological characteristics. The odds ratio was computed using the online statistical tool MedCal (https://www.medcalc.org/calc/) to assess the likelihood of a connection between *EGF* gene polymorphism and DFU, using the Pearson's Chi-square (X^2) test. Pearson correlation test was used to test correlation between continuous variables. SHEsis was used for evaluating genetic association at polymorphic loci and for identifying associations between genetic variations and DFU. Logistic regression was applied to adjust the confounding factors.

CHAPTER 4

RESULTS

4.1 Descriptive Statistics of Parameters

The normality of the data was assessed using the Kolmogorov-Smirnov tests. The findings revealed that the data for numerical variables, deviated from a normal distribution ($p < 0.05$).

Table 4.1.1 Baseline characteristics including age, height, and body mass index (BMI) in cases show significant difference in their values when compared with control group as shown in Table 4.1.1 ($p < 0.05$) whereas height and duration of T2D did not show any significant difference among cases with DFU and controls with T2D.

A Mann-Whitney U test was done for systolic and diastolic blood pressure for its non-parametrical distribution. And it revealed significant difference 0.025* for systolic blood pressure 130.78±13.822 but showed no significant difference 0.219 for diastolic blood pressure 83.36±7.573 among cases with DFU and controls with T2D as shown in table 4.1.2.

Comparison of total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides (TG) and genotype were made in controls and cases in Table 4.1.3 by Mann-Whitney U Test. It was noted that serum HDL and TG ($P<0.05$) were

significant in cases in comparison to controls, LDL was highly significant p<0.001 and genotype in controls and cases were non-significant.

In table 4.1.4 The Spearman's correlation coefficient test yielded results. Among both cases with DFU and controls with T2D, smoking and HDL were found to be weakly correlated, with a p-value of 0.129 (p<0.05), additionally, a correlation between BMI and TC, LDL, and TG, with p values as 0.112, 0.000**, and 0.004*, was found, respectively in table 4.1.5 and figure 4.1.1. Both LDL and TC showed strong correlations with each other, but only TG demonstrated a significant association for genotypes among cases with DFU and controls with T2D in table 4.1.6. TG is negatively or inversely correlated with *EGF* gene polymorphism rs4444903 which shows TG may reduce synthesis of EGF.

In table 4.1.7 and figure 4.1.2 reports the findings of univariate and multivariate model for estimating the risk of DFU cases with confounding variables using binary logistic regression analysis, results showed in univariate model for DFU cases increase in Age (years) gives positive association [OR=1.06,C.I(1.02-1.10)], Male gender gives positive association [OR=2.57,C.I(1.30-5.07)], in ethnicity Baloch gives positive association [OR=2.24,C.I(0.41-12.1)], Pathan gives positive association [OR=1.49,C.I (0.69-3.24)], Punjabi gives positive association [OR=3.59,C.I(1.04-12.3)], Saraiki gives positive association [OR=4.49,C.I(0.38-52.5)], and Sindhi gives positive association [OR=1.6, C.I (0.45-5.68)], for marital status Married samples gives positive association [OR=1.94, C.I (0.19-19.1)], in smoking status Smoker gives positive association [OR=1.13, C.I(0.49-2.59)], for increase in Duration of DM gives negative association [OR=0.99, C.I(0.94-1.04)], in co morbidities Hypertension + IHD gives positive association [OR=1.53,C.I (0.78-2.96)], increase in BMI (kg/m2) gives negative association [OR=0.89, C.I(0.82-0.97)], increase in SBP gives positive association [OR=1.01,C.I(0.99-1.03)], increase in DBP gives positive association [OR=1.01, C.I(0.97-1.05)], for lipid parameters TC gives positive association [OR=1,C.I(0.99-1.01)], HDL gives negative association [OR=0.96, C.I (0.93-0.99)], LDL gives negative association [OR=0.99, C.I(0.98-1.00)], TG gives negative association[OR=0.99,C.I(0.99-1.00)], in genetic parameters Genotype AG gives positive association [OR=1.56,C.I(0.70-3.48)], Genotype GG gives positive association [OR=1.47,C.I(0.56-3.86)], whereas DFU Allele A gives

positive association [OR=1.09, C.I(0.49-2.43)], and DFU Allele G gives positive association [OR=1.53,C.I(0.71-3.29)] for DFU cases in comparison of control group. Multivariate model also reported for DFU older Age (years) samples gives positive association [OR=1.07, C.I(1.02-1.13)], Male gender gives positive association [OR=1.9, C.I(0.66-5.43)], Baloch gives positive association [OR=11.1,C.I(0.81-152.)], Pathan gives positive association [OR=2.49,C.I (0.87-7.12)], Punjabi gives positive association [OR=19.3, C.I(2.69-139.)], Saraiki gives positive association [OR=16.3, C.I(0.27-975.)], and Sindhi gives negative association [OR=0.66,C.I(0.10-4.33)]. Married samples gives positive association [OR=4.89, C.I(0.15-150.)], Smoker gives positive association [OR=1.65, C.I(0.50-5.45)], Duration of DM gives negative association [OR=0.96, C.I(0.89-1.04)], Hypertension + IHD gives positive association [OR=1.31, C.I(0.44-3.87)], BMI (kg/m2) gives negative association [OR=0.86,C.I(0.76-0.97)], SBP gives positive association [OR=1.01,C.I(0.97-1.05)], DBP gives negative association [OR=0.99, C.I(0.93-1.06)], for lipid parameters TC gives positive association [OR=1.03,C.I(1.01-1.05)], HDL gives negative association [OR=0.89, C.I(0.84-0.95)], LDL gives negative association [OR=0.97, C.I(0.95-0.99)], and TG gives negative association [OR=0.99, C.I(0.98-0.99)], in genetic analysis Genotype AG gives positive association [OR=1.61, C.I(0.49-5.29)], Genotype GG gives positive association [OR=1.08, C.I(0.27-4.28)], and DFU Allele A gives positive association [OR=1.19,C.I (0.48-2.94)], DFU Allele G gives positive association [OR=1.73,C.I(0.74-4.06)] with DFU cases.

Table 4.1.1: Age, duration of T2D, weight, height and BMI in controls with T2D and cases with DFU and was analyzed by Mann-Whitney U Test.

Parameters	Controls with T2D n=116	Case with DFU n=58	Mann-Whitney U Test	2-tailed Test
Age	49.48±9.820	54.52±9.638	2397.5	0.002*
Duration of T2D	9.65±6.707	9.19±6.814	3092	0.384
Weight	72.972±13.59	71.41±9.09	3228.5	0.665
Height	161.426±10.153	163.276±13.454	2628.5	0.019*
BMI	28.105±5.159	25.853±3.375	2427.5	0.003*

*p<0.05 significant

**p<0.001 highly significant as compared to respective controls

Table 4.1.2: Systolic BP and diastolic BP in controls with T2D and cases with DFU was analyzed by Mann Whitney U test.

Parameters	Controls with T2D n=116	Case with DFU n=58	Mann-Whitney U Test	2-tailed Test
SBP	125.43±17.252	130.78±13.822	2397.5	0.025*
DBP	81.77±8.604	83.36±7.573	3092	0.219

*p<0.05 significant

**p<0.001 highly significant as compared to respective controls

Table 4.1.3: Comparison of TC, HDL, LDL, TG and genotype in controls with T2D and cases with DFU.

Parameters	Controls with T2D n=116	Case with DFU n=58	Mann-Whitney U Test	2-tailed Test
TC	166.94±45.716	159.07±37.969	2397.5	0.17
HDL	39.09±12.521	34.34±7.592	3092	0.028*
LDL	104.78±40.206	86.16±36.582	3228.5	0.001**
TG	188.25±87.441	155.19±80.663	2628.5	0.006*
Genotype	1.8707±0.740	2.00±0.675	2427.5	0.243

*p<0.05 significant
**p<0.001 highly significant as compared to respective controls

Table 4.1.4: Correlation of BMI and HDL in cases with DFU and controls with T2D.

Correlations

N= 174				
			BMI	HDL
Spearman's rho	BMI	Correlation Coefficient	1.000	-0.115
		Sig. (2-tailed)	.	0.129
	HDL	Correlation Coefficient	-0.115	1.000
		Sig. (2-tailed)	0.129	.

Table 4.1.5: Correlation of BMI with TC, LDL, and TG in cases with DFU and controls with T2D.

Correlations (r-value)

N= 174						
			BMI	TC	LDL	TG
Spearman's rho	BMI	Correlation Coefficient	1.000	0.121	0.293**	0.220**
		Sig. (2-tailed)	.	0.112	0.000	0.004
	TC	Correlation Coefficient	0.121	1.000	0.557**	0.374**
		Sig. (2-tailed)	0.112	.	0.000	0.000
	LDL	Correlation Coefficient	0.293**	0.557**	1.000	0.212**
		Sig. (2-tailed)	0.000	0000	.	0.005
	TG	Correlation Coefficient	0.220**	0.374**	0.212**	1.000
		Sig. (2-tailed)	0.004	0.000	0.005	.

**. Correlation is significant at the 0.01 level (2-tailed).

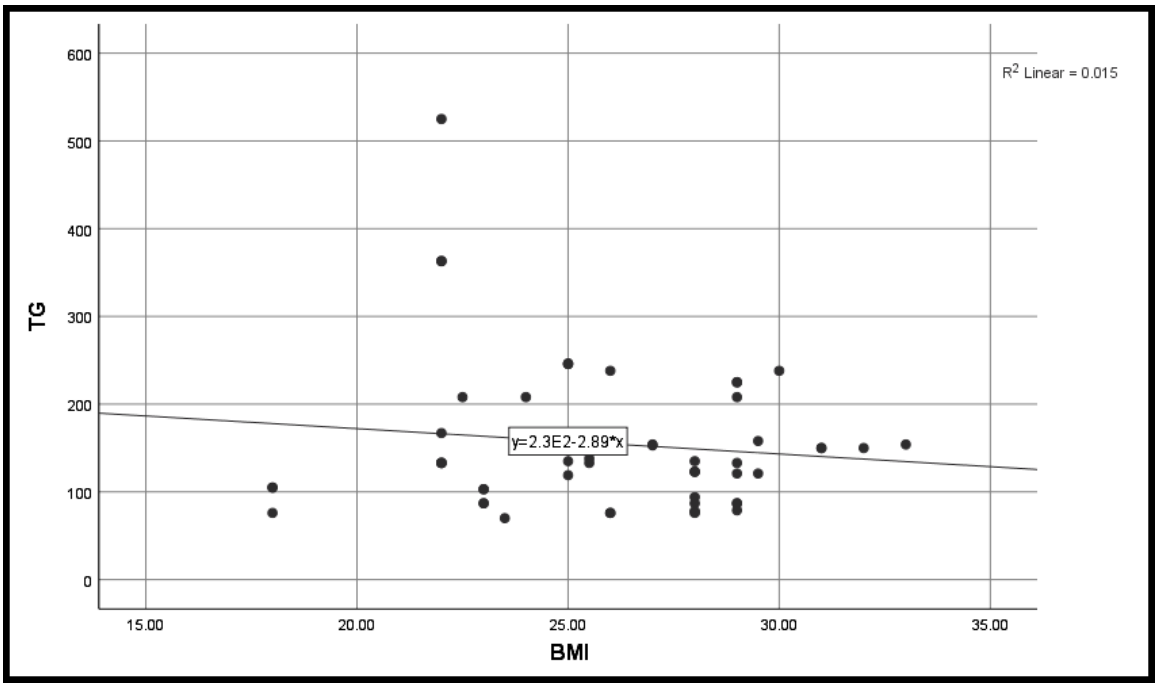


Figure 4.1.1: Correlation of BMI and TG

Table 4.1.6: Correlation of TG and genotype cases with DFU and controls with T2D.

Correlations				
N=174				
			TG	**Genotype**
Spearman's rho	TG	Correlation Coefficient	1.000	-0.219**
		Sig. (2-tailed)	.	0.004
	Genotype	Correlation Coefficient	-0.219**	1.000
		Sig. (2-tailed)	0.004	.

**. Correlation is significant at the p < 0.01 level (2-tailed).

Table 4.1.7. Risk assessment of *EGF* gene polymorphism rs4444903 cases with DFU and controls with T2D

Risk Factors	Univariate Model Odds Ratio (95% CI)	Multivariate Model Odds Ratio (95% CI)
Age (years)	1.06* (1.02-1.10)	1.07* (1.02-1.13)
Male	2.57* (1.30-5.07)	1.90 (0.66-5.43)
Baloch	2.24 (0.41-12.1)	11.1 (0.81-152.)
Pathan	1.49 (0.69-3.24)	2.49 (0.87-7.12)
Punjabi	3.59* (1.04-12.3)	19.3* (2.69-139.)
Saraiki	4.49 (0.38-52.5)	16.3 (0.27-975.)
Sindhi	1.60 (0.45-5.68)	0.66 (0.10-4.33)
Married	1.94 (0.19-19.1)	4.89 (0.15-150.)
Smoker	1.13 (0.49-2.59)	1.65 (0.50-5.45)
Duration of DM (years)	0.99 (0.94-1.04)	0.96 (0.89-1.04)
Hypertension + IHD	1.53 (0.78-2.96)	1.31 (0.44-3.87)
BMI (kg/m2)	0.89 (0.82-0.97)	0.86* (0.76-0.97)
Systolic blood pressure	1.01 (0.99-1.03)	1.01 (0.97-1.05)
Diastolic blood pressure	1.01 (0.97-1.05)	0.99 (0.93-1.06)
Total Cholesterol (mg/dl)	1.00 (0.99-1.01)	1.03* (1.01-1.05)
High density lipoproteins (mg/dl)	0.96* (0.93-0.99)	0.89 (0.84-0.95)
Low density lipoproteins (mg/dl)	0.99 (0.98-1.00)	0.97* (0.95-0.99)
Triglycerides (mg/dl)	0.99 (0.99-1.00)	0.99* (0.98-0.99)
Genotype AG	1.56 (0.70-3.48)	1.61 (0.49-5.29)
Genotype GG	1.47 (0.56-3.86)	1.08 (0.27-4.28)
Allele A	1.09 (0.49-2.43)	1.19 (0.48-2.94)
Allele G	1.53 (0.71-3.29)	1.73 (0.74-4.06)

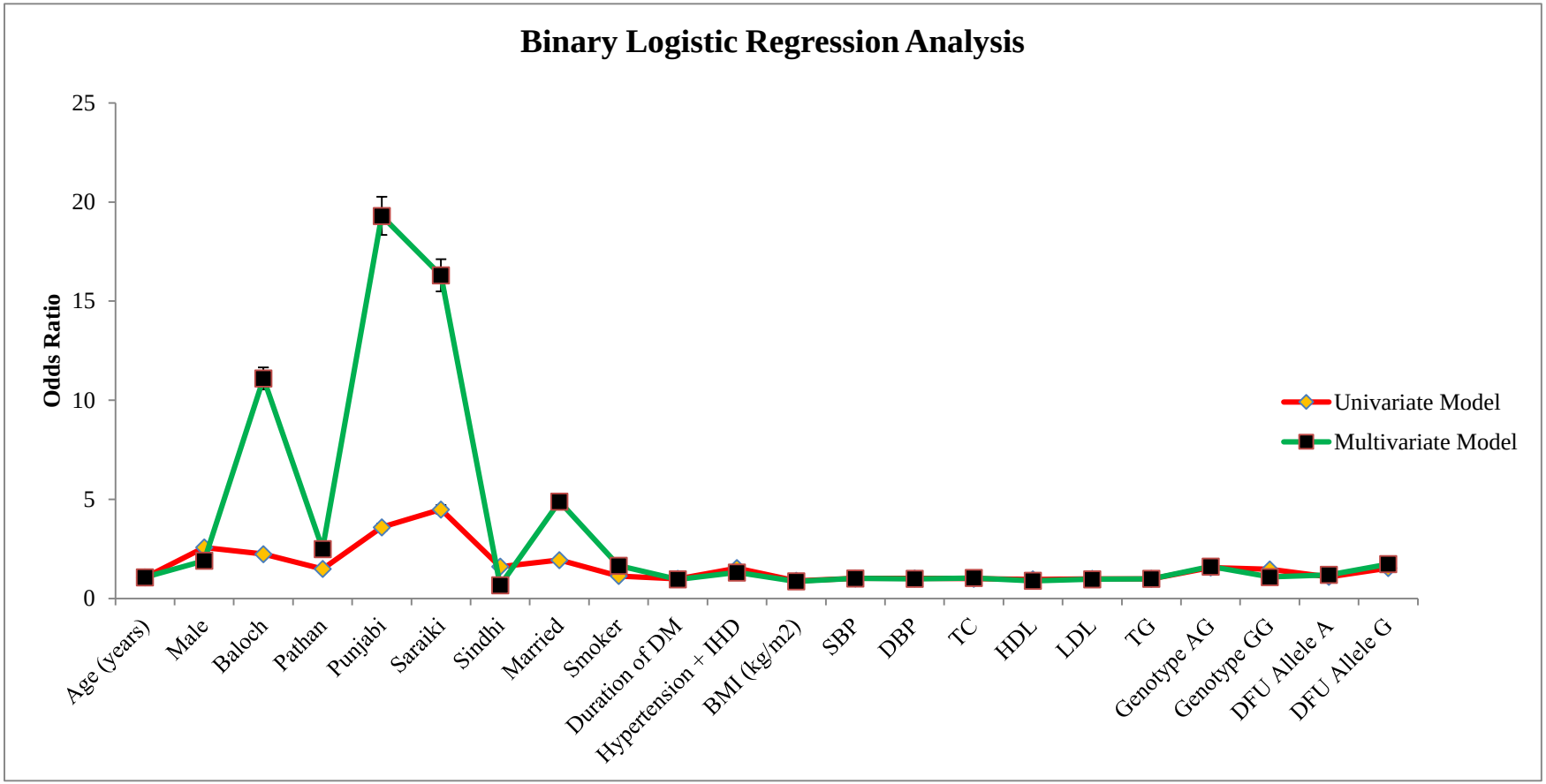


Figure 4.1.2: Binary Logistic Regression Analysis

4.2 Demographic Profile of the Cases with DFU and Controls with T2D

4.2.1 Age Distribution

The mean age of patients, along with the corresponding standard deviation, was 54.52 ± 9.554 years for the case group and 49.48 ± 9.777 years for the control group. The age distribution of targeted subjects lies between the 35 years to 70 years. It is observed that 20% of the cases were in age range of 51 to 55 years while 25% of the controls were in the age range of 35 to 40 as shown in figure 4.2.1.

4.2.2 Gender Distribution

The study was conducted on total 174 subjects in which 58 subjects diagnosed with diabetic foot ulcer while 116 subjects diagnosed with diabetes mellitus recruited as controls. The frequency of females were 21 (36.4%) & 68 (58.6%) in cases with DFU and controls with T2D respectively. The frequency of males were 37(63.7%) and 48 (41.3%) in cases with DFU and controls with T2D respectively, gender was matched among cases with DFU and controls with T2D as shown in figure 4.2.2.

4.2.3 Smoking Status

Among the cases, 12 individuals (20.63%) were smokers, while the majority, 46 individuals (79.3%), were non-smokers. Among the control group, 21 individuals (18.1%) were smokers, and 91 individuals (81.9%), were non-smokers as shown in figure 4.2.3.

4.2.4 Ethnicity

Of all, 78 (44.8%) were members of the Urdu-speaking community. Following this with 59 (33.9%) were the Pathan community, followed by Sindhi with 14 (8.1%) and Punjabi with comparable numbers. Six people (3.4%) belonged to the Baloch group and three (1.7%) to Saraiki as shown in figure 4.2.4.

4.2.5 Comorbidities

Among the cases with DFU and controls with T2D, a significant proportion of subjects exhibited hypertension and ischemic heart disease, with 67 (38.5%) and 10 (5.7%) individuals affected, respectively. However, it is worth noting that a majority of 97 (55.7%) participants did not report any other ailments as shown in figure 4.2.5

4.2.6 Classification of BMI

Figure 4.2.6 shows a graphical presentation, with class I being more prevalent among cases and class II in controls. About 62% of cases had class I obesity, whereas only 5% were underweight. Whereas class II was more prevalent, with 40.5% of controls having a lesser percentage, similar cases were among underweight subjects with 3.5%.

4.2.7 Etiology of DFU

In the group of patients with diabetic foot ulcers (DFU), there were 50 individuals with neuropathic conditions, accounting for 86.2% of the cases. There were 6 cases of ischemic DFU, representing 10.3% of the total. Only 2 cases were classified as mixed or neuroischemic, making up 3.4% of the cases as shown in figure 4.2.7.

4.2.8 DFU diagnosis using SINBAD Grading

The SINBAD grading system was utilized to assess the severity of diabetic foot ulcers (DFU). Out of the total cases, 38 subjects were diagnosed with grade <3 foot ulcers, accounting for approximately 65.5% of the cases. Grade ≥3 ulcers were found in 20 subjects, making up 34.4% of the cases as shown in figure 4.2.8.

4.2.9 Appearance of DFU

The most common occurrence of DFU is an infectious state, accounting for 70.6% of cases, followed by healing, cellulitis, and callus, which make up 20.7%, 5.2%, and 3.4% respectively as shown in figure 4.2.9.

4.2.10 Causes of DFU

DFU is primarily caused by trauma, which is responsible for 58.6% of cases with 34 instances. Bacterial infection and footwear contribute to 32.8% and 8.6% of cases, respectively as shown in figure 4.2.10.

4.2.11 Foot Side

In cases of diabetic foot ulcers (DFU), the right foot was more commonly affected, with 33 subjects accounting for 57% of the cases. Whereas, 25 cases presented with DFU in the left foot, making up 43% of the cases as shown in figure 4.2.11.

4.2.12 Surfaces of Foot

The most frequently observed location for foot ulcers among was the planter area, accounting for 82.7% of the cases (48 cases). This was followed by the non-planter area, which had 17.2% (10 cases) as shown in figure 4.2.12.

4.2.13 Location of Infection

The midfoot was found to be the most frequently affected area for foot ulcers, accounting for 70.6% of the cases. The forefoot was the second most common location, with 11 cases representing 19% of the total. The hind foot was less commonly affected, contributing 10.3% as shown in figure 4.2.13.

4.2.14 Temperature of Foot

Approximately 84.4% (49) of the cases had a normal temperature in their foot. There were 7 cases, accounting for 12%, that presented with a hot or raised temperature. Only 2 cases, making up 3.4%, reported a cold temperature as shown in figure 4.2.14.

4.2.15 Duration of T2D

Approximately 44.8% of the population presented with diabetes for 5 or less than 5 years, indicating that DFU is not significantly influenced by the duration of T2D. In contrast, 35.3% of individuals with diabetes had a duration of at least 6 years to more than 10 years, and the majority did not have DFU as shown in figure 4.2.15.

4.2.16 Duration of DFU

Out of the participants who were selected for this research, about 48.2% had been experiencing DFU for a period of less than six months. On the other hand, 34.5% of subjects had experienced DFU for more than six months, and 17.2% of subjects had experienced DFU for a period of more twelve months as shown in figure 4.2.16.

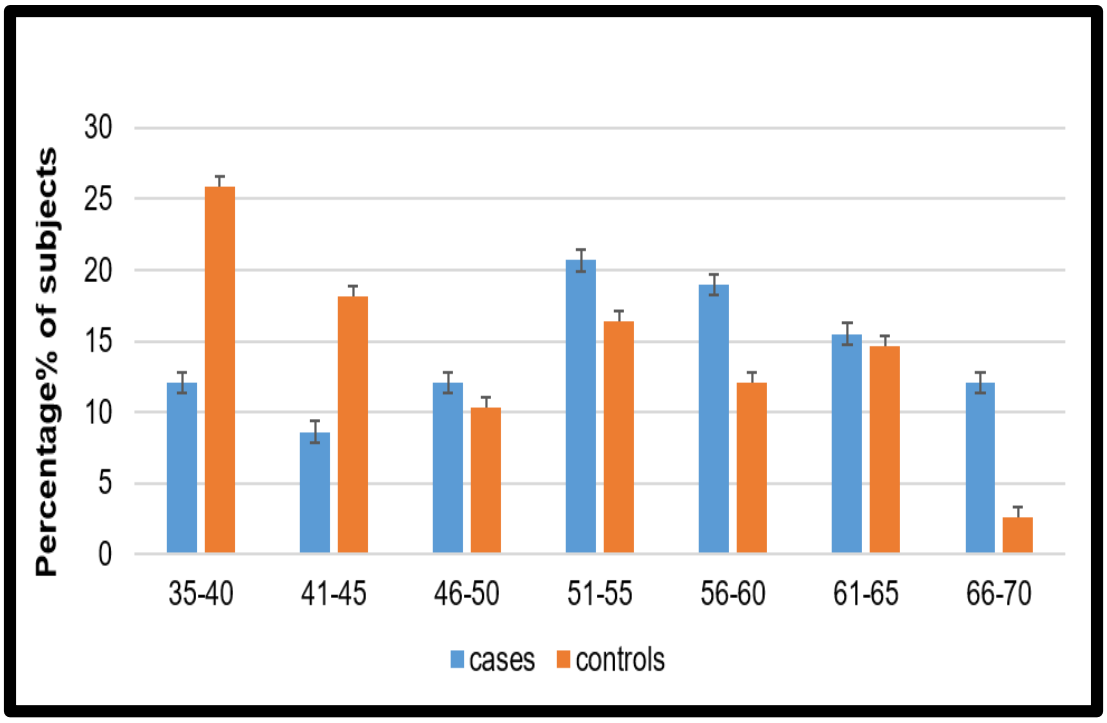


Figure 4.2.1: Age Distribution of cases with DFU and controls with T2D

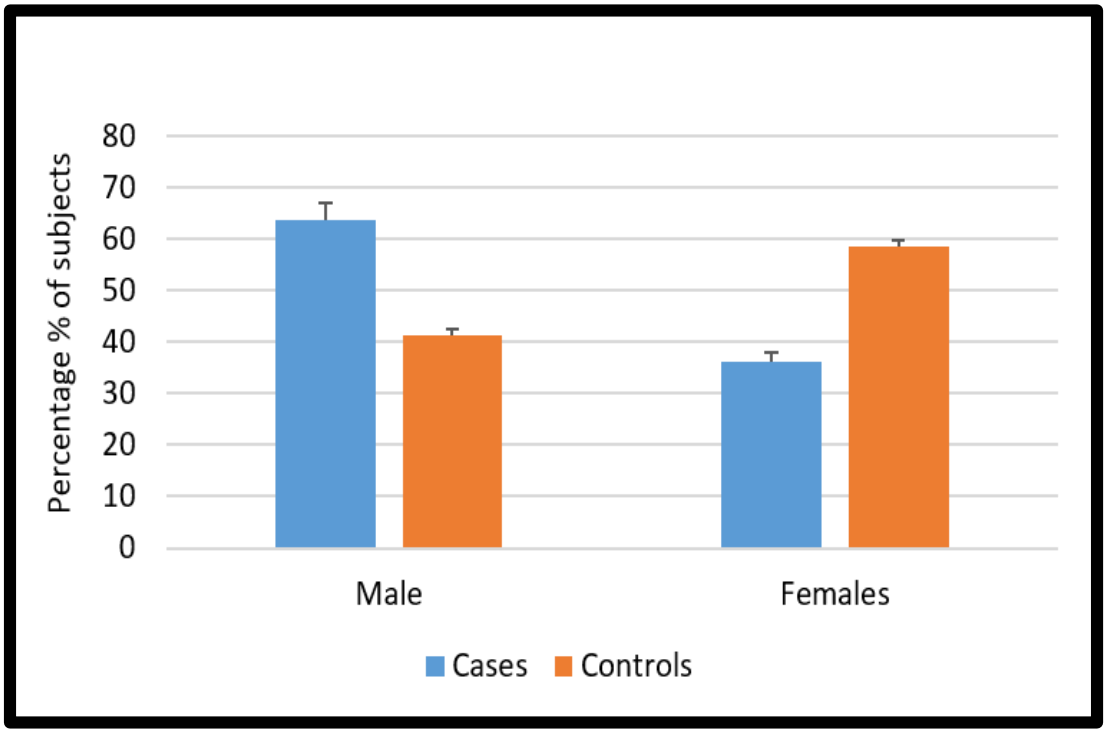


Figure 4.2.2: Gender Distribution amongst cases with DFU and controls with T2D

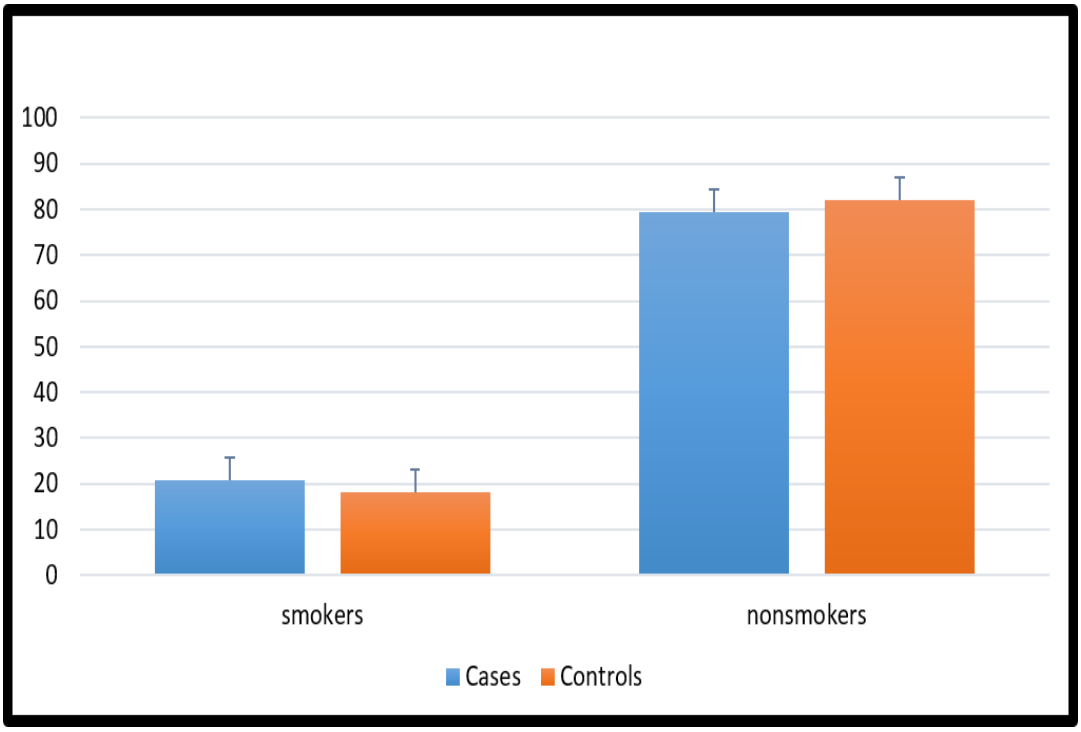


Figure 4.2.3: Smoking among cases with DFU and controls with T2D

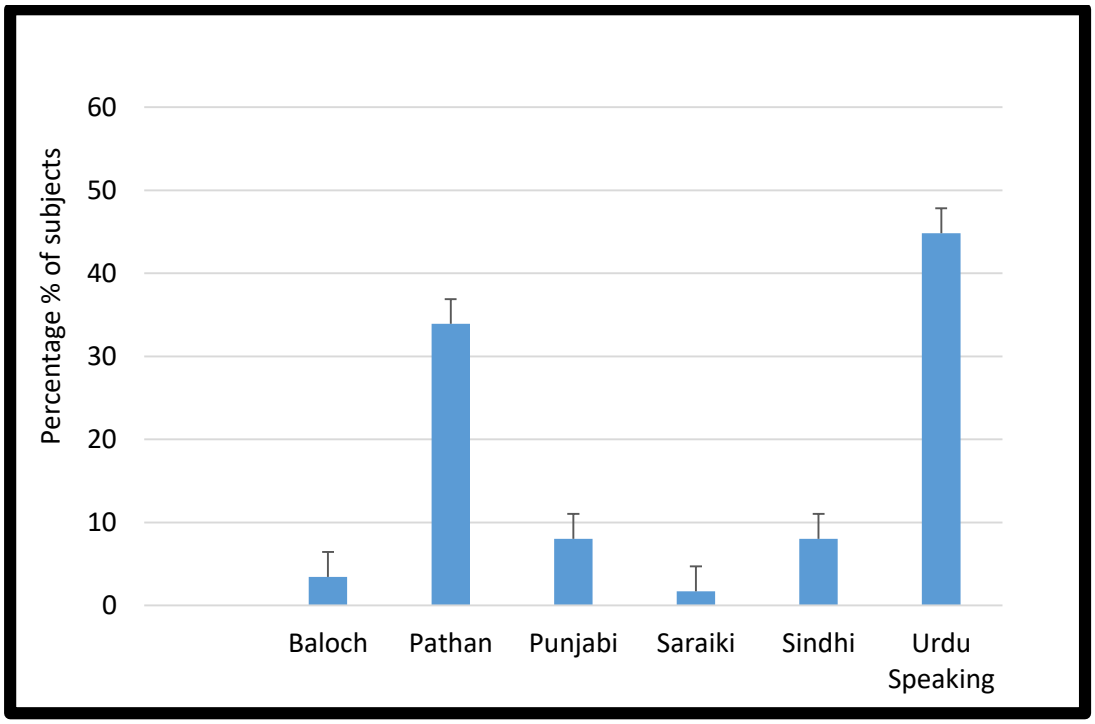


Figure 4.2.4: Ethnicity of recruited cases with DFU and controls with T2D

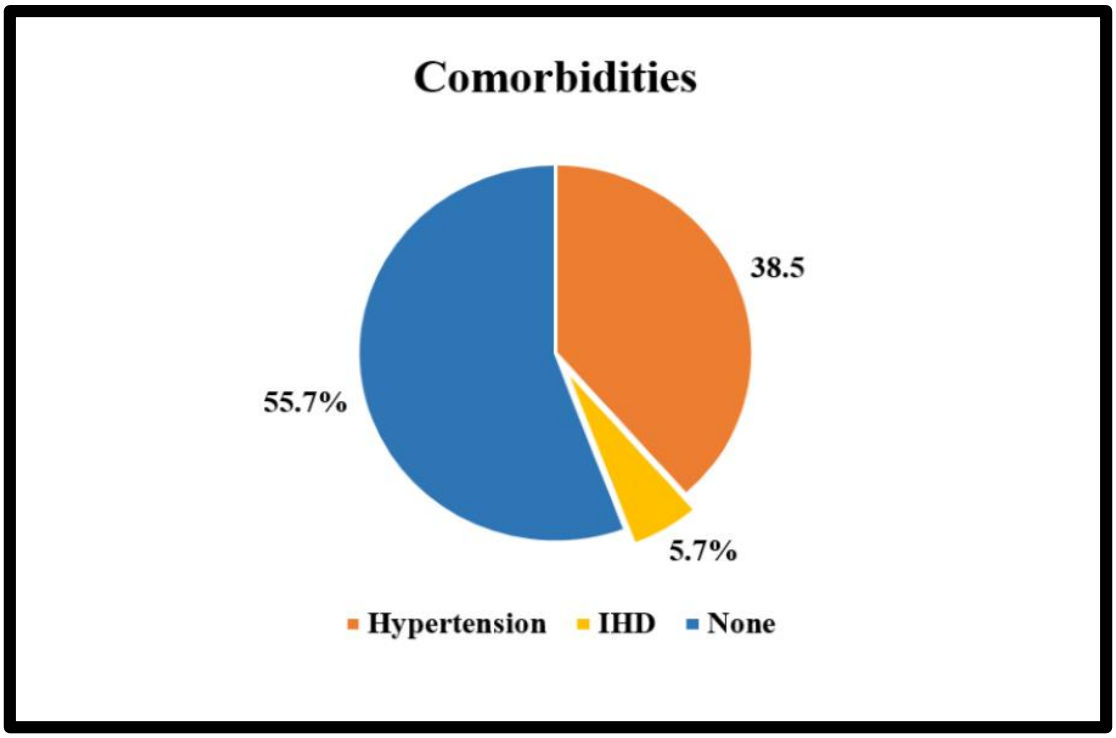


Figure 4.2.5: Comorbidities among cases with DFU and controls with T2D

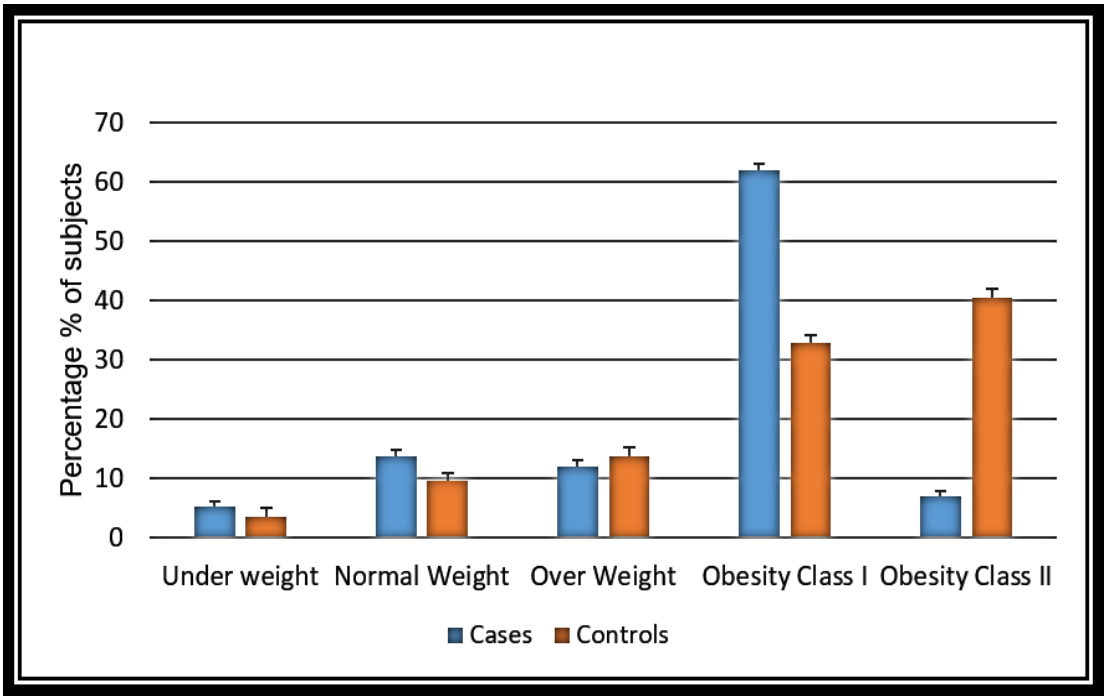


Figure 4.2.6: Classification of BMI

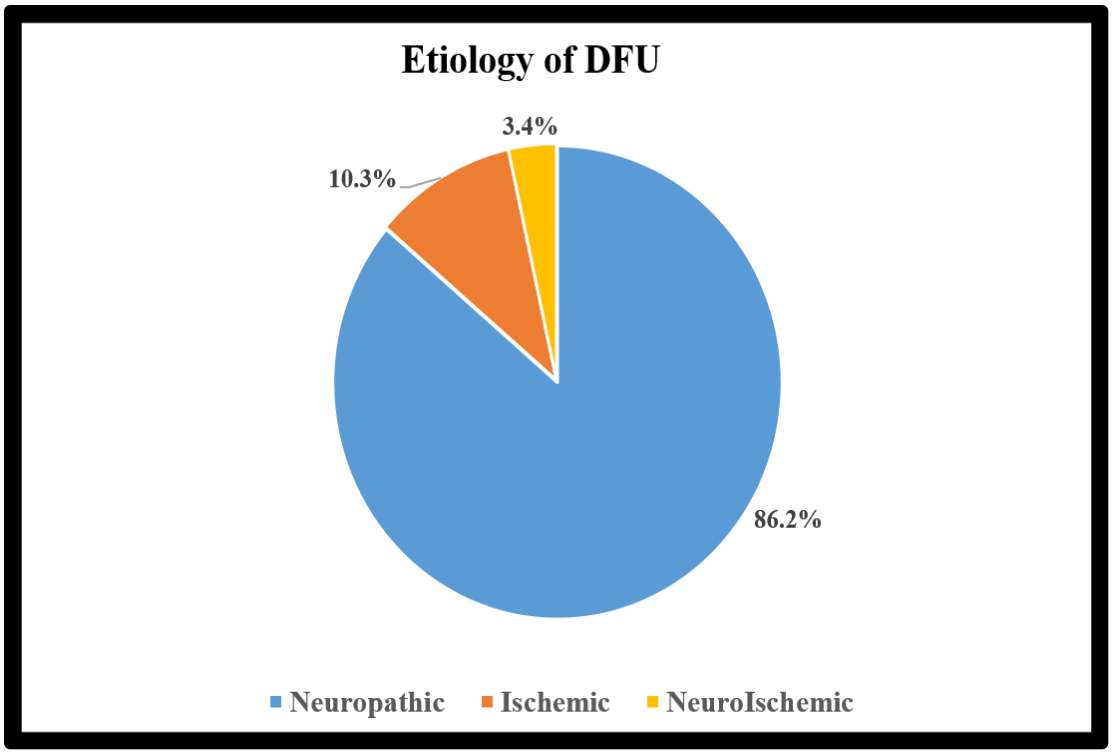


Figure 4.2.7: Etiology of DFU

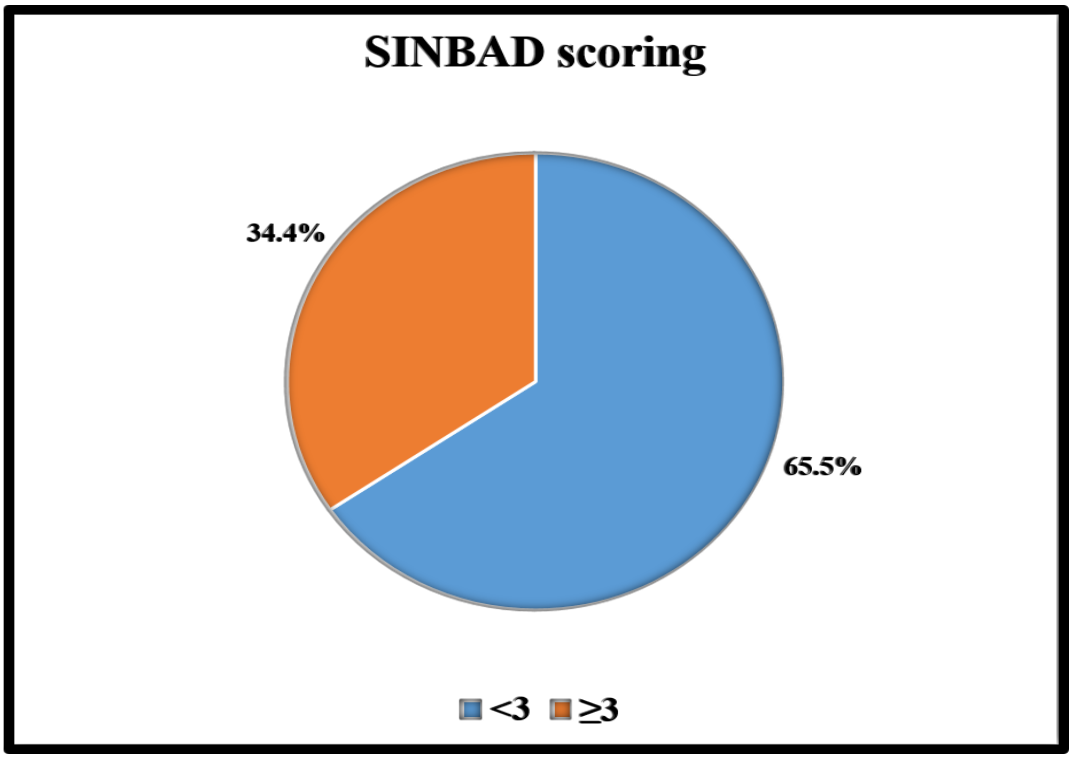


Figure 4.2.8: SINBAD scoring

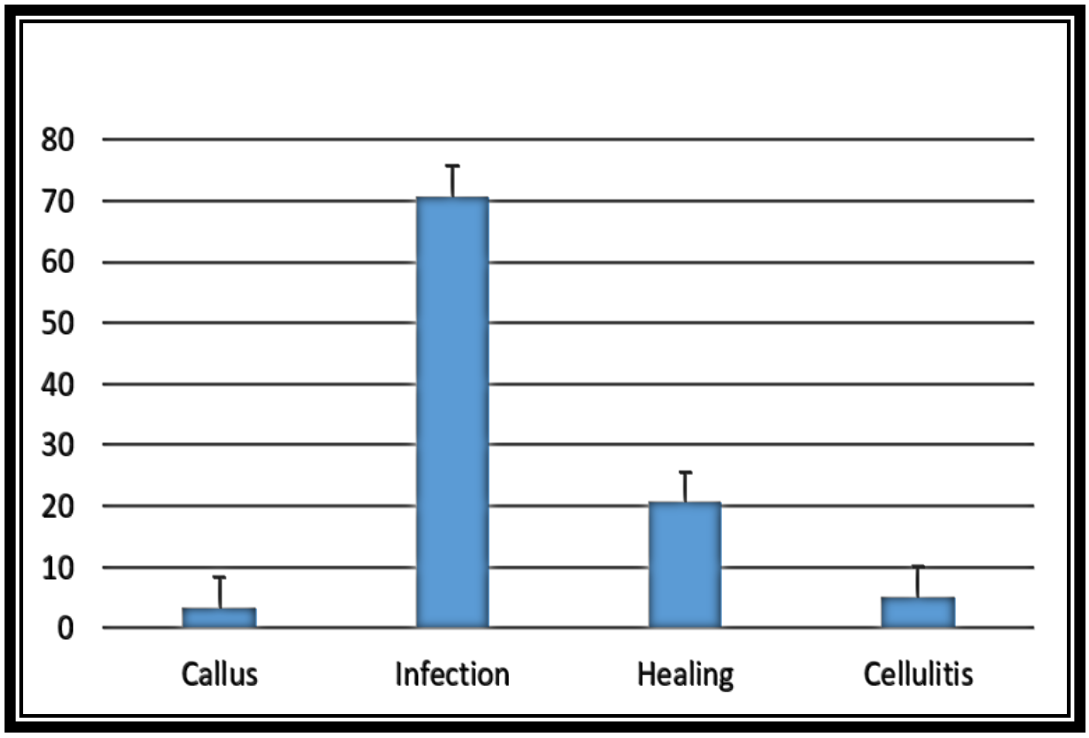


Figure 4.2.9: Appearance of DFU

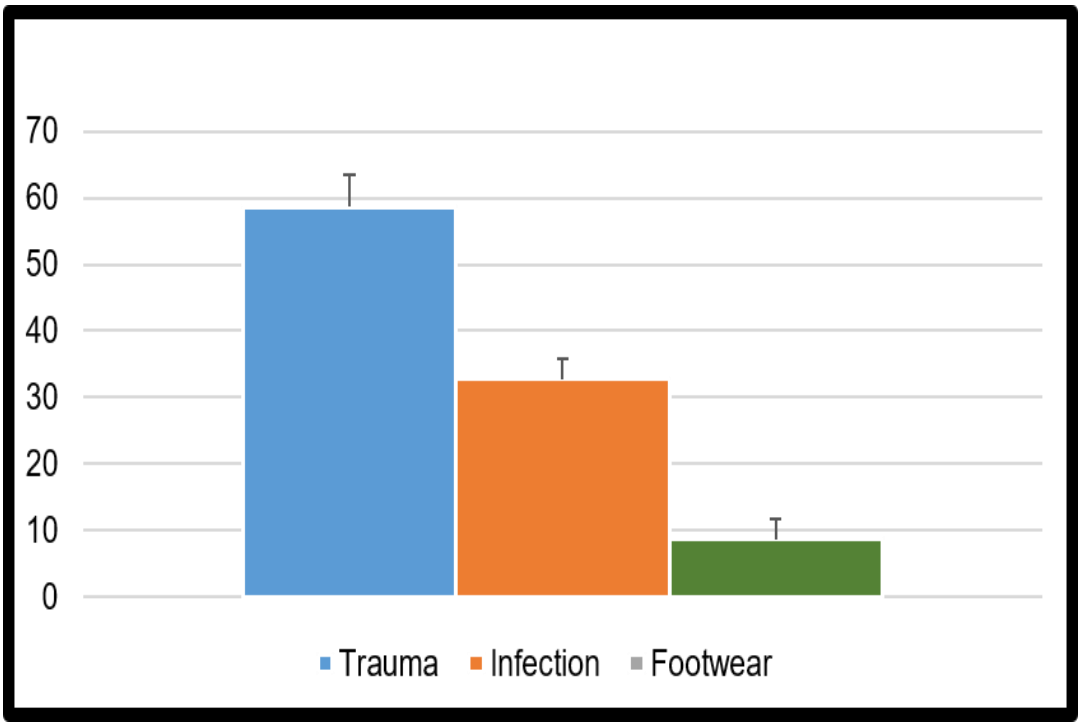


Figure 4.2.10: Causes of DFU

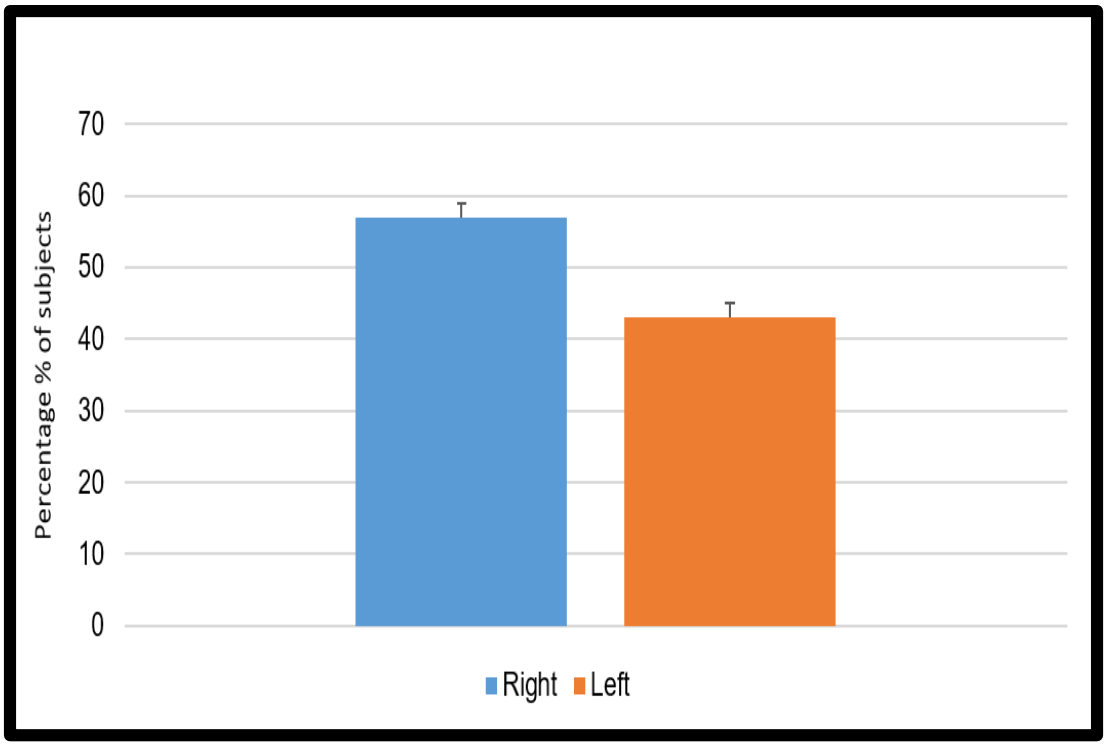


Figure 4.2.11: Foot Side

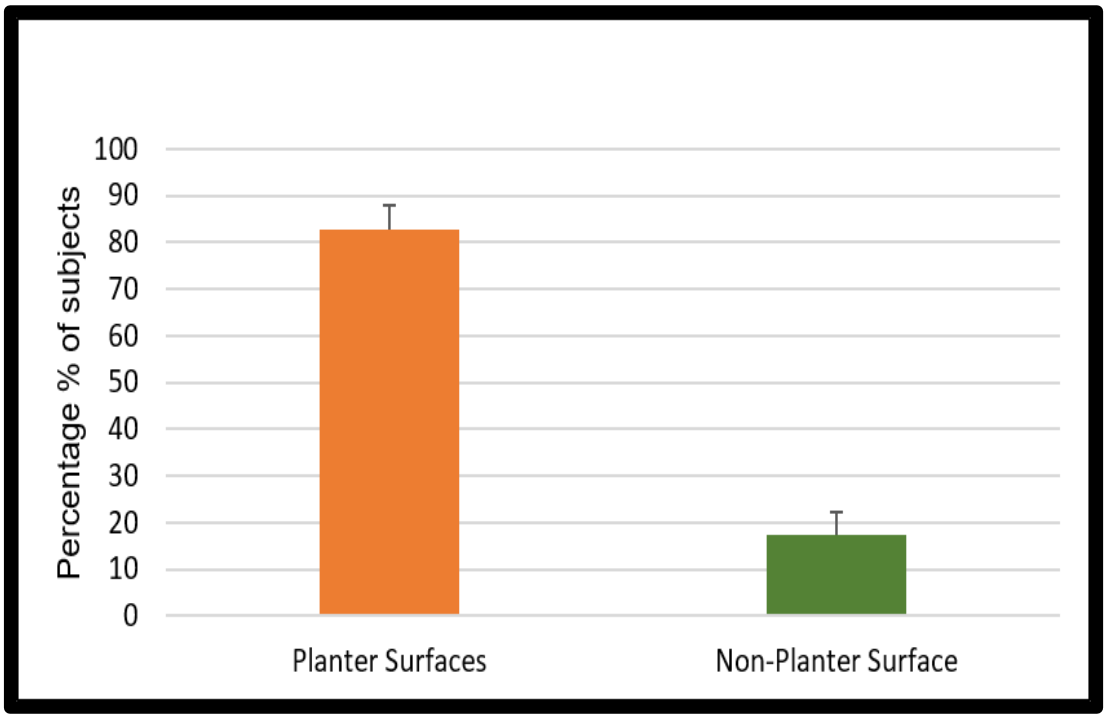


Figure 4.2.12: Surfaces of DFU

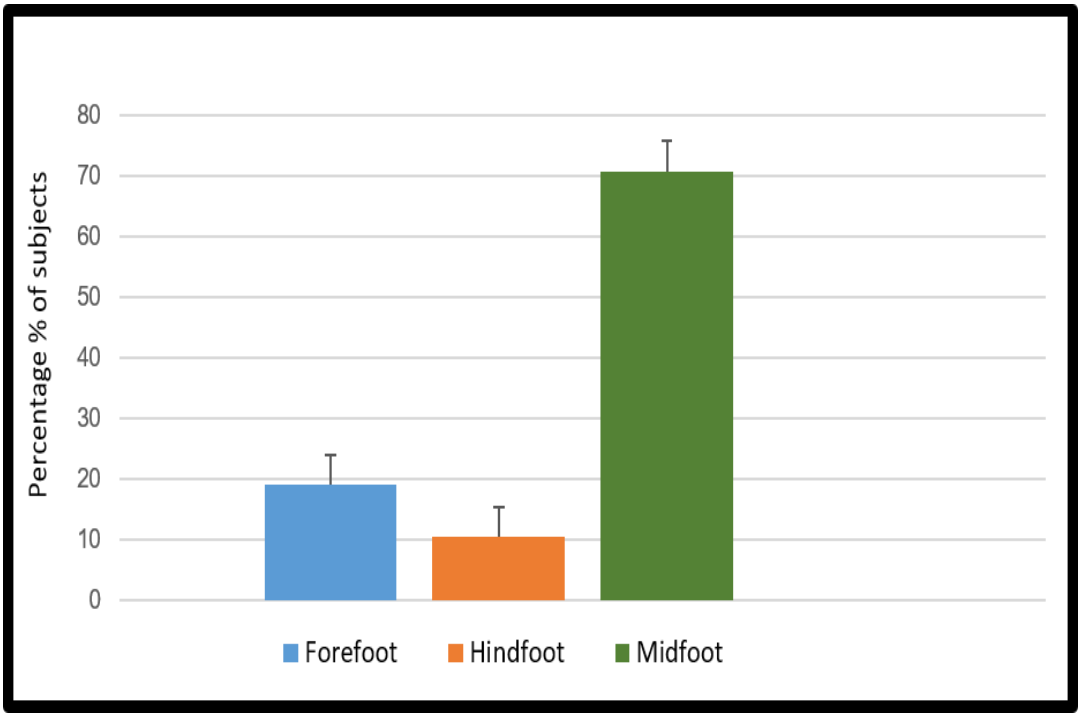


Figure 4.2.13: Location of DFU

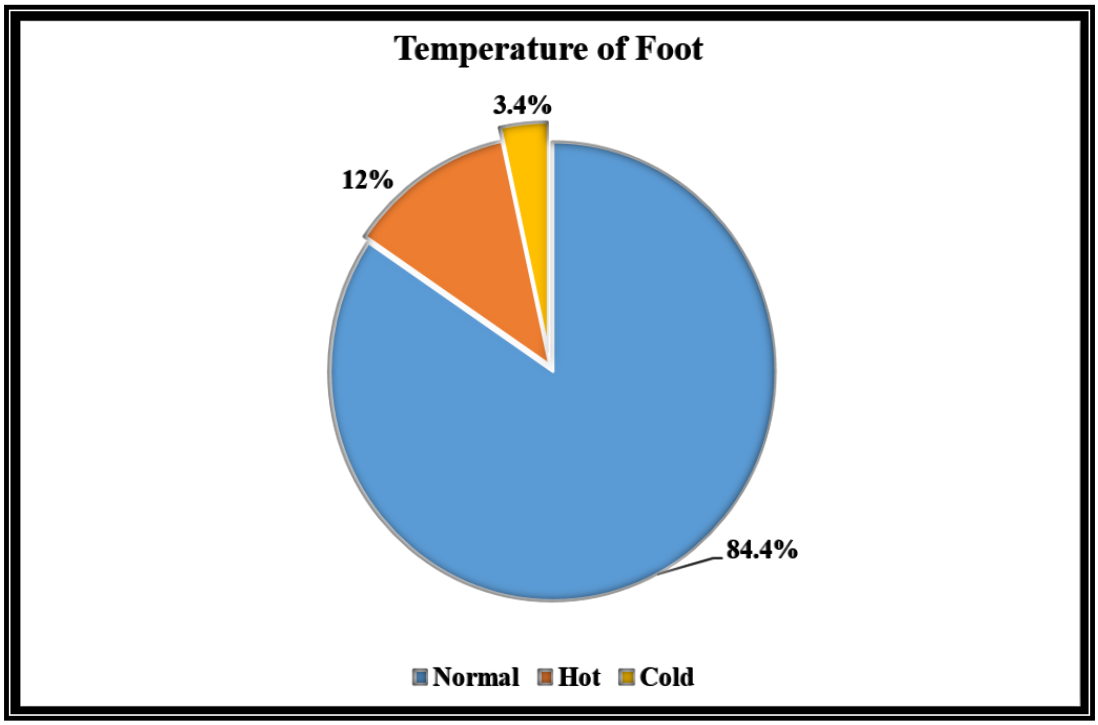


Figure 4.2.14: Temperature of Foot

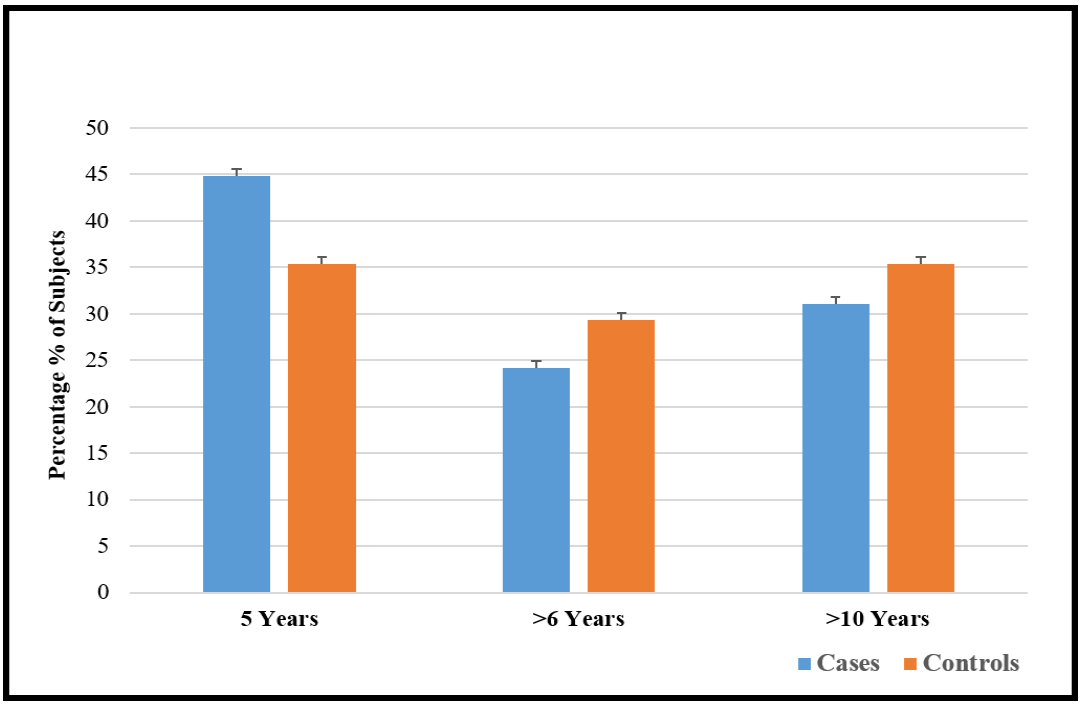


Figure 4.2.15: Duration of T2D

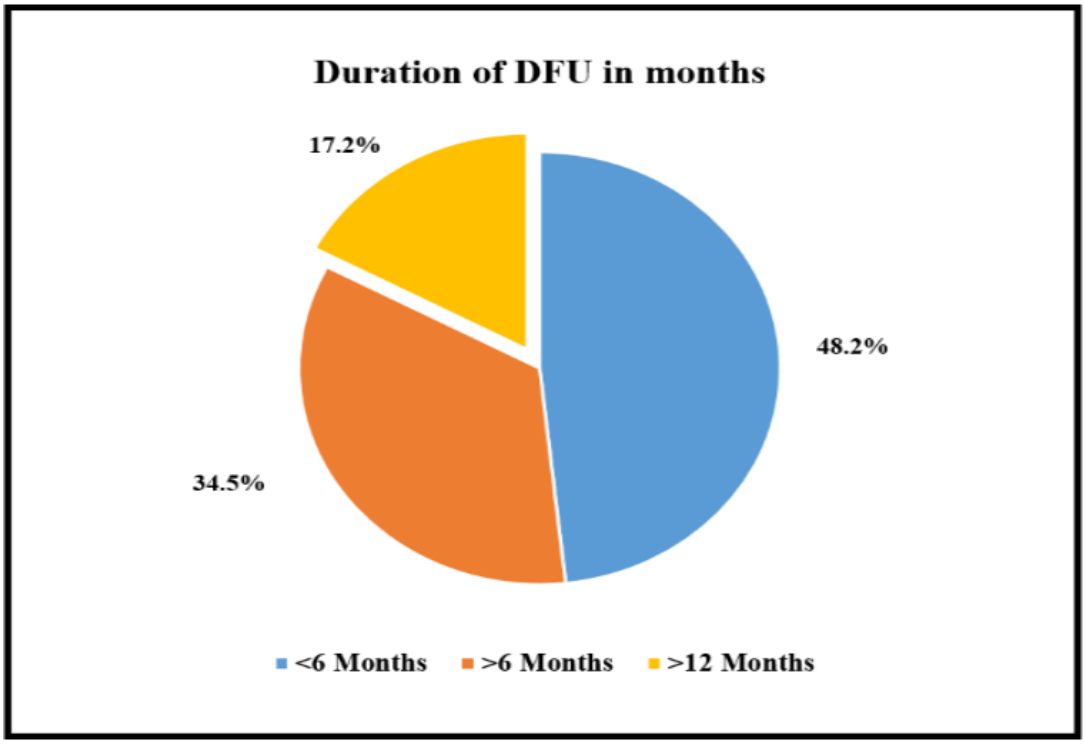


Figure 4.2.16: Duration of DFU

4.3 GENETIC DATA ANALYSIS

4.3.1 Genomic DNA

Using a NanoDrop spectrophotometer, DNA was quantified. Samples were pure with concentrations up to 1.8 (Figure 4.3.1(a)). The quality of the extracted genomic DNA was assessed using agarose gel electrophoresis. The gel picture in Figure 4.3.1(b) clearly shows discrete bands of genomic DNA, indicating the extracted DNA's integrity and excellent quality.

4.3.2 Gene Amplification of rs4444903 Polymorphism

The *EGF* gene was amplified using T-ARMS-PCR. Figure 4.3.2 displays a gel image of the amplified region of the *EGF* gene. The presence of discrete bands measuring 507bp confirms the presence of the targeted gene sequence. The band size of 296bp indicates the presence of the variant G allele, whereas 268bp represents the wild allele, which is the A allele.

4.3.3 Genotype Frequencies of *EGF* gene polymorphism rs4444903

The polymorphism rs4444903 was identified in three genotypes: heterozygous variant (AG), homozygous variant (GG), and homozygous wild (AA), with different rates seen in both patients and controls. Figure 4.3.3 indicated that 55.1% of patients exhibited heterozygous variant (AG) genotypes, whereas 22.4% displayed homozygous variant and wild-type genotypes. Nevertheless, the AG genotype was present in 38.8% of controls, whereas the AA genotype accounted for 35.3% and the variant GG genotype accounted for 25.8%. The wild type homozygous genotype AA was found to be more common in the control group (67%) compared to the case group (25%).

4.3.4 Allelic Frequencies of *EGF* gene polymorphism rs4444903

According to figure 4.3.4, the allelic frequency of wild type A allele was found to be greater among controls (54.4%) and cases 50% in comparison to variant allele G which happened to be 45.2% in controls and 50% in cases.

It indicates that this allele may provide some protective impact against the occurrence of DFU. It is possible that having this allele could be linked to a lower likelihood of developing DFU in individuals with T2D.

4.3.5 Association of *EGF* SNP rs4444903 with Diabetic Foot Ulcer

The association between the *EGF* gene polymorphism (rs44449003) and DFU was analyzed using Pearson Chi-square ($\chi 2$) in SPSS. Subsequently, this analysis is validated using the statistical software SHEsis plus. Table 4.3.1 demonstrated that the *EGF* SNP rs4444903 was not statistically associated with DFU. Significant association of the *EGF* gene polymorphism was demonstrated by the odds ratio when compared to their respective controls. With an odds ratio of 1.209, there is a potential indication of an elevated risk. However, it is important to note that the high p-value of 0.426 indicates that this association lacks statistical significance. The odds ratio values at p 0.426, which are more than 1, indicated the variant alleles were involved in the development of DFU suggesting that rs4444903 may have a risk factor against DFU [1.209, CI 95% (0.773-1.89) p=0.426].

4.3.6 Association of *EGF* SNP rs4444903 Genotypes

To check the further association of separate genotype, different genetic models were applied that is Dominant, recessive, co-dominant and over-dominant. In co-dominant model[OR 95% CI=2.24, (1.037-4.849, $p<0.05$)], the heterozygous AG genotype was significantly associated with the disease and odds Ratio also revealed that there is a risk

factor in getting DFU if person possess AG genotype, similar results were identified in over-dominant model[OR 95% CI=1.941, (1.025-3.376, p<0.05)], whereas dominant[OR 95% CI=1.89, (0.916-3.907, p=0.0847)] and recessive models[OR 95% CI=0.8281, (0.39-1.74, p=0.6194)] showed no significant association. The odds ration <1 revealed protective role of SNP, odds ratio >1 revealed risk association with the progression of disease, whereas the odds ratio 1 indicates that there is no relation among the SNP and disease. In this study the genotype AG showed a significant role against the risk of developing disease as shown in table 4.3.2.

Figure 4.3.1(a) Optical Density of DNA on NanoDrop

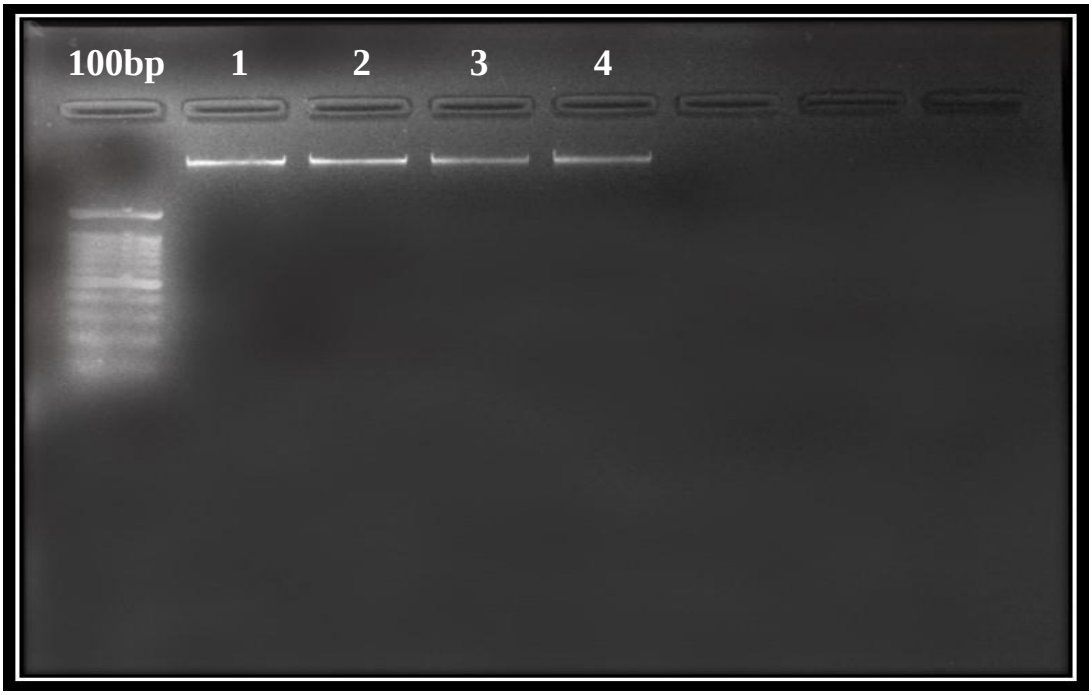


Figure 4.3.1(b): Gel Image of the Extracted Genomic DNA with 100bp ladder and Lane 1 to 4 of samples

Figure 4.3.2: Gel Image of the Amplified T-ARMS-PCR of *EGE* Gene

Representation of obtained genotypes of SNP rs4444903. 100bp ladder, whereas 1 to 7 shows amplified PCR product of targeted polymorphism on 1% agarose gel. The presence of two bands showed homozygous genotype, whereas three bands indicate the heterozygous genotype. The homozygous wild-type genotype appeared as two bands 507bp and 268bp, homozygous variant genotype appeared as two bands at 507bp and 296bp. The heterozygous genotype appeared as three bands at 507bp, 296bp, and 268bp. The band at 507bp showed inner control band from outer primer which is common for all genotypes.

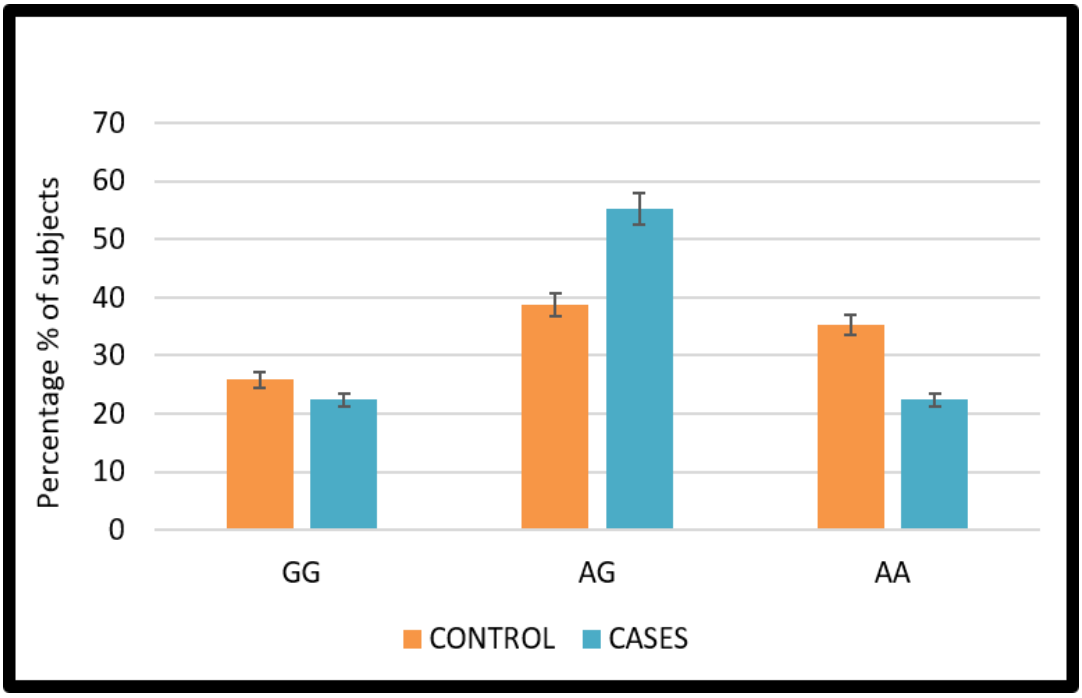


Figure 4.3.3: Genotypic Frequency for rs4444903 of *EGF* gene

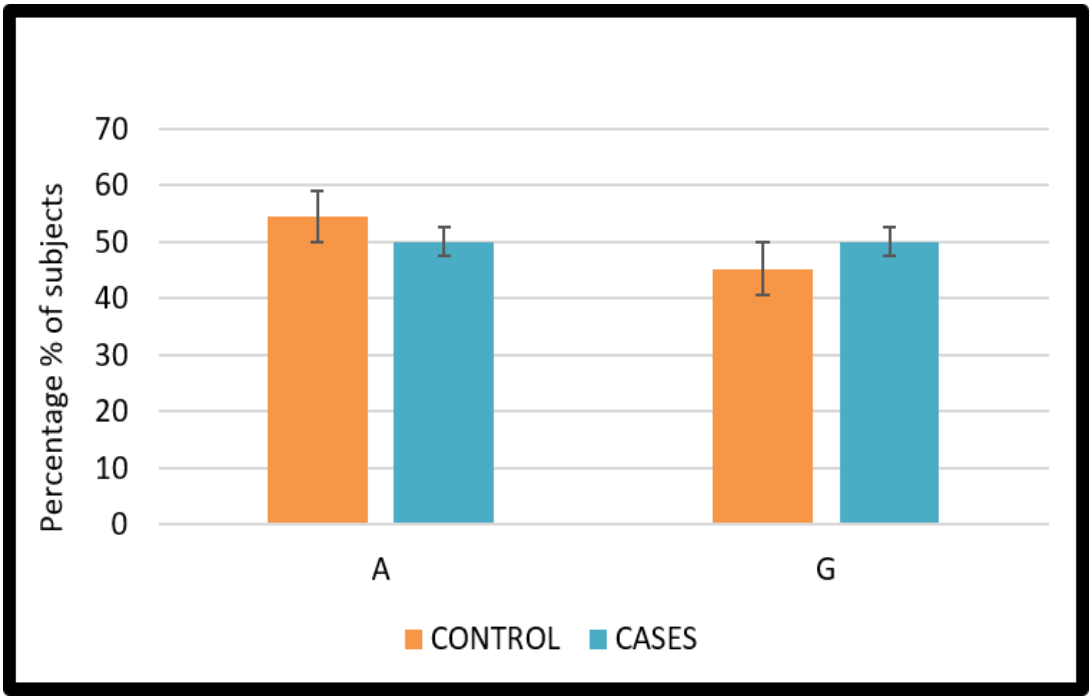


Figure 4.3.4: Allelic Frequency for rs4444903 of *EGF* gene

Table 4.3.1: Association Analysis of SNP in subjects

Gene	SNP	χ^2	Odds Ratio	95% Confidence Interval	p-value
EGF	rs4444903	0.698	1.209	0.773-1.89	0.426

Table 4.3.2: Genetic Model for rs4444903 of *EGF* Gene

rs4444903			
Genetic Models	Genotype	OR (95% CI)	p-value
Dominant	AA AG+GG	1 (Ref) 1.89 (0.916-3.907)	0.0847
Recessive	AG+AA GG	1 (Ref) 0.8281(0.39-1.74)	0.6194
Co-dominant	AA AG GG	1 (Ref) 2.24(1.037-4.849) 1.366(0.554-3.366)	<0.05 0.4971
Over-dominant	GG+AA AG	1 (Ref) 1.941(1.025-3.376)	<0.05

CHAPTER 5

DISCUSSION & CONCLUSION

5.1 Sequence of Discussion Experiment

Diabetes mellitus is a multifactorial epidemic, affecting both males and females globally. Genes, sedentary lifestyles, and environmental factors, such as stress and smoking, may all increase the incidence of DM. Although there are many therapeutic and interventional advancements in treating DFU, it's still highly challenging and debatable. Wounds that don’t heal become threatening for lower limb amputation (LEA) and thus increase the burden on both patients and the health care system. The pathophysiological events of DFU are ambiguous, as they may include peripheral neuropathy, peripheral vascular disease, improper cellular mechanisms involved in wound healing, and immunological disorders.

In Pakistan, where the majority of people have a low socioeconomic status, a metabolic disorder known as diabetes mellitus (DM) impacts 32.96 million people, or 26.7% of the population (Azeem *et al*., 2022). Poor medication compliance, inadequate foot care, and sociocultural factors specific to this region are the main causes of this condition. A lot of research has been done on single nucleotide polymorphisms (SNPs) of growth factors that are connected to DFU because they play a big part in epithelial growth and multiplication, making new blood vessels, cell proliferation, and tissue repair.

Epidermal growth factor (EGF) is a very important growth factor in healing DFU by promoting cell proliferation, chemotaxis, and tissue regeneration. As a result, it may protect cells from oxidative stress damage and reduce inflammation linked specifically to diabetes. Despite the clinical trials of topical and intra-lesional usage of EGF and rhEGF, high-risk patients have not yet incorporated it into their diabetes control regime.

The occurrence of diabetic foot ulcers (DFU) in Pakistan differs across various age groups. In the present study, there was a significant mean difference in the age of the subjects (51.16±10.01), contrary to another study by Eraydin (2023), whose mean age for subjects was 63.16±9.43. The current study found a significant link between age, height, and BMI in people with DFU and T2D. This finding is similar to those found by Asegid (2021) and Eltilib (2022), but different from the study by James (2022), which did not find any significant differences. In the case group, 20% of individuals were between 51 and 55 years old, which contrasts with a study by Akhtar *et al*., 2022, which found that the highest occurrence rate of diabetic foot ulcers (DFU) is 66.67% among those 75 years and older. Contrary to another study, there is a higher occurrence of DFU among individuals aged 56 to 65. Peripheral neuropathy and peripheral arterial disease develop due to age-related issues with arteries and lipid/glucose metabolism (Sukartini, 2020). In this study, there was no significant link between the length of T2D (mean of 9.49±6.72) and weight with DFU, since most DFU cases had T2D for less than five years. This is different from another study which found a significant link between T2D length (mean of 18.44±7.77) and weight with DFU (Eraydin, 2023).

There was an increased risk of systolic BP and DFU with a significant difference of 0.025* ($p<0.05$) whereas diastolic BP didn't show any statistical significance with $p = 0.219$ ($p<0.05$). Accumulation of advanced glycation end-products (AGEs) in DM may lead to arterial stiffening. This stiffening may increase with increasing age. The oxidative stress disrupts the endothelium (inner lining of blood vessels) which reduces the synthesis of nitric oxide, consistent with a study (Brennan *et al*., 2018).

In patients with diabetes, dyslipidemia is very common. Dyslipidemia can manifest as elevated triglycerides (TG), low-density lipoproteins (LDL), or total cholesterol (TC),

or as a decreased level of high-density lipoproteins (HDL), the protective lipoproteins. Dyslipidemia typically does not manifest when diabetes is well controlled. Usually, DFU may share risk factors and mechanisms with atherosclerosis, and dyslipidemia is one of the risk factors common in both processes. There was inverse correlation and non-significant observed between smoking and HDL levels among both cases with DFU and controls with T2D ($p=0.129$), showing smoking may reduce HDL levels in blood as well as can increase total cholesterol in blood. It can be inferred that BMI has an impact on the disruption of lipid profiles in both cases with DFU and controls with T2D. It was noted that smoking has the potential to lower HDL levels, and a robust correlation with TC levels. Thus smoking accelerate reduction of HDL in patients of diabetes and increase the risk factor for DFU and atherosclerosis. High levels of triglyceride in the blood may cause peripheral neuropathy and increase morbidity due to DFU and LEA consistent with a study by Sanja Vujčić (2022).

TG demonstrated a significant association between *EGF* genotypes among cases with DFU and controls with T2D. When triglyceride levels increase, the synthesis or manifestation of EGF decreases. Therefore, there is a negative link between TG and EGF points to the possibility that increasing levels of triglycerides may reduce the benefits that are associated with the higher amounts of EGF that are associated with the GG genotype. Song and Jiang (1994) conducted the study, which reveals a notable correlation between genotypes in the groups of EGF plays a significant role in activating cellular signaling pathways involved in lipid metabolism through the phospholipase C and D enzyme systems. An in-vitro study was conducted with goat mammary epithelial cells during lactation, which further supports this. EGF stimulates EGFR activity, which in turn activates phospholipase c-1 and Akt, leading to the synthesis of new genes involved in lipid synthesis and an increase in triglycerides within the cells (Huang *et al*., 2020).

Typically, in Pakistan, DFU may express a gender-specific association with varying prevalence among males and females. Males were more prevalent in cases of DFU (63.7%) than females (36.4%). Long-term diabetes, socioeconomic status, comorbidities, poor foot care, and smoking habits, which are more common in men than in women, could potentially explain this. Consequently, the proportion of male subjects is higher than that of female

subjects. Moreover, males are more susceptible to LEA and mortalities, whereas females are more responsive to ulcer healing. This corresponds to the many studies prominently studied by Iacopi *et al*. (2021) and Wang *et al*. (2021). However, this is in contrast to the findings reported where female subjects were recruited at 59.13% and males at only 40.86%. Overall, the demographic parameter, such as age, was statistically significant among all the cases with DFU and controls with T2D (Hussain *et al*. 2022; Khan and Junaid, 2017).

The current study recruited the majority of non-smokers, with smokers making up only 20.63% of the case groups. According to AbdElrahman (2021), nearly 79.3% of the respondents were non-smokers, which aligns with the study that recruited a larger proportion of non-smokers, with nearly 40% being non-smokers and 12% being smokers. Several studies, including one by researchers, found that smoking cigarettes was a major risk factor for DFUs because it impairs glucose metabolism, introduces the free radicals that promote the oxidative stress, slows down healing, and makes people more likely to get bacterial infections and become resistant to antibiotics (Li *et al*., 2022).

44.8% of the cases recruited were Urdu speaking, which was the majority. Separate research on atherosclerotic cardiovascular disease (ASCVD) revealed that 33.4% of the participants spoke Urdu, despite the lack of specific studies on the ethnicity of DFU (Ashraf, 2020). Comorbidities significantly increased the risk of developing diabetic foot ulcers, as proved by Khan (2018). One possible reason for this is that comorbidities often lead to a higher number of medications, which can in turn contribute to lower compliance rates. In contrast to the present study, which reported a lesser number of comorbidities, the outcome was suboptimal glycemic control, leading to an elevated risk of diabetic foot ulcers (DFU). In contrast, a study found no association between the presence of comorbid diseases and the development of diabetic foot ulcers (DFU). It is worth noting that this differs from the findings of other studies, which have shown a link between comorbidities and an increased incidence of DF (Mohammed 2016).

About 62% of cases were included in class I obesity, and 40.5% were obese with class II among controls, consistent with higher BMI being found to be associated with an

increased risk of DFU as studied by Khan (2018). A higher BMI brings insulin resistance and is also prone to the initiation of an inflapedismmatory cascade. As adipose tissues hold pro-inflammatory markers and lead the body responses in the state of chronic inflammation, this makes the glycemic control difficult. Thus, damage blood vessels and nerves (neuropathy) and increase the risk of DFU. And contrary to a study that shows patients who develop DFU and have a low body mass index (BMI) are at a higher risk of amputation and death. This may be because underweight individuals are more likely to be frail and have poor nutrition. Therefore, it is important to take BMI into account when managing and predicting outcomes for DFU patients (McDermott, 2022).

The majority of cases recruited were neuropathic (86.2%), while ischemic and mixed ulcers accounted for 10.3% and 3.4% of the cases, respectively. Neuropathic ulcers are more prevalent in DFU mainly due to prolonged hyperglycemia, which activates the polyol pathway, which causes accumulation of sorbitol in nerve cells, increasing osmotic pressure, and nerve cell damage. Chronic hyperglycemia also causes overproduction of advanced glycation end products (AGEs) and increases inflammation and oxidative stress to nerves. As in DFU, production of growth factors is also minimized, which delays the wound healing and impairs tissue repair. In another study conducted by Hafiza Ammarah Sadiq *et al*. (2023), it was discovered that a significant 70% of the participants had neuropathic ulcers, while 30% had neuroischemic ulcers. This starkly contrasts with the results of a study by Jiang *et al*. (2015), where only 21.2% had neuropathic ulcers, 23.5% had ischemic ulcers, and 53% had a different type. According to a study conducted, the majority of ulcers were found to be pure neuropathic ulcers (73%), with the remaining 27% being ischemic ulcers (Meloni *et al*., 2022).

The classification of DFU cases in this study was done using SINBAD grading. The subjects selected for the study exhibited varying degrees of low SINBAD scores (<3). The reported rates for these categories were 65.5%, respectively. The findings indicate that 34.4% of the individuals exhibited an ulcer grading more than equal to 3, aligning with the research conducted by Georges *et al*. (2023). Their study revealed that 61% of participants had a low SINBAD score, while 39% had a high SINBAD score. After awareness and screening for foot complications, early detection of ulcers is becoming common. SINBAD

classification criteria is highly reliable to detect the foot ulcers clinically. Low grading increases the likelihood of ulcer control by good glycemic control, offloading the ulcers and wound care thus minimizing the lower extremity amputation and mortalities. There is a significant disparity between the two groups. In contrast to the current study, a study conducted in Karachi recruited a majority of cases with high scores (65.3%) and a minority of cases with low scores (34.1%). The study was conducted by Ince *et al*. (2008).

In the current research, the most prevalent manifestation of a DFU was infection, which accounted for 70.6% of all cases. Healing was in second place, accounting for up to 20.7% of all cases. The research showed that infection (mono-microbial growth and Gram-negative organisms) was common in 92.31% of people with diabetic foot infections. This showed that bacteria had made their way into the feet of the participants. As the severity of DFI increased, there was a commensurate increase in hospital readmissions, as well as major and minor amputations and deaths (Seth *et al*., 2019).

In the present research, the most significant risk factor for DFU was trauma, which was responsible for 58.6% of the cases. Since neuropathy primarily causes trauma, a higher prevalence of trauma histories provides an explanation. Bacteria and footwear were responsible for 32.8% to 8.6% of the cases respectively. Factors such as the individual's general health, foot care habits, and the existence of peripheral neuropathy or ischemia play an important role in the development of bacterial growth. A research, where 95.71% of participants identified bacterial growth as the most prevalent risk factor, contrasts with the recruited respondents' reduced incidence of DFU due to footwear (Al-Hasnawi, 2023).

Based on the conclusions of the current study, it was observed that a significant proportion of individuals, 82.7%, exhibited dominant presence in the planter surface area or weight-bearing surface. Mainly as an outcome of nerve damage there is loss of sensations in the weight-bearing surface that causes tingling or lack of sensation when excessive pressure was applied. Pressure for longer duration without any proper relief injures the tissues; fissures may form and bacteria may grow there. In contrast, only 17.2% of individuals reported dominance in the non-planter surface area. Contrary to the findings of Younis *et al*. (2018), the study revealed that 61% of the participants had ulcers on the

plantar surface of their feet, while 31% had ulcers on the non-plantar side. Additionally, a cross-sectional study identified the non-planter surface of the foot as the dominant site for DFU (Vahwere *et al*., 2023). The study's findings revealed a prevalence rate of 70.6% for foot ulcers in the midfoot region among the population. The forefoot accounted for 19% of the total, while the hindfoot made up 10.3% and was lagging behind. Midfoot is involved more due to planter pressure, foot deformity, and loss of sensation. With loss of sensation and any protection, the injury continues to the weak portions of the midfoot that worsen and turn into foot ulcers. A separate study contradicted the present study's findings, revealing that only 18% of the population suffered from Charcot arthropathy, specifically affecting the midfoot joint (Martin & Davis, 2023).

In the current study, 84.4% of cases with DFU reported normal temperatures, whereas Bagavathiappan found a higher proportion of cases (60.6%) with warm temperatures. Although foot temperatures in healthy individuals without DFUs usually fall within the range of 27°C to 30°C, the diabetic neuropathic foot is characterized by warmth, palpable pulses, and distended veins, suggesting heightened blood flow in the affected limb. Studies indicate that blood flow to the foot may become sluggish, resulting in stable or reduced microcirculation (Bagavathiappan *et al*., 2010).

The long duration of T2D was thought to be significant for the appearance of DFU, but the current study disproved this theory by showing that 44% of people with less than or equal to 5 years of T2D presented with DFU. The majority of controls T2D were those with more than 10 years and >6 years of T2D. Delessa Hirpa and Tariku Bekela's study in Ethiopia (2023) aligns with these results, revealing that 58.3% of diabetic foot ulcer patients had diabetes for less than 5 years. In another study, cases were recruited with shorter duration of DFU (Younis *et al*., 2018).

This research thesis explored the association with the *EGF* gene polymorphism rs4444903 and DFU, and also checked the frequency of *EGF* alleles in Pakistan's T2D patients in a specific period of time. *EGF* is a protein-coding gene. A change from +61 A>G in the *EGF* coding region was thought to lower the expression of *EGF*, change the expression of EGFR and related enzymes, and stop the splicing of preproprotein, which

means that no EGF peptide is made. This, in turn, would deplete the EGF concentration required to heal the DFU (Gawish, 2023).

The single nucleotide polymorphism (SNP) rs4444903 in the *EGF* gene was the prime focus of the present research to determine its association with diabetic foot ulcers. The genotype frequency revealed that in cases with DFU and controls with T2D, the dominant genotype frequency was heterozygous (55.1% and 38.8%, respectively). The wild genotype AA was more prevalent in controls in comparison to cases (67% vs. 25%). In contrast, the variant allele GG did not show up very often in the case groups. This is different from a study, which found that the heterozygous variant or mutant genotype GA was more common in type 2 diabetes mellitus; the heterozygous variant or mutant was less common in cases of T2D with poor glycemic control compared to controls with good glycemic control. The allelic frequency of wild allele A was found more frequent in controls (54.4%) as compared to G variant allele (45.2%) whereas A and G are equal in both cases (50%) (Mahmoud *et al.*, 2024) and the findings of another study indicated same that the AG genotype was statistically significant and demonstrates a strong susceptibility to T2D (Trimal *et al.*, 2019).

The odds ratio analyzed the strength of association between the *EGF* gene single nucleotide polymorphism (SNP) rs4444903 and the risk of developing DFU. Although *EGF* gene polymorphism rs4444903 have no statistical significance with DFU but it may have risk of association with DFU. In consistent to a study, where variant allele A showed a highly significant association (Trimal *et al*., 2019). To check the further association of separate genotype, different genetic models were applied that is dominant, recessive, co-dominant and over-dominant. In co-dominant model, the heterozygous AG genotype was significantly associated with the disease and Odds Ratio also revealed that there is a risk factor in getting DFU if person possess AG genotype, similar results were identified in over-dominant model, whereas dominant and recessive models showed no significant association. To protect an allele from DFU in the recessive model, both alleles must be of the variant type, as the GG variant does not decrease the synthesis of EGF. Thus, the recessive model is protected from disease progression, contrary to a study that found a

significant association between the recessive model (GG/AG+AA) of *EGF* rs4444903 and colorectal carcinoma by Zhu (2019).

The co-dominant model separately elucidates each genotype and their respective effects (AA, GG, and AG). A co-dominant model with significant outcomes suggests that each genotype is independently influential for DFU. In the current study, the p value was significant at 0.05, indicating that the codominant model AG genotype was independently associated with DFU, consistent with a study where the co-dominant model's p value was highly significant and associated with the disease (Leal *et al*., 2020).

The heterozygous variants of *EGF* +61A>G rs4444903, or AG, to the homozygous wild type and variants, which are AA and GG, in an over-dominant model were compared. The p value was 0.05 in the over-dominant model, which shows heterozygous genotypes had statistical significance over homozygous genotypes.

5.2 Implication of Study

5.2.1 Theoretical Implication

This comprehensive investigation not only enhances our comprehension of DFU complexities but also adds to the wider scientific knowledge of the intricate connection between genetics and wound healing.

5.2.2 Practical Implication

Genetic polymorphisms within the *EGF* gene have the potential to be significant, according to the research. It might be used as a roadmap for creating personalized treatment

plans. Interventions targeting pathways connected to EGF may have a greater impact on individuals who have certain genetic variations.

5.2.3 Policy Implication

- Diabetes mellitus, the most frequent worldwide burden, increases DFU-related lower limb amputations. Encourage lifestyle changes and yearly lab testing for prediabetes, diabetes, and complications. Children and teens with obesity are more prone to have diabetes. Therefore, schools, colleges, and universities must offer diabetes mellitus risk education events. These initiatives should include behavioral change campaigns on sedentary lifestyles, smoking, BMI, stress management, late-night sleep, fast food, and unbalanced diet trends. Seminars, pamphlets, articles, brochures, radio, social media, and TV outreach may assist. As part of preventive healthcare, it is important to check TG levels regularly in groups of people who are likely to get diseases linked to EGF production.

- Smoking disrupts lipids, worsening atherosclerosis and DFU. Section 8 prohibits selling cigarettes and other tobacco products to minors. Ahsan *et al.* (2022) state that Section 9 bans tobacco sales, storage, and distribution within 50 meters of schools. These restrictions have been poorly implemented, with low public compliance and weak enforcement. New regulations should apply to all adults, and smoking should not be a choice. Electric cigarettes, vaporizers, and nicotine patches/gums should be banned. Thus, these regulations must be enforced to reduce DM and DFU.

5.3 Limitations & Strength of Study

(A) Limitations

Following limitations can be expected in the study is

- Small Sample size
- Single gene was targeted rather than multiple

(B) Strength of Study

- ARMS-PCR is less laborious and expensive as compared to gene sequencing by Sanger and Restriction Fragment Length Polymorphism-PCR. Where gene sequencing is quite expensive and RFLP-PCR is time consuming in running gel electrophoresis and enzyme incubation.
- To the best of my knowledge this association of *EGF* gene polymorphism rs4444903 with DFU is studied for the first time in Pakistan.
- This genetic variation could be a potential biomarker in diagnosing diabetic foot ulcer.

5.4 Future Research Direction/ Recommendations

- Studying both variations in *EGF* genes and the levels of EGF in the blood can offer valuable information about how these genetic variations impact the development of diseases.

- Additional research should be conducted on the related single nucleotide polymorphisms (SNPs) of the epidermal growth factor (*EGF*) gene in order to examine for any associated SNPs in diabetic foot ulcers (DFU).

- Further research, with greater population size should investigate the functional implications of *EGF* gene polymorphisms and gene expression analysis with protein expression, localization, and function in Pakistani population.

- Future research should focus on assessing the epigenetic alterations linked to *EGF* gene polymorphisms their possible influence on susceptibility to DFU.

- Animal models can be used to further explore the correlation between *EGF* variations and the development of DFU-like disease. These models may provide useful insights into the molecular pathways that contribute to the development of DFU.

- Future clinical trials on intra-lesional EGF administration can be planned to improve the process of healing of diabetic foot ulcer in Pakistani population based on the targeted gene polymorphism.

5.5 Conclusions

In this study, it was concluded:

EGF polymorphism is not significantly associated with the DFU but may still increase a risk.

The co-dominant and over-dominant models show a significant association with risk factors for DFU susceptibility.

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A

Bahria University
Health Sciences Campus, Karachi

No one left behind for research.

Institutional Review Board

BUHS-IRB # 036/23
Date: 01-12-23
Name of PI: Dr. Faizza Rasheed
Affiliation & Department: MPhil Candidate Biochemistry,
Address-BUHS,C Sailor Street, DHA Phase 2, Karachi

Subject: APPROVAL OF YOUR RESEARCH PROPOSAL:

Title of the research
"Association of *EGF* gene polymorphism rs 4444903 with Diabetic Foot Ulcer"

Dear Dr. Faizza Rasheed,

I am writing this letter at your request to confirm that we support theresearch project **"Association of *EGF* gene polymorphism rs 4444903 with Diabetic Foot Ulcer."** I know the research will determine the association of EGF gene polymorphism rs 4444903 (+61 A>G) in T2D patients with DFU as compared to T2D patients without DFU. The IRB will support the project under the proposed guidelines in the IRB application for six ***months***.

Suppose any unanticipated problems or adverse advents occur. In that case, it is up to Dr. Faizza Rasheed to report these events to the IRB as promptly as possible. The findings of the research have the potential to improve risk assessment, treatment strategies, and clinical management of DFUs by scientific communities, ultimately improving outcomes and quality of life for those affected. We will be happy tosupport this endeavor.

Sincerely,

Prof Dr. Inayat H. Thaver-
FCPS (Community Medicine); PhD (Public Health)
Chairperson, Institutional Review Board (IRB)
Bahria University Health Sciences Campus
Karachi

Prof. Dr. Inayat H. Thaver
Chair, Institutional Review Board.

B

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LETTER OF APPROVAL

Date: 08-01-2024

To,
Dr. Faizza Rasheed
MPhil – Student
Department of Biochemistry
BUHSCK

Subject: **Faculty Research Committee**
FRC-BUHSCK Approval of Research Study

Title of Study: Association of EGF gene polymorphism rs 4444903 with Diabetic foot ulcer.

Name of Student: **Dr. Faizza Rasheed**
Reference No: **FRC-BUHS 09/2024**

Dear Dr. Faizza Rasheed
Thank you for submitting research proposal to FRC-BUHSCK. The committee has approved your project.
Regards

PROF. DR. Shehla M. Baqai HI (M)
Maj. Gen (R)
Chairperson FRC-BUHSCK

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C

WRITTEN INFORMED CONSENT FORM OF PATIENT

You are giving your consent to participate voluntarily and at your own will in this research clinical trial project that aims to analyze "**Association of *EGF* Gene Polymorphism rs4444903 with Diabetic Foot Ulcer"**

You have been explained in detail the nature and significance of participating in the project and you understand the provided explanation.

You have been told that findings of your disease and your data will be kept strictly confidential and will be used only for the benefit of community, publications and paper presentations.

You have been explained that laboratory investigations will be conducted for the diagnosis of disease. For this purpose, you fully agree to give your blood samples at the beginning and end of study.

You also agree to give all relevant information needed, in full and to the best of your knowledge to the researcher. It is clarified to you that no incentive will be provided to you for participating in the study except the cost of lab investigations and drugs, whereas you do have the right to withdraw from the study at any time.

You are advised to contact Dr. Faizza Rasheed on mobile number: 0332-3494275 or visit PNS Shifa hospital in case of any query/ emergency related to your disease.

Name of Patient:_____

S/D/ W/o:_____

Signature / Thumb impression of patient:_____

Name of Researcher:_____

Signature of Researcher:_____

C

مریض کے لئے اجازت فارم

آپ رضاکارانہ طور پر اور اپنی مرضی سے اس **کلینکیکل** ٹرائل پر وجیکٹ میں حصہ لے رہے ہیں جس کا **مقصد زیابیطس کے مریض ذیابیطس کے پاؤں کے السر کے ساتھ** *EGF* **جین پولیمور فزم کی ایسوسی ایشن** کا خون میں تجزیہ کرنا ہے- آپ کو اس تحقیقاتی عمل میں حصہ لینے کیاہمیت کے بارے میں تفصیل سے وضاحت کی گئی ہے اور آپ فراہم کردہ وضاحت کو سمجھتے ہیں۔

آپ کو بتایا گیاہے کہ آپ کی بیماری اور آپ کے اعداد و شمار کو سختی سے خفیہ رکھا جائے گا اور صرف کمیونٹی کے فائدے، اشاعتوں اور آرٹیکل اشاعت کے لئے استعمال کیا جائے گا۔

آپ کو وضاحت کی گئی ہے کہ لیبارٹری کی تحقیقات، تشخیص کے لئے کئے جائیں گے اس مقصد کے لئے آپ اپنے خون کے نمونے کو، مطالعہ کے آغاز اور اختتام پر مکمل طور پر دینے کے لئے اتفاق کرتے ہیں اور جب بھی اس کے درمیان ضروری ہو۔

آپ پوری طرح سے اور بہتر علم کے ساتھ محقق کو تمام متعلقہ معلومات دینے پر اتفاق کرنے ہیں یہ آپ کو واضح کیا گیاہے کہ لیبارٹری کی تحقیقات اور ادویات کی قمیت کے علاوہ مطالعہ میں حصہ لینے کے لئے آپ کو کوئی ادائیگی نہیں کی جائے گی، جبکہ آپ کو کسی بھی وقت تحقیقاتی عمل کو چھوڑنے کا حق حاصل ہے۔

آپ کو مشورہ دیا جاتا ہے **کہ ڈاکٹر فائزہ رشید** کے موبائل نمبر:**0332-3494275** پر رابطہ کریں اور آپ کی بیماری سے متعلق کسی بھی سوال یا ہنگامی صورت حال کے معاملے پی این ایس شفا سے رابطہ کریں۔

مریض کا نام:_____

والد / شوہر کا نام:_____

مریض کا علاج:_____

مریض کے دستخط / انگوٹھے تاثر_____

محقق کا دستخط:_____

تاریخ:_____

D

SUBJECT EVALUATION FORM

Serial No: Date:_____ Name:_____

Contact #:_____ Father's/ Husband's Name _____

Age: _____Sex: _____ Hospital:_____

Reg.No:_____ Address:_____

Duration of diabetes: _____ Duration and progression of the Foot

Ulcer_____ Comorbidities and Risk Factors_____

Smoking History: _____

Drug history: _____

General Physical Examination

Height: _____Cm Weight: _____Kg

Body Mass Index:_____kg/m2

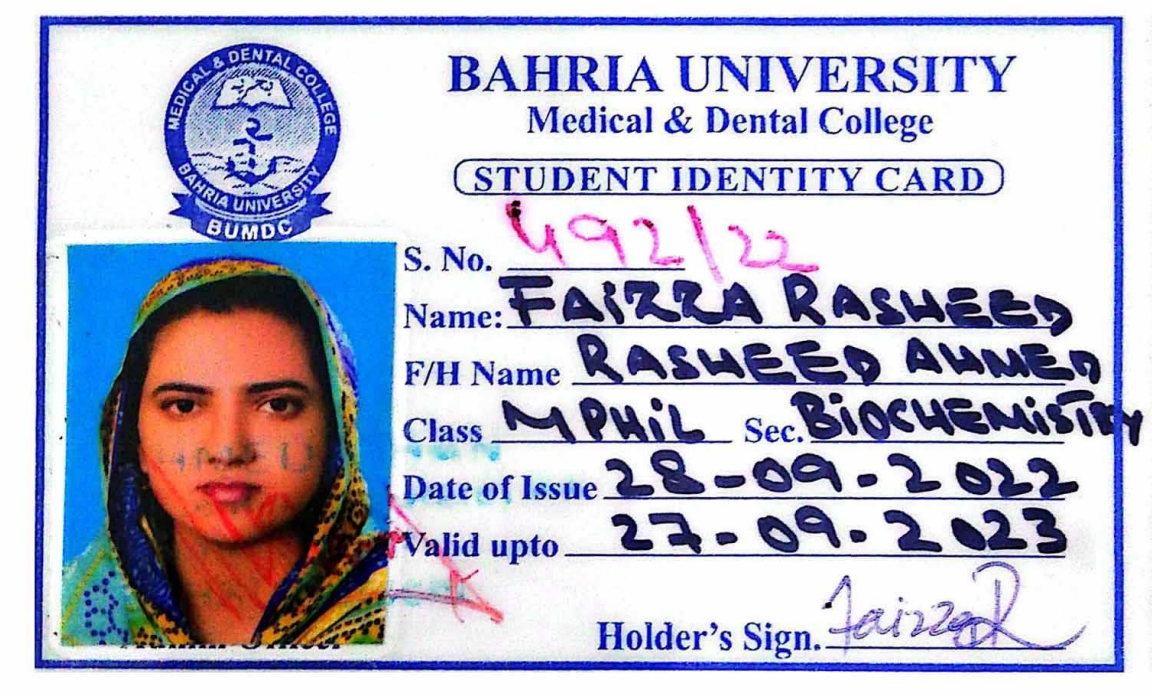
Blood pressure: _____mmHg Temperature of foot: _____ºC

Laboratory Investigations

Serum levels of total cholesterol_____ mg/dl TG_____mg/dl

HDL cholesterol____mg/dl LDL cholesterol _____mg/dl

E



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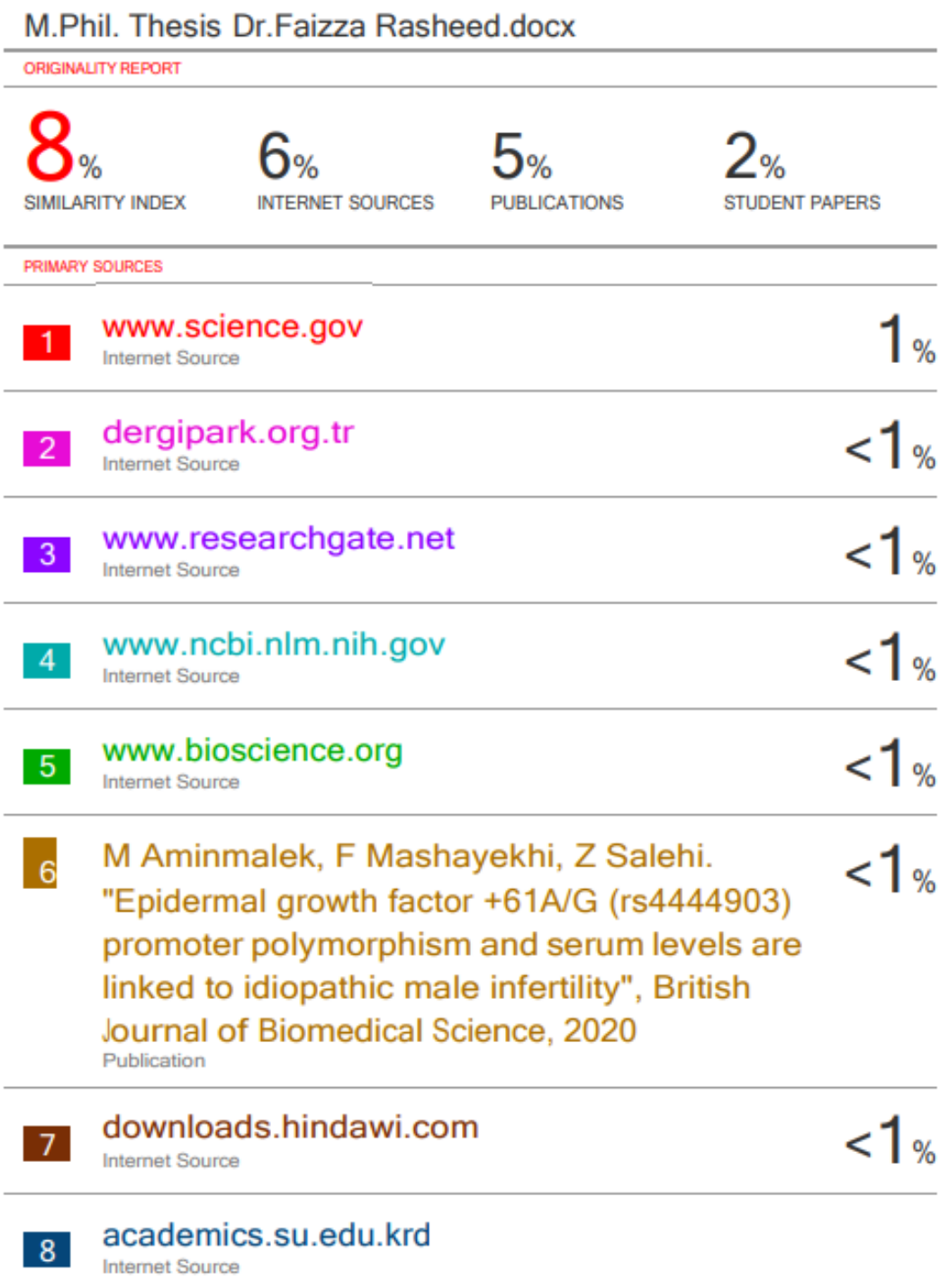

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