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A study for the Immunological role of Interleukin-37, 38 and 17A in Patients with Diabetic Foot Ulcer

A Thesis

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

(هُوَ الَّذِي جَعَلَ الشَّمْسَ ضِيَاءً وَالْقَمَرَ نُورًا وَقَدَّرَهُ مَنَازِلَ لِتَعْلَمُوا عَدَدَ السِّنِينَ وَالْحِسَابَ مَا خَلَقَ اللَّهُ ذَلِكَ إِلَّا بِالْحَقِّ يُفَصِّلُ الْآيَاتِ لِقَوْمٍ يَعْلَمُونَ)

صدق الله العلي العظيم

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A study for the Immunological role of Interleukin-37,38 and 17A in Patients with Diabetic Foot Ulcer.

Was prepared under our supervision in the College of Medicine/ University of Karbala, as a partial fulfillment of the requirements for the Degree of Master of Science in Medical Microbiology.

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Dedication

To ….

The dearest man in my life who lighted my way, to the bright star in my sky… guiding my nights for better tomorrows, my father.

To….

The kindest woman in my life with most pure and endless love in the universe, my mother.

To….

My companion… to the one who held my hand in every endeavor I pursue, with his endless support… with him I share every dream and success, My dear husband.

To….

The faces which the disease hide their smiles and to everyone who encouraged and helped me to make this work see the light …

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Summary:

Diabetes mellitus, a major cause of mortality around the globe, can result in several secondary complications, including diabetic foot syndrome, which is brought on by diabetic neuropathy and ischemia. Approximately 15% of diabetic patients suffer from diabetic foot complications, and results in high rates of morbidity and mortality of people with Diabetes mellitus. Among them25% are at risk of lower limb amputations due to impaired wound healing. Previous studies revealed that immune response and inflammatory processes play an important role in the pathogenesis of Diabetes.

A case control study was conducted for period of 6 months, starting from August /2022 to January/2023; the total number of participants were 193 subjects; they were divided into three groups: the first one includes patients with type 1 diabetes mellitus (29 with diabetic foot ulcer + 35 Non-diabetic foot), the second group includes type 2 diabetes mellitus patients (41 with diabetic foot ulcer + 38 Non-diabetic foot ulcer), and the third group includes (50) as apparently healthy control. Laboratory tests were done by serological techniques of sandwich ELISA, were tested for specific serum human IL-37, IL-38 and IL-17A. Swabs were taken from diabetic ulcer patients and performed for Aerobic bacteriological examination profile (culturing morphologically, microscopically and confirmative test by VITEK 2 compact system).

There were highly significant differences among the three studied groups regarding Age and DM duration. There were significant differences highly in FBS, HBA1c, Cholesterol, HDL, LDL and Triglycerides. As well as, there were a significant differences in CRP, PLT, Lymphocytes, Neutrophil and ESRlevels among the three studied groups. Whereas, no significant differences were found in Hb and WBCs.

Additionally, the result of the study revealed that IL-37 and IL-17A levels was highly significant ($P<0.01$) in all diabetic groups in compared to control healthy. While, the results of IL-38 show significant with T1DM and T2DM ($P<0.05$) than of control group. In addition to that, DFU group of T2DM illustrated higher levels of IL-37, IL-38 and IL-17A in comparisonswith other diabetic groups.

The highest incidence of diabetic foot ulcers was observed in patients aged 50-59 years. Of the 70 collected samples, 67 were positive for microbial growth, and 3 samples showed no growth. Of the 67 positive cultures, monomicrobial infection was found in 29 patients, and polymicrobial infection was found in 38 patients. Gram-positive pathogens were isolated from26 patients, and gram-negative microbes were isolated from 41 patients. Among all collected isolates (n=70), *Staphylococcus aureus* was the most predominant organism (20) and *Morganella morganii* species was the least common (only one isolate). Among the gram-negative bacteria, *E. coli* 15(36.58%) was predominant, followed by *K. pneumoniae* 11(26.83%), then *P.mirabilis* 7(17.07%) and *P.aeruginosa* 4(9.76%) and *A.baumannii* 3(7.32%), lastly, *Morganella morganii* 1(2.44%). Whereas, Gram-positive bacteria include *S. aureus* was the most isolated bacteria in this study with percentage 20(76.93%), followed by *streptococcus group B* 4(15.38%), then *Enterococcusspp.* 2(7.69).

In conclusion, in our studied the *S. aureus* is the most predominant bacteria in diabetic foot ulcers followed *by, E. coli.* On the other hand, it is the first study in Iraq that shed the light on the important role of a novel markers (IL-37, IL-38), as well as IL-17A serum level, and possibility of using them as diagnostic markers for diabetic patients and its complication such as DFU.

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List of Abbreviations

Code	Words
Anti-GAD65	Anti-Glutamic acid decarboxylase antibodies
AP-1	Activating protein-1
BAB	Blood agar base
BHIB	Brain heart infusion broth
CRP	CRP C-reactive protein
CVD	Cardiovascular disease
DCs	Dendritic cells
DFD	Diabetic Foot disorder
DFIs	Diabetic foot infections
DFU	Diabetic foot ulcers
EDTA	EDTA Ethylene Di amine Tetra Acetic Acid
ELISA	ELISA Enzyme –Linked Immunosorbent Assay
FOXP3	forkhead box
GDM	Gestational diabetes mellitus
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HbA1C	A glycated hemoglobin
HRP	Horseradish Peroxidase
IFN-γ	Interferon-γ
IL-17A	Interleukin IL-17A
IL-37	Interleukin IL-37
IL-38	Interleukin IL-38
JNK	c-Jun N-terminal kinase
LADA	latent autoimmune diabetes
LPS	Lipopolysaccharide
MHC	Human leucocyte antigens HLA
MLST	Multilocus sequence typing test
MODY	Maturity-Onset Diabetes of the Young
NDM	Neonatal Diabetes Mellitus

NF-κB	Nuclear factor kappa-B
OD	Optical density
PAD	Peripheral artery disease
PAMPs	pathogen-associated molecular patterns
ROS	Reactive oxygen species
SPSS	Specific Software Statistical Package for the Social Sciences
T1DM	Type I diabetes mellitus
T2DM	Type II diabetes mellitus
TGF-β1	Transforming growth factor-β1
Th17	T helper 17
TLR	Toll-like receptor
TMB	TMB Tetra methyl benzidine
TNF-α	Tumor necrosis factor-α
Tregs	Regulatory T cells

1.1 Introduction:

Diabetes mellitus (DM) is a major health problem worldwide. This metabolic disease is indicated by high blood glucose levels due to insufficient insulin production or action. The immune response to high blood glucose levels, as well as the presence of inflammatory mediators produced by adipocytes and macrophages in fat tissue, causing an inflammatory response. Low and chronic state of inflammation damages pancreatic beta cells, resulting in insufficient insulin production and hyperglycemia**.** Diabetes hyperglycemia is thought to cause immune response dysfunction, which fails to control the spread of invading pathogens in diabetic subjects. As a result, diabetic patients are known to be more susceptible to infections (El-hafez *et al.*, 2021, Afghahi *et al.*, 2022 and Soto-Chávez *et al.*, 2022).

The most commonly encountered micro-vascular complication of diabetes is diabetic neuropathy with the prevalence of 50-60%, neuropathy may cause decreased nerve functions and nerve blood perfusion with persistent nerve damage. Diabetic peripheral neuropathy increases development of foot ulceration risk (Soto-Chávez *et al.*, 2022).

Interleukin 37 is also known as IL-1 family member 7 (IL-1F7). It is a novel anti-inflammatory cytokine with immunomodulatory effects via three ways, i.e., by reducing the synthesis of pro-inflammatory cytokines, by lowering the expression of transcriptional cytokines, and by inhibiting the activation of kinase signaling (Zeng *et al.*, 2022).

Interleukin (IL)-38 is recently discovered, novel anti-inflammatory cytokine, which belongs to the IL-1β family, and inhibit subsequent signaling pathways; thereby regulating the differentiation and function of T cells, peripheral blood mononuclear cells, macrophages, and dendritic cells (Cao *etal.*, 2022(.

IL-17 secreted by Th17 cells initiates secretion of pro-inflammatory factors and further amplifies the inflammatory response in inflammatory and autoimmune diseases. Therefore, Th17 and IL-17 might be involved in pathogenesis of DM (Parhi *et al.,* 2019).

According to bacterial culture and molecular approaches in several studies, DFUs/Diabetic foot infections (DFIs) can be colonized by different aerobic bacteria such as Gram positive cocci in temperate climates, Staphylococcusaureus and (beta-haemolytic) Streptococcus are the most commonly identified pathogens in diabetic foot infections. Infections are usually acute, classified as mild to moderate and formation of pus is possible. In daily practice, coagulase- negative Staphylococcus (CNS), Corynebacterium, Enterococcus and Cutibacter ia (formerly Propionibacterium) are commonly present in ulcer cultures, but are usually considered colonizing, rather than pathogenic bacteria (Sadeghpour Heravi *et al.,* 2019 and Nagendra *et al.,* 2022).

Gram-negative bacteria cause approximately one third of DFIs, and anaerobes are found in a smaller minority of DFIs. Increased incidence and severity of bacterial infections in diabetes have been linked to impaired innate and adaptive immune responses within the hyperglycemic environment. There isa bidirectional relationship between diabetes and bacterial infections, while diabetes increases the susceptibility to bacterial infections and its complications (Gramberg *et al.,* 2022 and Nagendra *et al.,* 2022).

Aim of study:

In view of the aforementioned questions, this research is designed to compare between the levels of IL-37, IL-38 and IL-17A in (type1 and 2) diabetic mellitus patients with and without diabetic foot ulcers (DFUs), and a control group. Furthermore, investigation of aerobic bacterial etiology of

diabetic foot ulcers at species level using VITEK2 compact system as confirmative test for detection of pathogenic bacterial. These goals are achieved through the following objectives:

1. ELISA measurement of serum levels of IL-37, IL-38 & IL-17A indiabetic mellitus (type1 & 2) patients with and without diabetic foot ulcers complication and in control group as well as prediction of the possibility of using them as early biomarkers for diagnosis of diabetic mellitus complications & future prevention.

2. Investigation and isolation of aerobic bacterial etiology of diabetic foot ulcers patients at genus and species level using conventional and VITEK2 compact system techniques.

3. C-Reactive protein, lipid profile, HBA1C, white blood cells (lymphocytes and neutrophil), platelets and ESR for all diabetic patients.

4. Correlate levels of study markers between them and with etiological agents of diabetic complication.

1.2. Literature Review

1.2.1. Definition of Diabetes mellitus:

Diabetes mellitus is a collection of metabolic illnesses marked by an abnormal rise in blood glucose due to impaired insulin production, insulin action, or both (Seidu *et al.*, 2022). Diabetes damages, malfunctions, or fails several organs over time, most notably the eyes, kidneys, nerves, heart, and blood vessels. Several pathological conditions complicate the progression of diabetes, varying from autoimmune destruction of pancreatic β-cells, which results in insulin insufficiency, to anomalies that result in insulin resistance (Buse *et al.*, 2020).

The cause of diabetes-related abnormalities in carbohydrate, protein, and fat metabolism is insufficient insulin action on target tissues (Liu *et al.*, 2022).

Inadequate insulin action is caused by insufficient secretion of insulin and/or diminished or reduced tissue responses to insulin at one or more points along the complex hormonal action pathways (Adams *et al.*, 2021). Because abnormalities in insulin action or response frequently coexist, the lone cause of hyperglycemia may be unclear. Hyperglycemia is characterized by polyuria, polydipsia, and, in some cases, polyphagia, as well as weight loss and blurred vision. Hyperglycemia with ketoacidosis or nonketotic hyperosmolar syndrome are acute, life-threatening consequences of uncontrolled diabetes (Aldhaeefi *et al.*, 2022).

Long-term diabetes complications include retinopathy, which can cause vision loss; nephropathy, which can cause renal failure; peripheral neuropathy, which can cause foot ulcers, amputations, and Charcot's joints; and autonomic neuropathy, which can cause genitourinary, and cardiovascular symptoms, as well as sexual dysfunction (Bain & Bay, 2021). Diabetes increases the risk of developing atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular diseases. Diabetes patients may experience hypertension and changes in lipoprotein metabolism (Tian *et al.*, 2022).

1.2.2. Diabetes epidemiology

Diabetes mellitus has become one of the most pressing issues in recent decades, coinciding with the rising obesity crisis, and it is now the seventh leading cause of death worldwide, accounting for 5.2 million deaths and amortality rate of 82.4 per 100,000 people (Jiang *et al.*, 2022). Diabetes affects 366 million people worldwide, with half of those affected being unaware of theircondition. Six of the top ten diabetes-prevalence countries are in the Middle East(Kuwait, Lebanon, Qatar, Saudi Arabia, Bahrain, and the United Arab Emirates) (El-Kebbi *et al.*, 2021).

In the 20 Arab countries where data is available, nearly 20.5 million Arabs have diabetes, and another 13.7 million have prediabetes, or impaired glucose tolerance (Hamdy *et al.*, 2022). In developed countries, the majority of diabetics

are over the retirement age (Antal *et al.,* 2022). Nearly three-quarters (73.4 percent) of diabetics in Arab countries are under 60 years old and thus in their prime working years, adding to the burden of diabetes disability. According to recent study, diabetes is very common in Iraq, affecting one out of every five adults. A health problem of this magnitude would put a strain on health-care system's financial resources (SAl-Shaheeb *et al.,* 2022).

1.2.3. Types of Diabetes:

Diabetes is mainly divided into categories: type I, type II and gestational diabetes.

1.2.3.1. Type I diabetes

Type I diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by hyperglycemia due to insulin deficiency caused by the loss of pancreatic islet β-cells (Mathur *et al.,* 2022). The disease is most commonly diagnosed during childhood and adolescence, and it is characterized by a classic trio of symptoms (i.e., polydipsia, polyphagia, polyuria) along with a prominent hyperglycemia, positing the urgent need for exogenous insulin replacement therapy, a medicinal intervention that must be practiced for life (Mitsugashira *et al.,* 2022) who described several aspects that characterize type 1 diabetes mellitus as an autoimmune disease:

1. Presence of immune-competent and accessory cells in infiltrated pancreatic islets.

2. Association of susceptibility to develop the disease with the class II (immune response) genes of the major histocompatibility complex (MHC; human leucocyte antigens HLA).

3. Presence of unique autoantibodies to islet cell.

4. Alterations of T cell mediated immune-regulation, in especially in CD4+ T cell compartment.

5. The involvement of monokines and TH1 cells producing interleukins in the disease process.

6. Response to immunotherapy .

7. Incidence of other organ specific autoimmune diseases in the affected individuals or in their family members.

Monogenic diabetes is an unusual type of diabetes caused by a single gene mutation or change. Neonatal Diabetes Mellitus (NDM) and Maturity-Onset Diabetes of the Young (MODY) are the two most common types of monogenic diabetes. Diabetes that occurs before the age of 6 months is most likely NDM rather than autoimmune T1DM (Colclough *et al.,* 2022). T1DM is thought to be a chronic immune-mediated disease with a subclinical onset of variable duration. In genetically susceptible individuals, it is distinguished by the selective loss of insulin-producing β-cells in pancreatic islets. The most important genes that contribute to disease susceptibility are found on chromosome six , in addition to other ten genes or genetic regions that have been related to T1DM (Deutsch *et al.*, 2022).

Nonetheless, only a small percentage, less than 10%, of people with HLA-linked diabetes develop clinical disease. This indicates that other factors are required to initiate and drive β-cell eradication in genetically susceptible individuals. Clinical T1DM is end-stage insulitis, with only 10–20% of β-cells functional at the initial diagnosis (den Hollander & Roep, 2022). The process of β-cell degeneration can take more than three years before the disease manifests clinically, and its progression can occur in waves or cycles (Katsarou *et al.*, 2017 and Kaneto *et al.,* 2022).

Because of the scarcity of human pre-diabetic pancreas samples, little is known about the exact nature and kinetics of events occurring in humans during these early stages of T1DM, our understanding of the disease's natural progression is largely based on studies in nonhuman models. Because of the disease's complexity, it has been difficult to identify predictive factors,

environmental contributions, and immunological therapeutic targets (Nicolaus *et al*., 2022).

T cells with a high affinity for self-antigens are eliminated or their activity is controlled by different complementary mechanisms in a normally functioning immune system, resulting in immune 'tolerance.' Auto-reactive cells that have evaded these mechanisms are subject to regulation, which prevents clinicaldisease in the majority of people (Mitchell & Michels, 2022).

There are two types of regulation: intrinsic, which limits the size and duration of an immune response caused by a clone of lymphocytes, and 'extrinsic,' which results from the action of a specific subset of T cells known as regulatory T cells, or Tregs (Koranteng *et al.*, 2022). Many studies have implicated "immunological tolerance" defects in the onset and progression of autoimmune diseases, such as T1DM. Regulatory T cells have been well immunoregulators that can prevent effector cell proliferation (Schlöder *et al*., 2022).

Tregs are a type of T cell that expresses the transcription factor "forkhead box P3"(FOXP3) as suggested by (Khantakova *et al.,* 2022). According to epidemiological studies, adults with latent autoimmune diabetes (LADA) tally for 2–12% of all cases of diabetes. The existence of autoantibodies and islet- reactive T cells in LADA suggests that the disease is autoimmune (Hu *et al.*, 2022). LADA is assumed to be a type 1 diabetes subtype characterized by aslow autoimmune destruction of β-cells. It was observed that genomic DNA methylation was significantly higher in LADA patients' CD4+ T cells than in controls, and that the FOXP3 promoter region was hypermethylated in LADA patients' CD4+ T cells compared with controls (Zhang *et al.*, 2022).

Besides, it was discovered that FOXP3 expression was reduced in diabetic patients at the mRNA level (Goodwin *et al.*, 2022) .This regulation fails in T1DM, and Tregs are currently a significant subject of concern in the search for new therapies to prevent clinical disease (Goswami *et al.,* 2022). Although many

autoimmune disorders appear to target the female gender, T1DM appears to target both genders equally (Gottesman *et al.*, 2022). T1DM accounts for only about 10% of all diabetes cases worldwide, but it is becoming more common as people get older(Rittiphairoj *et al.*, 2022),.

Furthermore, while T1DM has typically been defined as a childhood disease, it has recently been recognized that the real number of new cases of T1DM diabetes in adults have been underreported (Wright, 2022). It seems that T1DM is more common in people who have no family history of the disease. Just 10–15% of patients have a first- or second-degree relative with the disease (Cerna, 2020). Figure (1.1) illustrate type 1 DM pathogenesis.

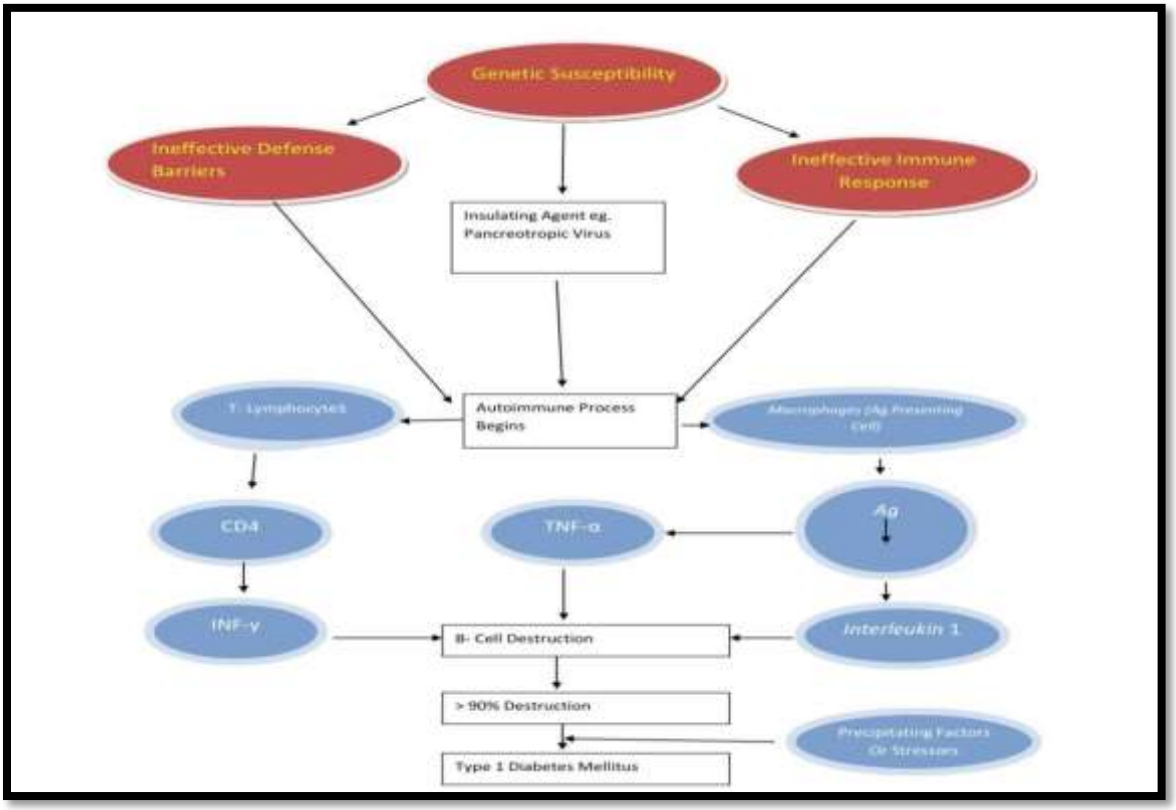


Figure (1.1): Pathogenesis of type 1 diabetes mellitus (Ozougwu, J. *et al*., 2013).

However, relatives of patients have a significantly higher lifetime risk of developing T1DM, with approximately 6% of children, 5% of siblings, and 50% of monozygotic twins having the disease, compared to a 0.4 percent prevalence in the general population. Environmental risk factors have long

been recognized as a critical trigger of β-cell destruction (Houeiss *et al.*, 2022). In early epidemiological studies, virus infections were mentioned as a possible cause of T1DM. The most likely candidate appears to be enteroviruses found in the pancreas of T1DM patients (Nekoua *et al.*, 2022).

1.2.3.2. Type II diabetes

Significant progress has been made in understanding the process involved in the pathogenesis of type 2 diabetes (T2DM). Obesity, aging, and inactivity are all associated with a decrease in insulin-stimulated glucose uptake "insulin resistance"(Lin *et al.*, 2022). Insulin resistance causes the pancreatic islets' cell mass and insulin secretory action to increase. However, if islet β-cell functional expansion is insufficient to compensate for the degree of insulin resistance, insulin deficiency and, ultimately, T2DM develop (Salib *et al.*, 2022).

The long-term consequences of T2DM, including macrovascular complications such as atherosclerosis and amputations, as well as associated complications such as retinopathy, nephropathy, and neuropathy (Ma *et al*., 2022).Chronic hyperglycemia and type II diabetes affect only about one-third of obese, insulin-resistant people. Although the causes of this heterogeneity are unknown, genetics and epigenetics are likely to play a role (Cefalu *et al.*, 2022).

The leading hypothesized mechanisms to explain insulin resistance and islet β-cell impairment in T2DM have been oxidative stress (Guo *et al*., 2022), endoplasmic reticulum stress (ER stress), Amyloid buildup in the pancreas, as well as ectopic lipid buildup in the muscle, liver, and pancreas, and lipotoxicity and glucotoxicity (Shrestha *et al.*, 2021).

Over-nutrition can cause all of these stresses, but it has been difficult to know which mechanism is most important in each tissue, model, or individual with T2DM (Pearson-Stuttard *et al.*, 2022). It is worth noting, however, that each of these cellular stresses is assumed to either induce or be aggravated by

or related to inflammation. Circulating inflammatory factors in obesity and T2DM provide evidence for T2DM being an inflammatory disease (Lee and Lee, 2022). In patients with T2DM, circulating levels of acute-phase proteins (such as C-reactive protein (CRP), haptoglobin, fibrinogen, plasminogen activator inhibitor, and serum amyloid A) and sialic acid, as well as cytokines and chemokines, are higher in cross-sectional and prospectivestudies(Khasanova *et al.*, 2022).

Most pro-inflammatory factors found in high concentrations in the blood of T2DM patients are IL-1 dependent, and inhibiting IL-1 activity has been shown to reduce their concentrations. Elevated levels of circulating IL-1β, IL- 6, and acute phase proteins in T2DM may reflect increasing nutrient concentrations activating innate immune cells; But these inflammatory markers may not necessarily reflect the degree of inflammation in individual tissues (Stanimirovic *et al.*, 2022). Variations in the inflammatory response that are genetically determined contribute to different susceptibility to establishing increased fat mass (FM), dyslipidemia, hypertension, and insulin resistance in healthy individuals (Fahed *et al.*, 2022).

Hyperglycemia promotes mitochondrial dysfunction and the formation of reactive oxygen species (ROS) in several tissues, including blood vessels and pancreatic beta cells, as diabetes progresses (Geest & Mishra, 2022). ROS damage to the mitochondria, as well as several macromolecules such as proteins, lipids, and nucleic acids, accelerates the aging process (Tirichen *et al.*,2021).

As a result, pancreatic β cells, which rely on functional mitochondria to maintain insulin synthesis, fail to produce enough insulin (Rungratanawanich *et al.*, 2021). Stress-responsive intracellular signaling molecules are activatedin the absence of compensatory mechanisms, resulting in cellular damage (Zuo *et al.*, 2022). Increased intracellular ROS levels and subsequent oxidative

stress play a significant role in the pro-atherosclerotic consequences of diabetes and the development of vascular complications (Pearson-Stuttard *et al.*, 2022).

Furthermore, glucose toxicity in several organs may occur as a result of chronic exposure of cells to high glucose levels in untreated T2DM patients (Sanches *et al.*, 2021). Nephropathy, cardiomyopathy, neuropathy, and retinopathy are all possible outcomes. Another important factor that can contribute to the onset and progression of metabolic diseases such as T2DM is dysbiosis of the gut microbiome (Pitocco *et al.*, 2020; Souptik *et al.*, 2022). The dysbiosis of the gut microbiome promotes insulin resistance in diabetic patients by altering the barrier functions of the intestine and the host's metabolic status (Thakur *et al.*, 2022). Diabetes also weakens the immune system, making patients more vulnerable to serious and long-term infections (Ching *et al.*, 2020).

1.2.3.3. Gestational diabetes

Since its first description in 1964, gestational diabetes mellitus (GDM), or glucose intolerance with first onset and recognition during pregnancy, has been a clinical entity fraught with controversy (Fu & Retnakaran, 2022). Whilethere has been an ongoing debate over the diagnostic criteria and the best protocol for screening and detection over the last half-century, there is universal agreement that GDM predicts the future risk of type 2 diabetes (Raz *et al*., 2022). Despite the fact that glucose tolerance typically returns to normal in the immediate postpartum period, women with GDM have a 20–70% chanceof developing type 2 diabetes within the first decade after delivery (Auvinen *et al.*, 2020). As a result, GDM is a well-established clinical predictor of future diabetes risk, with affected women having a more than sevenfold higher overallincidence of type 2 diabetes than their peers (Diaz-Santana *et al.*, 2022).

1.2.4. Tests for diagnosis of diabetes

The current protocol for diagnosis diabetes comprised of the following tests:

A. A glycated hemoglobin (HbA1C): a non-fasting blood test that determines the average blood sugar level over the previous two to three months. It calculates the amount of blood sugar that is attached to hemoglobin, the oxygen-carrying protein in red blood cells. The more sugar attached to hemoglobin, the higher the blood sugar levels. Diabetes is diagnosed when the A1C level is 6.5 percent or higher on two separate tests. An A1C of 5.7 to 6.4 percent indicates pre-diabetes. A value of less than 5.7 is considered normal (Ma *et al.*, 2022).

B. Random blood sugar: A blood sample will be drawn at random. A blood sugar level of 200 milligrams per deciliter (mg/dL) or 11.1 millimoles per liter (mmol/L) or higher, regardless of meal time, indicates diabetes (Genuth et al., 2015 and Gobena *et al.*, 2022).

C. Fasting blood sugar test: A blood sample will be taken after an overnight fast. A fasting blood sugar level of less than 100 mg/dL (5.6 mmol/L) is normal. A fasting blood sugar level from 100 to 125 mg/dL (5.6 to 6.9 mmol/L) is considered prediabetes. If it's 126 mg/dL (7 mmol/L) or higher on two separate tests, suggests diabetes (Goyal *et al*., 2020).

D. Oral glucose tolerance test: This test required fasting overnight, and the fasting blood sugar level is measured. Then glucose syrup is consumed, and blood sugar levels are tested periodically for the next two hours. A blood sugar level of less than 140 mg/dL (7.8 mmol/L) is normal. A reading of more than 200 mg/dL (11.1 mmol/L) after two hours indicates diabetes. A reading between 140 and 199 mg/dL (7.8 mmol/L and 11.0 mmol/L) indicates prediabetes (Chanda *et al*., 2020).

E. The most important differentiating tests to recognize between the two main types of diabetes are detection of the C-peptide levels and immune marker glutamic acid decarboxylase (anti-GAD65). C-peptide level below 5 µU/mL and

positive anti-GAD65 confirms the diagnosis of type-1 diabetes (Rao *et al.*, 2022).

F. Anti-Glutamic acid decarboxylase antibodies (Anti-GADA65): It is associated autoimmune conditions are increasingly diagnosed in outpatient clinics, it presents in 85-90% of individuals with a recent diagnosis of T1D.T1D is difficult to diagnose based on the mode of onset and requires the presence of anti-glutamic acid decarboxylase antibody (GADA) and/or islet cell antibodies (ICA). GAD is a major auto-antigen in the process leading to type 1 diabetes with both a clear cell-mediated immune response to GAD and auto-antibodies toGAD (GADA), which can be used as a predictor of diabetes. Administration of the isoform GAD65 can prevent autoimmune destruction of pancreatic beta cellsas confirmed in several studies (Couper *et al.*, 2018; Frommer & Kahaly, 2020).

1.2.5. Complications of diabetes:

1.2.5.1. Diabetic foot ulcers (DFU)

Diabetic foot ulcer is the most common complication of diabetes mellitus that usually fail to heal, and leads to lower limb amputation. Indeed, DFU can lead to infection, gangrene, amputation, and even death if necessary care is not provided. Overall, the rate of lower limb amputation in patients with DM is 15 times higher than patients without diabetes (Jiang *et al.*, 2022 and Tan *et al.*, 2022).

It is estimated that approximately 50%-70% of all lower limb amputations are due to DFU. The diabetic foot is associated with morbidity anddisability, leading to a significant impairment of quality of life. People with DM develop foot ulcers because of neuropathy (sensory, motor, and autonomic deficits), ischemia, or both (Asfonso *et al.*, 2021). The initiating injury may be from acute mechanical or thermal trauma or from repetitively or continuously applied mechanical stress. From foot ulcerations to neuropathy to peripheral vascular disease, the challenges are significant and can result in amputations and even premature death (Jan *et al.*, 2022).

1.2.5.2. Diabetic retinopathy

It is a chronic progressive retinal disorders, characterized by a wide spectrum of lesions, ranging from symptomless fine lesions to devastating lesions, leading to vision loss**.** It is the most prevalent cause of vision loss among diabetic patients, either of type-1 or type-2. Moreover, diabetic retinopathy reflects chronic uncontrolled hyperglycemia. Hyperglycemia of the retinal vasculature, even when controlled, remains the main metabolic event, intiating the pathophysiologic process to diabetic retinopathy (Das *et al.,* 2022 and Ansari *et al.,* 2022).

1.2.5.3. Diabetic nephropathy

It is a chronic progressive renal dysfunction, characterized by persistent albuminuria and progressive deterioration of the glomerular filteration rate (GFR). It is one of the leading causes of end stage renal failure world-wide, accounting for about 40% of prevalent renal failure. Similarly, about 40% (25%-45%) of diabetic patients develop nephropathy of various stages (de Sá *et al.,* 2022 and Yang *et al.,* 2022).

1.2.5.4. Cardiovascular complications

Cardiovascular disease (CVD) is the most serious complication of type-2 diabetes. More than half of the mortalities associated with type-2 diabetes are attributed to CVD. It has been reported that the diabetic patients are at an increased risk of acquiring CVDs compared to the general population (Mishra, 2022).

1.2.6. Diabetic Foot:

It is estimated that around 26 million people worldwide are affected by diabetes foot ulcers annually, importantly, not all diabetic patients are at a risk of diabetes foot ulcer (DFU) as there are many variables that contribute to them (Ertuğrul *et al.,* 2019).A diabetic foot is an infection, ulceration or destruction

of deep tissues associated with neurological abnormalities and various degrees of peripheral vascular diseases in the lower limb (Cardoso *et al.*, 2019).

The 50% of patients with diabetes foot ulcer suffer from Peripheral artery disease (PAD) which is occasionally caused by atherosclerosis, PAD is a risk factor in wound healing and lower limb amputations .Loss of protective sensation (LOPS), peripheral artery disease (PAD) and foot deformity combined with a history of foot ulceration and any level of lower extremity amputation are a major risk factor for DFU. The wound-healing process comprises complex biological mechanisms triggered by the injury, involving four key steps: (i) hemostasis; (ii) inflammation; (iii) proliferation and (iv) tissue remodeling factors affecting healing of diabetic foot ulcers that includes the location of ulcer, duration of diabetes, ulcer duration, the presence of heart failure and peripheral arterial disease (Ahluwalia *et al.*, 2021).

The ulcer can be superficial, i.e., a "full thickness lesion of the skin not penetrating any structure deeper than the dermis", or deep, i.e., "full thickness lesion of the skin penetrating below the dermis to subcutaneous structures involving fascia, muscle, tendon, or bone" (Bekele *et al.*, 2020).

Micro and macro-vascular anomalies play a role for the development of diabetic foot ulcers which can be neuropathic or ischemic. Compromised immunity, weak tissue reparative process and altered foot anatomy further deteriorate wound condition. Infection complicates the wound, slows its recovery and is significant cause of amputations (Eastman & Dreyer, 2022).

1.2.6.1. Epidemiology of Diabetic Foot:

Diabetes mellitus disease was considered as a global epidemic. Global prevalence of DFUs varies by region. In Iraq, the prevalence of diabetes mellitus is 9.3% in 2015 according to statistics published on International Diabetes Federation (Al Zahrani and Sehlo ,2013). Diabetic foot is one of the common complications of diabetes in developing countries including our country "Iraq".

Approximately two-thirds of the patients with diabetes have Diabetic Foot disorder (DFD) in Iraq and this health problem was associated with a serious social and financial impacts. A study by (Hamad *et al.*, 2021) in Babylon, Iraq, illustrated that incidence rate was increasing and more common among low educated females living in urban areas. As well as, in a study by (Alabbood & Marzoq, 2021) in Basrah, Iraq showed that DFD were strongly associated with long duration of diabetes and the female gender. The prevalence rates of DFU were highest in Saudi Arabia and Bahrain and lowest in Iraq in study by (Mairghani *et al.*, 2017). The reported annual incidence of diabetic footulceration varies between 2.1% to 7.4% and the lifetime risk of developing a diabetic foot ulcer has been estimated to be as high as 25% (Grennan, 2019).

The prevalence of DFUs among people with T2DM has been reported to be as high as 15 %. In addition, the prevalence of a DFU in the lifetime of a person with DM is estimated to be as high as 25% (Al-Mohaithef *et al.*, 2022). The overall prevalence is estimated to be 6.3% globally (95%), with the prevalence in North America reported to be 13.0%. In Asia, the prevalence is reported as 5.5 %, in Europe it is 5.1 %, in Africa it is 7.2 % and in the Oceania region it is 3.0 %. People who have DM for more than 10 years are also more likely to develop a DFU (Khunkaew, 2019 and Gong *et al.*, 2020). If not timely and properly managed, the ultimate endpoint of diabetic foot ulcer is amputationin 15% - 27% (Bafaraj, 2017). Furthermore, amputation is usually associated with significant morbidity mortality, in addition to social, psychological and financial consequences (Alzahrani & Sehlo, 2013).

1.2.6.2. Factors that contribute to Foot Ulceration in Diabetes:

The etiology of DFU involves combination of the patient's genetics, microbiome, and environmental factors that together affect the severity, response to treatment, and outcome of ulcers. Herein, the etiology of DFU involves both host-related disturbances and pathogen-related factors as in (figure1.3). Indeed, skin health is an interdependent relationship between the human

host and the skin microbiome (Tola *et al.*, 2021). In a healthy state, commensal microbes co-exist with their human hosts and protect the host from being colonized by pathogens. In the context of a skin injury, the host immune system triggers an inflammatory cascade to initiate the healing process and prevent pathogen invasion (Kaminski *et al.*, 2019 and Liu *et al.*, 2022).

Importantly, commensal microbes can be distinguished from pathogens based on pathogen-associated molecular patterns (PAMPs) or molecules that are associated with infectious agents in the affected tissue. In DM patients, the long-term hyperglycemia decreases elasticity of blood vessels and causes them to narrow (Pereira *et al.*, 2022).

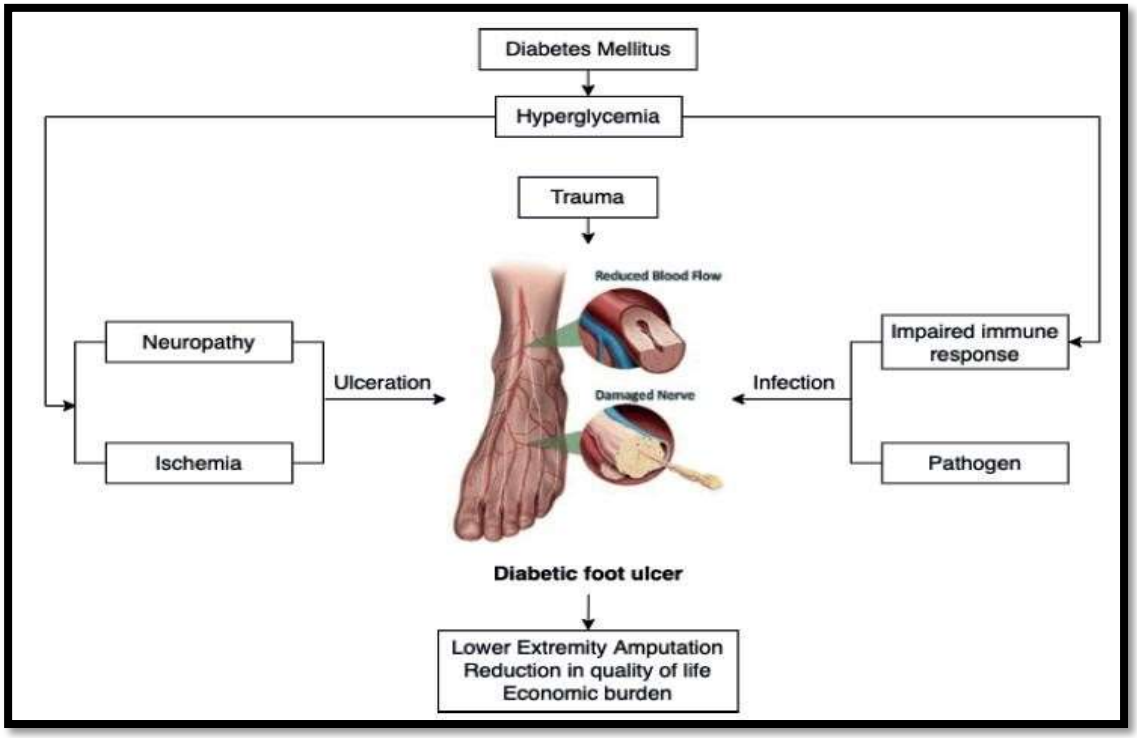


Figure (1.2): Illustrates of diabetic foot ulcers (Liu, 2022).

This poor blood flow, known as ischemia, can impair immune function and delay wound healing. At the same time, excess blood sugar damages the small blood vessels that supply the nerves resulting in nerve damage called neuropathy, which leads to a loss of feeling and may cause wounds to go undetected (Schilrreff & Alexiev, 2022). Hyperglycemia also contributes tochanges in immune system function such as increased inflammatory responses

and reduced bactericidal activity. The combination of peripheral neuropathy, ischemia, and hyperglycemia impedes the process of healing in DM patients by reducing the immune response and natural defense mechanisms (Choudhury *et al.*, 2021 and Giese *et al.*, 2021).

1.2.6.3. Classification of diabetes foot ulcers:

Diabetic foot ulcers are classified as either neuropathic, neuroischaemic or ischaemic (Turns, 2011). Doctors also use the Wagner Grades to describe the severity of an ulcer. The purpose of the Wagner Grades is to allow specialists to better monitor and treat diabetic foot ulcers. This grading system classifies Diabetic foot ulcers using numbers, from 0 to 5 as in table (1-2).

Table (1-1) Classification of diabetic foot ulcers suggested via Wagner (Wagner *et al*., 1983): -

Grade-0	No ulcer in a high-risk foot.
Grade - I	Superficial ulcer involving the full skin thickness but not underlying tissues.
Grade - II	Full-thickness ulcers, penetrating through fat to tendon, or joint capsule without causing a deep abscess or osteomyelitis.
Grade - III	Deep ulcer with abscess formation, often with osteomyelitis.
Grade - IV	Localized gangrene.
Grade - V	Extensive gangrene that involves the entire foot

1.2.7. Bacteriological Profile of Diabetic Foot Ulcer: Diabetic foot wounds are commonly infected due to aerobes, anaerobes, and hence infection leads to the formation of microthrombi causing further ischemia, necrosis, and progressive gangrene then amputation (Mudrik-Zohar *et al.*, 2022). The impaired microvascular circulation in patients with diabetic foot limits the access of phagocytes favoring development of infection. Several studies illustrated at present a diverse range of pathogens isolated from diabetic foot infections,

reflecting the chronic, open, nature and anatomical location of these infections. DFIs can be either mono- or polymicrobial, with polymicrobial being common among chronic infections that have undergone previous antibiotic treatment (Perez-Favila *et al.,* 2019).

A great diversity of pathogenic and non-pathogenic microorganisms live on the skin of humans. Generally, three to five species of different microorganisms are found in an infected DFU, including the following: gram- positive aerobes (*Staphylococcus aureus, Staphylococcus epidermidis, Corynebacterium spp.*); gram-positive anaerobes (*Enterococcus spp., Propionibacterium spp., Streptococcus spp., Peptostreptococcus spp., Peptococcus spp.*); gram negative aerobes (*Pseudomonas aeruginosa*, *Acinetobacter spp.*); gram negative anaerobes (*Proteus mirabilis, Escherichia coli, Bacteroides spp.*); and fungi (*Candida spp*.). In low-income countries, thereis higher prevalence of gram-negative pathogens, the most common of which is *Pseudomonas aeruginos*. Gram positive cocci, particularly *Staphylococci,* are frequently isolated and *Streptococcus* (Perez-Favila *et al.,* 2019 and Atlaw *et al.*,2022).

Deeper DFIs have been shown by some studies to be associated with the presence of anaerobic organisms. Patients with diabetes are susceptible to foot infection because of neuropathy, vascular insufficiency, and diminished neutrophil function. Peripheral neuropathy has a central role in the development of a foot infection and it occurs in about 30 to 50 percent of patients with diabetes (Macdonald *et al.,* 2021 and Jepsen *et al.,* 2022).

Moreover, bacteria frequently form biofilms that resist immune clearance and promote antimicrobial resistance; in one study 78.2% of chronic wounds showed evidence of biofilm production. A greater understanding of the microbiology of DFIs is important to help inform antimicrobial therapy and direct the development of novel therapeutics (Ghoreishi *et al.,* 2022).

1.2.8. Managements of diabetic foot ulcers/ infections:

Numerous studies have shown that proper management of DFU can greatly reduce, delay, or prevent complications such as infection, gangrene, amputation, and even death. The primary focus of DFU management is achieving wound healing. Management should consist of treating theunderlying disease process, achieving and maintaining adequate perfusion, wound care and infections control, and offloading. Identifying the underlying cause, correcting and eliminating it, is essential to effective management of DFU (Doğruel *et al.*, 2022).

Interventions for ischemia are the most essential to achieving wound healing and limb preservation. Patients with critical ischemia should have emergent consultation for interventions which will reestablish and maintain blood supply. In addition to glycemic control managing, other risk factors such as hypertension, high cholesterol, smoking and malnutrition are essential to keep DFU's on a healing trajectory. Identifying the cause of trauma or potential for repetitive trauma is important as well. This would alsoinclude assessing the patient's footwear for proper fit, possible foreign bodiessuch as rocks, pieces of glass, pins, needles, or pet dander which have the potential to induce trauma (Phipps & Cronin, 2020).

Optimal wound care is also essential and should include a focus on initial and serial debridement to keep the wound in an acute state free of debris and non-viable necrotic tissue, frequent assessment, and dressing interventions that aid in bacterial control and maintain a moist healing environment without the risk of maceration. Additionally, offloading of at risk and ulcerated areas ofthe foot aids in maximizing pressure redistribution across the plantar surface ofthe foot, thus helping maintain perfusion and aid healing (Armstrong, 2022).

Although there are multiple devices available to provide offloading, total contact casting is the gold standard for offloading DFU. This is a padded foot and lower leg cast with an opening over the DFU. The cast provides the offloading and the opening in the cast provides access to the wound for dressing changes. It also provides a method of ensuring patient compliance since it is very difficult to remove. Total contact casting has also been found to reduce healing time by as much as six weeks. Contraindications for totalcontact cast include infected DFU's and osteomyelitis, due to difficult observation, and those with critical ischemia due to the risk of increasing ischemia (Colodetti *et al.*, 2021).

1.2.9. Antimicrobial therapy of diabetic foot infections:

For the successful treatment of DFIs, the administration of antimicrobial agents alone is insufficient without accompanying proper wound care. Nevertheless, the choice of appropriate empirical antibiotics is important to reduce treatment failure, the likelihood of antimicrobial resistance, adverse events, and costs. Foot ulcers in diabetes require multidisciplinary assessment, usually by diabetes nurse specialist, a tissue viability nurse, podiatrists, diabetes specialists and surgeons. An aim to improve glycaemic control, forms part of the management, to slow disease progression (Ramirez-Acuña *et al.*, 2019 and Islamuddin *et al.*, 2022).

When osteomyelitis is suspected to be involved in the foot ulcer, but not evidenced on an x-ray, an MRI scan should be obtained. With regards to infected foot ulcers, the presence of microorganisms is not enough to determine whether an infection is present. Signs such as inflammation and purulence arethe best indicators of an active infection. The most common organism causing infection is staphylococcus.The treatment consists of debridement, wound management , managing peripheral arterial disease and appropriate use of antibiotics against (pseudomonas aeruginosa, staphylococcus, streptococcus and Klebsiella), and arterial revascularization (Albadri, 2021).

DFUs are often treated with broad-spectrum oral antibiotics while severe infections require parenteral treatment. The definitive treatment is modified according to the results obtained in the microbiological culture and the response of the empirical treatment. Its duration depends on the severity of the infection; for example, a mild infection could remain for 1–2 weeks, or 2–4weeks for a severe infection, or longer if there is osteomyelitis (Rodriguez- León *et al.*, 2021).

The recommended empiric antibiotic therapy according to severity is dicloxacillin, cephalexin, clindamycin, or amoxicillin/clavulanate for mild-moderate cases; vancomycin + ampicillin/sulbactam, moxifloxacin, cefoxitin, or cefotetan for moderate cases; and vancomycin + piperacillin/tazobactam, imipenem/cilastatin, meropenem, or doripenem for severe cases. Anti-microbial peptides are potent agents against a wide spectrum of pathogens, including viruses, fungi, and antibiotic-resistant bacteria, and have antitumor activity, which represents an alternative treatment to conventional antibiotic therapy (Sanz-Corbalán *et al.,* 2021).

Antibiotic resistance is an emerging problem globally so that antibiotic sensitivity tests of bacterial isolates from DFUs revealed a high resistance of commonly used antibiotics such as ampicillin, Augmentin, co-trimoxazole, penicillin, gentamycin, erythromycin and oxacillin (Sannathimmappa et *al.*, 2021). Current recommendations state that antibiotics are only used when there is evidence of infection and continued until there is evidence that the infection has cleared (Le Vavasseur & Zeller, 2022). Microbiological investigation is of value in cases of osteomyelitis; most ulcer infections involve multiple microorganisms (Lucidarme *et al.,* 2022).

1.2.10. Therapeutic Options:

The most common treatments for a foot ulcer are off-loading, medication, cleaning, debridement, and surgery if foot ulcer treatments have not been successful. With technological advancement, a number of biological healing products have been developed to aid in the healing process of DFUs. Tissue engineering represents a novel treatment for DFUs, including the use of dressings for wounds designed with living tissues to function as skin substitutes. These skin substitutes use living cells such as fibroblasts, keratinocytes, and stem cells, alone or in combination with extracellular matrices, and growth factors. Even though the use of these new therapies is expensive, they are supported by several clinical trials, encouraging their use, and representing potential new therapies for the future (Perez-Favila *et al.,* 2019 and Barakat, 2021).

1.2.11. Immunological Markers in Diabetic patients:

1.2.11.1. Interleukin-37 (IL-37)

IL-37 is also known as IL-1 family member 7 (IL-1F7), IL-1H4, and IL-1RP1. Subtypes of the IL-1 family and their different functions illustrates in figure (1.2). IL-37 is a novel anti-inflammatory cytokine with immunomodulatory effects. Specifically, it reduces the production of anti- inflammatory cytokines and thereby inhibits the inflammatory and immune responses by reducing the production of anti-inflammatory cytokines. IL-37 functions in three ways, i.e., by reducing the synthesis of pro-inflammatory cytokines, by lowering the expression of transcriptional cytokines, and by inhibiting the activation of kinase signaling (Brunt *et al.*, 2022 and Elsaid *et al.*, 2022). IL-37 is widely expressed in multiple human tissues and organs, including the skin, heart, kidney, gut, lymph node, thymus, bone marrow, lung, testis, placenta, and uterus (Jaramillo-Rangel *et al.*, 2021). However, the expression of distinct subtypes differs according to the specific tissues andorgans involved. Under physiological conditions, IL-37a is mostly found in the

lymph nodes, thymus, bone marrow, placenta, colon, lung, testicles, and brain, whereas IL-37b is mainly found in the peripheral blood, lymph nodes, placenta, colon, lung, testicles, and kidney. IL-37c is mostly expressed in the lymphnodes, placenta, colon, lung, testis, and heart, whereas IL-37d is predominantly expressed in the testis, bone marrow, blood system, umbilical cord tissue, and adipose tissue mesenchymal stem cells (Li *et al.*, 2022 and Zeng *et al.*, 2022).

The testicles and bone marrow are the primary sites of IL-37e expression. Cells from the aforementioned tissues may express IL-37 in a number of ways; for instance, monocytes, macrophages, B cells, plasma cells, endothelial cells, and skin keratinocytes are all capable of producing IL-37(Zhao*et al.*, 2020). IL-37 is expressed at low levels under physiological conditions, butcan be upregulated in response to inflammatory stimuli and pro-cytokines. For example, IL-37 is mainly produced by macrophages in response to Toll-like receptor (TLR) activation, and lipopolysaccharide (LPS) (Su & Tao, 2021).

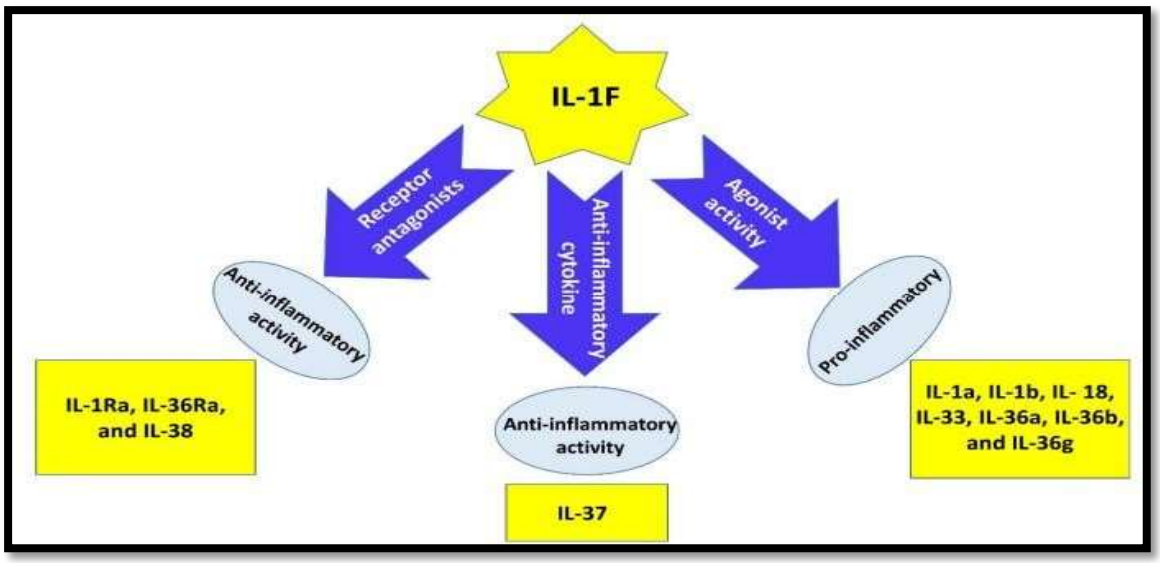


Figure (1.3): The IL-1 family subtypes and their functions depend on their receptor (Esmaeilzadeh *et al.*, 2021).

In different cells, such as peripheral blood mononuclear cells (PBMCs), dendritic cells (DCs), epithelial cells, endothelial cells, and T cells, IL-37 can be up-regulated by various pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-1β, transforming growth factor-β1 (TGF-β1; low concentrations), IL-4, and IL-6. IL-12, IL-32, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are known to limit IL-37 production (Zhu *et al.*, 2021 and Schröder *et al.,* 2022). In vivo evidence has shown that IL-37 can block the activity of Th1/Th2/Th17 cells via PBMCs, M1 macrophages, and DCs (Li *et al.,* 2019), while activating the function of Tregs (Osborne *et al.,* 2022).

IL-37 primarily reduces innate and acquired immune responses through intracellular and extracellular inhibition by reducing the secretion of pro-inflammatory chemokines (Majnarić *et al.,* 2022). IL-37 is a transcription factor that can be used to regulate gene expression in cells. Caspase-1 cooperates with the signal transduction protein Smad3 to regulate its transcription. The primary function of IL-37, an anti-inflammatory cytokine, is the secretion of proteins to the exterior of cells, which acts as ligands for a functional receptor structure on the target cell membrane via engaging the IL-18 receptor (Rafiei *et al.,* 2022 and Gritsenko *et al.,* 2022).

1.2.11.2. Interleukin 38 (IL-38)

Interleukin (IL)-38, a novel anti-inflammatory cytokine in the IL-1β family, has an important role in various autoimmune diseases. IL-38 is secreted by various immune cells, for instance, B cells. Notably, as a B cell product, the relative deficiency of IL-38 is related to increased systemic inflammation in metabolic diseases, cardiovascular, and aging (Iznardo *et al.,* 2022). Generally, IL-38 is expressed in human heart, thymus etc., but not in T cells in the tonsil. Additionally, IL-38 binds to the receptors via nuclear factor kappa-B (NF-κB), activating protein-1 (AP-1), and c-Jun N-terminal kinase (JNK) signaling

pathways to regulate the inflammatory cytokines generation (Manzanares-Meza *et al.*, 2022).

This data indicated that IL-38 might be related to autoimmune diseases. Furthermore, IL-38 may affect the mechanism of autoimmune diseases in regulating the balance of anti-inflammatory and pro-inflammatory. A study by Aravindhan *et al.*, (2022) showed significantly decreased serum levels of IL-38 in diabetes subjects. Another study by Liu *et al.*, (2020) showed that the IL-38 was highly expressed in the group sensitive to insulin therapy and thus associated with the increased insulin sensitive in children with type 2 DM and overexpressing of IL-38 could suppress the expression of IL-36, a pro- inflammatory factor, and also the diabetes development (Yu *et al.*, 2017). Immunobiological activities of IL-38 are shown in figure below (Esmaeilzadeh *et al.*, 2022 and Luo *et al.*, 2022).

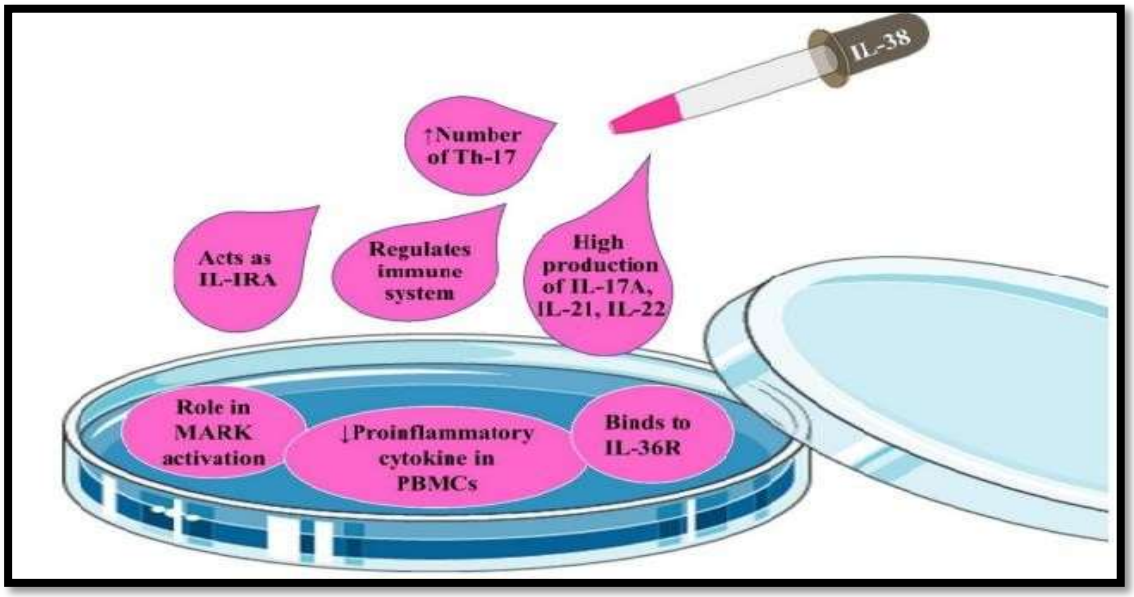


Figure (1.4): Immunobiological activities of IL-38 in host immune system (Esmaeilzadeh *et al.*, 2021).

It has been demonstrated that major functions of IL-38 are apoptosis-mediated phagocyte regulation, and inhibition of the induction of responses related to T Helper17 (TH17) cytokines (Karpisheh *et al.*, 2022). Eventually,

pre-inflammatory and anti-inflammatory effects of IL-38 were considered as dose-dependent immunological responses.

1.2.11.3. Interleukin-17A (IL-17A)

IL-17A is a newly identified inflammatory cytokine of the IL-17 family, which consist of six cytokines (IL-17A to IL-17F), including IL-17A and IL-17F that are the predominant isoforms (Ni *et al.*, 2022). IL-17A is produce byactivated and memory T lymphocytes such as TH17 cells. Other IL-17 producing cells, including PMNs, CD8 + T cell cells, macrophages and fibroblasts. However, in recent years have increased evidence that any defect in gene expression in cytokines (IL-17) may play a role in numerous inflammatory disorders (Fu *et al.*, 2022).

IL-17A has pleiotropic activities including the induction of diverse inflammatory cytokines (e.g. IL-6 and TNF-α) and chemokines (e.g. CCL2/MCP-1, CXCL1/KC, and CXCL2/MIP-2) from a large variety of cells. IL-17 acts as highly potent inflammatory cytokine that initiates tissueinflammation and induces the infiltration of other inflammatory cells into the target organs. Increasing evidence suggests that IL-17 plays a crucial role in various inflammatory responses and autoimmune diseases (Zwicky *et al.*, 2020 and Abdel-Naby *et al.*, 2022).

The pathogenicity of IL-17 has been well recognized in several diseases, including psoriasis, rheumatoid arthritis, multiple sclerosis, cancer and diabetes. A studies by (Ma *et al.*, 2019) showed elevated plasma IL-17 levels compared to healthy individual in patients with diabetic (Țiburcă *et al.*, 2022).

Another study showed the level of IL 17 in complicated diabetics that was higher than the patients with T2DM without complications. Multiple logistic regression analysis showed positive correlation of IL-17 with Diabetic Retinopathy and Diabetic Neuropathy (Parhi *et al.*, 2019).

Indian subjects with T2DM with or without complications had higher values of IL 17 as compared to healthy controls (Parhi *et al.,* 2019). Also diabetic neuropathy and diabetic retinopathy were positively correlated to levels of IL 17 (Zheng *et al.,* 2020). Recently, there has been increasing evidence of the proinflammatory interleukin 17 (IL 17) playing an important role in patients of diabetes mellitus and its complications (Lavos *et al.,* 2020).

IL-17A activates the JAK1, JAK2, PI3K and NF-κB pathways which up-regulate inflammatory gene expression. It also activates MAPK, CEBP cascade which increases proinflammatory gene expression of cytokines like TNFα, IL1, IL6, G-CSF, and MMP which mediate tissue infiltration and destruction. Also a recent study described ongoing β-cell destruction by IL1b and TNFα (Bai *et al.,* 2021 and Druszczyńska *et al.,* 2022). Also there have been studies regarding increased levels of IL 17 in vitreous humour, and increased Th 17 cells in the renal tissues indicating significant role of IL 17 in the complication of diabetes (Yuan *et al.,* 2022).

2.1. Study design & setting:

This is a case control study that was done at private centers in several provinces. All patients were registered in Diabetes centers in several provinces from August (2022) to January (2023).

2.2. Subjects Group:

One hundred ninety-three (193) participants were enrolled in this study including three groups involved in this case-control study according to clinical diagnosis by a clinician: the first one includes patients with type 1 diabetes mellitus [29 (17 male, 12 female) with diabetic foot ulcer + 35 (19 male, 16 female) Non-diabetic foot]. The second group includes type 2 diabetes mellitus patients [41 (25 male, 16 female) with diabetic foot ulcer + 38 (21 male, 17 female) Non-diabetic foot ulcer], and the third group includes [50 (29 male, 21 female)] healthy control group. All of the groups' ages range from 18 to 73 years as in figure (2.1). Detailed case information sheets involving age, gender, full history and other variables were carried out for each patient by a questionnaire as in (Appendix 1).

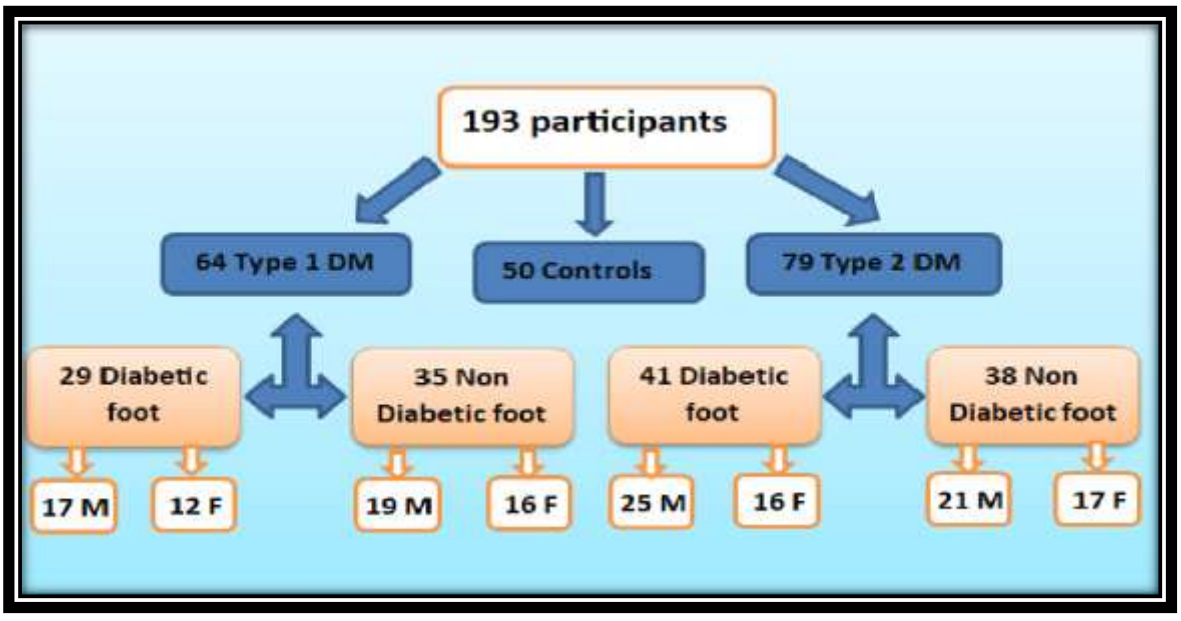


Figure (2.1): study design groups.

2.3. Bacterial Study Design:

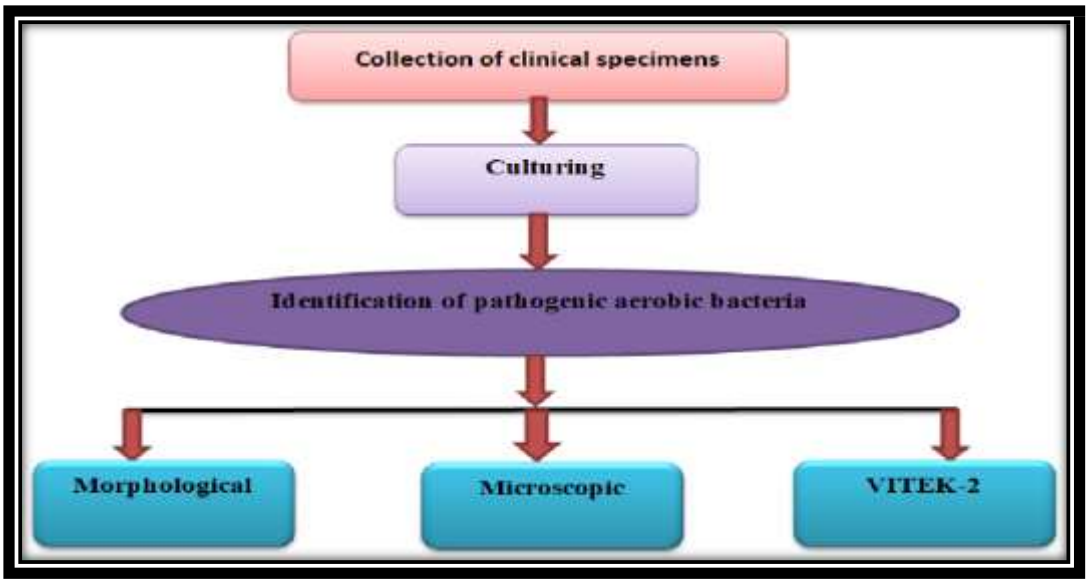


Figure (2.2): Bacterial study design.

2.4. Inclusion Criteria:

Every case with clinical diagnosis of type 1 and type 2 diabetes with and without Diabetic foot ulcers (DFUs) complications with their duration was included in the study. For the control group included, age (18 - 73) years and gender matching with the patient group, Fasting Blood Sugar (FBS) < 140 mg/dl.

2.5. Exclusion Criteria:

If a different diagnosis was documented include, pregnancy, patient with multiple autoimmune diseases and history of current inflammation &infection, patients with cardiovascular disease (CVD), patients taking immunosuppressive therapy, renal failure patients, liver failure patients and patients with age group below 18 years.

2.6. Designation of questionnaire for patients:

Those patients were registered at several diabetic centers and were questioned with a specially designed questionnaire. This questionnaire contains demographic data including age, gender, residency, Duration of DM, Family

history of diabetes, type of Rx whether oral, insulin or mixed, smoking Hx, Usage of other drugs and many exclusion criteria also listed in (appendix 1).

The complication of diabetes was also looked for in diabetic foot ulcers and their duration. Results of Fasting Blood sugar, lipid profiles, CRP, Hb and White Blood Cells with their differential including (Lymphocytes and Neutrophils) and platelets were collected from patients' records (Appendix 1).

2.7. Ethical Approval:

Patients involved in this study were informed about the detailed aim of study and the agreement was obtained verbally from each one before the collection of samples. This study was approved by the Scientific Council of Karbala Medical College.

2.8. Materials

2.8.1. Equipment and Instruments Utilized in the Study:

In the present study, the following equipment and instruments were used (table 2.1).

Table (2.1): Devices and Instruments.

Equipment & Instruments	Manufacturing Company	Origin
Autoclave	Hirayama HVE-50	Japan
Incubator	Memmert	Germany
Refrigerator	Panasonic	Korea
Para-film	Bemis	USA
Vortex	Clay Adams	Germany
Water distillatory	GFL	Germany
VITECK® 2 compact system	Bio merieux	France
Syringe 5 ml	Arrow	Egypt
Gel Tubes 6 ml	ALS	China

Eppendorf tube 0.5 ml	ALS	China
Centrifuge	Kokusan	Japan
Deep freezer	Hettich	Korea
Cool box	VB	China
Graduated glass cylinder	Supc orior	Germany
Light microscope	Olympus	Japan
Electric oven	Olympus	Japan
Burner	Amal	Turkey
Sensitive balance	Sartorius	Germany
Slides	Himedia	India
Swab media	Himedia	India
Flasks (different size)	Jlassco	India
Biological safety cabinet	EuroClone Safemate	Italy
Loop	Himedia	India
Para-film	Bemis	USA
Water bath	Polyscience	USA
Gloves	ALS	China
Cotton	BDH	England
Pipette tip	ALS	China
Micropipette set	SLAMED	Germany
Multichannel micropipette set	SLAMED	Germany
ELISA Devices (washer & reader)	Human	Germany
ELISA printer	Epson	Japan

2.8.2. Chemicals and Biological materials

The chemicals and biological materials are listed in table (2.2).

Table (2.2): Chemicals and biological materials which are used in the study.

Chemicals and biological materials	Company	Country of origin
Absolute alcohol	Bioneer	Korea
Normal saline (0.9 %)	choueifat	Lebanon
Gram's stain kit	Biolife	Italy
Glycerol	Biolife	Italy
Oil immersion	BDH	England

2.8.3. Culture media

The culture media used in the present study are in table (3-4).

Table (2.3): Culture media used in the current study.

Culture media	Company	Country of origin
Blood agar base (BAB)	Himedia	India
Brain heart infusion broth (BHIB)	Oxoid	England
MacConky agar	Oxoid	England

2.8.4. Commercial kits

The commercial kits used in the present study are in table (2.4).

Table (2.4): The Commercial kits which are used in the study.

Kits	Company
Human Interleukin 38 (Interleukin 37) ELISA Kit	BT LAB / China
Human IL-37 (Interleukin 37) ELISA Kit	Elabscience / USA
Human IL-17 (Interleukin 17) ELISA Kit	Elabscience / USA
ID (VITEK2) cards cassette	BioMerieux

Table (2.5): Reagents and Quantity (IL-38) ELISA Kits.

Components	Quantity
Standard Solution (240ng/L)	0.5ml x1
Pre-coated ELISA Plate	12 * 8 well strips x1
Standard Diluent	3ml x1
Streptavidin-HRP	6ml x1
Stop Solution	6ml x1
Substrate Solution A	6ml x1
Substrate Solution B	6ml x1
Wash Buffer Concentrate (25x)	20ml x1
Biotinylated Human IL-38 Antibody	1ml x1
User Instruction	1
Plate Sealer	2 pics
Zipper bag	1 pic

Table (2.6): Reagents of Human IL-37 ELISA Kits

Components	Storage
Micro ELISA Plate (Dismountable)	-20℃, 6 months
Reference Standard	
Concentrated Biotinylated Detection Ab (100×)	
Concentrated HRP Conjugate (100×)	-20℃(shading light), 6 months
Reference Standard & Sample Diluent	2-8℃, 6 months
Biotinylated Detection Ab Diluent	
HRP Conjugate Diluent	
Concentrated Wash Buffer (25×)	
Substrate Reagent	2-8℃(shading light)
Stop Solution	2-8℃
Plate Sealer	

Table (2.7): Reagents of Human IL-17 ELISA Kit

Components	Storage
Micro ELISA Plate (Dismountable)	-20℃, 6 months
Reference Standard	
Concentrated Biotinylated Detection Ab (100×)	
Concentrated HRP Conjugate (100×)	-20℃, 6 months
Reference Standard & Sample Diluent	4℃, 6 months
Biotinylated Detection Ab Diluent	
HRP Conjugate Diluent	
Concentrated Wash Buffer (25×)	
Substrate Reagent	4℃(Protect from light)
Stop Solution	4℃
Plate Sealer	

2.9. Method

2.9.1. Sample collection

1. Blood Sample collection

From each patient two syringes of five milliliters of venous blood using 5 ml disposable syringe was taken. The blood sample was immediately transformed into gel tube and left to clot for 15 minutes in room temperature (20-25) °C. Then, after collecting, it was centrifuged at (3000 rpm) for approximately (15) min period to obtain serum, the isolated serum of samples was distributed into 3 aliquots (0.5ml) in tightly closed Eppendorf tubes, and then the tubes were stored at -20 C until assayed for immunological markers testing by ELIZA.

2. Bacteriological sampling

After thorough wound cleansing by normal saline to remove surface contaminants, ulcers were scarped before sampling to get exudate; pus, or discharges from the base of the ulcer, under an aseptic technique the samples were collected from the deepest part of the ulcer using sterile swabs from diabetic's foot ulcers patients by passing over the wound area in circular or a zigzag motion while twisting the swab so that the entire head of the swab comes into contact with the wound surface and then was passed from the center of the wound outward to the edge of the wound. The swabs were sent to the laboratory as soon as possible. These samples were inoculated on different culture media including brain heart infusion broth as enrichment media, MacConkey and Blood agar then incubated aerobically at 37°C for 24 hours for isolation of aerobic bacteria (Bhatia, 2008) after that the plates were examined for growth in the next day and then pure colony for all bacteria types was prepared. Isolated bacteria were identified according to morphological, microscopic characteristics and specific cards of the automated VITEK2 system.

2.9.2. Sterilization Methods

A. Sterilization of the culture media used in the present study by autoclave at 121C^{o} for 15 minutes.

B. Sterilization of the glass wares are done by dry heat in an electric oven at 180 C^{o} for 2 h.

2.9.3. Media Preparation

The culture media that used in this study, which are listed in table (3-4), were prepared according to the manufacturer's instructions and then autoclavedsterile at 121℃ for 15 minutes. After sterilization, blood agar base was supplemented with 5% blood, and the media was poured into a petri dish. Teh eniinhiniah medium was prepared by using brain heart infusion broth media and then adding 15% glycerol, and all media were incubated at 37C^{o} for 24

hours to ensure sterility before being ready for culture and storage in the refrigerator (Brown and Smith, 2017).

2.9.4. Preparation of Solutions and Reagent

A. Gram stains solutions:

The solutions were prepared according to the required microbiological methods. The solutions included four solutions crystal violate, iodine, absolute alcohol and safranine (Colle *et al*., 1996; (Leboffe and Pierce, 2012).

B. Blood agar medium:

Blood agar medium performed according to the manufacturer by dissolving 40 gm blood agar base in 1000 ml D.W. The medium was autoclaved at 121ºC for 20 minutes, cold to 45ºC and 5% of fresh human bloodwas added. It was used as an enrichment medium for the bacterial isolates and to determine their ability to hemolysis RBCs (MacFadden, 2000).

C. Brain heart infusion (BHI) broth:

Brain-heart infusion broth was made according to the manufacturing company by dissolving 37 gm in 1 liter of distilled water and autoclaved at 121ºCfor 20 minutes, used for bacterial reactivation if necessary (MacFadden, 2000).

D. MacConky agar medium:

This medium can be prepared by dissolving 40gm of agar in 1000 ml of D.W and then sterilizating in autoclave at 121C°for 20 minutes. After cooling, it was poured to the plates, this type of media used selective gram-negative media (MacFadden, 2000).

2.10. Bacterial Profile identification

A. Morphological Tests

Colonial characteristics were tested such as the shape of the colonies, size, color, borders, and texture of colonies.

B. Microscopic Characteristics

Bacteria were examined by the light microscope after being stained with Gram stain. A small amount from a colony of the bacterium was spread with a drop of normal saline on a clean slide, fixed by flashing it on the flame and smeared with crystal violet, treated with Iodine, decolorized with alcohol and counterstained with safranine finally examined under oil immersion (Tille, 2017).

C. Identification by using automated methods [VITEK2] system:

Automated methods are the quickest and most accurate bacterial identification methods. The VITEK2 system consists of plastic reagent cards consisting of microliter quantitative of several biochemical test media in 30 wells to supply a biochemical profile that is used for organism diagnosis (Maina & Kagotho, 2014). An inoculum taken from cultured samples isautomatically transferred into the card, and a photometer intermittently measures the color altered in the card that outcome from the metabolic activity of the microbe. The data were analyzed, stored, and printed in a computerized data base; several types of cards are available, including those for Gram-negative identification GN, and Gram-positive identification GP (Maina & Kagotho, 2014). The VITEK 2 compact system steps mentioned in (appendix 7).

2.11. Maintenance of bacterial isolates:

The bacterial isolates' maintenance was done as the following:

A. Short-term storage

The pure bacterial isolates were maintained for a few months in screw-capped universal tubes containing brain heart infusion agar slant, and incubated at 37ºC for 24 hours. The slants were tightly wrapped with Parafilm and then kept at 4ºC for three months (Benson, 2002. (

B. Long-term storage

A brain heart infusion broth was inoculated by a loop of overnight pure bacterial culture and incubated at 37°C after 18 hours. Glycerol was added to inoculate in the final concentration (20%) and stored at -20°C for 2-8 months (Green, 2015).

2.12. Biomarkers Profile Assay by ELISA Technique:

The serum level of interleukin **38**, interleukin **37** & interleukin **17A** were determined by classic sandwich-ELISA using ELISA research kits.

2.12.1. Determination of the level of Human Interleukin 38 (Cat. No E3276Hu):

2.12.1.1. Principle of Sandwich ELISA technique:

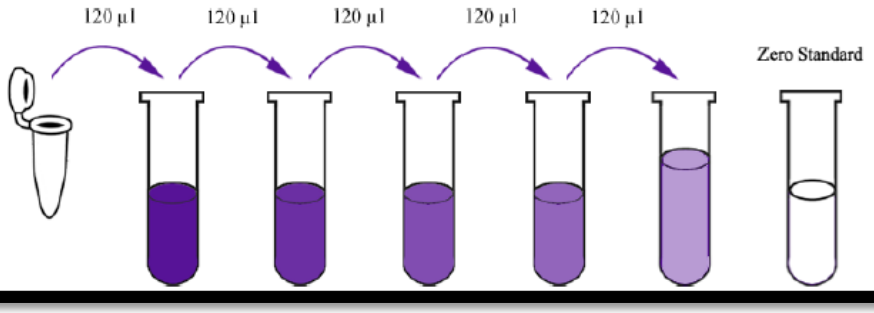
This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with a Human IL-38 antibody. IL-38 present in the sample is added and binds to antibodies coated on the wells. There, biotinylated Human IL-38 Antibody is added and binds to IL-38 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL-38 antibody. After incubation unbound Streptavidin-HRP has washed away during a washing step. A substrate solution is then added and color developed in proportion to the amount of Human IL-38. The reaction is terminated by the addition of acidic stop solution and absorbance is measured at 450 nm.

2.12.1.2. Reagent Preparation

1. All reagents brought to room temperature before use.

2. Standard reconstituted the 120µl of the standard (240ng/L) with 120µl of standard diluent to generate a 120ng/L standard stock solution. Allowed the standard to site for 15 minutes with gentle agitation prior to making dilutions. Prepared duplicated standard points by serially diluting the standard stock solution (120ng/L) 1:2 with standard diluent to produce 60ng/L, 30ng/L, 15ng/L and 7.5ng/L solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution had frozen at -20°C and used within onemonth. Dilution of standard solutions suggested are as the following:

120ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
60ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
30ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
15ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
7.5ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
240ng/L	120ng/L	60ng/L	30ng/L	15ng/L	7.5ng/L

Figure (2.3): Concentration of standards of IL-38.

Wash Buffer: Diluted 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. Where noticed if the crystals had formed in the concentrate, mixed gently until the crystals had completely dissolved.

2.12.1.3. Assay Procedure:

1. Prepared all reagents, standard solutions, and samples as instructed. Brought all reagents to room temperature before use. The assay was performed at room temperature.

2. Determined the number of strips required for the assay. Inserted the strips in the frames for using. The unused strips were stored at 2-8°C.

3. We added 50µl standard to the standard well. Note: Don't addition antibodies to the standard well because the standard solution contains a biotinylated antibody.

4. Addition of 40µl sample to sample wells and then we added 10µl anti-IL-38 antibody to sample wells, then added 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mixed well and covered the plate with a sealer. Incubated 60 minutes at 37°C.

5. We removed the sealer and washed the plate 5 times with washing buffer. Soaked wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each washing. For automated washing, aspirate or decant each well and washed 5 times with wash buffer. Blotted the plate onto paper towels or other absorbent material.

6. 50µl substrate solution was added to each well and then adds 50µl substrate solution B to each well. Incubated plate covered with a new sealer for 10 minutes at 37°C in the dark condition.

7. We added 50µl Stop Solution to each well, the blue color would change into yellow immediately.

8. Determined the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after addition of the stop solution.

2.12.1.4. Calculation of Result

Construction of the standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X)

axis and draw a best fit curve through the points on the graph. These calculations could be best performed with computer-based curve-fitting software and the best fit line could be determined by regression analysis. The standard curve of IL-38 in (appendix 2).

2.12.2. Determination the level of Human IL-37(Interleukin 37) (Cat. No: E-EL-H2571):

2.12.2.1. Principle of Sandwich ELISA technique:

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human IL-37. Samples (or Standards) are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human IL-37 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away.

The substrate solution is added to each well. Only those wells that contain Human IL-37, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human IL-37. You can calculate the concentration of Human IL-37 in the samples by comparing the OD of the samples to the standard curve.

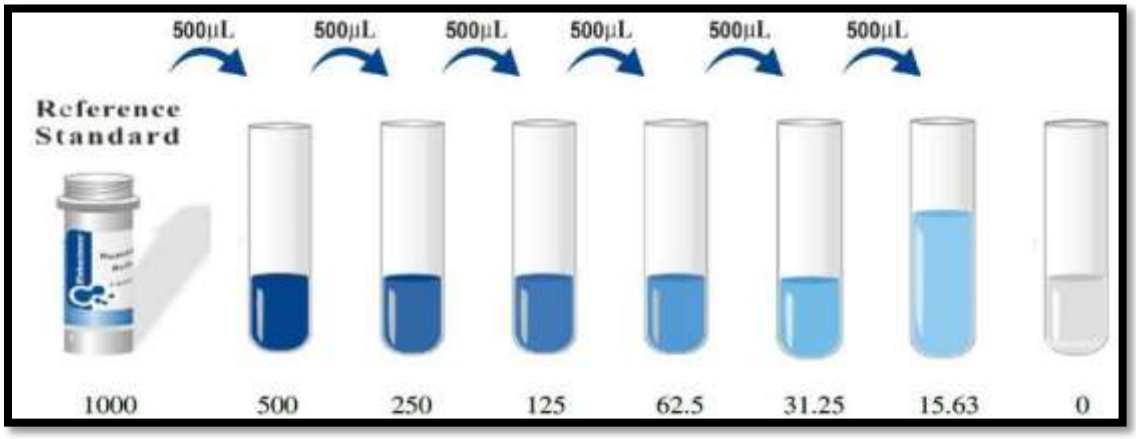
2.12.2.2. Reagent preparation

1. Brought all reagents to room temperature (18-25℃) before use. When the kit would not be used up in one assay, taken out the necessary strips and reagents for present experiment, and stored the remaining strips and reagents at required condition.

2. Wash Buffer: Diluted 30 mL of concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: when crystals had formed in the concentrate, warming it in a 40℃ waterbath and mixed gently until the crystals had completely dissolved.

3. Standard working solution: We centrifuged the standard at 10,000×g for 1 min. Addition 1.0 mL of Reference Standard and Sample Diluent, let it stand for 10 min and invert it gently several times. After it is dissolved fully,mixed it thoroughly with a pipette. This reconstitution produces a working solution of 1000 pg/mL(or add 1 mL of Reference Standard and Sample Diluent, let it stand for 1-2 min and then mixed it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then made serial dilutions as needed. The recommended dilution gradient is as the following: 1000, 500, 250, 125,62.5, 31.25, 15.63, 0 pg/mL.

Dilution method: Taken 7 EP tubes, and added 500uL of Reference Standard & Sample Diluent to each tube. Pipetted 500uL of the 1000 pg/mL working solution to the first tube and mixed it up to produce a 500 pg/mL working solution. Pipetting 500uL of the solution from the former tube into the latter one according to this step. The illustration below is for reference.



4. Biotinylated Detection Ab working solution: Calculated the required amount before the experiment (100μL/well). In preparation, slightly more than

calculated was prepared. Centrifuged the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then diluted the 100× Concentrated Biotinylated Detection Ab to 1× working solution with Biotinylated Detection Ab Diluent (Concentrated Biotinylated Detection Ab: Biotinylated Detection Ab Diluent= 1:99).

5. Concentrated HRP Conjugate working solution: Calculation the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated was prepared. Centrifuged the Concentrated HRP Conjugate at 800×g for 1 min, then diluted the 100× Concentrated HRP Conjugate to 1× working solution with HRP Conjugate Diluent(Concentrated HRP Conjugate: HRP Conjugate Diluent= 1: 99).

2.12.2.3. Assay procedure

1. Determined wells for **diluted standard, blank** and **sample**. We add 100 μL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate). Covered the plate with the sealer provided in the kit. Incubated for 90 min at 37℃. Note: solutions were added to the bottom of the micro ELISA plate well, avoided touching the inside wall and causing foaming as much as possible.

2. Decanted the liquid from each well, did not wash. Immediately added 100 μL of **Biotinylated Detection Ab working solution** to each well. Covering theplate with a new sealer. Incubated for 1 hour at 37°C.

3. Decanted the solution from each well, add 350 μL of **wash buffer** to each well. Soaked for 1 min and aspirated the solution from each well and pat it dry against cleaned absorbent paper. This wash Repeated step 3 times. Note: a microplate washer could be used in this step and other wash steps. Made the tested strips in use immediately after washing step. Did not allow wells to be dry.

4. Added 100 µL of **HRP Conjugate working solution** to each well. Covered the plate with a new sealer. Incubated for 30 min at 37°C.

5. Decanted the solution from each well, the wash process repeated for 5 times as conducted in step 3.

6. Addition 90 µL of **Substrate Reagent** to each well. Covered the plate with a new sealer. Incubated for about 15 min at 37°C. Protecting the plate from light. Note: the reaction time could be shortened or extended according to the actual color changing, but not more than 30 min. Pre-heated the Microplate Reader for about 15 min before OD measurement.

7. We added 50 µL of **Stop Solution** to each well. Note: adding the stop solution was done in the same order as the substrate solution.

8. Determination of the optical density (OD value) of each well was at once with a micro-plate reader set to 450 nm.

2.12.2.4. Calculation of results

Average the duplicated readings for each standard and samples, then subtract the average zero standard optical density. Plotted a four parameter logistic curve on log-log axis, with standard concentration on the x-axis and OD values on the y axis. If the OD of the sample surpasses the upper limit of the standard curve re-tested it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor. The standard curve of IL-37 in (appendix 2).

2.12.3. Evaluation the level of Human Interleukin 17A (IL-17A): (Catalog No: E-EL-H0105):

2.12.3.1. Principle of Sandwich ELISA technique:

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to HumanIL-17.Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then, a biotinylated detection antibody specific for HumanIL-17and Avidin-Horseradish Peroxidase (HRP)

conjugate are added successively to each micro plate well and incubated. Free components are washed away.

The substrate solution is added to each well. Only those wells that contain HumanIL-17, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of HumanIL-17. You can calculate the concentration of HumanIL-17in the samples by comparing the OD of the samples to the standard curve.

2.12.3.2. Reagent preparation

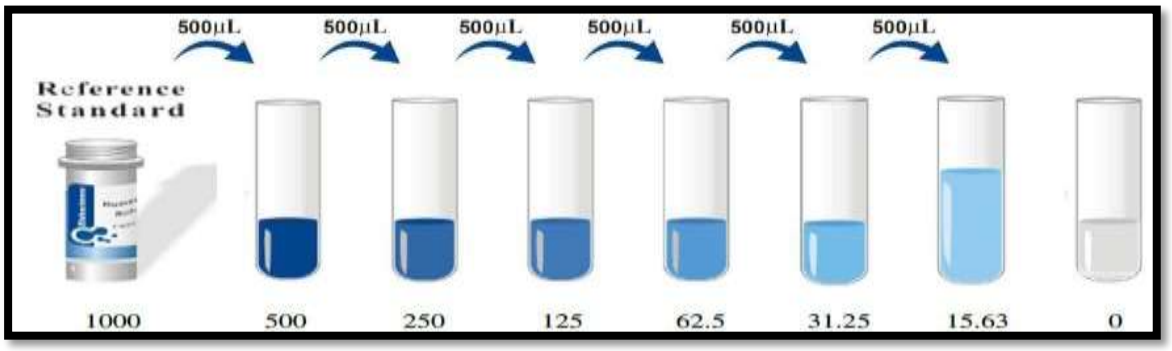
1. Brought all reagents to room temperature (18~25℃) before use. Followed the Microplate reader manual for set-up and preheat it for 15 min before OD measurement.

2. Wash Buffer: Diluted 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to preparing 750 mL of Wash Buffer. Note: when crystals had formed in the concentrate, warmed it in a 40℃ water bath and mixed gently until the crystals had completely dissolved.

3. Standard working solution: Centrifuged the standard at 10,000×g for 1 min. Added 1.0 mL of Reference Standard &Sample Diluent, let it stand for 10 min. and inverted gently several times. After that dissolved fully, then mixed thoroughly with a pipette. This reconstitution produced a working solution of 2000pg/mL then, make serial dilutions as needed. The recommended dilution gradient is as the following: 2000、1000、500、250 125、62.5、31.25、0 pg/mL.

Dilution method: 7 EP tubes was taken, add 500uL of Reference Standard & Sample Diluent to each tube. Pipetted 500uL of the 2000pg/mL working solution to the first tube and mixed up to produce a 1000pg/mL working

solution. Pipetted 500uL of the solution from the former tube into the latter one according to this step. The illustration below is for reference.



4. Biotinylated Detection Ab working solution: Calculated the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated was prepared. Centrifuged the stock tube before use, diluted the 100× Concentrated Biotinylated Detection Ab to 1×working solution with Biotinylated Detection Ab Diluent.

5. Concentrated HRP Conjugate working solution: Calculated the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated was prepared. Diluted the 100× Concentrated HRP Conjugate to 1× working solution with Concentrated HRP Conjugated Diluent.

2.12.3.3. Assay procedure

1. Added the **Standard working solution** to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (100 uL for each well). Added the samples to the other wells (100 uL for each well). Covered the plate with the sealer provided in the kit. Incubated for 90 min at 37°C. (Note: solutions were add to the bottom of the micro ELISA plate well, avoided touching the inside wall and causing foaming as much as possible.

2. Removed the liquid out of each well, did not wash. Immediately added 100 μL of **Biotinylated Detection Ab working solution** to each well. Covered with the Plate sealer. Gently mixed up. Incubated for 1 hour at 37°C.

3. Aspirated the solution from each well added 350 uL of **wash buffer** to each well. Soaked for 1~2 min and aspirated the solution from each well and pat it dry against clean absorbent paper. Repeated this wash step 3 times. Note: a microplate washer could be used in this step and other wash steps.

4. We added 100 μL of **HRP Conjugate working solution** to each well. Covered with the Plate sealer. Incubated for 30 min at 37°C.

5. Aspirated the solution from each well, repeating the wash process for five times as conducted in step 3.

6. Addition 90 μL of **Substrate Reagent** to each well. Covered with a new plate sealer. Incubated for about 15 min at 37°C. Protected the plate from light. Note: the reaction time could be shortened or extended according to the actual color change, but not more than 30min.

7. Addition 50 μL of **Stop Solution** to each well. Note: addition the stop solution was done in the same order as the substrate solution.

8. Determined the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

2.12.3.4. Calculation of results

Average duplicated readings for each standard and samples, then subtract the average zero standard optical density. Plotted a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis andOD values on the y-axis. If the samples had been diluted, the concentration calculated from the standard curve and multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor. The standard curve of IL-17A as in (appendix 2).

2.13. Statistical analysis:

Data of diabetic patients and controls were checked, managed and analyzed using the Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA) and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020(. Descriptive statistics was presented as mean, standard deviation, frequencies, and percentages according to the variable types. All variables of scale (continuous) type were checked as numerical means of assessing normality by the Shapiro-Wilk test. The distribution of the data was checked using $\chi 2$-test, T test to compare between groups. As well as, we used the ANOVA test to compare more than 2 groups.

Biomarkers were compared using Pearson's r correlation test to evaluate the relationship within the case study.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p- values <0.05 (two-side) were considered to be statistically significant.

The optimal threshold with high specificity and sensitivity for diabetic cases was detected using receiver operating characteristic (ROC) analysis. It was found out that all the values of P were two-sided, and a $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Demographic characteristics of study groups: Baseline characteristics of the studied groups are shown in (table 3.1).

Table (3.1): Baseline Characteristics of Study Participants.

		Group										P. value*
		T1DM (N=64)				T2DM (N=79)				Control (N=50)		
		DFU		Non		DFU		Non				
		No	%	No	%	No	%	No	%	No	%	
Age (year)	< 20	0	0.00	13	37.14	0	0.00	1	2.63	14	28	0.009 Sig.
	20 - 29	3	10.34	9	25.71	1	2.44	3	7.89	20	40	
	30 - 39	6	20.69	10	28.57	3	7.32	4	10.53	11	22	
	40 - 49	8	27.59	2	5.72	5	12.20	7	18.43	5	10	
	50 - 59	12	41.38	1	2.86	19	46.33	15	39.47	0	0.00	
	≥ 60	0	0.00	0	0.00	13	31.71	8	21.05	0	0.00	
	Total	29	100	35	100	41	100	38	100	50	100	
Gender	Male	17	58.62	19	54.29	25	60.98	21	55.26	25	50	1.57 ns.
	Female	12	41.38	16	45.71	16	39.02	17	44.74	25	50	
	Total	29	100	35	100	41	100	38	100	50	100	
DM duration	0-10 years	4	13.79	17	48.57	5	12.19	20	52.63	0	0.00	0.01 Sig.
	11-20 years	9	31.03	9	25.72	11	26.83	11	28.95	0	0.00	
	21-30 years	13	44.84	6	17.14	21	51.22	3	7.89	0	0.00	
	Missing	3	10.34	3	8.57	4	9.76	4	10.53	0	0.00	
	Total	29	100	35	100	41	100	38	100	50	100	
Family history of DM	Positive	17	58.62	22	62.86	20	48.78	19	50.00	6	12.0	2.09 ns.
	Negative	9	31.04	10	28.57	17	41.46	15	39.47	44	88.0	
	Missing	3	10.34	3	8.57	4	9.76	4	10.53			
	Total	29	100	35	100	41	100	38	100	50	100	
DFU duration	>30 days	17	58.62	0	0.00	22	53.66	0	0.00	0	0.00	1.03 ns.
	≤ 30 days	12	41.38	0	0.00	19	46.34	0	0.00	0	0.00	
	Total	29	100	35	100	41	100	38	100	50	100	

*Chi square used in comparison, sig: significant, ns; not significant P. value (≤ 0.05), (≤ 0.01).

In the current study there were highly significant differences (P<0.01) between studied groups according to the age when compared among them and with the control group. There were non-significant differences according to the gender when compared between the studied groups. However, according to duration of DM there were highly significant differences (p < 0.01) when compared among duration level between both patient's groups and with control group. In relation to the family history of DM patients, the results of study showed that there were non-significant differences between the patients group and control group. Likewise, the results of this study showed that there were non-significant differences in relation to DFU duration for the patient's groups and control group.

3.2. Laboratory parameters of the studied groups:

3.2.1. Biochemical Parameters of study groups:

Table (3.2) illustrated that DFU group of T1DM showed highly significant increase (p<0.01) in fasting blood sugar when compared with other groups, whereas least value was in Non-group of T2DM.

Table (3.2): Biochemical Properties of Diabetic groups.

Parameter	Group				P. value	Normal Value
	T1DM (N=64)		T2DM (N=79)			
	DFU(N=29)	Non(N=35)	DFU(N=41)	Non(N=38)		
	Mean± SD	Mean± SD	Mean± SD	Mean± SD		
FBS(mg/dL)	283.7± 26.2^{a}	243.7±18.2^{b}	213.9± 16.8^{c}	197.6± 17.2^{d}	**0.008 Sig.**	65-125
HbA1c%	11.7± 2.9^{b}	10.1± 1.8^{c}	12.1±2.2^{a}	9.23± 1.9^{d}	**0.01 Sig.**	4.5-6.4
Cholesterol (mg/dL)	197.5± 18.8^{b}	167.9±17.6^{d}	213.3±14.1^{a}	186.7± 16.3^{c}	**0.009 Sig.**	< 200
HDL(mg/dL)	51.2± 11.1^{b}	43.8± 7.1^{d}	61.5± 12.4^{a}	47.2± 7.3^{c}	**0.01 Sig.**	35-60
LDL(mg/dL)	88.6±14.8^{b}	82.8±9.4^{c}	133.5±16.3^{a}	87.9± 19.7^{b}	**0.01 Sig.**	< 120
Triglycerides (mg/dL)	211.1±13.9^{b}	199.4±19.1^{c}	232.6±23.2^{a}	214.8±28.1^{b}	**0.01 Sig.**	< 203
Welch's t-test used in comparison, sig : significant, P≤ 0.01.						

On the other hand, HbA1c levels and Cholesterol were significantly higher ($p<0.01$) in DFU group of T2DM when compared with other study groups, while lowest value was in Non-group of T2DM. The same, HDL and LDL and Triglycerides levels were highly elevated ($p<0.01$) in DFU group of T2DM whilst the lowest level was in Non-group of T1DM. Generally, in comparisons among all groups of diabetic patients the results showed significantly higher differences ($p<0.01$) as illustrated in the table above.

3.2.2. Inflammatory and Hematological parameters of studied groups:

As revealed in (table 3.3), T1DM and T2DM had higher mean CRP, Hb, PLT, WBC, lymphocytes, neutrophils and ESR levels according to DFU than Non-DFU groups. On the other hand, the results showed highly significant differences ($p <0.01$) according to PLT count, as well as a significant differences ($P<0.05$) in relation to CRP, lymphocytes, neutrophils and ESR levels when compared between T1DM and T2DM groups.

Table (3.3): Inflammatory and Hematological Parameters among groups.

Parameter	Group				P. value	Normal Value
	T1DM (N=64)		T2DM (N=79)			
	DFU(N=29)	Non(N=35)	DFU(N=41)	Non(N=38)		
	Mean± SD	Mean± SD	Mean± SD	Mean± SD		
CRP (mg\L)	18.2± 2.1^{b}	15.4±3.9^{d}	20.3±3.1^{a}	17.5±4.6^{c}	**0.045 sig.**	8-10
Hb (g\dL)	15.3±4.3	15.6± 2.4	14.7±3.7	15.1±4.6	**0.71 ns.**	Male: 13.8-17.2 Female: 12.1-15.1
PLT× 10^3/µl	378.9 ±38.2^{a}	289.6± 36.4^{c}	301.5± 18.2^{b}	267.3± 24.2^{d}	**0.009 sig.**	100-400
WBC ×10^3/µl	12.9±3.5	9.3±1.7	13.7±3.1	8.3±1.9	**0.37 ns.**	4.5-11.0
Lymph ×10^3/µl	4.8±0.97^{b}	3.7±1.13^{c}	5.3±0.92^{a}	4.2±0.87^{b}	**0.047 sig.**	1-4
Neu ×10^3/µl	7.1±1.2^{a}	5.8±1.9^{c}	6.2±1.3^{b}	5.1±0.7^{d}	**0.05 sig.**	2.5-8
ESR mm/hr	64.3±13.1^{a}	53.2±14.9^{c}	67.4±18.1^{a}	59.2±14.7^{b}	**0.039 sig.**	Male: 0-10 Female: 0-20
Welch's t-test used in comparison, sig : significant, ns: not significant P. value (≤ 0.05), (≤ 0.01).						

Conversely, Hb level and WBC counts illustrated non-significant differences between studied groups when compared between them in diabetes type 1 and 2 groups (P>0.05) as shown in (table 3.3).

3.2.3. Immunological parameters of study groups:

3.2.3.1. IL-37 level in diabetes groups.

Comparison of IL-37 among the studied groups revealed a highly significant difference (P<0.001) in mean level of IL-37 across the studied groups of T1DM and T2DM. The mean IL-37 was 28.20 and 23.29 both in DFU and Non groups of T1DM, respectively. On the other hand, T2DM had 30.94 and 25.02 mean both in DFU and Non groups of T2DM patients, while the mean of control group was 16.09. Generally, in all comparisons the results illustrated higher level of IL-37 in T1DM and T2DM patient groups in compared with the control group (table 3.4).

Table (3.4): IL-37 levels among different study groups.

Parameter	Group				Control (N=50)	P. value
	T1DM (N=64)		T2DM (N=79)			
	DFU(N=29)	Non(N=35)	DFU(N=41)	Non(N=38)		
IL-37 pg/mL	Mean± SD	Mean± SD	Mean± SD	Mean± SD	Mean± SD	
	28.20±8.05[b]	23.29±7.06[d]	30.94±9.95[a]	25.02±7.74[c]	16.09±4.10[e]	0.007 Sig.
Multiple pairwise comparisons./ least significant difference (LSD) post hoc test						

Subgroups	P. value		
	DFU	Non	DFU vs Non
T1DM			0.049
T2DM			0.042
T1DM vs. T2DM	0.008	0.009	
T1DM vs. controls	0.004	0.006	
T2DM vs. controls	0.002	0.005	

SD; Standard Deviation of mean, sig: significant. P. value (≤ 0.05), (≤ 0.01).

As shown in the table above there were many significant differences when compared with study groups. There were significant differences ($p < 0.05$) when compared between DFU and Non groups in T1DM and T2DM. In addition, there are highly significant differences ($p < 0.01$) when compared both T1DM and T2DM with control group. As well as, there are highly significant differences ($p < 0.01$) when compared among DFU and Non groupsof T1DM and T2DM diabetic groups.

3.2.3.2. IL-38 level in diabetic and control group.

Comparison of IL-38 among the studied groups revealed that all groups of T1DM and T2DM had higher significantly level of IL-38 than control groups. As well as, the DFU group of T2DM patients had higher meanlevel of IL-38 (3.57) than DFU group of T1DM (3.05). On the other hand, there were a significant difference ($P < 0.05$) in IL-38 between the studied groups as illustrated in (table 3.5).

Table (3.5): IL-38 levels in studied groups.

Parameter	Group				Control (N=50)	P. value
	T1DM (N=64)		T2DM (N=79)			
	DFU(N=29)	Non(N=35)	DFU(N=41)	Non(N=38)		
	Mean± SD	Mean± SD	Mean± SD	Mean± SD		
IL-38 ng/L	3.05±1.90[b]	2.13±1.87[d]	3.57±1.66[a]	2.55±1.75[c]	1.13±0.40[e]	0.05 Sig.
Multiple pairwise comparisons./ least significant difference (LSD) post hoc test						

Subgroups	P. value		
	DFU	Non	DFU vs Non
T1DM			0.009
T2DM			0.008
T1DM vs. T2DM	0.13 ns	0.24 ns	
T1DM vs. controls	0.007	0.01	
T2DM vs. controls	0.006	0.008	

SD; Standard Deviation of mean, sig: significant. P. value (≤ 0.05), (≤ 0.01). NS: not significant.

3.2.3.3. IL-17A level in studied groups:

The results summarized in table (3.6) indicated that there was a highly statistically significant difference (P<0.01) in levels of IL-17A in all diabetes cases groups when compared with control group. On the other hand, the level of IL 17 was highest in the group with chronic complication (DFU) with a mean value (37.44), followed by DFU of T1DM with mean level (25.19), then Non-group of T2DM with mean level (23.87) and lastly Non-group of T1DM (21.05), while, the mean level of control is (6.12). However, there are significant higher differences (P<0.01) when compared between studied diabetic groups as shown in (table 3.5).

Table (3.6): Mean levels of IL-17A in different groups.

Parameter	Group				Control	P. value
	T1DM (N=64)		T2DM (N=79)			
	DFU(N=29)	Non(N=35)	DFU(N=41)	Non(N=38)		
	Mean± SD	Mean± SD	Mean± SD	Mean± SD		
IL-17A pg/mL	25.19±10.84[b]	21.05±8.19[d]	37.44±10.90[a]	23.87±11.24[c]	6.12±3.80[e]	0.005 Sig.
Multiple pairwise comparisons./ least significant difference (LSD) post hoc test						
Subgroups	P. value					
	DFU	Non	DFU vs Non			
T1DM			0.045			
T2DM			0.038			
T1DM vs. T2DM	0.007	0.05				
T1DM vs. controls	0.002	0.000				
T2DM vs. controls	0.006	0.004				
SD; Standard Deviation of mean, sig: significant. P. value (≤ 0.05), (≤ 0.01).						

3.2.4. Roc Analysis of immunological markers:

3.2.4.1. ROC curve (DFU Vs Control group) of IL-37 in type 1DM.

The ROC curve was analyzed in the current study to determine the performance of the immunological cytokines (IL-37) in diagnosing and separating DFU from control group in T1DM. The results showed the presence of statistically significant difference in IL 37 between DFU and control group (Area under curve (AUC) = 0.949, p value 0.000), as shown in table (3.7) and figure (3.1).

Table 3.7: ROC Analysis of IL-37 (DFU and Control) of T1DM.

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.949	.023	.000	.904	.995
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

The sensitivity, specificity, positive, and negative predictive value of IL-37 illustrated in (table 3.8).

Table 3.8. Sensitivity, specificity, positive, and negative predictive value of IL-37 in DFU of T1DM.

Values of IL 37	Percentage
Cutoff point	13.42
Area under ROC curve (AUC)	0.949
Sensitivity	100.0%
Specificity	83.0%
Accuracy	92.0%
Positive predictive value	86.4%
Negative predictive value	100.0%

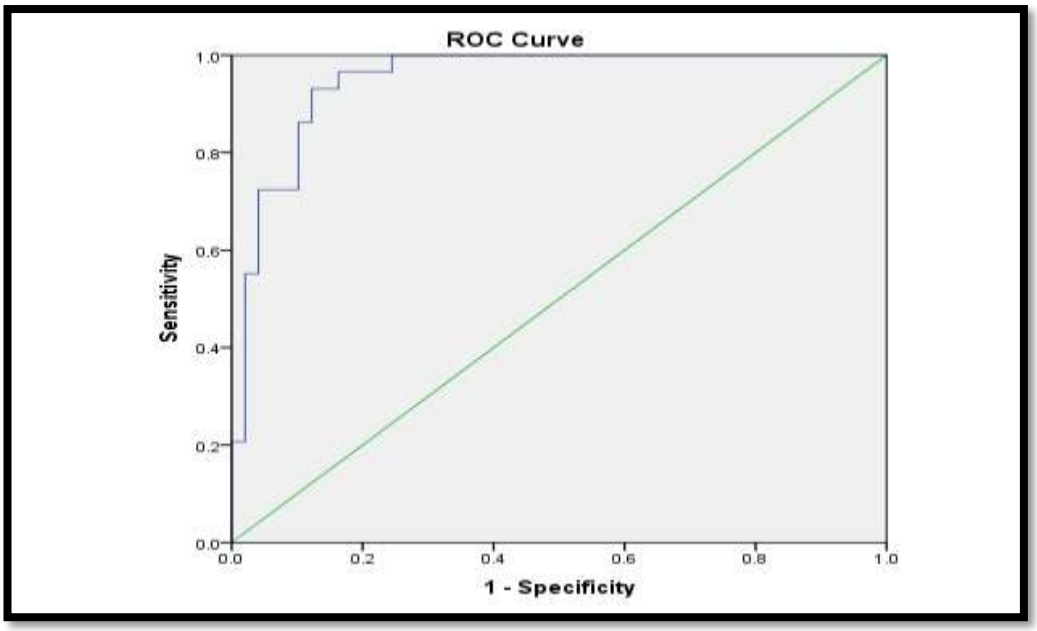


Figure 3.1: Receiver Operating Characteristic Curve and Area under the ROC Curve of IL-37 (DFU and Control) of T1DM.

3.2.4.2. ROC curve (DFU Vs Control group) of IL-37 in type 2DM.

As illustrated in table (3.9) and figure (3.2), there was a significant difference was observed in case of IL-37 in in diagnosing and separating DFU from control group in T2DM (AUC=0.893, p value = 0.000).

Table 3.9: ROC Analysis of IL-37 (DFU Vs Control) of T2DM.

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.893	.042	.000	.811	.976
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Table 3.10. Sensitivity, specificity, positive, and negative predictive value of IL-37 in DFU of T2DM.

Values of IL 37	Percentage
Cutoff point	22.54
Area under ROC curve (AUC)	0.893
Sensitivity	83.7%
Specificity	93.4%
Accuracy	94.2%
Positive predictive value	98.0%
Negative predictive value	88.3%

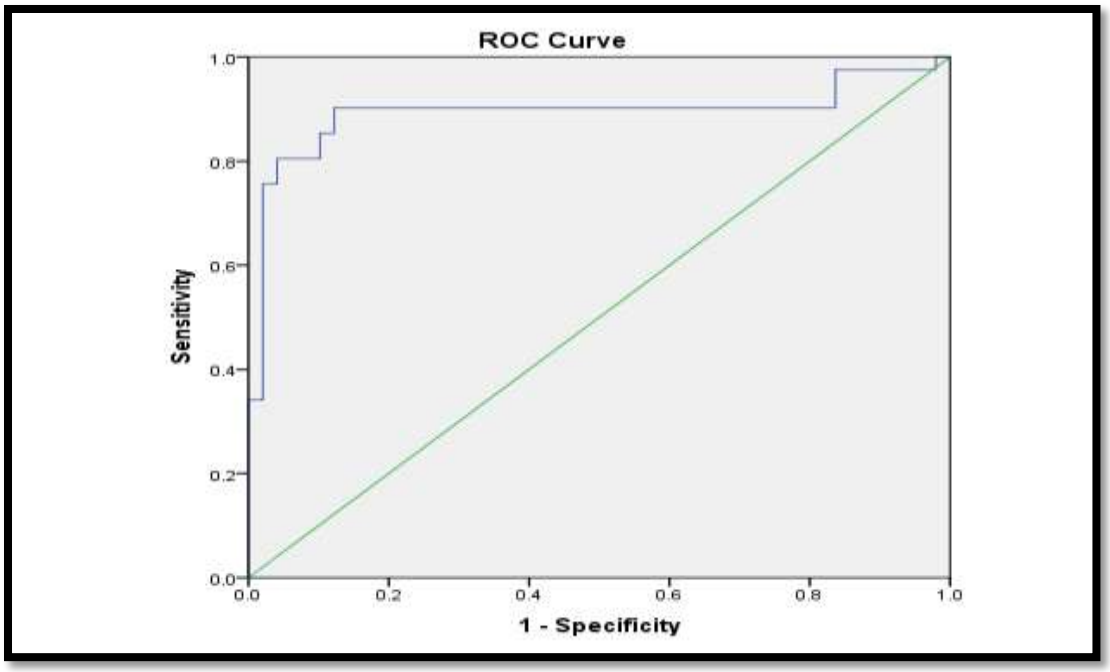


Figure 3.2: Receiver Operating Characteristic Curve and Area under the ROC Curve of IL-37 (DFU and Control) of T2DM.

3.2.4.3. ROC curve (DFU Vs Control group) of IL-38 in type 1DM.

However, a significant difference was observed in case of IL-38 in T1DM (AUC=0.889, p value 0.000) as shown in table (3.11).

Table 3.11: ROC Analysis of IL-38 (DFU Vs Control) of T1DM.

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.889	.050	.000	.792	.987
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Table 3.12. Sensitivity, specificity, positive, and negative predictive IL-38 in DFU of T2DM.

Values of IL 38	Percentage
Cutoff point	19.4
Area under ROC curve (AUC)	0.889
Sensitivity	93.5%
Specificity	94.8%
Accuracy	91.4%
Positive predictive value	93.9%
Negative predictive value	96.7%

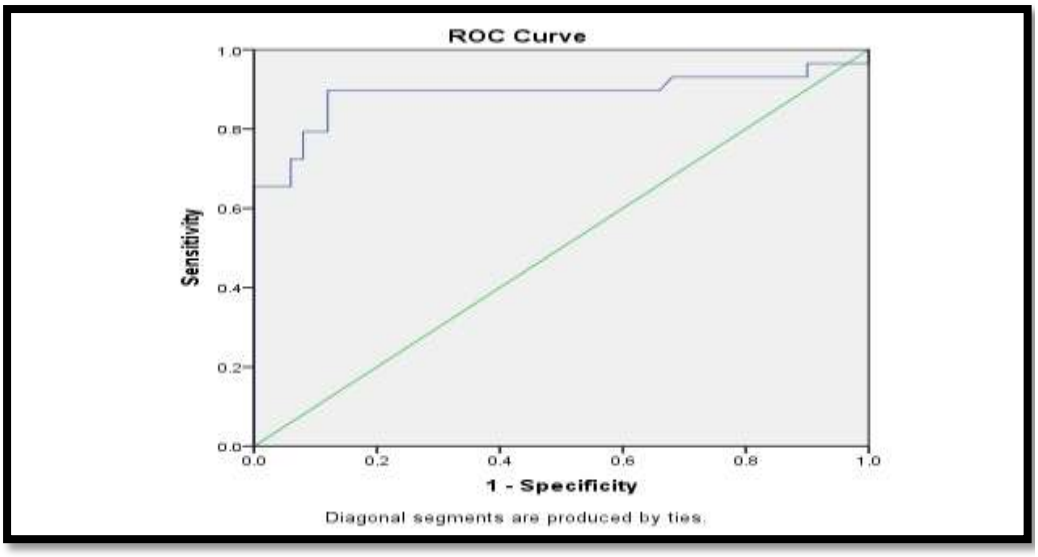


Figure 3.3: Receiver Operating Characteristic Curve and Area under the ROC Curve of IL-38 (DFU and Control) of T1DM.

3.2.4.4. ROC curve (DFU Vs Control group) of IL-38 in type 2DM.

ROC curve was analyzed in the current study to determine the performance of the inflammatory cytokines (IL-38) in diagnosing and separating DFU from control group in T2DM. The results showed the presence of statistically significant difference in IL 38 between DFU and control group (Area under curve (AUC) = 0.940, p value 0.000), as showed in (table 3.13 andfigure 3.4).

Table 3.13: ROC Analysis of IL-38 (DFU Vs Control) of T2DM.

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.940	.028	.000	.885	.994
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Table 3.14. Sensitivity, specificity, positive, and negative predictive value of IL-38 in DFU of T2DM.

Values of IL 38	Percentage
Cutoff point	17.9
Area under ROC curve (AUC)	0.940
Sensitivity	99.5%
Specificity	90.7%
Accuracy	93.2%
Positive predictive value	96.1%
Negative predictive value	98.9%

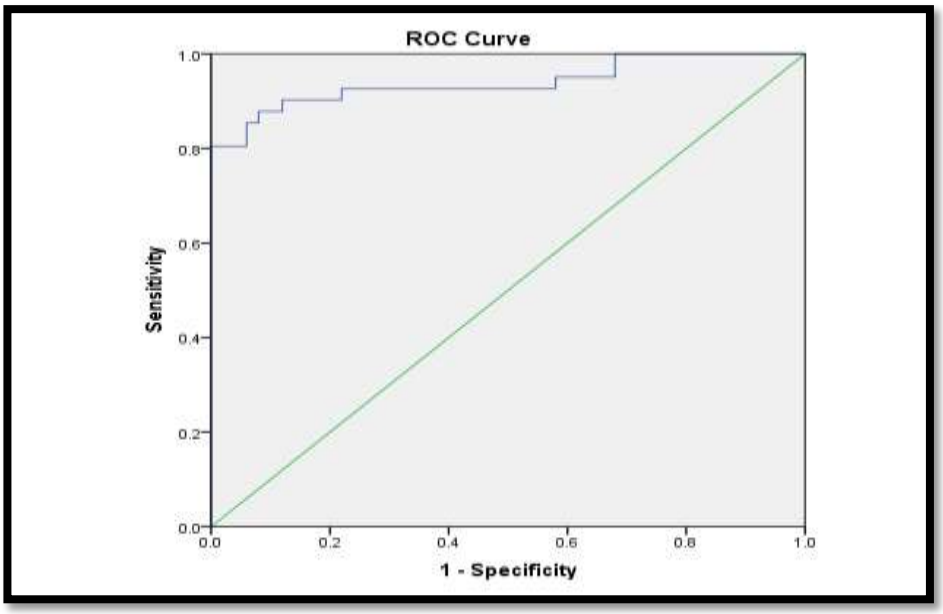


Figure 3.4: Receiver Operating Characteristic Curve and Area under the ROC Curve of IL-38 (DFU and Control) of T2DM.

3.2.4.5. ROC curve (DFU Vs Control group) of IL 17A in type 1DM.

ROC curve was analyzed in the current study to determine the performance of the inflammatory cytokines (IL-17A) in diagnosing and separating DFU from control group in T1DM. The results showed the presence of statistically significant difference in IL 17A between DFU and control group (Area under curve (AUC)= 0.970, p value 0.000).

Table 3.15: ROC Analysis of IL 17A (Non Vs Control) of T1DM.

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.970	.016	.000	.939	1.000
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Table 3.16. Sensitivity, specificity, positive, and negative predictive value of IL-17A in DFU of T2DM.

Values of IL 17	Percentage
Cutoff point	642.44
Area under ROC curve (AUC)	0.968
Sensitivity	83.9%
Specificity	100.0%
Accuracy	91.15%
Positive predictive value	100.0%
Negative predictive value	85.4%

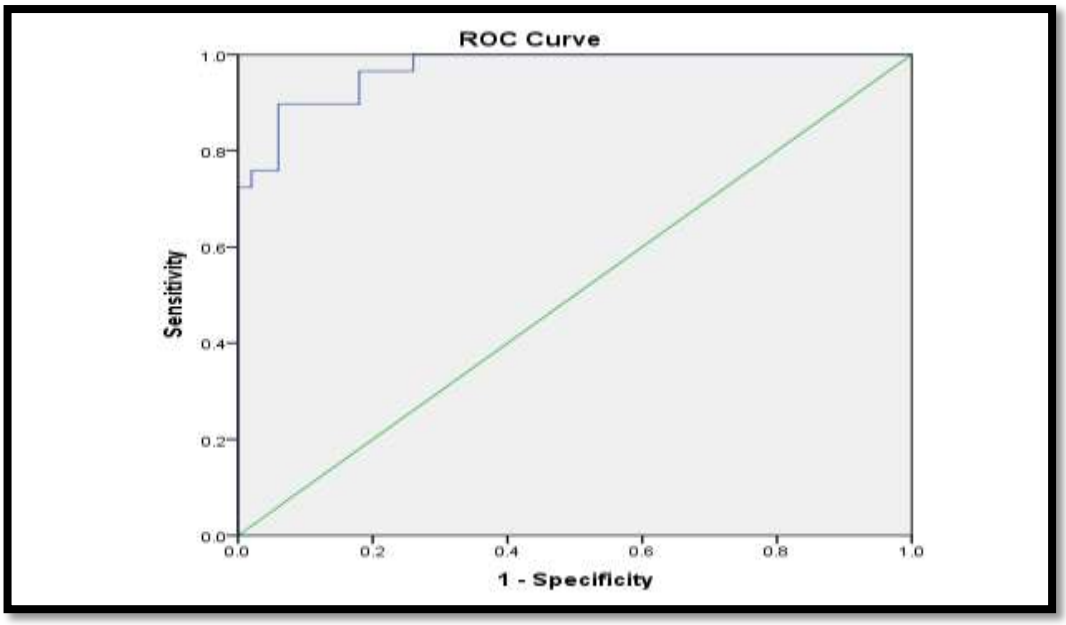


Figure 3.5: Receiver Operating Characteristic Curve and Area under the ROC Curve of IL 17A (DFU and Control) of T1DM.

3.2.4.6. ROC curve (DFU Vs Control group) of IL 17A in T2DM.

ROC curve was analyzed in the current study to determine the performance of the inflammatory cytokines (IL-17A) in diagnosing and separating DFU from control group in T1DM. The results showed the presence of statistically significant difference in IL 17A between DFU and control group (Area under curve (AUC)= 1.000, p value 0.000), as shown in table (3.17).

Table 3.17: ROC Analysis of IL 17A (DFU Vs Control) of T2DM.

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
1.000	.000	.000	1.000	1.000
a. Under the nonparametric assumption				
b. Null hypothesis: true area = 0.5				

Table 3.18. Sensitivity, specificity, positive, and negative predictive value of IL-17A in DFU of T2DM.

Values of IL 17	Percentage
Cutoff point	243.36
Area under ROC curve (AUC)	1.000
Sensitivity	89.1%
Specificity	100.0%
Accuracy	95.15%
Positive predictive value	88.9%
Negative predictive value	92.8%

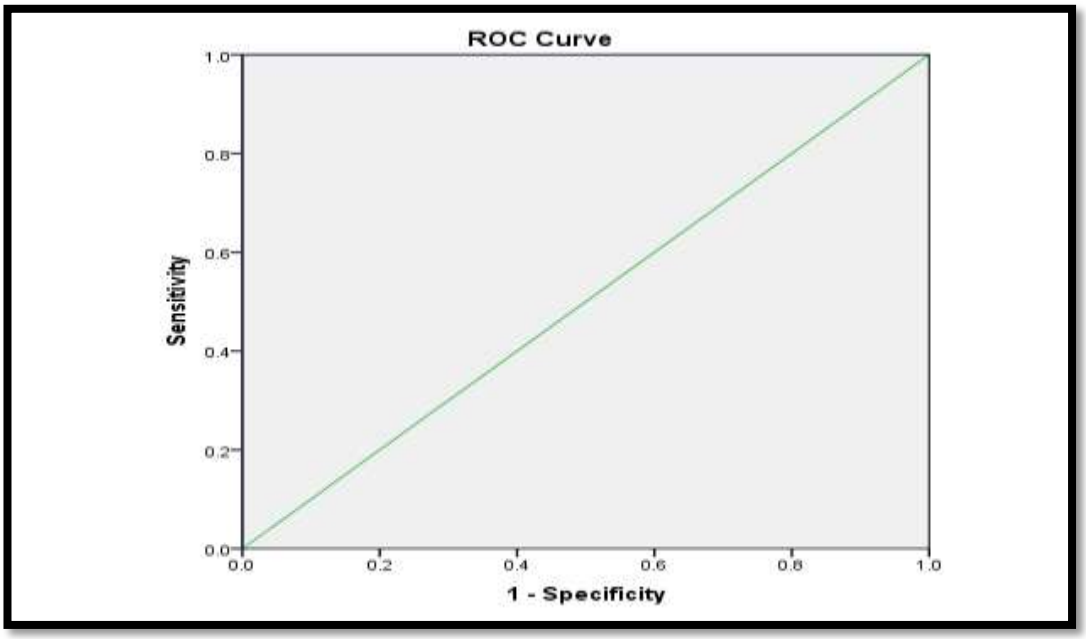


Figure 3.6: Receiver Operating Characteristic Curve and Area under the ROC Curve of IL 17A (DFU and Control) of T2DM.

3.3. Isolation of Pathogenic Bacteria of diabetic foot ulcer (DFU):

A total of seventy specimens were collected from patients with diabetic foot ulcer, 67 (95.71%) were positive for microbial growth, and 3(4.29%) samples showed no growth. Out of 67 positive cultures, monomicrobial infection was found in 29 patients, and polymicrobial infection was found in 38 patients as in (table 3.19 and figure 3.13).

Table (3.19): Characteristics of aerobic bacterial culture.

Gram staining property	Type of Bacteria	Percentage
Gram Negative Bacteria	*E. coli*	36.58 %
	K. pneumoniae	26.83 %
	P.mirabilis	17.07 %
	P.aeruginosa	9.76 %
	A.baumannii	7.32 %
	Morganella morganii	2.44 %
Total		**61.19 %**
Gram Positive Bacteria	*S. aureus*	76.93%
	streptococcus group B	15.38%
	Enterococcus spp.	7.69%
Total		**38.81%**
Number of isolated Bacteria	Mono-infection	43.28%
	Mono microbial infection with gram positive bacteria	34.48%
	Mono microbial infection with gram negative bacteria	65.52%

Culturing investigation depending on morphological and VITEK compact system results showed that Gram negative bacteria reveals a high rate 41(61.19%) which includes *E. coli* that show a high percentage 15(36.58%), then *K. pneumoniae* 11(26.83%). Followed by, *P.mirabilis* 7(17.07 %) and *P.aeruginosa* 4(9.76%) and *A.baumannii* 3(7.32%), lastly, *Morganella morganii* 1(2.44%). Whereas, Gram-positive bacteria recorded 26(38.81%),which include *S. aureus* was the most isolated bacteria in this study with percentage 20(76.93%) followed by *streptococcus group B* 4(15.38%) then *Enterococcus* 2(7.69%) as shown in (table 3.19 and figure 3.7).

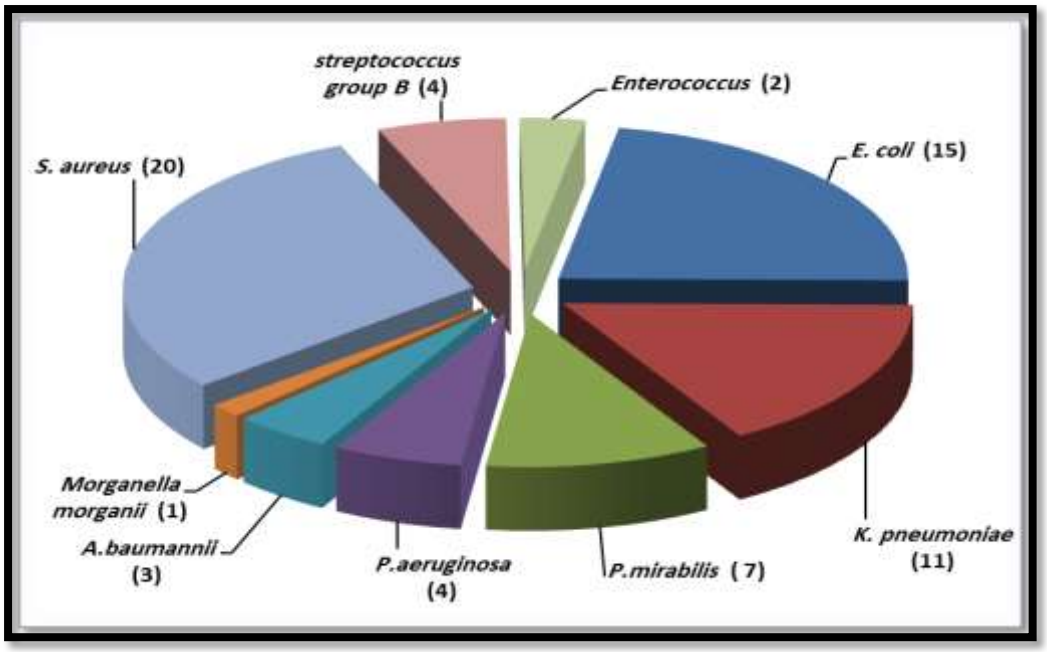


Figure (3.7): Distribution of Bacterial Species isolates.

3.4. Distribution of Isolated Bacteria according to gender and participant groups:

Bacterial types isolated according to test diabetic population groups are shown in table (3.20). The most frequent pathogen isolated from all diabetic groups was *S. aureus* (20 isolates) with male predominance in type 1 DM, followed by *Escherichia coli* (15 isolates) with female predominance in type 2 DM. Also, *Klebsiella pneumonia* (11 isolates) with male predominance of type 1 DM percentage (54.55%). *Proteus mirabilis* was the fourth microorganism isolated (7 isolates) in this study with (85.71%) percentage in type 1 DM. *Pseudomonas aeruginosa* uncommon diabetic pathogen were isolated from diabetics group (4 isolates) with approximately prevalence in type1DM, as well as *streptococcus group B* uncommon diabetic pathogen were isolated from diabetics group (4 isolates) with equal prevalence in T1DM and T2DM. Also, least isolated diabetic pathogens were; *Enterobacter* spp. (2 isolate) isolated from both T1DM and T2DM and *Morganella morganii* (1 isolates) in T2DM only. *Acinetobacter baumanni* (3 isolates) in T1DM females.

Table (3.20): The results of DFU culturing in investigated groups.

Bacterial Species		T1DM male	T1DM female	T2DM male	T2DM female	Total
S. aureus	NO.	8	3	5	4	20
	% within Bacterial type	40.0 %	15.0 %	25.0 %	20.0 %	100.0%
E. coli	NO.	0	5	3	7	15
	% within Bacterial type	0.0%	33.33 %	20.0 %	46.67 %	100.0%
K. pneumoniae	NO.	6	0	2	3	11
	% within Bacterial type	54.55 %	0.0%	18.18 %	27.27 %	100.0%
P.mirabilis	NO.	2	4	0	1	7
	% within Bacterial type	28.57 %	57.14 %	0.0%	14.29 %	100.0%
P.aeruginosa	NO.	0	3	0	1	4
	% within Bacterial type	0.0%	75.0 %	0.0%	25.0 %	100.0%
streptococcus group B	NO.	1	1	2	0	4
	% within Bacterial type	25.0 %	25.0 %	50.0 %	0.0%	100.0%
A.baumannii	NO.	0	2	1	0	3
	% within Bacterial type	0.0%	66.67 %	33.33%	0.0%	100.0%
Enterococcus	NO.	0	1	1	0	2
	% within Bacterial type	0.0%	50.0 %	50.0 %	0.0%	100.0%
Morganella morganii	NO.	0	0	0	1	1
	% within Bacterial type	0.0%	0.0%	0.0%	100.0%	100.0%
No growth	NO.	1	0	0	2	3
	% within Bacterial type	33.33 %	0.0%	0.0%	66.67%	100.0%
Total	NO.	18	19	14	19	70
	% within Bacterial type	25.72 %	27.14 %	20.0 %	27.14 %	100.0%

No growth also reported accounting for 4.29% of the entire population under study with the majority being T2DM female, table (3.20) indicates the positive and negative diabetic culture in investigated groups.

3.5. Distribution of age groups based on results of bacterial growth:

The present study findings of bacterial growth showed that all age groups have bacterial growth with age level of (≥ 60) years were the most associated with infection as recorded in table (3.21) both in T1DM and T2DM, while, some persons in age groups (30 - 39 Years) showed negative bacterial growth results in T1DM. Besides, the (<20+50-59Years) age groups revealed negative growth results in T2DM patients. However, the data of bacterial growth analyzed compared to the age groups revealed there were a significant variation between all the age intervals of study patients as in table (3.21).

Table (3.21): Distribution of age groups based on results of bacterial growth.

Variable	Categories	Bacterial Growth				P-Value
		T1DM		T2DM		
		Positive	Negative	Positive	Negative	
Age	< 20	3(10.71%)	0 (0.0%)	5 (12.82%)	1(50.0%)	0.01 Sig.
	20 - 29 Years	7(25.0%)	0 (0.0%)	8 (20.51%)	0 (0.0%)	
	30 - 39 Years	2(7.15%)	1 (100%)	2 (5.13%)	0 (0.0%)	
	40 - 49 Years	3(10.71%)	0 (0.0%)	4 (10.26%)	0 (0.0%)	
	50-59 Years	5(17.86%)	0 (0.0%)	11(28.21%)	1(50.0%)	
	≥ 60	8(28.57%)	0 (0.0%)	9 (23.07%)	0 (0.0%)	
Total		28 (100.0%)	1(100.0%)	39 (100.0%)	2(100.0%)	

Chi-square test, significant difference at P ≤ 0.01.

3.6.Correlations between demographic characteristics & study parameters:

3.6.1.Correlations between demographic characteristics & study parameters in DFU of T1DM:

Further analysis was performed to assess the correlation between demographic variables from one side as independent variables and each of IL-37, IL38 and IL-17A as dependent variables using bivariate spearman's test which revealed non-significant correlation between these three dependent parameters and all of demographic variables in DFU of T1DM, in all correlations, (P. value > 0.05), except to that, the (DM and DFU duration) illustrated a positive significant correlation with IL-17A, where (p. value =0.044 and 0.049. respectively). In addition, DFU duration illustrated a significant positive correlation with IL-38 (p. value= 0.047), as demonstrated in (table 3.22).

Table 3.22. Pearson's r correlation analysis of demographic variables with biomarkers in DFU of T1DM.

Variable	IL-37		IL-38		IL-17A	
	R value	P. value	R value	P. value	R value	P. value
Age	0.128	0.301	0.119	0.477	0.702	0.318
Gender	0.046	0.739	0.236	0.236	0.072	0.739
DM duration	0.192	0.161	0.145	0.145	0.992	**0.044**
Family history	0.162	0.614	0.023	0.912	0.132	0.616
DFU duration	0.097	0.560	0.945	**0.047**	0.871	**0.049**
R: Spearman's correlation coefficient						

3.6.2.Correlations between demographic characteristics & study parameters in DFU of T2DM:

From another point of view, Pearson's bivariate correlation analysis revealed a significant correction in (DM and DFU duration) with IL-17A (p. value=0.046 and 0.048, respectively). Also, DFU duration and IL-37 (p. value=0.043), while, all other relationships between laboratory variables and

each of immunological markers were non-significant, (P. values >0.05), as in (tables 3.23).

Table 3.23. Pearson's r correlation analysis of demographic variables with biomarkers in DFU of T2DM.

Variable	IL-37		IL-38		IL-17A	
	R value	P. value	R value	P. value	R value	P. value
Age	0.199	0.207	0.029	0.817	0.502	0.417
Gender	0.049	0.839	0.313	0.109	0.079	0.937
DM duration	0.199	0.156	0.167	0.209	0.921	**0.046**
Family history	0.173	0.415	0.066	0.817	0.163	0.721
DFU duration	0.997	**0.043**	0.645	0.092	0.950	**0.048**
R: Spearman's correlation coefficient						

3.7.Correlations between laboratory variables & immunological markers:

3.7.1.Correlations between laboratory variables & immunological markers in DFU of T1DM:

Using Pearson correlation to find any association between markers revealed positive significant correlation between Cholesterol and IL-17A, also between LDL and IL-17A, as well as between ESR and IL-17A, p-value were= (0.048, 0.038 and 0.040, respectively). All other correlation was non- significant as demonstrated in table 3.24.

Table 3.2. Pearson's r correlation analysis of laboratory variables & immunological markers in DFU of T1DM.

Variable	IL-37		IL-38		IL-17A	
	R value	P. value	R value	P. value	R value	P. value
FBS	0.166	0.273	0.267	0.421	0.156	0.202
HBA1C	0.033	0.798	0.175	0.211	0.035	0.680
Cholesterol	0.068	0.644	0.301	0.158	0.877	**0.048**
HDL	0.187	0.123	0.187	0.281	0.190	0.163
LDL	0.057	0.493	0.107	0.555	0.917	**0.040**
Triglycerides	0.059	0.768	0.219	0.084	0.068	0.662
CRP	0.199	0.347	0.131	0.380	0.185	0.401
Hb	0.298	0.088	0.090	0.535	0.267	0.158
PLT	0.186	0.133	0.170	0.201	0.192	0.141
WBC	0.359	0.169	0.329	0.094	0.721	0.062
Lymphocyte	0.314	0.128	0.188	0.390	0.148	0.319
Neutrophil	0.309	0.155	0.060	0.705	0.567	0.101
ESR	0.185	0.104	0.402	0.101	0.992	**0.038**
R: Pearson's correlation coefficient						

3.7.2.Correlations between laboratory variables & immunological markers in DFU of T2DM:

From another point of view, Pearson's bivariate correlation analysis revealed non-significant correlation between laboratory variables and each of IL-37, IL-38 and IL-17A, in all correlations, were (P. values >0.05) (table 3.25), except in relationships between LDL and IL-17A, as well as between CRP and IL-17A, in addition, there was positive correlation between ESR and IL-17A level, were (p. value= 0.047, 0.041 and 0.037, respectively).

Table 3.25. Pearson's r correlation analysis of laboratory variables & immunological markers in DFU of T2DM.

Variable	IL-37		IL-38		IL-17A	
	R value	P. value	R value	P. value	R value	P. value
FBS	0.455	0.102	0.217	0.331	0.577	0.099
HBA1C	0.211	0.465	0.215	0.399	0.389	0.213
Cholesterol	0.189	0.413	0.329	0.159	0.679	0.082
HDL	0.187	0.123	0.187	0.281	0.190	0.163
LDL	0.444	0.107	0.561	0.098	0.899	**0.047**
Triglycerides	0.259	0.366	0.301	0.205	0.211	0.199
CRP	0.521	0.102	0.277	0.306	0.974	**0.041**
Hb	0.395	0.204	0.080	0.605	0.317	0.128
PLT	0.206	0.173	0.184	0.311	0.172	0.231
WBC	0.459	0.119	0.439	0.194	0.521	0.102
Lymphocyte	0.434	0.118	0.238	0.301	0.288	0.279
Neutrophil	0.379	0.235	0.081	0.615	0.677	0.071
ESR	0.495	0.133	0.502	0.113	0.933	**0.037**
R: Pearson's correlation coefficient						

4. Discussion

Diabetes mellitus (DM) represents one of the significant health problem worldwide that affects up to 11% of people globally (Davies *et al.*, 2020). According to recent studies in Iraq, about 1.4 million people have DM (Abusaib *et al.*, 2020).

4.1. Demographic characteristics of study groups:

Concerning age, the present study found that T1DM was younger than those with T2DM, this due to earlier onset and diagnosis of T1DM as it occurs and diagnosed earlier in childhood and adolescence life(Ang, 2020). These results showed there were highly significance differences among the studied groups and several previous studies documented that maximum number ofDFU patients occur within age range 40-60 years (Saleh & Hadi, 2019, Anyim *et al.*, 2019 and Kadhim, 2021), as shown in Table (3.1).

According to gender, the distribution of the DM group and DFU group found that males were relatively dominant than females but statistically non-significant in the studied groups, these findings consistent with that reported in previous studies (Abdissa *et al.*, 2020, Kadhim, 2021 and AL-khadi, 2021). Although females are more affected by autoimmune diseases including type 1 diabetes due to hormonal effects. The explanation for dominant males could be more attention for male health in Iraqi society. However, several previous studies documented higher frequency of DFU patients among males but no to statistically difference level (Datta *et al*., 2019, Singh *et al*., 2020). This might possibly have attributed to the higher level of outdoor activity, have better access to health care facility, lack of foot care and poor compliance to foot carepractice as compared to females. Furthermore, male predominance in DFU could be linked to factors such as gender-related differences in life styles, improper hygiene, type of foot wear, and professional roles that require the feet

to tolerate more pressure as a result of work and make them more subjected to trauma (Amjad *et al.*, 2017, Tamali *et al.*, 2015).

Diabetic patients with DFUs were older than patients without DFUs, this might be because older age poses the risk of diminished ability for self-care due of poor vision and impaired mobility (Hameed and Baras, 2020). The predominance of diabetic foot ulcers in this age range may be due to the fact that patients in this age range have nutritional deficiencies, decreased immunity (Saleh and Hadi, 2019) and with their increasing ages and disease course, elderly patients with diabetes frequently also have peripheral neuropathy and vascular lesions, leading to diabetic foot ulcers that poorly heal over a long treatment time (Shahbazian *et al*., 2013).

The duration of diabetes was significantly higher in the DFU patients group compared with diabetic patients without foot ulcer; as in study (Nongmaithem *et al*., 2016). The possible explanation might be the fact that diabetic patients for long time were presumed to be at more risk due to the development of long term diabetic complications such as neuropathy, nephropathy and retinopathy which could lead to the occurrence of foot ulcer in the diabetic patients (Deribe *et al*., 2014). Mohammed et al stated that older age, long diabetes duration, multiple and bad foot care are the common risk factors for the development of DFUs in Iraq (Mohammed *et al*., 2016). It can be seen that as the duration of diseases increases so is the incidence of the ulceration, the reason for the same could be as follows, as the duration of disease increases so is the degree/severity of neuropathy and vasculopathy and thus predisposing the patients to ulceration that are resistant to heal (Maharaul *et al*., 2021).

The current study found that family history appeared to be more frequent in T1DM patients than T2DM, and these results are consistent with Salomon and Church study which stated that autoantibodies that attack insulin producing

cells contributes to T1DM rather than family history as compared to T2DM (Salomon, S. H., & Church, K. 2020).

Table (3.1) showed the presence of 44.84% and 51.22% of patients (DM and DFU) had diabetes type 1 and 2 more than 20 years ago, respectively. This is in contrast to a recent study done by (Kene *et al*., 2021) who reported that the majority of the patients involved in his study had DM for less than 10 years.

4.2. Laboratory parameters of the studied group:

4.2.1. Biochemical Parameters of study groups:

In the present study, there were highly significant differences between HBA1C values in patients with DFU with higherlevels as recorded compared to normal values reported previously by (American Diabetes Association, 2017).

Regarding to the fasting blood sugar and HBA1C the results show in table (3.2). There was an increase in level of fasting blood sugar and HbA1c levels in both Type 1 and Type 2 diabetic patients when compared between studied groups, with levels being higher in DFU of Type 1 diabetic study group as compared to Type 2 diabetic study group. The HbA1c was higher in DFU of Type 1 diabetic subjects as compared to other diabetic groups (Chutani & Pande, 2017) illustrated increase in FBS and HbA1c in diabetic patients. Also, Kaleli *et al* documented the presence of significant difference in HbA1c level (Kaleli *et al*., 2019).

The HbA1c test is a strong predictor of diabetes complications (SAl-Shaheeb *et al.,* 2022). Raised blood sugar has been considered as a risk factor for the development of diabetic foot ulcers because of its contribution toward the development of peripheral neuropathy and microvascular complications (Al Kafrawy *et al.,* 2014). Poor glycemic control is the main risk factor for

developing diabetic complications. Consequently, optimal control of plasma glucose will halt the progression of all complications including DFUs (Marzoq *et al.*, 2019).

Comparison of mean value of lipid profile between type 1, type 2 and control group was shown in (table 3.2). In the present study, hypercholesterolemia is obvious with significantly more higher in DFU of T2DM in compared with others. One of the possible explanations of this hypercholesterolemia was loss of affinity for Apo B receptors of the glycated LDL may contribute to the increase in serum cholesterol level in diabetics. Other cause that intake of saturated fatty acid is positively associated with high serum cholesterol.

It is a well-known fact that low HDL is common in Non-complicated group of type 1 and may be a strong factor for coronary heart disease (CHD). Which agrees with our result that low HDL is evident in both types. High serum LDL is noted in DFU of T2DM. It could be explained that patients have an increased level of small dense LDL and reduced clearance due to glycosylation of ligand proteins. Serum Triglycerides have been increased in DFU of Type 2 diabetic patients in present study. The most common lipid abnormality in DM is the hypertriglyceridemia which is known to be an independent risk factor of CHD. It is due to increase in VLDL synthesis and an impaired VLDL catabolism. However this phenomenon is less evident in type 1 with different mechanism which is based on the fact that in type 1 DM the reduced chylomicron and VLDL catabolism occur as a direct consequence of reduced lipoprotein lipase activity due to insulin deficiency (Ismail, 2010 and Arab Sarhadi *et al.*, 2019).

We found significant variations in the level of lipid profile components between diabetics as results in the table (3.2). Several factors are involved in

the alteration of lipid profile following DM. The increased FBS level in diabetics promotes lipid peroxidation through stimulation of non-enzymatic glycation of proteins and formation of advanced glycated end-products (Suryawanshi *et al.*, 2006).

Similarly, the increase in the lipid components such as LDL that are susceptible to peroxidation which is inconsistent with (Marin *et al.*, 2015). In the present study, both HDL and LDL levels were significantly higher in DFU patients with T2DM compared to other groups. In such circumstances, the positive effect of high HDL may be masked by LDL elevation.

4.2.2. Inflammatory and Hematological parameters of studied groups:

As revealed in (Table 3.3), the result of CRP showed statistically significant differences between DFU of T2DM compared with others patients groups, this agrees with (Kahraman *et al.*, 2014). So inflammation caused by bad habits with aging and obesity lead to diabetes , and elevated CRP levels under the effect of cytokines especially (IL6) & (TNFά) which express CRP gene. Also, the elevations of CRP may be associated withsystemic inflammation and cardiovascular complication occasioned by Hyperglycemia seen in T2DM (Ehiaghe *et al*., 2013).

CRP is acute phase protein and considered as the main inflammatory factor produced by liver during acute infection or inflammation. Plasma concentration could be increased as much as 1000 fold during injury and infection. Additionally, high level of CRP could predict the development of T2DM and cardiovascular disease (Kareem et *al.*, 2012).

In study by (Abd-Elrahman *et al*., 2013) found that ESR was higher in diabetic patients with foot ulceration than diabetic patients without foot ulceration. Also, the ESR and CRP levels increased in the presence of infection and they were the two potentially valuable biochemical markers.

ESR and C-reactive protein (CRP) were reported to play a role in monitoring the treatment of diabetic foot osteomyelitis (DFO) (van Asten *etal*., 2017). (Lavery *et al*., 2019) showed that both the ESR and the CRP level were inflammatory biomarkers for evaluating a foot infection.

The investigation of some of the important hematological markers among the studied groups are presented in Table (3.3). The Lymphocytes was significantly higher in DFU of T2DM in compared with another groups. Regarding WBC indices, this count was higher in DFU group of T2DM butthis increase is in non-significant level. On the other hand, the mean of neutrophils in DFU of T1DM patients was significantly higher than that of others DM groups. This is in corroboration with the report of previous studies conducted by (Kaleli *et al.*, 2019 and Pourkazemi et al., 2020).

Similarly, Ubeid has found that the count of neutrophils in diabetic female higher than that of non-diabetic patients (Ubeid, 2020). Likewise, Platelets counts were higher significantly in DFU of T1DM in comparisons with other diabetic groups. Similar to this study, studies conducted by (Akinsegun et al., 2014 and Olana et al., 2019) found that platelet counts were significantly higher in the diabetes group. The reason might be that PLT counts are indicatorof thrombotic potential and risk of vascular complications in diabetes. There might be a release of S100A8/A9 by neutrophils which triggers IL-6 production and thrombopoietin synthesis from hepatocytes, leading to bone marrow stimulation to recruit greater numbers of reticulated platelets, which are associated with both atheroprogression and atherothrombosis (Lee andBergmeier, 2017).

Diabetic platelets are larger with denser granules and they are enzymatically and functionally hyperactive to produce more prothrombotic factors like thromboxane A2, platelet factor 4, serotonin, and P-selectin than smaller platelets and hence cause an increased tendency to thrombotic events (Randriamboavonjy, 2015).

Platelet hyperactivity in diabetics patients is also attributed to a multitude of factors including insulin resistance, oxidative stress, endothelial dysfunction, inflammation, and hyperglycemia (Kaur *et al.,* 2018). On the other hand, the mechanism underlying this increase of the total and differential WBC counts in diabetic patients might be explained by the effect of hyperglycemia and the pathogenesis of DM. The DM is an inflammatory disease. Although defects in insulin action on the peripheral tissues lead to a chronic low-grade inflammatory state and induce the secretion of pro-inflammatory cytokines, which promote differentiation and maturation of leukocytes (Jiang *et al.*, 2014).

Additionally, in hyperglycemic state leukocytes are activated by AGEs, oxidative stress, and cytokines that increase the state of inflammations and the development of vascular complications in diabetes. Neutrophils are also suggested to be a marker of inflammation, which is associated with the progression of complications (Hamed, 2016). The DFU group had higher WBC counts when results compared with the DM group without foot ulcer; this finding is in coherence with the report of studies conducted by (Gunes *et al.,* 2017). Current result showed that the hemoglobin level was higher in non- significant levels in Non-DFU group of T1DM in compared with other groups.

This agreement with studies by (Biadgo *et al.,* 2016 and Arkew *et al.,* 2021) and this might be explained by the effect of insulin resistance, which is associated with the stimulation of erythroid progenitors increasing RBC count, and increased levels of Hgb (Arkew *et al.,* 2021). This finding is inconsistent to previous studies carried out in Bangladesh (Alam *et al.,* 2015), India (Harish Kumar *et al.,* 2017) and Libya (Salhen *et al.,* 2017). This discrepancy might be due to a difference in the characteristics of the study population and sample size variation. In our study the participants were adults and most of them were males. It has been documented that hematological change is a common

complication of DM and represents a significant and under-recognized burden in these patients.

4.2.3. Immunological parameters of study groups:

4.2.3.1. IL-37 level in diabetes groups:

IL-37 is of the IL-1 family. It has anti-inflammatory property. It appears widely. IL-37 is usually expressed in granule cells and T-cells, with the best degree of statement in regulatory T-cells (Treg cells) (Alhayali *et al.*, 2021). In this study, comparison of İL-37 level between different study groups IL-37 was significantly higher ($P<0.01$) in patients with T1DM and T2DM when compared with control group. This finding similar to the findings of Alhayali *etal.*, who found that the mean of IL-37 was higher in patients with DM than of control healthy (Alhayali *et al.*, 2021).

Serum IL-37 levels were highest in DFU of T2DM patients and lowest in healthy control group; as in study by (Cao *et al.*, 2021) illustrated that IL-37 inhibits the pathogenesis of rheumatoid arthritis (RA) via downregulating proinflammatory cytokines and corrects the established metabolic disorders caused by diabetic and thus contributes to improved systemic insulin sensitivity. In addition in diabetic, the production of pro-inflammatory cytokines and chemokines increased that can attract and activate macrophages and other immune cells; this causes chronic low-grade inflammation and promote of diabetic complications (Velikova *et al.*, 2021).

Inversely, reduced production of IL-37 may promote to the T2DM as the absence of IL-37 may promote inflammation that interferes with insulin signaling (Alhayali *et al.*, 2021). Herein, it suggests that IL-37 being novel inflammatory mediator might be responsible for some underline changes which may donate for the progression of DM.

4.2.3.2. IL-38 level in diabetic and apparently healthy control groups:

In the present study, comparison of IL-38 among the studied groups revealed that T1DM and T2DM patients had higher level of IL-38 than controls. Likewise, level of IL-38 was higher in DFU of T2DM in compared with others diabetic groups. There results consistent with study of (Gurău *et al.*, 2021) these found the plasma IL-38 was higher in T2DM patients.

On the other hand, the findings of this study contrast with that reportedby (Zhao *et al.*, 2022) these finding illustrated that the Serum IL-38 levels in T2DM patients were significantly lower than those in controls.

Another study by (Yu *et al.*, 2017) which studied gestational diabetes showed; IL-38 was increased 3.3, 2.6, or 2.6 fold in chorionic villi ($P<0.01$), umbilical artery ($P<0.05$), umbilical vein ($P<0.05$) from GDM women, respectively, compared to that from non-GDM women and herein, IL-38 produced in the chorionic villi and umbilical cords may be a response to local inflammation during the development of GDM. So that, such a dysregulated micro-environment may contribute to the development of GDM via an immune-mediated mechanism.

Interleukin 38 (IL-38) is a new member of the IL-1 family, and it has anti-inflammatory activity. However, its role in type 2 diabetes mellitus (T2DM) has not been reported. In the present study, we found that the plasma concentration of IL-38, a natural suppressor of the inflammatory response, was significantly increased in T2DM, in line with the few available reports showing the upregulation of IL-38 in placentas from GDM patients (Yu *et al.*, 2017) andin sera from pediatric T2DM patients (Liu *et al.*, 2020). The higher circulating IL-38 levels observed in patients with diabetic patients with and without DFU complication represent the first evidence of IL-38 modulation in a T2DM.

The IL-38 inhibits the production of T-cell cytokines IL-17 and IL-22. IL-38 also inhibits the production of IL-8 induced by IL-36γ, thus inhibiting inflammatory responses. IL-38-related cytokines, including IL-1Ra and IL-

36Ra, are involved in the regulation of inflammation and immune responses. These data may provide useful information for the intervention for DM and suggest that IL-38 may be a new biomarker of T2DM.As well as, mightprovide new insights for developing anti-inflammatory treatments in the near future.

4.2.3.3. IL-17A level in studied groups:

IL-17, which is a pro-inflammatory cytokine, has been studied in development of diabetes**.** The present data revealed highly significant increase in IL17A serum level in all diabetic group patients in compared with healthy group. It is believed that IL17A had a crucial role in development of diabetic mellitus and its complications such as DFU. As well as, DFU group of T2DM showed higher mean levels. These findings are consistent with the studies of Parhi *et al*. which showed increase in level of IL-17 in newly diagnoseddiabetes than the healthy controls (Parhi *et al.,* 2019) and one of the possible mechanisms for that is the binding of IL17A with its receptor may enhance activation of metalloproteinase, hypertensive and vascular dysfunction. Another mechanism is through the activation of JAK/STAT pathway that lead to hepatic insulin resistance, beta and liver cell apoptosis and down regulation of gluconeogenesis related molecules (Yousefidaredor *et al*., 2014).

Likewise, the level of IL 17A was even more in the group of patients with diabetic complications (DFU), this finding is in corroboration with Yousefidaredor *et al.* who found that IL 17 plays an important role in development of T2DM and its complications via the up regulation of several inflammatory molecules including angiotensin II type I receptor and JAK 2 STAT 3 pathway related molecules (Yousefidaredor *et al.,* 2014 and Parhi *et al.,* 2019). Thus, higher IL17A level in patients might possibly due to inflammation in the ulceration, deterioration of skin integrity and various types of bacteria causing infection (Kaleli *et al.,* 2019). These results indicate IL17A as a contributory factor to the inflammatory process in T2DM and its

complications. However this finding does not corroborate with the findings of Roohi *et al.* and Vasanthakumar *et al.* that showed no significant difference in the level of IL 17A between type 2 diabetes mellitus and controls (Roohi *et al.*, 2014 and Vasanthakumar *et al.*, 2015).

So that, IL-17 has been found to be an independent marker of severity of degree of diabetic. Thus IL-17A can act as a marker of inflammation and complications in patients of diabetes mellitus.

4.2.4. Roc Analysis of IL37, IL-38 and IL-17A immunological markers:

This might possibly reflect that higher level of IL-37, IL-38 and IL-17A in both DFU and Non- groups of T1DM and T2DM patients compared to control group gives the possibility of using of these parameters as a marker in diagnosis of DFU of T1DM and T2DM, as well as in diagnosing of diabetic patients of both types above.

Persistently elevated levels of IL-37, IL-38 and IL-17A family members may be associated with wound chronicity (Hadian *et al.*, 2019). Interestingly, another study found decreased in levels of these cytokines in other diseases such as in IL-17A levels in wound fluid from chronic diabetic foot ulcers when compared to acute surgical wounds (Bekeschus *et al.*, 2017(.

Rodero *et al.* found that genetic deletions in IL-17A or administration of IL-17A antibody accelerated wound healing in association with increased expression of a pro-healing macrophage population (Rodero *et al.*, 2013). Conversely, recombinant IL17A administration to wild-type wounds produced delayed healing due to increased inflammatory cell infiltration (Takagi *et al.*, 2017).

4.2.5. Isolation of Pathogenic Bacteria of diabetic foot ulcer:

Swabs were obtained from the infected area of ulcer and cultured using laboratory standard protocols and some samples taken from the infected area revealed positive culture growth while others showed negative culture growth.

The result of negative growth may be as a result of the other causative agents of infection, anaerobic bacteria, fungi ,and viruses (Jain and Barman, 2017), or may be as a result of either the wounds were not infected at the time of the study or the antibiotics prescribed were effective (Turhan *et al* .,2013).

Bacterial isolates that were obtained from the clinical specimens have been initially characterized based on the cultural morphology. Results of culture showed colonies on MacConkey agar Pink color with precipitation of bile salt around colonies; this refers to *E. coli* isolates. This results diagnostic for *E. coli* (MacFaddin, 2000 and Tille, 2015). Further confirmation done using Vitek2. Similar results was recorded by Abdul-hussein *et al.,* (2018) as shown in the figure (5) appendix 3 and figure (5,6) appendix 4.

Results of identification of *K. pneumoniae* were showed pink lactose fermenter mucoid colonies on MacConkey agar (Forbes *et al.*, 2007). Further confirmation done using Vitek2 give similar results. These results likewise result was recorded by Raheema, (2016a) as shown in the (Figure 7 andappendix 5).

On mannitol *S.aureus* fermenters as yellow colonies; *S.aureus* was not the only mannitol positive species many Staphylococcus species .Also give yellow colonies bounded by yellow regions on the mannitol salt agar (Raheema and Abed, 2019).Similar finding were recorded by Raheema, (2016b) andblood hemolysis in blood agar (MacFaddin, 2000) as shown in the (Figure 8 and appendix 5). In addition, the shape and culturing growth images for *Acinetobacter* are illustrated in the (figure 9, 10 and appendix 6).

In study by, AL-muhana *et al.,* (2020) observed that gram negative bacteria were isolated in 64.5% whereas gram positive bacteria were isolated in 35.5% of DFU patients. The predominance of gram negative bacteria more

than gram negative has been noted in the current study, and these agree with (Al-Allak *et al.*, 2019 and Qadir *et al.*, 2020).

An explanation for the difference in the nature of microbes infecting the diabetic foot infection has been due to variation in environmental factors such as sanitary habits, e.g., use of water for perianal wash (ablution) after defecation that can often lead to contamination of hands with fecal flora that is rich in Gram-negative bacteria (Ramakant *et al.*, 2011). In our study *E. coli* and *S. aureus* are the most frequently isolated of gram negative and positive bacteria followed by *K. pneumoniae and proteus* and this is similar to (Kaimkhani *et al.*, 2018 and Albadri, 2021).

Also similar result was found by Qadir *et al*. who showed that S. aureus was the most prevalent gram-positive bacteria and *E. coli* was the prevalent gram-negative bacteria responsible for DFUs (Qadir *et al.*, 2020 and Selvarajan *et al.*, 2021). Additionally, similar result were documented by (shaya AL-Hemedawi *et al.*, 2006 and Sekhar *et al.*, 2014) in Iraq and India, respectively. Inversely, a study from Pakistan documented a bacterial profile of *Staphylococcus aureus* (25%), followed by *Pseudomonas aeruginosa* (18.18%), *Escherichia coli* (16.16%) by (Chaudhry *et al.*, 2016). The variation in bacterial profiles isolated from patients with DFUs could be attributed to variation in sample collection method, geographical region, treatment therapy and severity of infection (Al-Rubeaan *et al.*, 2015).

Regarding Mono-microbial infection versus polymicrobial infections,the present study demonstrated that 56.72% of DFU patients revealed polymicrobial infections (isolation of two or more of bacteria), similarly (Kaimkhani *et al.*, 2018, Al Benwan *et al.*, 2012) who documented that 83% and 75% of patient had poly microbial infection, respectively. Inversely, in study by (Singh *et al.*, 2020), illustrated that 48.57% of sample revealed single

organism, 28.57% revealed two organisms and 15.24% more than 2 organisms, another study done by (Otta *et al.*, 2019) that found, 62.2% of wound cultures had monomicrobial growth and 27.1% had polymicrobial growth. These findings could be explained based on the duration of DFIs, ulcer grade and empirical antimicrobial agents. Furthermore, in the first episode of diabetic foot infections, the monomicrobial etiologies arise as common infections, and with the time progress, the polymicrobial infections emerge significantly (Hassan *et al.*, 2019).

Mono-bacterial infections are easy to manage as compared to poly-bacterial infections (Citron *et al.*, 2007). Poly-bacterial infections are difficult to cure because these bacteria form biofilms and impede the activity of antibiotics, release virulence factors that increase inflammation and act synergistically to cause a chronic wound infection (Lipsky *et al.*, 2013). The microbiological yield of diabetic foot wounds varies according to the extent of infection and foot involvement. The superficial diabetic foot infections are usually secondary to aerobic gram-positive cocci. However, the ulcers which are deep, chronic, or previously managed with antibiotics are more likely to be polymicrobial. Such wounds may bear the Enterococci, Enterobacteriaceae, Pseudomonas aeruginosa and anaerobes in addition to commonly seen organisms in diabetic foot (Senneville *et al.*, 2006).

4.2.6. Distribution of Isolated Bacteria according to gender and participant groups:

Overall, gram-negative bacteria (61.19%) were predominantly isolated (41/67) compared to gram-positive isolates (38.81%). This finding is in agreement with an earlier study done in the same study site, where gram-negative bacteria were isolated in 88.55% (54/61) versus 7% (11.47%) gram-positive bacteria (15). Similarly, a study from Egypt reported 56% gram-

negative and 27.7% gram positives, while 79% gram-positive and 21% gram negatives were isolated in northeast India (Ismail *et al.*, 2021 and Dwedar *et al.*, 2015).

In the present study, *S. aureus* was the predominant isolate 76.93% (20), unlike a previous study in Ethiopia which reported, *Klebsiella species* 23.9% (22/92) as the predominant bacteria followed by *Proteus species* 18.47% (17/92) (Amogne *et al.*, 2011). In Egypt, *P. mirabilis* (16.8%) is the most common isolate (Dwedar *et al.*, 2015), in Saudi Arabia Pseudomonas species 15.6% (n = 134) (27), and in South America Pseudomonas species (18.8%)was the most common isolate (28).

Similarly in agreement with studies in Kenya 17.5% (14/80 (Al Ayed *et al.*, 2018), and in Iran 28% (n=92) (Akhi *et al.*, 2015). A recent study also reported that the growth rate was 81.7% (98/120), and no growth of 22% (18.34%), respectively (Ismail *et al.*, 2021) in accordance with our study with negative growth results was 3(4.29%) and positive growth results 67 (95.71%).

This variation may be due to the sample size differences of the different studies and other unique characteristics of each study site. This shows that the predominant bacteria causing DFU infections could vary in different settings.

The current study confirmed that both gram-positive and gram-negative aerobic pathogenic bacteria cause DFU infection in the study sites and can lead to more complications such as osteomyelitis, and possibly amputation of the limbs.

Conclusions

The study concluded the following points:

1. There is increase in inflammatory state in diabetic patients (T1DM and T2DM) regardless foot ulcers.

2. The DFU of all diabetic patients type 1 & 2 illustrated higher serum levels of interleukins 37, 38 & 17A compared to non-DFU group especially in type 2 diabetic groups so that might be using them as novel predictive targets for preventing and treating DFU and possible prevention of further damage as well as had a good diagnostic utility for differentiate diabetic patients with or without DFU from control group.

3. The study markers (IL-37, IL-38 and IL-17A) had a direct positive proportion with duration of DFU, IL-37 & IL-17A also directly proportionate with duration of disease.

4. Based on the bacteriological profile of diabetic foot ulcers, ***S. aureus*** among the gram-positive isolates and ***E. coli*** among the gram-negative isolates were the predominant pathogens.

Recommendations

1. More multicentric studies with a larger number of patients and longer duration are highly suggested for further assessment of the value of markers IL- 37, IL-38 and IL-17A in the prediction of DFU and other diabetes mellitus complications.

2. Further studies are needed for isolation and identification of another microorganism such as anaerobic bacteria, fungi and virus.

3. Patients with DFUs should have microbial swabs for culture and better- quality methods of antimicrobial susceptibility testing are required in Iraqi hospitals for correct management and antibiotic stewardship.

4. Using of molecular approaches such as Multilocus sequence typing test (MLST) which are more reliable than traditional method in the study the source DFUs pathogenic bacterial profile.

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Appendix 1: Questionnaire

Case No.:

Name: Age: gender:

Residency: Rural: Urban:

Family history of DM: Type1 Positive…….. Negative…….

Type2 Positive…….. Negative…….

Type of Rx: Oral…… insulin……. Mixed……

Duration of diabetes mellitus

Smoking Hx: Yes ……. No ……..

Usage of other drugs: Yes…… No……

Current Inflammation & infection Yes…… No……

CVD Yes…… No……

Immunosuppressive therapy Yes…… No……

Multiple autoimmune disease Yes…… No……

Pregnancy Yes…… No……

Renal failure Yes…… No……

Liver failure Yes…… No……

Recent history of trauma to lower limb Yes…… No……

Past medical history:

Diabetic foot ulcer Positive…….. Negative…….

Duration of DFU:

Appendix 2

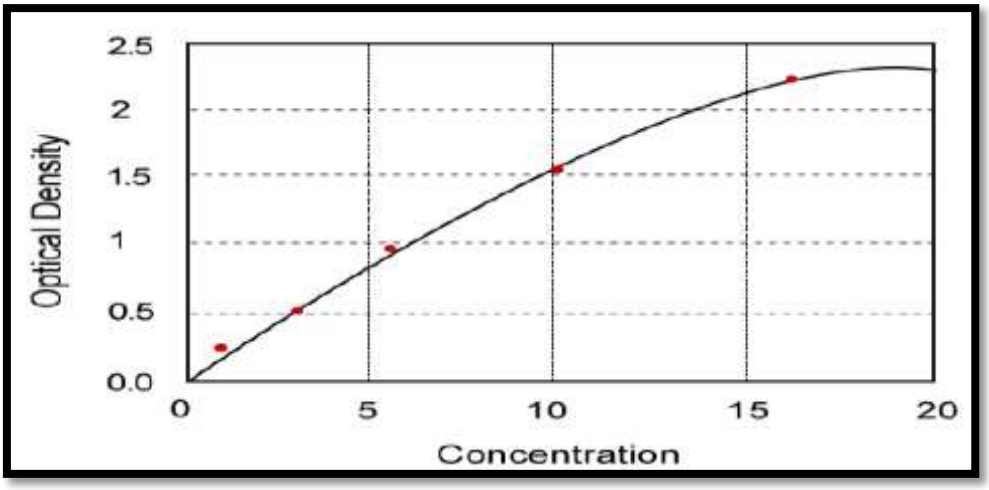

Figure (1): The standard curve of IL-38.

pg/mL	OD	Corrected OD	Standard Curve
1000	2.376	2.315	
500	1.697	1.636	
250	0.978	0.917	
125	0.476	0.415	
62.5	0.233	0.172	
31.25	0.162	0.101	
15.63	0.113	0.052	
0	0.061	-	

Figure (2): The standard curve of IL-37.

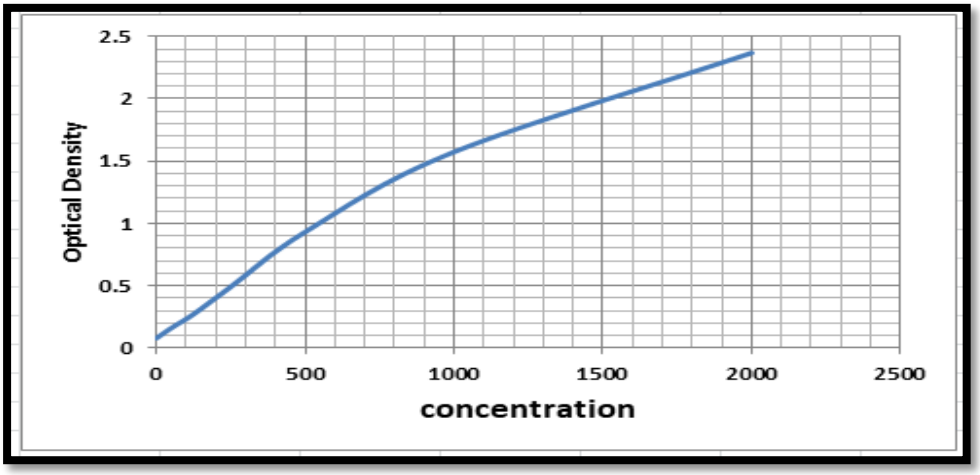

Figure (3): The typical standard curve of IL-17A.

Appendix (27

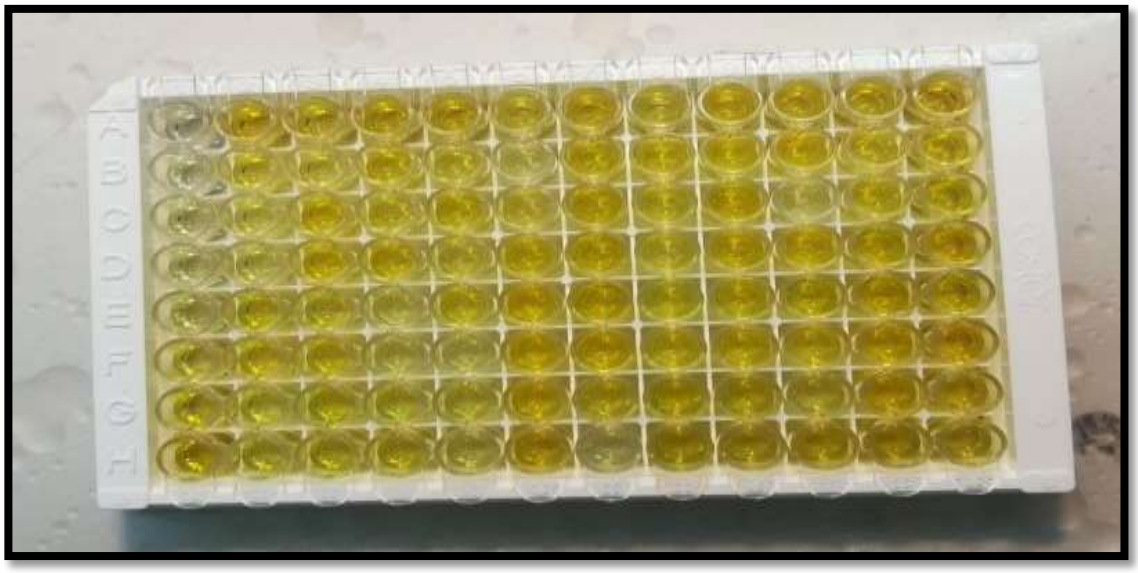

Figure (4): Microtiter plate final reaction (after adding the stop solution).

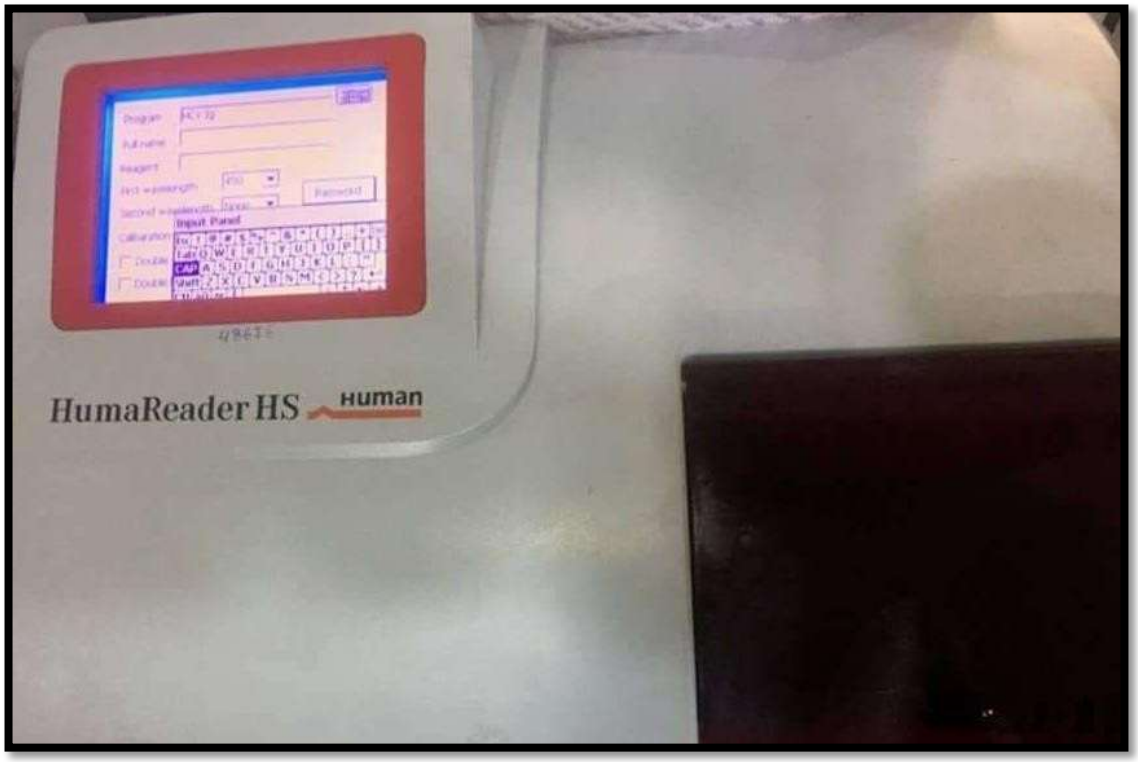

Figure (5): ELISA Human Reader system.

Appendix (28

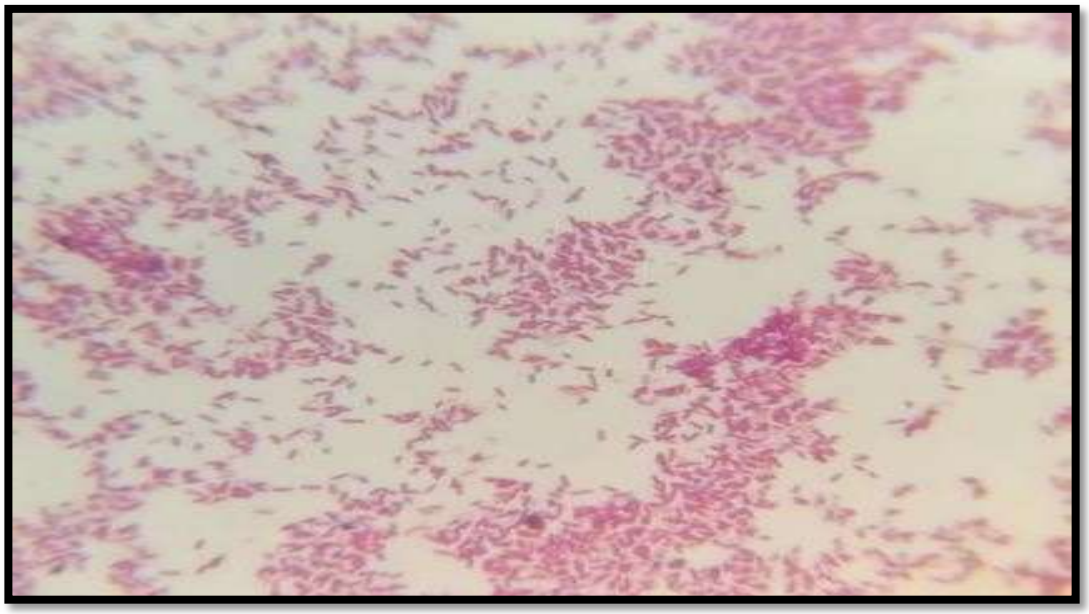

Figure (5): A Gram's negative *Escherichia coli* isolate under microscope (40X).

Figure (6): The *Escherichia coli* Isolate on MacConkey agar Lactose fermenter.

Appendix (29

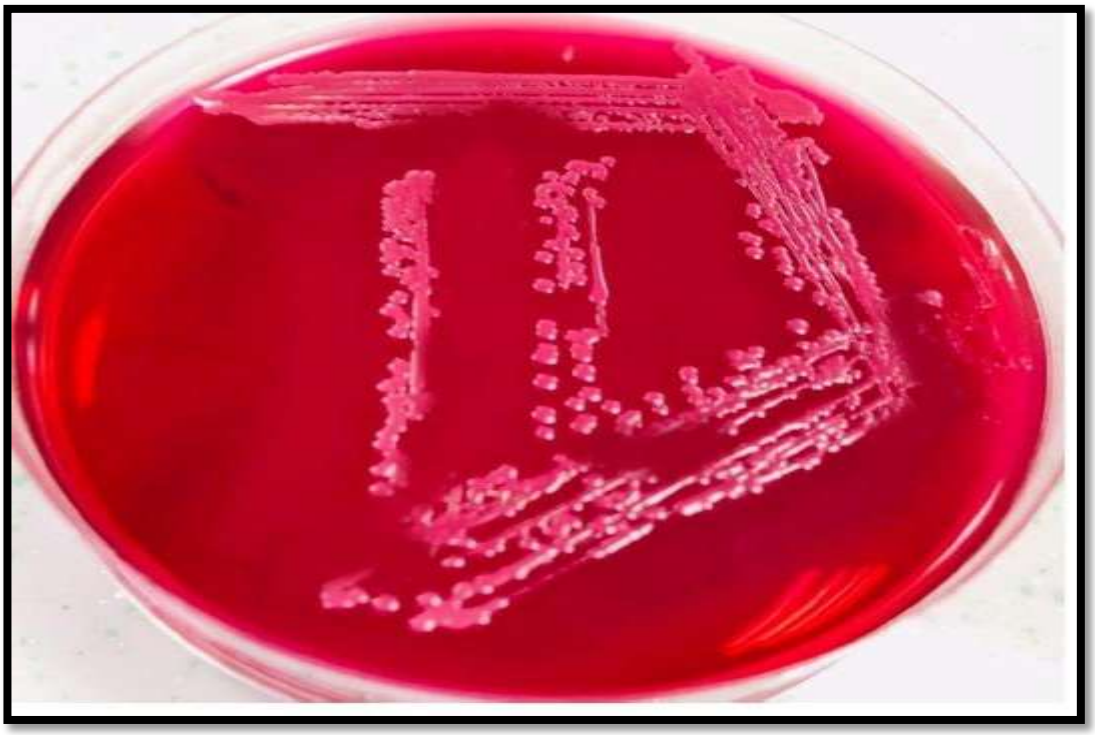

Figure (7): The culture of *E. coli* Isolate on MacConkey agar lactose fermenter (pink shiny colonies).

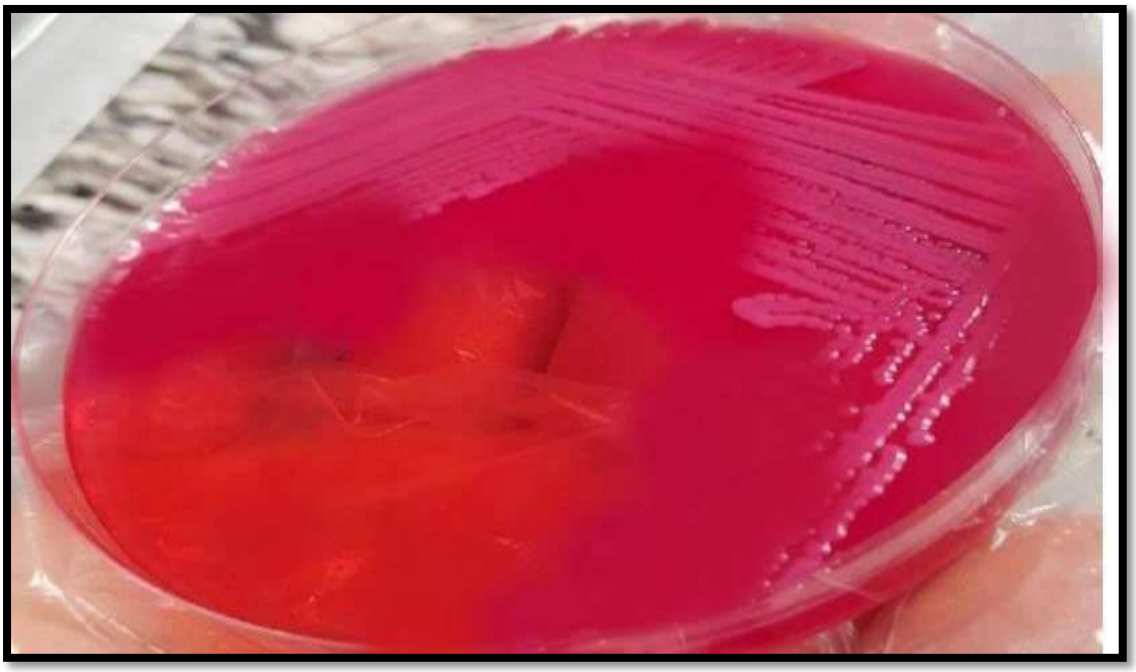

Figure (8): The culture of *k. pneumoniae* Isolate on MacConkey agar lactose fermenter (pink mucoid shiny colonies).

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Appendix (30

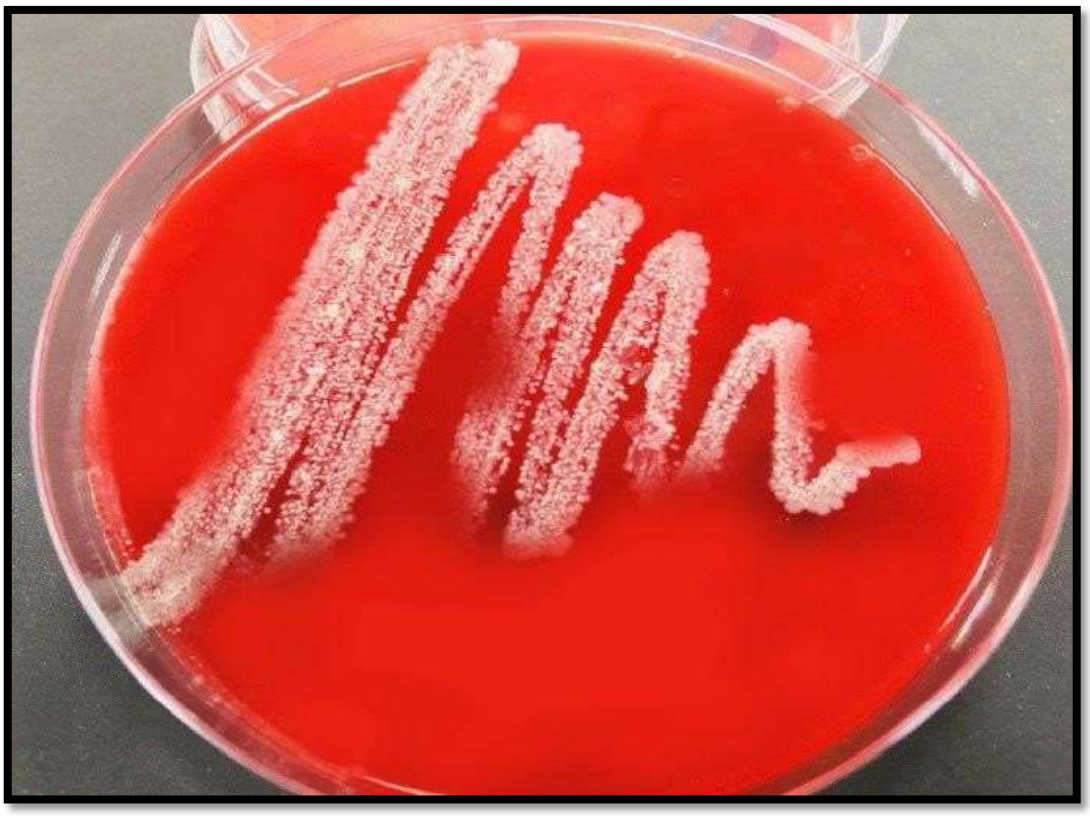

Figure (9): The culture of *S. aureus* Isolate on Blood agar.

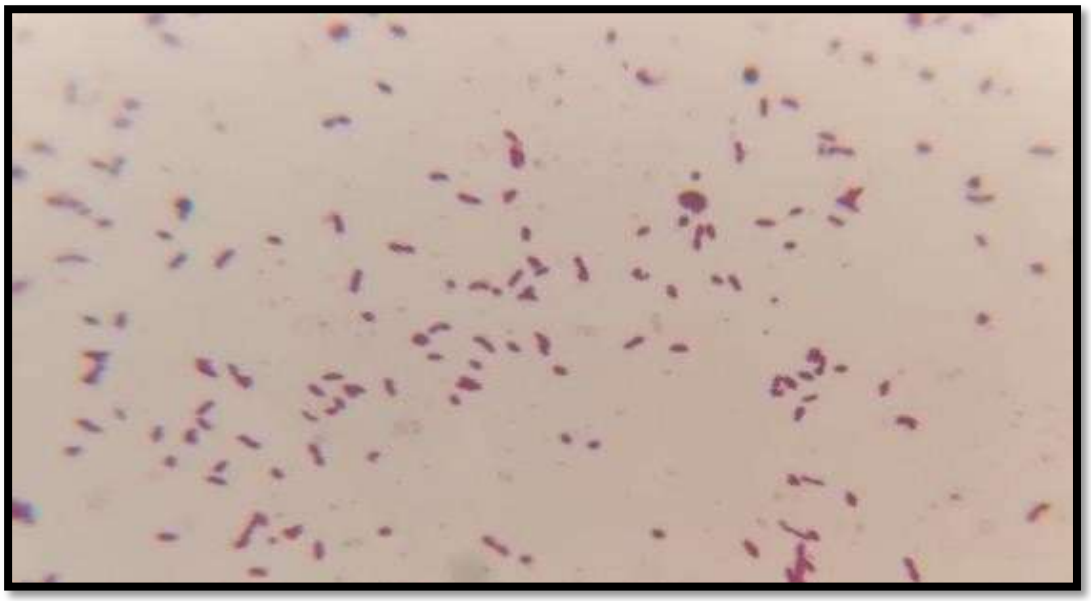

Figure (10): The *Acinetobacter* Isolate image under microscope (40X).

Appendix (31

Figure (11): *The Acinetobacter* Isolate on MacConkey agar.

1. VITEK 2 compact system was performed following the manufacturer's instructions by several steps as the following:

1. The study isolates that reveal positive result in culturing on MacConkey agar, Mannitol salt agar and blood agar and incubated at 25°C for 24 hours after the incubation a slide of the isolates were stained by gram's stain to verify the gram-negative reaction besides checking the purity of our isolates.

2. By using a sterile swabs 1-2 colony taken from the culture grown on blood agar 24 hours and suspended in special clear plastic polystyrene test tube (a 12x 75 mm) of manufacturer company containing 3 ml sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) supplied by the company and mixed by vortex.

3. The turbidity of the bacterial suspension was adjusted according to the turbidity of standard table turbidity range via turbidity meter (DensiChek TM) supplied by the company in which the final concentration of the suspension

should be prevailed between 0.5-0.63 which is required for GN Identification card.

4. Inoculation of GN Identification card; the ID card was warmed to room temperature, the test tube containing the microorganism suspension is placed into a special rack (cassette) and the identification card is placed in the neighboring slot while inserting the transfer tube into the corresponding suspension tube. The filled cassette is placed into filling chamber (a vacuum chamber station). After the vacuum is applied and air is re-introduced into the station, the organism suspension is forced (by negative pressure) through the transfer tube into micro-channels that fill all the test wells after finishing the operator given blue signal.

5. Bar Code Scanner for the ID cards was done for Data Entry.

Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the incubator chamber. All card types are incubated on-line at 35.5 + 1.0ºC. Each card is removed from the carousel incubator once every 15 minutes, transported to the optical system for reaction readings (measure either turbidity or colored products of substrate metabolism), and then returned to the incubator until the next read time. Data are collected at 15-minute intervals during the entire incubation period. The test data were compared to the respective database in the computer attached to the VITEK 2 operator system.

Appendix (8)

Figure (12): The IL-38 ELIZA kit.

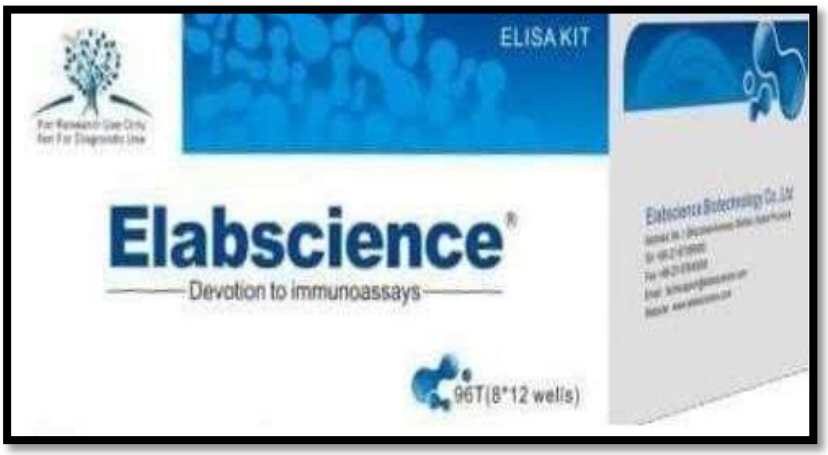

Figure (13): The IL-17A ELIZA kit.

Figure (14): The IL-37 ELIZA kit.

الخلاصة

داء السكري هواحد الأسباب الرئيسية للوفيات في جميع أنحاء العالم ،ممكن ان يؤدي داء السكري إلى العديد من المضاعفات الثانوية ، بما في ذلك متلازمة القدم السكري ، والتي تحدث بسبب الاعتلال العصبي ونقص التروية الدموية. يعاني ما يقرب من 15٪ من مرضى السكري من مضاعفات القدم السكري ، مما يؤدي إلى ارتفاع معدلات الإصابة بالعدوى البكتيرية. 25٪ من الاشخاص المصابين بالقدم السكري معرضون لخطر بتر الأطراف السفلية بسبب ضعف التئام الجروح. كشفت الدراسات السابقة أن الاستجابة المناعية والعمليات الالتهابية تلعب دورًا مهمًا في التسبب في امراضية داء السكري.

اجريت الدراسة لمدة 6 أشهر ابتداء من آب / 2022 الى كانون الثاني/2023. بلغ العدد الإجمالي للمشاركين في الدراسة 193 شخصًا؛ تم تقسيمهم إلى ثلاث مجموعات: المجموعة الأولى تضم مجموعة مرضى السكري من النوع الأول (29 مصابًا بقرحة القدم السكرية + 35 غير مصابًا بالقدم السكري) ، المجموعة الثانية تشمل مجموعة مرضى السكري من النوع 2 (41 مصابًا بقرحة القدم السكرية + 38 غير مصابًا بقرحة القدم السكرية) والمجموعة الثالثة تضم (50) شخصا كمجموعة سيطرة تخضع للمراقبة الصحية. تم استخدام تقنية الاليزا (ELISA) لتحديد مستويات ال IL-37 و IL-38 و IL17A في عينات مصل الأشخاص في مجموعات الدراسة. وتم أخذ مسحات من المرضى المصابين بقرحة القدم السكري لغرض تشخيص مسببات العدوى البكتيرية للبكتريا الهوائية باستخدام الطرق المختبرية التالية لتشخيصها (من خلال زراعتها على الاوساط الزرعية المختلفة وتشخيصها و تشخيصها تحت المجهر بعمل صبغة كرام ومن ثم استخدام جهاز الفايتك لتشخيص المسببات البكتيرية المتواجدة في منطقة الإصابة على مستوى النوع ويعتبر طريقة تأكيدية لغرض التشخيص).

أوضحت نتائج الدراسة الحالية أن هناك فروق ذات دلالة إحصائية بين المجموعات الثلاث المدروسة فيما يتعلق بالعمر ومدة الاصابة بالسكري. كما كانت هناك فروق ذات دلالة إحصائية عالية في FBS، HBA1c ، الكوليسترول ، HDL، LDL والدهون الثلاثية. بالإضافة إلى ذلك ، كانت هناك اختلافات معنوية في مستويات CRP و PLT و Lymphocytes و Neutrophil و ESR بين المجموعات الثلاث المدروسة. في حين بينت النتائج بعدم وجود فروق ذات دلالة إحصائية في مستويات ال Hb و WBCs.

علاوة على ذلك ، أظهرت الدراسة الحالية أن مستويات IL-37 و IL-17A كانت ذات دلالة أحصائية عالية ($P < 0.01$) في جميع المجاميع لمرضى السكري قيد الدراسة بالمقارنة مع مجموعة السيطرة. بينما أظهرت النتائج بأن مستويات IL-38 كانت ذات دلالة معنوية في مرضى السكري من النوع الاول والثاني مقارنة مع الاشخاص الاصحاء. بالإضافة إلى ذلك ، أوضحت مجموعة قرحة القدم السكري DFU في النوع الثاني من السكري T2DM مستويات مرتفعة لكل من IL-37 و IL-38 و IL-17A اذا ما قورنت مع مجاميع السكري الاخرى.

أظهرت نتائج الفحص البكتريولوجي للمسحات المأخوذة من القدم المصاب ان أعلى معدل للإصابة بقرح القدم السكرية في المرضى الذين تتراوح أعمارهم بين (50-59) سنة. من بين 70 عينة تم جمعها ، كانت 67 عينة موجبة للنمو الميكروبي و 3 عينات لم تظهر أي نمو. من بين 67 عينة إيجابية الزرع

الميكروبي ، تم العثور على عدوى أحادية المسبب البكتيري في 29 مريضًا ، وتم العثور على عدوى متعددة المسبب البكتيري في 38 مريضًا. كان عدد الأنواع البكتيرية إيجابية الجرام 26 ، و سالبة الجرام 41. من بين جميع العزلات التي تم جمعها (n = 70) ، كانت *Staphylococcus aureus* هي أكثر الانواع البكتيرية انتشارًا (20) وكانت *Morganella morganii* هي الأقل شيوعًا (عزلة واحدة فقط). وسجلت الدراسة من البكتيريا سالبة الجرام*Escherichia coli* 15(36.58٪) هي السائدة ، يليها *K. pneumoniae* 11) (%26.83) ثم *P. mirabilis* 7 (%17.07)، ثم *P.aeruginosa* 4 (%9.76)، و*A.baumannii* 3 (7.32٪) ، وأخيراً *Morganella morganii* 1 (%2.44). في حين أن البكتيريا موجبة الجرام تشمل المكورات العنقودية الذهبية *S. aureus* كانت البكتيريا الأكثر عزلة في هذه الدراسة بعدد 20 ونسبة (76.93٪) ، تليها المكورات العقدية 4(15.38)٪ واخيرا المكورات المعوية بنسبة 2(7.69%). نستنتج من الدراسة الحالية أن بكتيريا *E. coli* هي أكثر المسببات شيوعا لقرح القدم السكري بين المصابين بداء السكري بعد بكتيريا *S. aurus*.

أضف الى ذلك إنها الدراسة الأولى في العراق التي تسلط الضوء على الدور المهم للانترلوكينات الجديدة (IL-37 و IL-38) ودور الانترلوكين (IL 17A) ، وإمكانية استخدامها كاختبارات تشخيصية لمرض السكري ومضاعفاته مثل تقرح القدم السكري.

وزارة التعليم العالي والبحث العلمي
جامعة كربلاء / كلية الطب
فرع الاحياء المجهرية الطبية

دراسة الدور المناعي للانترولكين 37, 38 و 17A في الاشخاص المصابين بقرحة القدم السكري

رسالة مقدمة الى
مجلس كلية الطب وهي جزء من متطلبات نيل شهادة الماجستير في الاحياء المجهرية الطبية
من قبل الطالبة
مروة محمد علي
بكالوريوس علوم حياة (2010)
بأشراف

الاستاذ المساعد الدكتورة
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