1.1 *Introduction*

Blood is a bodily fluid in animals that delivers necessary substances such as nutrients and oxygen to the cells and transports metabolic waste products away from those same cells; it is composed of blood cells suspended in blood plasma (The Franklin Institute, 2009), about 55% of blood is blood plasma, a fluid that is the blood's liquid medium, which by itself is straw-yellow in color. In average the blood plasma volume totals of 2.7–3.0 liters. It is essentially an aqueous solution containing 92% water, 8% blood plasma proteins, and trace amounts of other materials. Plasma circulates dissolved nutrients, such as glucose, amino acids, and fatty acids (dissolved in the blood or bound to plasma proteins), and removes waste products, such as carbon dioxide, urea, and lactic acid (O'Neil, 1999).

The blood cells include three main types, the first type are erythrocyte (red blood cells), leucocytes (white blood cells) and thrombocytes (platelets) (Medical Encyclopedia, 2007). Red blood cells (RBCs) contain the blood's hemoglobin and distribute oxygen, mature RBCs lack a nucleus and organelles in mammals. The red blood cells (together with endothelial vessel cells and other cells) are also marked by glycoproteins that define the different blood types. The proportion of blood occupied by red blood cells is referred to as the hematocrit, and is normally about 45%. The combined surface area of all red blood cells of the human body would be roughly 2,000 times as great as the body's exterior surface (Tallitsch et al., 2006), the second type of cells is leukocytes (white blood cells) are part of the body's immune system; they destroy and remove old or aberrant cells and cellular
debris, as well as attack pathogens and foreign substances. There are five types of leucocytes, these are neutrophils, lymphocytes, monocytes, eosinophils and basophil, the third type is thrombocytes (platelets) are take part in blood clotting (coagulation) (Ganong and William, 2003).

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period, symptoms of high blood sugar include frequent urination, increased thirst, and increased hunger. Untreated diabetes can cause many complications (WHO, 2014), diabetes is due to either the insufficient insulin produced by pancreas cells or the improperly response to the insulin produced (Shoback, 2011).

In medicine neutrophil to lymphocyte ratio (NLR) is used as a marker of subclinical inflammation. It is calculated by dividing the number of neutrophils by number of lymphocytes, usually from peripheral blood sample, but sometimes also from cells that infiltrate tissue, such as tumor (Wang, 2014).
1.2 Literature review

1.2.1 White blood cells

Leucocytes, are the cells of the immune system that are involved in protecting the body against both infectious disease and foreign invaders. All leukocytes are produced and derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell. Leukocytes are found throughout the body, including the blood and lymphatic system (Maton et al., 1997).

1.2.2 White blood cells (WBCs) classification

All white blood cells are nucleated but are otherwise distinct in form and function. White cells are best classified into two major lineages; the myeloid leukocytes and the lymphoid leukocytes; Myeloid lineage include neutrophils, monocytes, eosinophils and basophils, while the lymphocytes include T cells, B cells and natural killer cells (Orkin and zon, 2008).

1.2.2.1 Neutrophil

Are the most abundant white blood cell, constituting 60-70% of the circulating leukocytes (Alberts et al., 2002), They defend against bacterial and fungal infections. They are usually first responders to microbial infection. And commonly referred to as polymorphonuclear (PMN) leukocytes, although, in the technical sense, PMN refers to all granulocytes. They have a multi-lobed nucleus, which consists of three to five lobes connected by slender strands (Saladin and Kenneth, 2012), this gives the neutrophils the appearance of having multiple nuclei, hence the name polymorphonuclear leukocyte. The cytoplasm may look transparent because of fine granules that are pale lilac when stained. Neutrophils are active in phagocytosing bacteria and are present in large amount in the pus of wounds. These cells are not able to renew their lysosomes (used in digesting microbes) and die after having phagocytosed a few pathogens (Wheater et al., 2002). Neutrophils are the most common cell type seen in the early stages of acute inflammation. The
life span of a circulating human neutrophil is about 4-5 days (Pillay et al., 2010).

1.2.2.2 Eosinophil

Eosinophils compose about 2-4% of the WBCs. This count fluctuates throughout the day, seasonally, and during menstruation. It rises in response to allergies, parasitic infections, collagen diseases, and disease of the spleen and central nervous system. They are rare in the blood, but numerous in the mucous membranes of the respiratory, digestive, and lower urinary tracts; They primarily deal with parasitic infections. They secrete chemicals that destroy these large parasites, such as hook worms and tapeworms, that are too big for any one WBCs to phagocytize. In general, their nucleus is bi-lobed and connected by a thin strand, the cytoplasm is full of granules that assume a characteristic pink-orange color with eosin staining (Saladin and Kenneth, 2012).

1.2.2.3 Basophil

Basophils are chiefly responsible for allergic and antigen response by releasing the chemical histamine which causing the dilation of blood vessels. Because they are the rarest of the WBCs (less than 0.5% of the total count) and share physicochemical properties with other blood cells, they are difficult to study (Franco et al., 2000). They can be recognized by several coarse, dark violet granules, giving them a blue hue. The nucleus is bi- or tri-lobed, but it is difficult to see due to the number of coarse granules that hide it; They excrete two chemicals that aid in the body's defenses, histamine and heparin. Histamine is responsible for widening blood vessels and increasing the flow of blood to injured tissue. It also makes blood vessels more permeable so neutrophils and clotting proteins can get into connective tissue more easily. Heparin is an anticoagulant that inhibits blood clotting and promotes the movement of white blood cells into an area. Basophils can also release chemical signals that attract eosinophils and neutrophils to site of infection (Saladin and Kenneth, 2012).
1.2.2.4 Monocyte

Monocytes are the largest of leukocyte cells. They are part of the innate immune system of vertebrates including all mammals (humans included), birds, reptiles, and fish. They are amoeboid in shape, having a granulated cytoplasm (containing azurophil granules), and have unilobar nuclei, which makes them one of the types of mononuclear leukocytes. They play multiple roles in immune function. Such as replenishing resident macrophages under normal states and response to inflammation signals, monocytes can move quickly (approximately 8–12 hours) to sites of infection in the tissues and differentiate into macrophages and dendritic cells to elicit an immune response. Half of them are stored in the spleen (Swirski et al., 2009).

1.2.2.5 Lymphocyte

Lymphocytes are much more common in the lymphatic system than in blood. Lymphocytes are distinguished by having a deeply staining nucleus that may be eccentric in location, and a relatively small amount of cytoplasm. Lymphocytes include B cells which produce antibodies that can bind to pathogens, block pathogen invasion, activate the complement system, and enhance pathogen destruction. And T cells (CD4+ helper T cells, CD8+ cytotoxic T cells and γδ T cells) T cells displaying co-receptor CD4 are known as CD4+ T cells. These cells have T-cell receptors and CD4 molecules that, in combination, bind antigenic peptides presented on major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (Robert et al., 2003). Helper T cells make cytokines and perform other functions that help coordinate the immune response. T cells displaying co-receptor CD8 are known as CD8+ T cells. These cells bind antigens presented on MHC I complex of virus-infected or tumor cells and kill them. Nearly all nucleated cells display MHC class I. γδ T cells possess an alternative T cell receptor (different from the αβ TCR found on conventional CD4+ and CD8+ T
cells). Found in tissue more commonly than in blood, γδ T cells share characteristics of helper T cells, cytotoxic T cells, and natural killer cells (O’Neil, 1999).

**Natural killer cells** are able to kill cells of the body that do not display **MHC class I** molecules, or display stress markers such as **MHC class I polypeptide-related sequence A** (MIC-A). Decreased expression of MHC class I and up-regulation of MIC-A can happen when cells are infected by a virus or become cancerous (Charles *et al.*, 2001)

### 1.2.3 WBCs disorders

There are two types of WBCs disorders, benign and malignant.

#### 1.2.3.1 Benign leucocyte disorders

These can be numerical, functional or morphological. Numerical leucocyte disorder causes deviation of the number from normal range (4-11×10⁹/L) either increase (leukocytosis) or decrease (leucopenia). Functional leucocyte disorder may be acquired or congenital. Morphological leucocyte disorder include nucleus abnormalities and cytoplasmic abnormalities (Hoffbrand and Moss .,2010).

#### 1.2.3.2 Malignant leucocyte disorders

These are Leukemia, lymphoma and plasma cell myeloma. Leukemia is the malignant proliferation of the WBC, with the presence of immature forms in the peripheral circulation. Lymphoma tends to localize in lymph tissues. Often disseminated to other sites at diagnosis. Characterized by malignant cells found in lymphoid cells. Plasma cell myeloma is the malignant transformation of B-cell plasma cells. Likes to form tumors in bony structures (McPhee *et al.*, 2003)

#### 1.2.3.3 Leukocytosis

Leukocytosis is white blood cells (the leukocyte count) above the normal range in the blood. It is frequently a sign of an inflammatory response (*Porth and Mattson, 2011*), most commonly the result of infection, but may also occur
following certain parasitic infections or bone tumors. It may also occur after strenuous exercise, convulsions such as epilepsy, emotional stress, pregnancy, labour, anesthesia, and epinephrine administration (Rogers and Kara, 2011). Neutrophilic leukocytosis (neutrophilia) can be due to either bacterial infection (especially pyogenic infection) or sterile inflammation (tissue necrosis, myocardial infarction and burns). Lymphocytosis can be due to chronic infection, viral infection, pertussis and some forms of malignancy such as lymphocytic leukemia. Eosinophilic leukocytosis (eosinophilia) can be due to allergic disorders, parasitic infections, some forms of malignancy, systemic autoimmune diseases, some forms of vasculitis and cholesterol embolism. Monocytosis can be due to chronic infections, systemic autoimmune diseases and inflammatory bowel diseases. Basophilic leukocytosis (basophilia) is rare but may be seen in myeloproliferative disease (Zorc and Joseph, 2011).

1.2.3.4 Leukopenia

Leukopenia (also known as leukocytopenia or leucopenia) is a decrease in the number of white blood cells (leukocytes) found in the blood, which places individuals at increased risk of infection. The causes of leucopenia are either due to medical condition or medication. Medical condition (low white blood cell count may be due to acute viral infection, human immunodeficiency virus, systemic lupus erythematosus, Hodgkin's lymphoma, some types of cancer, typhoid, malaria, tuberculosis, dengue, rickettsial infections, enlargement of the spleen, folate deficiencies, psittacosis, sepsis and Lyme disease deficiency in certain minerals (such as copper and zinc) (Nicholson et al., 2010). Medication (Some medications can have an impact on the number and function of WBCs include clozapine, antipsychotic, the anticonvulsant drug, lamotrigine, immunosuppressive drugs such as sirolimus, mycophenolate mofetil, tacrolimus, cyclosporine, Leflunomide (Arava) and TNF inhibitors,
Interferons used to treat multiple sclerosis and chemotherapy (Nicholson et al., 2010).

1.2.4 Neutrophil lymphocyte ratio

Neutrophil to lymphocyte ratio (NLR) is a new addition to the long list of the inflammatory markers. NLR, which is calculated from complete blood count with differential, is an inexpensive, easy to obtain, widely available marker of inflammation, Previous studies have demonstrated that leukocyte subtype, and neutrophil/lymphocyte (N/L) ratio are indicators of systemic inflammation (Wang, 2014).

Many epidemiological studies have determined that DM is associated with chronic inflammation (Pitsavos et al 2007), which may contribute to the acceleration of diabetic microangiopathy and the development of macroangiopathy, patients with Type 2 DM are in a state of low-degree chronic inflammation that induces hyper secretion of inflammatory factors, such as CRP, IL-6, TNF-α, and MCP-1, which results in a constantly elevated neutrophilic granulocyte count (Tabák et al 2010). Moreover, the N/L ratio has been revealed to predict cardiovascular mortality and survival in malignancies (Azab et al., 2010), a high neutrophil-to-lymphocyte ratio (NLR) has been reported to be a poor prognostic indicator in cardiovascular disease and several malignancies such as esophageal cancer or pancreatic cancer (Chua et al., 2011), higher NLR is independent predictor of mortality in patients undergoing angiography or cardiac revascularization (Wang , 2014). NLR has been recently defined as a novel potential inflammation marker in cardiac and noncardiac disorders, NLR can easily be calculated using the neutrophil-to-lymphocyte ratio in peripheral blood count. Calculating NLR is simpler and cheaper than measuring other inflammatory cytokines, such as IL-6, IL-1β and TNF-α (Bhutta, 2011)

Recent studies have shown that NLR as microvascular complications of diabetes is
related with the presence of nephropathy and it has been correlated as an indicator of end stage renal failure with albuminuria levels (Afsar, 2013)

1.2.5 Inflammation

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (Miliani et al., 2007). Inflammation is a protective response that involves immune cells, blood vessels, and molecular mediators. The purpose of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair, the classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function; inflammation is a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen (Abbas and Lichtman, 2009). Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues; prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (Werner, 2009).

1.2.6 Diabetes mellitus

1.2.6.1 Definitions

Diabetes mellitus (sometimes called "sugar diabetes") is a condition that occurs when the body can’t use glucose (a type of sugar) normally. Glucose is the main source of energy for the body’s cells. The levels of glucose in the blood are controlled by hormone called insulin, which is made by the pancreas. Insulin helps glucose enter the cells (WHO, 2006).
1.2.6.2 Types of diabetes mellitus

There are three main types of diabetes mellitus

1.2.6.2.1 Type 1 diabetes mellitus

Type 1 DM results from the pancreas's failure to produce enough insulin. This form was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes". The cause is unknown (Rother, 2007).

1.2.6.2.2 Type 2 diabetes mellitus

Type 2 DM begins with insulin resistance, a condition in which cells fail to respond to insulin properly. As the disease progresses a lack of insulin may also develop. This form was previously referred to as "non-insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". The primary cause is excessive body weight and not enough exercise (Risérus et al., 2009).

1.2.6.2.3 Gestational diabetes

Gestational diabetes is the third main form and occurs when pregnant women without a previous history of diabetes develop a high blood-sugar level (Kitabchi, 2009).

1.2.6.3 Complications of diabetes mellitus

Are far less common and less severe in people who have well-controlled blood sugar levels (Nathan et al., 2005).

1.2.6.3.1 Acute complication

1.2.6.3.1.1 Diabetic ketoacidosis

Diabetic Ketoacidosis (DKA) is an acute and dangerous complication that is always a medical emergency. Low insulin levels cause the liver to turn fatty acid to ketone. Elevated levels of ketone bodies in the blood decrease the blood's pH, leading to DKA, diabetic ketoacidosis is much more common in type 1 diabetes than type 2 (Deshpande et al., 2008)
1.2.6.3.1.2 **Hyperglycemia**

Hyperosmolar nonketotic state (HNS) is an acute complication sharing many symptoms with DKA, but an entirely different origin and different treatment. A person with very high blood glucose levels (usually above than 300 mg/dl), water is osmotically drawn out of cells into the blood and the kidneys eventually begin to dump glucose into the urine. This results in loss of water and an increase in blood osmolarity. If fluid is not replaced, that will eventually lead to dehydration (Ahmed et al., 2008).

1.2.6.3.1.3 **Hypoglycemia**

Hypoglycemia is an acute complication of several diabetes treatments, the patient may become agitated, sweaty, weak, and have many symptoms of sympathetic activation of the autonomic nervous system resulting in feelings akin to dread and immobilized panic. Consciousness can be altered or even lost in extreme cases, leading to coma, seizures, or even brain damage and death. In patients with diabetes, this may be caused by several factors, such as too much or incorrectly timed insulin, too much or incorrectly timed exercise or not enough food. The variety of interactions makes cause identification difficult in many instances. In most cases, hypoglycemia is treated with sugary drinks or food (Mealey, 2006).

1.2.6.3.1.4 **Diabetic coma**

It is a medical emergency in which a person with diabetes mellitus is comatose is comatose (unconscious) due to one of the acute complications of diabetes either Severe diabetic hypoglycemia, Diabetic ketoacidosis or Hyperosmolar nonketotic coma (Lakschevitz, et al., 2011).

1.2.6.3.1.5 **Respiratory infections**

The immune response is impaired in individuals with diabetes mellitus. Cellular studies have shown that hyperglycemia both reduces the function of immune cells and increases inflammation. The vascular effects of diabetes also tend to alter lung
function, all of which leads to an increase in susceptibility to respiratory infections such as pneumonia and influenza among individuals with diabetes (Ahmed et al., 2008).

1.2.6.3.1.6 **Periodontal disease**
Diabetes is associated with periodontal disease (gum disease) and may make diabetes more difficult to treat; Gum disease is frequently related to bacterial infection by organisms such as Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans (Lakschevitz et al., 2011)

1.2.6.3.2 **Chronic complication**
Chronic elevation of blood glucose level leads to damage of blood vessels which can cause series of complication, damage of small blood vessels leads to a microangiopathy, which can cause one or more of diabetic cardiomyopathy, diabetic nephropathy, diabetic neuropathy, diabetic retinopathy and diabetic encephalopathy (Centofani, 1995). **Macrovascular disease** leads to cardiovascular disease, to which accelerated atherosclerosis is a contributor for (Coronary artery disease, Diabetic myonecrosis, Peripheral vascular disease, Or Stroke) (Weiss and Sumpio , 2006). Menstrual irregularities (especially oligomenorrhea), mild hyperandrogenism, polycystic ovarian syndrome and lipohypertrophy (Codner et al, 2012). Increases rates of skin ulcers (diabetic foot ulcers) and infection and, in serious cases, necrosis and gangrene (Scott, 2013).

1.2.7 **Diabetic foot ulcer** (DFU)
Diabetic foot ulcer is a major complication of diabetes mellitus, and probably the major component of the diabetic foot. It occurs in 15% of all patients with diabetes and precedes 84% of all diabetes-related lower-leg amputations (Brem and Canic, 2007).
1.2.7.1 Risk factors
Risk factors implicated in the development of diabetic foot ulcers are diabetic neuropathy, peripheral vascular disease, cigarette smoking, poor glycemic control, previous foot ulcerations or amputations, diabetic nephropathy, and ischemia of small and large blood vessels (Scott, 2013). Diabetic patients often suffer from diabetic neuropathy due to several metabolic and neurovascular factors. Peripheral neuropathy causes loss of pain or feeling in the toes, feet, legs and arms due to distal nerve damage and low blood flow. Blisters and sores appear on numb areas of the feet and legs such as metatarso-phalangeal joints, heel region and as a result pressure or injury goes unnoticed and eventually become portal of entry for bacteria and infection (wu et al., 2007).

1.2.7.2 Aetiopathogenesis
DFU is characterized by a classical triad of neuropathy, ischemia, and infection. Due to the impaired metabolic mechanisms in DM, there is an increased risk of infection and poor wound healing due to a series of mechanisms which include decreased cell and growth factor response, diminished peripheral blood flow and decreased local angiogenesis. Thus, the feet are predisposed to peripheral vascular disease, damage of peripheral nerves, deformities, ulcerations and gangrene (Pendsey, 2010).

1.2.7.2.1 Neuropathy
Neuropathy causes more than 60% of the foot ulcers and affects patients with both type 1 and type 2 DM. Rise in blood glucose levels leads to increased enzyme production such as aldose reductase and sorbitol dehydrogenase. These enzymes convert glucose into sorbitol and fructose. As these sugar products accumulate, the synthesis of nerve cell myoinositol is decreased, affecting nerve conduction. Furthermore, hyperglycaemia induced microangiopathy leads to reversible metabolic,
immunologic and ischemic injury of autonomic, motor and sensory nerves (Clayton and Elcasy., 2009). This causes a decrease in peripheral sensation and damages the nerve innervations of small muscles of the foot and fine vasomotor.

control of the pedal circulation. When the nerve gets injured, the patient is at a higher risk of getting a minor injury without noticing it until it becomes an ulcer. The risk of developing foot ulcers in patients with sensory loss is increased up to seven-fold, compared to non-neuropathic patients with diabetes (Jeffcoate and Harding, 2003).

1.2.7.2.2 Vasculopathy

Hyperglycemia causes endothelial cell dysfunction and smooth cell abnormalities in peripheral arteries. Endothelial cells synthesize nitric oxide which causes vasodilation and protects the blood vessels from endogenous injury. Hence, in hyperglycemia, there is perturbation of the physiological properties of nitric oxide which usually regulates the endothelial homeostasis, anticoagulation, leukocyte adhesion, smooth muscle cell proliferation and antioxidant capacity. Endothelium-derived vasodilators and nitric oxide are decreased hence leading to constriction of the blood vessels and propensity for atherosclerosis, eventually leading to ischemia. Ischemia can also occur even in the presence of palpable pedal pulses. The microcirculation is also disturbed due to arteriolar-venular shunting, reducing the blood circulation to the area of need. Hyperglycemia in DM is also associated with increase in thromboxane A2 leading to plasma hypercoagulability. Clinically the patient may have signs of vascular insufficiency such as claudication,
night pain or rest pain, absent peripheral pulses, thinning of skin, loss of limb hair etc (Armstrong and Lavery, 1998)

1.2.7.2.3 Immunopathy
Compared to a healthy person’s immune system, diabetics patients is much weaker. So, foot infection in diabetics patients with diabetes is a limb threatening and debilitating condition. The hyperglycemic state causes an elevation of pro-inflammatory cytokines and impairment of polymorph nuclear cell functions like chemotaxis, adherence, phagocytosis and intracellular killing. Also that, high blood glucose is a good medium for the growth of bacteria (Gupta et al., 2007). The predominant organisms in diabetic foot infections are mainly aerobic gram positive cocci like S. aureus and β-hemolytic streptococci but in one research conducted in India, gram-negative aerobes were the common microorganisms in diabetic foot (Lipsky et al., 2004). The soft tissues of foot like plantar aponeurosis, tendons, muscles sheaths and fascia cannot resist infections. Furthermore, several compartments in the foot are interconnected and could not limit the spread of infection from one into another. This soft tissue infection can rapidly spread to the bones, causing osteitis. This a simple ulcer on the foot can easily result in complications such as osteitis/osteomyelitis and gangrene without appropriate care (Gadepalli et al., 2006).

1.2.7.2.4 Neuroarthropathy
Charcot neuroarthropathy (CN) is a chronic painless progressive degenerative arthropathy resulting from the disturbance in sensory innervations of the affected joint. The impairment of the autonomic nervous system due to DM causes an increase in local blood supply and the resting blood flow much higher than in the normal patients (Madan and Pai, 2013). The sudden increase in blood flow causes calcium to dissolve, leading
to osteoclastic activity of the bone and thus damaging the bone. Another theory is that the repetitive minor trauma to the insensate joints leads to fracture and disintegration. The production of proinflammatory cytokines leads to uncontrolled osteolysis in CN. The cytokines such as tumor necrosis factor-α and interleukin-1β increase the expression of receptor activator of nuclear factor-κb (RANKL), which in turn causes maturation of osteoclasts by triggering production of nuclear factor-κb. The hallmark deformity associated with this condition is midfoot collapse, also known as “rocker-bottom” foot. There might be hallux valgus deformity and loose bodies in the joint cavity. The deformities associated with CN also predispose for recurrent ulcerations (Rogers et al., 2011).

1.3 Previous studies
A number of studies were conducted on this topic, one of them was done in Turkey at 2014, where 31 diabetics with ulcers and 31 without ulcer were included. There was no statistically significant difference between groups by means of female/male gender. Furthermore, there was statistically significant differences observed according to HbA1c, serum urea, neutrophil percent, lymphocyte percent and NLR among the 2 groups. Pearson correlation analysis revealed that NLR was correlated with age, serum urea, creatinine, WBC, neutrophil percent, lymphocyte percent and total cholesterol (Kahraman et al., 2014).

Another study showed that the NLR values of the patients with diabetes were significantly higher than those of the healthy controls, and the NLR values of the patients with early-stage diabetics nephropathy (DN) were higher than those of the patients without DN. Logistic regression analysis showed that the risk predictors of DN include NLR, creatinine, total cholesterol, systolic blood pressure, HbA1c and insulin resistance. NLR levels positively correlated with DN (Huang et al., 2015).

Also a study done at 2013 conducted among 104 diabetic patients who had coronary lesion with a diameter stenosis of at least 50% and 64 diabetic patients who had normal coronary anatomy matched with age and sex were retrospectively selected and classified Coronary Artery Disease (CAD) (+) and CAD (-) group respectively. Baseline NLR in two groups was compared. The NLR was higher in CAD (+) group compared to group without CAD (-). C reactive protein (CRP) was higher in CAD (+) group compared to group without CAD (-). Multivariate analysis indicated CRP and NLR were an independent indicator of presence of CAD in diabetic patients (Sahin et al., 2013).

Another study was done at 2015 found that the mean NLR were significantly higher in patients with diabetic retinopathy (DR) compared with patients without DR (p.value=0.02) (Yue et al., 2015).
1.4 Rationale
Neutrophil lymphocyte ratio could be an important measure of systemic inflammation as it is cost effective, readily available and could be calculated easily. Little is known and published about neutrophil lymphocyte ratio and its relationship with prevalent chronic conditions among general population. Therefore, the current study was conducted to investigate the neutrophil lymphocyte ratio as a measure of systemic inflammation in diabetics patients with foot ulcer.
1.5 Objectives

1.5.1 General Objective
To evaluate Neutrophil/Lymphocyte ratio as a marker of systemic inflammation in patients with diabetic foot ulcer.

1.5.2 Specific Objectives
- To determine TWBCs count, absolute lymphocyte count and absolute neutrophil count among study population.
- To compare neutrophil to lymphocyte ratio in diabetic patients with and without foot ulcer.
- To correlate NLR with patients age, demographic data and inflammatory marker CRP.
Materials and Methods

2.1 Study design
This study was prospective and case-control study, carried out in Best care Hospital at Khartoum state during the period from May to September 2015.

2.2 Study population
Sudanese patients with type 2 diabetes mellitus.

2.3 Inclusion criteria
Patients with type 2 diabetes mellitus and approved to participant in this study were included.

2.4 Exclusion criteria
Patients with active infection, inflammation, and malignancy were excluded from the study. Also immunocompromised Patients were also excluded.

2.5 Sample collection
Two and half milliliter(ml) venous blood sample was collected in Ethylene Diamine Tetra Acetic acid (EDTA) container and mixed gently.

2.6 laboratory methods
The sample was analyzed by Sysmex KX-21N automated hematology analyzer for CBC, and for CRP and HbA1c using fluorescence immunoassay (i-CHROMA, Korea).

2.7 Principles of Sysmex KX-21N
Measurement of blood cells (RBCs, WBCs, and platelet). And hemoglobin concentration obtained by aspiration of small volume of well mixed (k2EDTA) blood by sample probe and mixed with isotonic diluents in nebulizer. Diluted aspiration delivered to RBCs aperture bath for providing information about RBCs and platelet. Other portion of aspirated sample induced in to WBCs bath in which hemolytic reagent (stromatolyzer) added to break down (RBCs) and release of
hemoglobin which measured in build colorimeter, based in cyanomethemoglobin method (HICN). The through three sensing apertures for each cell type, cell counted and size information generated in triplicate pulses acting to electronic conductively. Mentioned pulses converts in to digital number using in build calculator 25 programmed and designed for RBCs, WBCs counts. Some portion of diluted sample delivered to in build hemoglobin meter at the same time, hence three values directly measured (RBCs, WBCs, Hb) and displayed on (LCD). Other values of red cell indices, leukocyte differential and absolute count calculated from given information, the result printed out according to the setting mode.

On the other hand, platelet count and histogram determined from pulses acting to size of the platelet (Dacie and lewis, 2006).

2.8 Principle of HbA1c

Is based on the fluorescence immunoassay technology, specifically the competition immune-detection method. Whole blood is added to the mixture of hemolysis buffer and detection buffer, which results in hemolysis of red blood cells. The mixture containing HbA1c from the hemolyzed red blood cells and fluorescence-labeled HbA1c peptides from detection buffer is loaded onto the sample well of the Cartridge. The mixture then migrates through the nitrocellulose matrix of the test strip by capillary action. HbA1c from the blood competes with fluorescence-labeled HbA1c peptides for binding sites on HbA1c antibodies fixed on the nitrocellulose matrix. As a result, the higher concentration of HbA1c produces a lower fluorescence signal from HbA1c-peptides. The signal is interpreted and the result displayed on i-CHROMA™ reader in units of percentage.
2.8.1 Test procedure

1. A Test Device was placed on a clean, dust-free flat surface.
2. ID chip well be checked and inserted into the instrument. Make sure that Test Device lot # matches ID chip lot #.
3. A Detector Buffer vial was been taken out from the refrigerator and leaved at room temperature for 20 minutes.
4. 100 μL of Detection Buffer was taken from the vial, and puted into the pre-dispensed Hemolysis Buffer tube and mixed well.
5. A fingertip Pricked and blood sample prepared in a test tube.
6. 5 μL of Whole Blood was drawn with a glass capillary.
7. The glass capillary was putted into Hemolysis Buffer tube and shaken the tube up and down ten times to taken the blood out of the capillary.
8. 50 μL of the mixture was applied onto the sample well and 100 μL onto the Hb sample well of Test Device (refer to the picture above).
9. Test Device was leaved at room temperature for 12 minutes before inserted the device into the holder.
10. To start scanning, test Device was inserted onto the holder of i-CHROMA Reader and pressed “SELECT” button. The direction of Test Device was checked.
11. The instrument was automatically started to scan Test Device immediately.
12. The results was read on the display screen of i-CHROMA Reader or the printer output.

2.9 Principle of CRP

The test uses a sandwich immunodetection method, such that the detector antibody in buffer binds to CRP in sample and antigen – antibody complex are captured to another CRP antibody that has been immobilized on test strip as sample mixture migrates nitrocellulose matrix. Thus the more CRP antigen in sample, the more antigen-antibody complex accumulated on the test strip. Signal intensity of
fluorescence on detector antibody reflects the amount of antigen captured and is processed by ichroma™ reader to show CRP concentration in specimen.

2.9.1 Test Procedure
1. The plasma was prepared, a puncture was made on the top of the detector tube by inserted an empty blood collection capillary.
2. The sample was drawn with the blood collection capillary, the capillary and tube were assembled in to one, and the assembled tube was shaken 10 times by inversion to taken the blood out of capillary.
3. The cap was removed off of the top of tube, two drops of reagent were discarded, and two drops of the sample were applied onto the well of a cartridge.
4. The test cartridge was inserted into holder of the ichroma™ reader.
5. Select button was selected on the ichroma™ reader to started the scanning process (read within 3 minutes) the test result was read on display screen of the ichroma™ reader. Ichroma™ reader calculated the result automatically and displayed CRP concentration in terms of mg/l.

Working range of ichroma™ CRP is 2.5-300 mg/l.

2.10 Ethical considerations
Approval to conduct this research was obtained from Ethical Committee of Faculty of Medical Laboratory Sciences, Sudan University for Science and Technology. Verbal Informed consent was obtained from each patient before sample collection.

2.11 Data collection and analysis
Data was collected using structured interview questionnaire and analyzed using Statistical Package for Social Sciences (SPSS) version, 15 the independent samples T-test was used to compare means between two groups and correlation between Neutrophil/Lymphocyte ratio and quantitative variables were analyzed using Pearson correlation.
Chapter Three

Results

One hundred patients with type 2 diabetes mellitus were enrolled in this study, 50 diabetics with foot ulcer (23/50 46% patients were male and 27/50 54% patients were female) and 50 without foot ulcer (22/50 44% of patients were male and 28/50 56% were female) (Figure 3.1).

Figure 3.1 Distribution of gender in study subjects
Table (3-1): Patients age

<table>
<thead>
<tr>
<th>Variables</th>
<th>Diabetics with foot ulcer</th>
<th>Diabetics without foot ulcer</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Age</td>
<td>57.92</td>
<td>10.8</td>
</tr>
</tbody>
</table>

Table (3-2): Distribution of total leukocyte, absolute neutrophil and absolute lymphocyte among study population

<table>
<thead>
<tr>
<th>Variables</th>
<th>Diabetic with ulcer</th>
<th>Diabetic without ulcer</th>
<th>p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWBC Mean ± SD</td>
<td>8.8±2</td>
<td>6.8±1.8</td>
<td>0.000</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>6.3±1.9</td>
<td>4±2</td>
<td>0.000</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>1.6±0.4</td>
<td>2.2±0.7</td>
<td>0.000</td>
</tr>
</tbody>
</table>

There was statistically significant increase total white blood cells (TWBCs) count and absolute neutrophil count in patients with foot ulcer than those without foot ulcer, but absolute lymphocyte count was show no statistical correlation in patients with foot ulcer than those without foot ulcer.

Table (3-3): Comparison of HbA1c in patients with and without foot ulcer

<table>
<thead>
<tr>
<th>Variables</th>
<th>Diabetics with ulcer</th>
<th>Diabetics without ulcer</th>
<th>p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c Mean ± SD</td>
<td>6.3±1.3</td>
<td>7.3±1.8</td>
<td>0.007</td>
</tr>
</tbody>
</table>
There was statistically decrease HbA1c in patients with foot ulcer than those without foot ulcer.

**Table (3-4): Comparison of CRP in patients with and without foot ulcer**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Diabetics with ulcer</th>
<th>Diabetics without ulcer</th>
<th>p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP Mean ±SD</td>
<td>18.9±18.8</td>
<td>5.2±2.9</td>
<td>0.000</td>
</tr>
</tbody>
</table>

There was statistically significant increase in CRP in patients without foot ulcer than those with foot ulcer.

**Table (3-5) Correlation between NLR and patients demographics and laboratory findings.**

<table>
<thead>
<tr>
<th>variables</th>
<th>r</th>
<th>p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>age</td>
<td>0.259</td>
<td>0.045</td>
</tr>
<tr>
<td>TWBC</td>
<td>0.650</td>
<td>0.001</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>0.817</td>
<td>0.001</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>-0.553</td>
<td>0.001</td>
</tr>
<tr>
<td>CRP</td>
<td>0.441</td>
<td>0.001</td>
</tr>
</tbody>
</table>
The correlation analysis showed statistically significant positive correlation between NLR and each of age, T.WBCs, Absolute neutrophil count, and CRP, and significant negatively correlation with Absolute lymphocyte count.

Chapter Four

Discussion, conclusion, recommendation

4.1 Discussion

NLR represents a combination of two markers where neutrophils represent the active non specific inflammatory mediator initiating the first line of defense, whereas lymphocytes represent the regulatory or protective component of inflammation (Bhutta et al., 2011).

The study aimed to evaluate N/L ratio as a marker of systemic inflammation in patients with foot ulcer.

Our results showed that mean T.WBCs, absolute neutrophil count and absolute lymphocyte count there were statistically significant difference in patients with foot ulcer than those without ulcer. While absolute neutrophil count was
significantly higher in patients with foot ulcer, the absolute lymphocyte count was significantly lower, this agrees with Kahraman et al at Dumlupinar University School of Medicine, who stated that values of neutrophil percent and lymphocyte percent have significantly statistic differences (Kahraman et al.,2014). There was no statistically significant difference between groups by means of female / male gender ratio (P.value = 0.346).

The present study showed that, the NLR values of the diabetic patients with foot ulcer were higher than those without foot ulcer (P.value = 0.000), this result agrees with Kahraman, et al when comparing diabetics with foot ulcer to those without ulcer (Kahraman et al.,2014).

The subject of N/L ratio is recently examined and evaluated, number of studies conducted on the diabetics with ulcer syndrome, diabetics nephropathy and coronary artery disease, all the result of the researches reveals that, the inflammation have strong relationship with complication (Kahraman et al.,2014, Huang et al.,2015, Sahin et al.,2013).

Huang et al study reported that NLR values of the patients with early-stage diabetic nephropathy (DN) were higher than those of the patients without DN (Huang et al.,2015), Sahin, et al at 2013 results showed that the NLR was higher in diabetic patients with coronary artery disease (CAD) group compared to group without (CAD) (Sahin et al.,2013), Yue et al at 2015 revealed that the mean NLR was significantly higher in patients with diabetic retinopathy (DR) compared with patients without DR(Yue et al.,2015).

NLR is superior to other leukocyte parameters (e.g. Neutrophil, lymphocyte, and total leukocyte counts) because of its better stability compared with the other parameters that can be altered by various physiological, pathological, and physical factors (Matthews et al.,1985),(Gibson et al.,2007).
HbA1c was lower in patients with foot ulcer compared to those without foot ulcer ($P\text{.value} \ 0.002$). This means diabetics patients may suffer from foot regardless of diabetics control. This result similar to that reported by Kahraman, et al (Kahraman et al .,2014) and disagree with Sahin et al (Sahin et al .,2013).

For the CRP the result showed statistically significant differences ($p\text{.value}=0.000$), this agree with Kahraman et al (Kahraman et al .,2014).

The correlation analysis showed statistically significant positive correlation between NLR and each of age ($r = 0.259 , p = 0.045$), T.WBCs ($r = 0.650, p = 0.001$), absolute neutrophil count ($r = 0.817, p = 0.001$), and CRP ($r = 0.441, p = 0.001$), and significant negative correlation with absolute lymphocyte count ($r = -0.553, p = 0.001$), these results were similar to Kahraman et al who also reported a significant correlation.

### 4.2. Conclusion

- NLR is a simple, rapid and reliable method for evaluation of the extent of systemic inflammation.
- NLR is significantly higher in Diabetics with foot ulcer than those without foot ulcer.
- NLR is positively correlated with age, total leukocyte count, absolute neutrophil count and CRP, and negatively correlated with absolute lymphocyte count.
4.3. Recommendation

- NLR can be used routinely as a marker of systemic inflammation.
- NLR can be used as predictor marker for foot ulcer in diabetics patients.
- Further extensive studies should be carried out on a larger number of samples to verify our findings concerning the role of NLR in the inflammatory process.
References


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Neutrophil to Lymphocyte ratio in diabetic mellitus type 2 patients with foot ulcer

Questionnaire

Personal data:
Serial No:.................................................................

Gender:

1. Male (...)  2. Female (...)

Age:.................................................................

Medical history:

Diabetic          yes........  No ...........
Foot ulcer        yes........  No ..........
Medication        yes........  No ..........
Type of medication ..................................................
Other diseases    ..................................................