ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL BIOCHEMISTRY

Assessment of Neutrophil-lymphocyte Ratio as an Inflammatory Marker among type 2 Diabetes Mellitus patients with and without Diabetic Nephropathy

By: Mesfin Zewude (BSc)

A thesis submitted to the Department of Medical Biochemistry, College of Health Science, Addis Ababa University, in partial fulfillment of the requirement for the degree of Master of Science in Medical Biochemistry

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Co-advisors: Dr. Solomon Tebeje Gizaw (PhD)

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This is to clarify that this thesis prepared by Mesfin Zewude entitled, “Assessment of Neutrophil-lymphocyte Ratio as an Inflammatory Marker among Type 2 Diabetes Mellitus patients with and without Diabetic Nephropathy," is submitted in partial fulfillment of the requirement for the degree of Master of Science in Medical Biochemistry, complies with the regulations of the University and meets the accepted standards concerning the originality and quality.

Signed by the board of examiners committee:

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Chair of the Department of Graduate Program Coordinator

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LIST OF ABBREVIATIONS AND ACRONYMS

ADA .....American diabetic Association
AGEs .....Advanced glycation end products
ALC .....Absolute lymphocyte count
ANC..... Absolute neutrophil count
BMI..... Body mass index
CKD..... Chronic kidney disease
DM…..... Diabetes Mellitus
DN .....Diabetic Nephropathy
DBP .....Diastolic blood pressure
eGFR..... estimated GFR
ESRD…..... End-stage renal disease
GFR .....Glomerular filtration rate
HIV… .....HumanImmunovirus
MDRD… .....Modification of diet in renal disease study
NLR .....Neutrophil lymphocyte ratio
SBP .....Systolic blood pressure
SGOT…..... serum glutamate oxaloacetate transaminase
SGPT…… serum glutamate pyruvate transaminase
TWBC..... Total white blood cell
T1DM… .....Type 1 diabetes mellitus
T2DM… .....Type 2 diabetes mellitus
UAE…..... Urinary albumin excretion
WHO..... World Health Organization

ABSTRACT

Background: Diabetic nephropathy (DN) is one of the microvascular complications of diabetes mellitus manifested as increased albumin in urine excretion. Total leucocyte count is a crude, but a sensitive indicator of inflammation and studied in many non-communicable diseases such as acute myocardial infarction, stroke, heart failure and cancer as index of inflammation. In this study, the neutrophil-lymphocyte ratio (NLR) was evaluated between T2DM patients with and without DN.

Objective: To assess the neutrophil-lymphocyte ratio as an inflammatory marker among Type 2 diabetes mellitus (T2DM) patients with and without diabetic nephropathy.

Methodology: A cross-sectional study was conducted to compare NLR between type 2 diabetic patients with and without DN at Bole 17 Health Center, Addis Ababa, Ethiopia from October 15, 2019 to April14, 2020. Convenient sampling technique was used to recruit the study participants. The data was entered in to Epi-data statistical software version 3.1 and then exported to SPSS software version 22.0 for analysis. Simple descriptive statistics such as mean, standard deviation, and percentages were used to present socio-demographic characteristics and magnitude of patients with DN, without DN and NLR ratio. Appropriate statistical tests, including student t-test, chi-square test, and Pearson correlation were done to analyze the statistical data. The p-values < 0.05 were considered statistically significant.

Results: Out of the 199 DM patients, 22.6% and 77.4% were found with diabetic nephropathy (albumin positive) and without diabetic nephropathy (albumin negative), respectively. Interestingly, the mean NLR value (2.66±0.49) was found higher in diabetic patients with DN compared to the mean NLR (1.65±0.20) of diabetes patients without DN ($p < 0.0001$).

Conclusion: The NLR was significantly increased in T2DM patients with DN, suggesting that inflammation and endothelial dysfunction could be an integral part in the pathogenesis of DN, and NLR may be considered as a predictor and a prognostic biomarker of DN.

Keywords: Diabetic nephropathy, inflammatory biomarker, Neutrophil-lymphocyte ratio, Type 2 Diabetes mellitus

1. INTRODUCTION

Diabetes mellitus (DM) refers to a group of common metabolic disorders that are characterized by hyperglycemia. It results from defects in insulin secretion, insulin action, or both. It also causes major morbidity and mortality in the society. DM has recently assumed epidemic proportions and affects more than 460 million individuals world wide. Global diabetic prevalence in 2019 was estimated to be 9.3% (463 million people), rising to 10.2% (578 million people) by 2030 and 10.9% (700 million) by 2045, with the greatest increases in the developing countries of Africa, South America, and, Asia (Wild *et al.*, 2019). DM is a systemic disease having serious microvascular and macrovascular complications. While microvascular complications include diabetic nephropathy (DN), diabetic retinopathy, and diabetic neuropathy, macrovascular complications include stroke, cardiovascular diseases (CVDs), and peripheral vascular diseases (Rathmann and Giani, 2004).

DM is also the leading cause of blindness in adults, and is the most frequent cause of chronic kidney failure in both developed and developing countries. Type 2 diabetes mellitus (T2DM) is a chronic disease associated with many complications. It is a complex metabolic disease that affects multiple organs in the body. DN is one of its complications with a typical feature of albuminuria (greater than 300mg/24 hour) confirmed on at least twice in a 3-6 months apart, permanent and irreversible decrease in glomerular filtration rate (GFR), and arterial hypertension (Gariani *et al*., 2012). DN is indicated by the presence of continuous macroalbuminuria existing together with insulin or non-insulin-dependent diabetes. It is diagnosed by continuous raised of albumin or protein in urine without other known renal disease (Giorgino *et al*., 2004).

Total leucocyte count is a crude, but a sensitive indicator of inflammation and studied in many non-communicable diseases as an inflammatory marker such as acute myocardial infarction, stroke, and heart failure (Jones *et al*., 2005).

1.2. Literature review

1.2.1. Introduction

Type 2 Diabetes mellitus (T2DM) is a metabolic disorder which described by the presence of chronic hyperglyecia because of resistance to insulin action or inadequate secretion of insulin (Gastaldelli *et al*., 2004). The long-term complications of T2DM includes retinopathy with a potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulceration, amputations, and Charcot's joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms (Evans and Capell, 2017). Furthermore, it can be considered as a risk factor for atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease (Ouellet *et al*., 2016). Diabetes is one of the biggest health problems of the 21st century. According to the World Health Organization (WHO) report, hyperglycemia is globally the third highest risk factor for premature mortality, after high blood pressure, and tobacco use. It accounts for 5 million (14.5%) of global all-cause mortality among people aged 20–79 years, which is higher than the combined number of deaths from the three major infectious diseases (1.5 million deaths from HIV/AIDS, 1.5 million from tuberculosis, and 0.6 million from malaria in 2013) (Dilektasli *et al*., 2016).

1.2.2. Screening for T2DM

T2DM was defined following the criteria of the WHO (WHO, 2016). The patients are considered to have T2DM when their fasting blood sugar (FBS) level is ≥126mg/dl and are currently using antidiabetic medications (Alberti and Zimme, 1998). The American Diabetic Association (ADA) recommends that screening for DN must be initiated at the time of diagnosis in patients with T2DM. If albuminuria is absent, the screening must be repeated annually. To screen and diagnose DN, it is important to detect albumin in the first-morning urine specimen, collected during a medical visit. This method is accurate, easy to perform, and recommended by the ADA guidelines (Gross *et al*., 2005). The measured albumin can be reported either as urinary albumin concentration (mg/l) or urine ACR (mg/g or mg/mmol). A urine ACR of 2.0 mg/mmol in men and 2.8 mg/mmol in women or higher suggests the presence of albuminuria. Because of variability in urinary albumin excretion (UAE), two of three specimens should be abnormal before the diagnosis of DN is made. When specific UAE measurements are not available, alternative methods like semi-quantitative dipstick measurements of albuminuria

(e.g.micral test II) and a qualitative test for proteinuria (dipstick) or quantitative measurement of protein in a morning urine sample can be used (Gross *et al.*, 2005).

1.2.3. Diabetic Nephropathy

DN is also known as Kimmelseteil Wilson syndrome, as it was first discovered in 1936 by Clifford Wilson and Paul Kimmelstiel who described the classic lesions of nodular glomerulosclerosis associated with proteinuria and hypertension (Arya *et al*., 2012). It is both the major cause of the end-stage renal disease (ESRD) in developed and developing countries as well as the leading cause of chronic kidney disease (CKD) in patients starting renal replacement therapy (RRT). It is highly anticipated that DN will be the most frequent cause of ESRD in the developing country (Locatelli *et al*., 2003). About 20-30 percent of people with Type 1 diabetes mellitus (T1DM) and T2DM develop DN, and its incidence increases with the duration of illness. Ziyad proved that up to 35% of all patients with either T1DM or T2DM eventually develop nephropathy after 25 – 30 years of suffering from diabetes (Ziyad, 2009).

There are five phases of development towards DN. These are phase I – phase V. Phase I is characterized by hyperfiltration with increased glomerular filtration rate (GFR) with no evidence for albuminuria and renal hypertrophy. Phase II is reflected with relatively normal albumin excretion (<30mg/24 hours), increased albumin after physical exercise and stressful condition, perpetuated renal structural change, with high risk to develop into DN. Phase III, an initial phase of DN, is marked with microalbuminuria (30-300mg/24 hours), high or normal GFR, and there is, histopathologically, swelling in the glomerular basal membrane; In phase IV proteinuria is acquired, albumin excretion is >300mg/24 hours, decrease in GFR, and usually accompanied by hypertension. The last phase (phase V) is characterized by an ESRD with very low GFR that indicates a sign for uremia (Shikata and Makino, 2013).

According International Diabetes Federation (IDF) report, diabetes cases were 460 million in 2019, and estimated rise to 578 million by 2030 and 700.2 million by 2045 (IDF Diabetes Atlas, 2019). Reports from WHO indicated that the number of diabetic patients in Ethiopia will be more than double by 2030 (WHO, 2000). Diabetic nephropathy is proposed to be

more common among African patients than in the developed world. From DM clinics reports, the prevalence of proteinuria in diabetes patients (i.e., DN) ranges from 2 – 53.1% worldwide (Ahn *et* al., 2014 and Heydari *et al.*, 2010), 5.3 – 53.1% in Africa (IDF Diabetes Atlas, 2014), and 15.7– 29.5% in Ethiopia (Worku and LejaHamza, 2010). Tikur Anbessa Specialized Hospital report showed that 15% of DM patients developed DN and 70,000 new cases of DM patients with DN in Ethiopia were added to those patient populations (Abejew *et al*., 2015 and Tefera, 2014). Clinically, DN is described as a continuous excretion of albumin in the urine (> 300mg/24 hour), a persistently reduction in glomerular filtration rate (GFR), and hypertension. DN is rare during the first five years of diabetes, increasing to a peak at 15 to 20 years after the onset of disease. Albuminuria (>30mg/24 hour), which is the clinical evidence of kidney damage, strongly predicts the development of DN (Walker and Shah, 2004).

1.2.4. Assessment of renal function

Once the diagnosis of DN is made, the renal function must be assessed to measure the progression of DN or eventually to confirm the need for treatment of ESRD (Rossing, 2004).

1.2.4.1. Measurement of glomerular filtration rate (GFR)

The measurement of GFR is considered as the best parameter of kidney function. The clearance of inulin during constant infusion has been accepted as the gold standard for the determination of GFR. However, this method is not used because the procedures are cumbersome, costy, and limited availability of inulin (Rossing, 2004).

1.2.4.2. Estimation of GFR of (eGFR)

Estimation of GFR can be obtained from some or all of the following variables: serum creatine, age, sex, race, and body size. It is the most frequently used measure of kidney function in clinical practice because of the low cost and more available for patients. Hence, estimation of GFR was calculated, a renal function test of all patients was carried out, and estimated GFR (eGFR) was calculated by Modification of diet in renal disease study (MDRD) formulae (Stevens *et al*., 2006).

Table1. Stages of CKD relating to eGFR According to the Canadian Diabetes Association, 2008.

Stage	Qualitative description	eGFR (mL/min)
1	kidney damage, normal GFR	≥90
2	kidney damage, mildly decreased GFR	60-89
3	moderately decreased GFR	30-59
4	Severely decreased GFR	15-29
5	End-stage renal disease	< 15 (or dialysis)

1.2.5. Clinical studies on NLR as a novel marker for DN in T2DM patients

Several scholars that have described the association between inflammation and vascular disease showed that chronic inflammation can accelerates the development of micro and macrovascular complication in patients with diabetes. Leucocyte count is one of the useful inflammatory biomarkers in clinical practice. Leucocyte count is a crude, but a sensitive and cost-effective indicator of inflammation which can be done easily in diagnostic laboratories. However, the sub-types, particularly the neutrophils, lymphocytes, and subsequent calculation of NLR are relatively more stable than individual leukocyte parameters. Moreover, NLR as a inflammatory marker is better than other leukocyte paramaters due to its stability compared with other absolute counts that could be altered by various physiological, pathological, and physical factors (Gross *et al.*, 2005). The NLR is studied in many non-communicable diseases as an inflammatory marker that used to predict the prognosis of diseases like acute myocardial infarction (MI), stroke, and heart failure (Rudiger *et al*., 2006). Furthermore, NLR is used as an indicator of an inflammatory process progression in various diseases such as appendicitis, cardiac diseases, cancer, and DN (Zahorec, 2001).

Additional factors involved in the development of DN include glomerular hyperfiltration, oxidative stress, advanced glycation end product (AGE) activation, protein kinase activation, excessive expression of transforming growth-factor-β (TGF-β), followed by increased extracellular matrix. Exposure of cells and bio-molecules to high levels of glucose for long periods leads to glycation end products, which is a non-enzymatic reaction of glucose with

amino groups of proteins and other molecules that cause oxidation of protein and lipids which leads to tissue damage. There is evidence that indicates accumulation of AGEs in tissues are responsible for many complications of DM, particularly the microvascular complications. They appear to accumulate especially in tissues affected badly by diabetes, which can directly interact with various cellular components and can also trigger numerous intracellular signaling pathways; elicit abnormal immune responses, leading to cellular damage (Rojas and Morales, 2004). Hyperglycemia triggers the occurrence of renal damage that can result in hemodynamic changes, metabolic changes, endothelial dysfunction, and altered vascular factor expression resulting in inflammatory cell activation (increased white blood cells including neutrophils) (Skikata and Makino, 2013).

Persistent and perpetuated inflammation occurs in diabetic nephropathy results in an increase in inflammatory mediators including interleukin-1 (IL-1), interleukin 6 (IL-6), interferon-γ, and tumor necrosing factor-α (TNF-α). This inflammatory process will cause neutrophils activation and reduces lymphocyte production. In DN, IL-6, as one of the released inflammatory mediators, can stimulate neutrophil production from progenitor cells called colony-stimulating factor (CSF) in the bone marrow. Neutrophil will adhere to the target cell, namely the endothelial cells in blood vessels, resulting in disturbed endothelial function in blood vessels (Lim and Tesch, 2012; Nakhavani *et al.,* 2015; Skikata and Makino, 2013). In another way, an inflammation that occurs in DN reduces lymphocyte production. Hence, the increase in inflammatory mediators accelerates the muscular protein catabolism, increases positive stenosis of acute-phase protein C-reactive protein (CRP), and suppresses protein/albumin production in addition to the loss of protein through proteinuria. This cause protein deficiency, which in turn affects the normal hematopoiesis process and consequently results in bone marrow dysfunction, and insufficient lymphocyte production. Leptin produced by adipose tissue inhibition against T helper 2 (Th2) proliferations will cause the lymphocyte count to decrease (Makino, 2013; Nakhavani *et al.,* 2015).

Increased neutrophils and decreased lymphocytes cause increase in the NLR. Therefore, NLR can be a marker for a systemic inflammatory process. As discussed above, leukocytes play an important role in the initiation and progression of kidney disease via inflammatory mechanisms independent of infection (Chung *et al.,* 2005). Leukocytes in diabetic patients

may be activated by advanced glycation end products or reactive oxygen radicals or cytokines (Pertynska-Marczewska *et al.,* 2004*).* Activated leukocytes secrete many kinds of cytokines and transcription factors that promotes inflammation and thereby contribute to glomerulosclerosis (Chung *et al.,* 2005). Moreover, the released superoxide and proteases will encourage increased formation of oxidative stress (Kocyigit *et al.,* 2013).Therefore, hyperglycemia promotes oxidative stress that play crucial role in the development of DN (Evans *et al.,* 2002). And an increased urinary protein excretion is the earliest clinical manifestation of DN (Gross *et al.,* 2005).

The number of immune cells has a potential role in indicating the status of immune system dysfunction. Increased neutrophils are generally considered as a marker of a destructive non-specific inflammatory response. Decreased lymphocyte shows a failure of immune regulation and also related with a risk of heart disease (Ommen *et al.,* 1998*).* The NLR represents two interdependent immune pathways; neutrophils that are the active nonspecific inflammatory mediator form and the first line of defense, where as lymphocytes are the regulatory or protective component of inflammation (Bhutta *et al.,* 2011). It is stable, inexpensive, and easy to detect compared with other inflammatory factors. Many inflammatory markers are related to DN, such as interleukin-1 (IL1), interleukin-6 (IL6), interleukin-8 (IL8), transforming growth factor-beta 1, tumor necrosis factor-alpha (TNF-α), and cytokines. However, their measurement is not used routinely as it is not easy to do it (Núñez *et al.,* 2008). In this respect, NLR has emerged as a novel surrogate marker.

The study done in Indian patients with DN and without DN showed the mean age of DN vs. without DN patients was (52.29 ± 11.45) years and (50.05 ± 11.29) years, respectively. Distribution of age (p= 0.294), gender (p=0.951), BMI (P=0.8208), total cholesterol (p=0.7423) and FBS (P=0.092) was not statistically significant between the two groups. But significant correlation was found between the two groups within the following parameters; NLR ($P < 0.001$), absolute neutrophil count (ANC), ($P < 0.001$), absolute lymphocyte count (ALC) ($P < 0.001$), absolute lymphocyte count (ALC) ($P < 0.001$) and eGFR ($P = 0.047$). Patients with albumin positive had low eGFR (mean eGFR = 85.71 ± 27.72) than normal group (mean eGFR = 96.2 ± 28.23). However, other parameters such as blood urea and serum creatinine had no significance association in these two groups. Liver function tests for all

patients were carried out, and serum glutamate pyruvate transaminase (SGPT) (mean SGPT = 40.89 ± 36.62) was found to be significantly raised in DN patients' group as compared to normal patients' group (mean SGPT = 9.74 ± 13.03), which was highly statistically significant ($P < 0.001$) but serum glutamate oxaloacetate transaminase (SGOT) value had no difference indication between two patients. Insulin resistance in type 2DM patients can cause non-alcoholic fatty liver; liver inflammation which can alter liver enzymes like SGOT, SGPT, and alkaline phosphate (Alkouri *et al.,* 2012 and Khandare *et al.,* 2017).

The exact biochemical pathway of DN is unknown. But, a pathological sequences (that start with glomerular damage, results in proteinuria, succeeded by progressive renal damage, fibrosis, inflammation, and lastly loss of functional nephrons) are known to play an important role in the development and progression of DN (Gai *et al.,* 2006 and Lim *et al.,* 2012). Hence, renal inflammation in the setting of DN is known to play a critical role (Gai *et al.,* 2006). Leucocyte count and its subclasses are among the easily available and cost effective exemplary of inflammatory index. Many studies have explained that inflammatory markers such as increased neutrophils and decreased lymphocytes are independent markers of many diseases, especially complications of DM, such as DN (Ulu *et al.,* 2014). Diagnosis with individual leucocyte has its own limitations, but NLR based diagnosis, which is a dynamic parameter has a higher diagnostic value (Azab *et al.,* 2011). Moreover, diagnosis based on NLR has also positive relationship with presence and severity of the metabolic syndrome (Buyukkaya *et al.,* 2014). Similarly, different researchers have proved that NLR value is significantly correlated with chronic diseseses such as hypertension, diabetic complications, systemic inflammation, severity of glucose intolerance, and insulin tolerance (Imtiaz *et al*., 2012 and Shiny *et al*., 2014).

Further more, study done by Azab *et al.* indicated that NLR can be used as a prognostic marker to asses severity of renal function (Azab *et al.,* 2012). Afsar has explained that NLR was related to DN and also associated with ESRD **(**Afşar, 2014). Another study has proved that NLR was correlated with albumin positive in diabetic patients, which indicated there was a relationship between inflammation and endothelial dysfunction in DN (Akbas *et al.,* 2014). A study done by Huang *et al.* has shown that NLR values of DN was significantly higher than without DN and healthy controls which were (2.48±0.59), (2.20 ± 0.62) and (1.80 ± 0.64)

respectively. ANC and ALC values were also significantly raised in DN (Huang *et al.*, 2015). A study done in Egypt has indicated that NLR levels were significantly raised in diabetic patients with micro vascular complications such as nephropathy (P < 0.001), neuropathy (P = 0.025) and retinopathy (P <0.001) than diabetic patients without any microvascular complications and healthy individuals (Moursy EY *et al.*, 2015). Moreover, study done in Turkey, indicated that NLR was meaningfully raised in diabetic patients with albuminuria as compared with moderately increased albuminuria or normoalbuminuria patients. Besides, there was a strong and significant correlation between the NLR and albuminuria (Ünal *et al.*, 2015).

A study published in the African Journal of Medical Sciences indicates there was a significant correlation between DN patients and control group participants in neutrophil to lymphocyte ratio (p = 0.00). However, there was insignificant correlation between DN patients and control group participants in total WBCs and neutrophils counts (p = 0.14 and 0.96), respectively. Also, there was insignificant correlation between the N/L ratio and the age incidence of participants investigated (p = 0.05). Furthermore, there was no significant correlation between N/L ratio, gender, and frequency rate of hemodialysis and other renal diseases (p = 0.06, 0.41, and 0.16), respectively (Moawia *et al.*, 2018).

Cuneyt *et al.* compare three groups of T2DM patients; normoalbuminuria, microalbuminuria and macroalbuminuria within their different parameters. In this study no significant association was seen among the three groups with respect to gender, duration of illness, total cholesterol and TWBC. Howevre, statistically significant association was observed among the three groups with respect to age (p = 0.013), creatinine (p < 0.001), neutrophil count (p = 0.022), lymphocyte count (p < 0.001), NLR (p < 0.001), and 24-hour UAE (p = 0.001). The NLR did not differ between genders, but correlated with GFR (r = –0.298, p = 0.002), neutrophil count (r = 0.500, p < 0.001), lymphocyte count (r = –0.634, p < 0.001), urea (r = 271, p= 0.005**),** creatinine (r = 0.304, p = 0.001), spot urine microalbumin (r = 0.396, p < 0.001), and total cholesterol (r = –0.214, p = 0.025) (Cuneyt *et al.*, 2016).

Further more, a study done on parameters related to DM patients shows that an increase in age and duration of DM after diagnosis, poor adherence to blood sugar measurement at home,

having systolic hypertension and overweight determines the presence of nephropathy in diabetic patients.

As age of diabetic patients increases by one year, probability of developing DN increases by 3.7%; which means for ten years increases in the duration of illness after diagnosis, the probability of developing diabetic microvascular complication increases by 1.43 times (43%). Many researchers described that patients with older age is at high risk of developing DN. Hence, as age of diabetic patients increases, there will be higher probability of developing chronic kidney disease which are mostly characterized by albuminuria (Hintsa *et al.,* 2017).

With the diabetes inter-playing, older age patients will be at increased risk to develop nephropathy as evidenced elsewhere (Abeje *et al.,* 2015; Noubiap *et al.,* 2015; Worku and Leja Hamza, 2010). A study conducted in Africa concluded that 95% of diabetic patients developed albuminuria with ten years of diagnosis, 35% developed the ESRD within five years of diagnosis, while 18% die of DN after 20 years of diagnosis. This study revealed that the duration of diabetes, blood pressure, advancing age, obesity, and glucose control were statistically related to DN (Noubiap *et al.,* 2015). Studies done in India, Cameron, and Ethiopia also reported similar results (Noubiap *et al.,* 2015; Tesfaye, 2010; Vijay and Sk, 2012).

1.3. Statement of the problem

Diabetes mellitus usually manifests with hyperglycemia that has been one of the global health burdens and leads to another sequel of diseases, which diabetic nephropathy one of the common complications. It was reported that about 25-40% of patients with T2DM suffered from DN (Lingling *et al.,* 2017). DN is a serious microvascular complication and has been considered as the major cause of the end-stage renal disease (ESRD) in developed and developing countries as well as the leading cause of chronic kidney disease (CKD) in patients starting renal replacement therapy (RRT), (Locatelli *et al.,* 2003). Many developed countries use different bio-markers for diagnosis and follow-up of DN, but in Ethiopia, no bio-markers are available to diagnose DN. In addition, available diagnosis and treatments are costy, time-consuming, and inconvenient for patients. To the best of my knowledge, there are limited studies that determined the inflammatory marker associated with diabetic nephropathy in

Ethiopia. Therefore, the present study was aimed to assess the NLR as an Inflammatory Marker among type 2 Diabetes Mellitus patients with and without Diabetic Dephropathy.

1.4. Significance of the Study

NLR can be used as early predictors of DN by which we can predict the disease and can halt its progression. NLR as described to be a simple and cheap laboratory tools that provides important information regarding inflammation. In Ethiopia, inflammatory markers have not been used to diagnose and follow-up of diabetic complications. Therefore, this study can be used as baseline data and may deliver information to clinicians about DN micro-angiopathic complications in patients with diabetes and may contribute to improving its management in the early stages.

2. OBJECTIVES

2.1. General Objective

To assess the neutrophil-lymphocyte ratio (NLR) as an inflammatory marker among Type 2 Diabetes mellitus patients with and without diabetic nephropathy.

2.2. Specific Objectives

- To determine whether NLR can serve as an inflammatory marker among T2DM with diabetic nephropathy
- To compare some renal function tests (creatinine, urea, eGFR, and albumin) between T2DM patients with DN and without DN
- To evaluate liver function tests (serum glutamate oxaloacetate transaminase and serum glutamate pyruvate transaminase) between T2DM patients with DN and without DN

3. MATERIALS AND METHODS

3.1. Study area and period

The study was conducted at Bole 17 health center, Bole sub-city, Addis Ababa, Ethiopia. The health center was established in 1972. The number of staff working at the health center include: 22 nurses, 1 physician, 10 health officers, 8 pharmacists, and 8 medical laboratory technologists. It also has 32 administrative and support staff. It serves about 100,000 patients per year in its outpatient department and about 6,000 in both the inpatient and emergency departments. The Laboratory services in the institution are organized for outpatient, inpatients, and emergency. The study was conducted from October 15, 2019 to April 14, 2020.

3.2. Study design

A facility-based comparative cross-sectional study design was conducted.

3.3. Population

3.3.1. Source population

The source population includes all DM patients who attending Bole 17 health center during the study period.

3.3.2. Study population

All T2DM patients who attending Bole 17 health center during the study period and who were included in the study.

3.4. Eligibility criteria

3.4.1. Inclusion criteria

- ✓ All diagnosed T2DM patients who attending the health center and voluntary to participate.
- ✓ All patients aged ≥30years (Since T2DM is common in adult age)

3.4.2. Exclusion criteria

❖ **Patients with chronic infections like;**

- ✓ Urinary tract infection (UTI),
- ✓ Upper respiratory tract infection,
- ✓ Lower respiratory tract infections,

- ✓ Viral hepatitis,
- ✓ Tuberculosis,
- ✓ HIV/AIDS
- ✓ Cardiovascular disease (CVD)
- ✓ Chronic kidney disease (CKD)
- ✓ Chronic liver disease
- ✓ Blood disorders and
- ✓ Malignancy

❖ **Patients on anti-inflammatory drugs like**;

- ✓ Systemic or topical steroids
- ✓ Smokers and alcoholic patients

3.5. Sample size determination

The sample size was estimated using the general formula for a single population proportion. The value of proportion (p) for the study was taken from a study conducted in Ethiopia, which was 29.5 % (Worku and LejaHamza, 2010; Abejew *et al*., 2015 and Tefera, 2014), 95% confidence interval, and 1.96 for Z and 5% for d, the sample size will be: $n= Z\alpha/2^{2} P (1-P) / d^{2}$. Using this formula the calculated sample size is 319. Averagely 25 patients are admitted to the diabetic outpatient department daily, so monthly around 550 patients. Since the study population is less than 10,000 finite population correction formula was used to calculate the actual sample size. So, corrected sample size = (sample size)*(population)/sample size+ (population-1) = 202.

3.6. Sampling procedure

A convenient sampling technique was employed while recruiting the study participants.

3.7. Study variables

3.7.1. Independent variable

- ❖ T2DM
- ❖ Age,

- Sex
- Marital status
- Residence
- Income status
- Educational level
- Body mass index
- Blood pressure (systolic and diastolic blood pressure)
- Absolute neutrophil
- Absolute lymphocyte
- Neutrophil- lymphocyte ratio (NLR)
- Leukocyte count(WBC)
- Liver Function tests; serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase(SGPT)
- Total cholesterol
- Duration categories

3.7.2. Dependent Variable

- Urinary albumin status
- Renal functional tests (creatinine, urea, and eGFR)

3.8. Socio-demographic data, urine and blood sample collection procedure

The training was given to the data collectors on the data collection tool and sampling techniques. The principal investigator did close supervision throughout the data collection process. Data were collected by using questionnaire and laboratory test request forms. The collected data were checked regularly for completeness and consistency.

English version questionnaire was translated to the Amharic language then retranslated to English. Socio-demographic characteristics and other data related to T2DM was collected from participants using the Amharic version structured questionnaire by experienced health professionals. Prior to data collection the questionnaire was pretested in 5% of the sample size in Garji Health center. Patients' history (duration of illness, types of DM, BP, weight, and height, BMI) were collected from their medical record and patient's socio-demographic

information (marital status, residence, education, occupation, and income) was collected by trained clinicians. As confirmed during the data collection, professionals in the diabetic clinic measured weight, height, and blood pressure as follows. Weight of the patient was measured in light closing and bare foot in kilograms (kg) as per recommended (CDC, 2007). Height was measured using Stadiometer (Seca, Germany) in centimeter (cm) in an erect position that the head, shoulder blades, buttocks, and heels make contact with the backboard at a precision of 0.1cm with shoes removed as per recommended but not repeated measurements and body mass index (BMI) was calculated as the ratio of weight (kg) to height square (m^2), (CDC, 2007; ISAK, 2001). Blood pressure was measured using a mercury sphygmomanometer with a cuff deflation rate of2mmHg. Two measurements from the left-arm 5 minutes apart in the sitting position was averaged to be recorded (CDC, 2007; ISAK, 2001). During urine collection, a patient was advised well by laboratory professionals and 10ml of urine was collected in clean plastic specimen containers. Approximately 4 or 5 ml of venous blood was withdrawn in the EDTA vacutainer tube (use pink test tube without anti-coagulant for clinical chemistry test). For clinical chemistry analysis left the sample to clot (in serum separator test tube) at room temperature for 30 min. Then the whole blood was centrifuged at 800–1600 revolution per minute (RPM) for 20 minutes at room temperature as serum or plasma was required. Finally the sample was stored at -80°C until analysis.

3.9. Test principles of the laboratory analytes

3.9.1. Measurement of fasting blood sugar (FBS)

FBS test was measured to know the blood glucose level of participants. It is enzymatic methods, in which the enzyme glucose oxidase reacts with glucose in patient sample, water, and oxygen to form gluconic acid and hydrogen peroxidase. The hydrogen peroxidase can then be used to oxidize a chromogen or the consumption of oxygen measured to estimate the amount of glucose present (Dietz and Smith, 1980). The test result was displayed either in milligrams per deciliter (mg/dl) or in mill moles per liter (mmol/l).

Test principle: The amount of sugar present in the blood sample is reacted with the reagent of the test strip. Then meter estimates the current and shows the equivalent blood sugar level.The intensity of the current generated by the reaction relies on the quantity of sugar in the blood sample.

3.9.2. Measurement of creatinine

Creatinine and non-creatinine chromagen in the sample react with picric acid in alkaline media to form an orange-red colored complex solution. During the first step (approximately 30sec), mostly the rapid non-creatinine chromagen reacts where as during the following phase (approximate 2min) mainly true creatinine reacts, and finally the slow non-creatinine chromagen. The change in absorption during the second step is measured at 492nm.The absorbance of this complex is proportional to the creatinine concentration in the sample.

Creatinine + Picric acid $\xrightarrow{NaOH}$ Creatinine-picrate complex

Clinical utility; this test is used in:

- as a crude basic screen of renal function
- for establishing an individual's baseline creatinine range
- monitoring potentially nephrotoxic drugs, particularly in the elderly NSAIDs

    - ✓ ACE inhibitors
    - ✓ Aminoglycosides
    - ✓ Diuretics Lithium and others

- Monitoring potential nephropathy e.g. in diabetics
- Monitoring established renal disease monitoring renal transplant rejection
- Monitoring renal dialysis (Tietz, 2001).

3.9.3. Measurement of blood urea

Urea is the main breakdown product of protein catabolism. Malnutrition is therefore associated with low urea concentrations, whilst a high protein diet can elevate blood urea. Patients with muscle breakdown caused by sepsis, burns, or corticosteroids may also have high urea concentrations. Liver disease decreases the production of both urea and creatinine.

Principle: urea breakdown into ammonia and carbondioxide which is catalayzed by urease in the presence of water. Then ammonia combines with 2-oxoglutarate and NADH that catalayzed by glutamate-dehydrogenase (GLDH) to produce glutamate and NAD+. The

reaction is optimized since the GLDH is the rate-limiting enzyme. As the amount NADH depletes, the absorbance is decreased hence decrease in absorbance is directly proportional to the urea concentration within the given time intervals.

As the kinetics is very fast this test is preferably designed for analyzer application.

$$\text{Urea} + 2H_2O \xrightarrow{\text{Urease}} 2NH_4^+ + CO_3^{2-}$$

$$\text{2-Oxoglutarate} + 2NH_4 + NADH \xrightarrow{\text{GLDH}} \text{L-glutamate} + H2O + NAD^+$$

Quality Control: Reconstituted one vial of control solution which contains both normal and pathological with 5ml of distilled water. Controls must be kept at room temperature before use and also must be within range. If out of range, repeat the run. If still out of the range investigate for a root cause. (Reagent, calibration and quality control preparation….etc). Solve the problem, document, and rerun the quality controls and patient samples. Controls must be within range (Tietz., 2001).

3.9.4. Measurement of eGFR

Estimation of GFR was obtained from serum creatinine concentration and some or all of the following variables: age, sex, and race, and body size. Hence estimation of GFR was calculated, renal function tests of all patients were carried out, and estimated GFR (eGFR) was calculated using Modification of diet in renal disease study (MDRD) formulae as follows: $(GFRmL/min/1.73m^2 = 175x\ (s_{cr})^{-1.154}x(Age)^{-0.203}x$ (.742 if female), (1.212 if black) (conventional units) , where scr is serum creatinine (Stevens *et al*., 2006).

3.9.5. Measurement of neutrophil, lymphocyte, and leukocyte

Leukocyte, neutrophil, and lymphocyte counts were carried out using the automated hematology analyzer (HUMACOUNT PLUS) and expressed as × 1000 cells/mm^3. The test for blank was ≤ % 0.2 × 10^9 cells/l. Then, by dividing the neutrophil by the lymphocyte count, the NLR was calculated.

Test Principle: HUMACOUNT PLUS hematology analyzer uses two independent measurement methods; they are:

- The electrical impedance method was used for determining white blood cell (WBC), red blood cell (RBC), platelet (PLT) and differential count. As the cells pass through a small aperture, a change in electrical resistance occurs generating an equivalent voltage pulse. The number of pulses sensed during each cycle corresponds to the number of

WBC, red blood cell (RBC) and platelet (PLT) cell counted. The intensity of each pulse was essentially proportional to the cell volume.

- Cyan methaemoglobin Method for determining hemoglobin through photometric measurement. The HUMACOUNT PLUS measures the ability of the dilution to absorb light at a wavelength of 540nm (nanometers) (HUMACOUNT PLUS 1800 Hematology analyzer User manual).

3.9.6. Measurement of Urine albumin or macroscopic examination

To perform a chemical analysis using a urine dipstick the testing may consist of any or all of the following: pH, protein (semi-quantitative), glucose, bilirubin, ketone, hemoglobin (blood), urobilinogen, albumin, nitrite, leukocytes (leukocyte esterase) and specific gravity. Albuminuria was tested by MICRAL-II TEST strips by the dipstick method. Urinary albumin excretion (UAE) of greater than 300 mg/dl was assessed as macroalbuminuria. It is a semi- quantitative method of urine albumin analysis. The test was repeated within three months of interval.

Principle of the test: it is based on an immunological test principle using gold-labeled monoclonal antibodies with a chromogenic color indicator enabling confidence in results. The specific chromogen immobilized onto a pad reacts with albumin present in the urine and changes the color of the strips from light yellow to blue-green. Note that a clinical DN is indicated when urine albumin (>300mg/dl) is present in at least two of the three-morning urine samples (Henry, 1996).

3.9.7. Measurement of serum SGOT

Aspartate transaminase (AST) or SGOT is that was formerly known as glutamate oxaloacetate transaminase (GOT). When damage to heart or liver cells occurs, intracellular enzymes, such as ALT, are released into the peripheral blood. Since AST is located in the parenchymal hepatic cells and heart muscle, this enzyme is used to assess damage to these areas. In myocardial infarctions, AST rises in the serum 6-8 hours after onset and peaks within 2 days and returns to normal in 4-5 days after the injury. Increases in AST can be seen in hepatitis, liver necrosis, cirrhosis, and liver metastasis.

Principle: Aspartate transaminase catalyzes the transamination of L-aspartate to 2-oxoglutarate forming L-glutamate and Oxaloacetate. The oxaloacetate formed is reduced to malate by malate dehydrogenase (MD) with simultaneous oxidation of reduced NADH to NAD. The change in absorbance with time is due to the conversion of NADH to NAD which is directly proportional to AST activity. The measurement is taken by a spectrophotometer at 340 nm using continuous kinetic produced.

2-Oxoglutarate + L- aspartate $\xrightleftharpoons{AST}$ L-glutamate + Oxaloacetate

Oxaloacetate + NADH + H^+ $\xrightleftharpoons{MDH}$ L-malate + NAD^+

Quality Control**:** Reconstituted one vial of control solution which contains both Normal and Pathological with 5ml of distilled water. Controls must be kept at room temperature before use and also must be within range. If out of range, repeat the run. If still out of the range investigate for a root cause. (Reagent, calibration and quality control preparation….etc). Solve the problem, document, and rerun the quality controls and patient samples. Controls must be within range (Lothar, 1998 and Tietz, 2001).

3.9.8. Measurement of serum SGPT

Alanine transaminase (ALT) or SGPT is an enzyme involved in the metabolism of the amino acid alanine. This test is used to determine if a patient has liver damage. ALT found in different tissues but its highest amount is seen in liver. Hepatocyte damage causes the release of this enzyme in to the blood.

Principle: Kinetic method for measurement of ALT is used as recommended by the Expert Panel of the International Federation of Clinical Chemistry (IFCC). ALT is measured by the reagent rate analysis by the coupled reaction with lactate dehydrogenase (LDH) to reduce NADH (measured at a wavelength of 340nm) to NAD+. The reduction in absorbance at 340 nm due to NADH depletion is is equivalent to the concentration ALT in the sample.

2-oxoglutarate + L-alanine $\xrightleftharpoons{ALT}$ L-glutamate + pyruvate

Pyruvate + NADH + H+ $\xrightleftharpoons{LDH}$ L-lactate + NAD+

Quality Control: Reconstituted one vial of control solution which contains both normal and pathological with 5ml of distilled water. Controls must be kept at room temperature before use and also must be within range. If out of range, repeat the run. If still out of the range investigate for a root cause. (Reagent, calibration and quality control preparation….etc). Solve the problem, document, and rerun the quality controls and patient samples. Controls must be within range (Lothar, 1998; Tietz, 2001).

3.9.9. Measurement of Total Cholesterol

Cholesterol is measured enzymatically in serum or plasma in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reaction by products, H_2O_2 is measured quantitatively in a peroxidase catalyzed reaction that produces a color. Absorbance is measured at 500 nm. The color intensity is proportional to cholesterol concentration. The reaction sequence is as follows:

Cholesteryl ester + H_2O $\xrightarrow{\text{cholesteryl ester hydrolase}}$ cholesterol + fatty acid

Cholesterol + O_2 $\xrightarrow{\text{cholesterol oxidase}}$ cholest-4-en-3-one + H_2O_2

$2H_2O_2$ + 4-aminophenazone + phenol $\xrightarrow{\text{peroxidase}}$ 4-(p-benzoquinonemonoimino)-phenazone + 4 H_2O (Seguchi *et al* ., 1995).

3.10. Data processing and analysis

Simple descriptive statistics such as; mean, standard deviation, percentiles, and percentages were used to present socio-demographic characteristics, clinical parameters, NLR ratio, and other laboratory tests, and the value were compared between DN and without DN patients. Student t-test, a Chi-square test, and Pearson correlation were applied to analyze associated factors of two groups. The data was being entered by using Epi-Data statistical software version 3.1 and then exported to SPSS software version 21.0 for analysis and the p-value <0.05 was considered to be statistically significant in all analyses.

3.11. Data Quality control and management

- The socio-demographic characteristics and related data were collected by trained health professionals.
- All the laboratory procedures were handled by laboratory technologists and all

laboratory tests and measurements were performed based on the standard operational procedures (SOPs)

- ❖ Tools that were used to measure and analyze the test were standardized and automated.
- ❖ Data coding, entering, verifying, and cleaning was performed with great care.

3.12. Ethical consideration

Before starting data collection and preliminary study, an ethical clearance letter was obtained from the Department of Research and Ethics Review Committee, Department of Medical Biochemistry, College of Health Science, Addis Ababa University (DRERC), by approval letter with Ref. No. of SOM/BCHM/152/2011, meeting No. of DRERC 01/19 and protocol No. of M.Sc. 07/19 issued on June 25/2019 G.C. In addition, permission was obtained from Addis Ababa Health Bureau Bole Sub-city and Bole 17health center medical director to conduct the study. The purpose of the study was briefly explained for the study participants and they are informed that their responses were treated with strict confidentiality. Samples and data were collected after the study participants gave full consent by signing on the form. Confidentiality, anonymity, neutrality, and accountability were maintained throughout the study.

4. RESULTS

4.1. Socio-demographic Analysis

In this study, diagnosed T2DM patients (age 30 and above) were screened for DN with their informed consent. A total of 202 subjects have been enrolled in the present study; but, 3 patients were excluded because of default. Data from 202 patients (98.5%) were included in the analysis. Based on their albumin content, the subjects were divided into T2DM patients with albumin positive – with diabetic nephropathy (22.6%) and patients with albumin negative – without diabetic nephropathy (77.4%).

Subjects with microvascular complications were older than subjects without microvascular complications (60.36±10.30 vs.53.85±11.900 years) with longer duration of disease. Duration of disease category of patients with microvascular complication versus without microvascular complication was such that for ≤5 years it was 0% vs. 19.1%, for 6-10 years it was 4.4% vs. 39%, for 11-15 years it was 15.6% vs. 44.2%, for 16-20 years it was 42.2% vs.3.2%, for 21-25 years it was 17.8% vs. 1.9% and for 26 years and above it was 20% vs. 2.6%. From a total of 199 type 2DM diagnosed patients urban dwellers account for 95.6% and rural 4.4% for DN while urban dwellers account for 94.8% and rural 5.2% for the group without DN. Concerning education status DN patients scored lower education level than without DN patients. Education level of DN versus without DN group was: illiterate 37.8% vs. 7.1%, primary school 24.4% vs.16.9%, secondary 17.8% vs. 19.5%, College 13.3% vs. 27.3% and University and above attended were 6.7% vs. 29.2% respectively. In the present study, the mean age of patients with DN and the normal group was 60.36±10.30 and 53.85±11.90 years respectively. Hence subjects with DN were older than normal subjects, which was a statistically significant difference (p=0.001). There was also a statistically significant difference between DN and normal group with duration categories (p <0.0001) and educational level (p <0.0001). However, other demographic parameters; marital status (P =0.084), occupation (P =0.076), gender (p= 0.207), residence (p =0.839) and monthly income (p=0.368) did not differ in the two groups. The mean values ±SD and p-value of demographic parameters of the two groups is shown in Table 1.

Table-1:-The comparison of demographic parameters of T2DM with and without DN.

Variables	Category	Patients with DN (n=45)	Patients without DN (n=154)	P- value
Age (years)		60.36±10.30	53.85±11.900	0.001
Gender	Female	62.2%	53.9%	0.207
	Male	37.8%	46.1%	
Duration categories	≤ 5	0%	9.1%	<0.0001
	6-10	4.4%	39%	
	11-15	15.6%	44.2%	
	16-20	42.2%	3.2%	
	21-25	17.8%	1.9%	
	≥ 26	20%	2.6%	
Marital status	Single	17.8%	10.4%	0.084
	Married	62.2%	79.2%	
	Others	20%	10.4%	
Residence	Urban	95.6%	94.8%	0.839
	Rural	4.4%	5.2%	
Educational level	Illiterate	37.8%	7.1%	<0.0001
	Primary school	24.4%	16.9%	
	Secondary school	17.8%	19.5%	
	College	13.3%	27.3%	
	University and above	6.7%	29.2%	
Occupation	Unemployed	8.9%	7.1%	0.076
	Housewife	24.4%	24.0%	
	Merchant	20%	9.1%	
	Civil servant	13.3%	30.5%	
	Others	33.4%	29.2%	
Monthly income	≤ 1500	20%	26.6%	0.368
	≥ 1500	80%	73.4%	

4.2.Measurement of total leukocyte and differential count

In this study, the mean NLR values for T2DM with DN (2.66±0.49) was found to be higher compared to T2DM without DN (1.65±0.20; P <0.0001). Similarly, higher values were recorded with absolute neutrophil count (ANC) (p <0.0001) and absolute lymphocyte count (ALC) (p <0.0001) in DN than in without DN, which was statistically significant even though the total white blood cell count (TWBC) did not differ between the two groups (Table2).

Table-2:-The comparison of NLR and other leukocyte counts of T2DM with and without DN

Variables	Patients with DN (n=45)	Patients without DN (n=154)	P- value
TWBC	8528.89±11245.33	6905.19±495.84	0.338
ANC	5371.11±1013.68	4220.78±395.01	< 0.0001
ALC	1973.33±276.67	2585.71±357.08	< 0.0001
NLR	2.66±0.49	1.65±0.20	< 0.0001

4.3. Measurement of renal function tests and some clinical parameters

The renal function tests (serum urea, creatinine, and eGFR) of both groups were carried out, and estimated GFR (eGFR) was calculated by a modification of diet in renal disease (MDRD) study formula. There was a significant difference between the two groups in eGFR (p =0.048). Patients with albumin positive had a significantly low eGFR (97.51±62.61) than albumin negative group (126.03±89.87). However, the mean values of blood urea, serum creatinine, FBS, total cholesterol were not found significantly different between the two groups. While SBP and DBP records were higher in T2DM with DN compared to T2DM without DN (p <0.001), their BMI did not show any significant difference (Table-3).

Table-3:-The comparison of renal function tests and some clinical parameters of T2DM with and without DN

Variables	Patients with DN (n=45)	Patients without DN (n=154)	P-value
Serum urea	28.27±4.41	27.64±3.18	0.288
Serum creatinine	0.92±0.37	0.87±0.31	0.451
eGFR	97.51±62.61	126.03±89.87	0.048
FBS	207.07±75.50	194.64±28.15	0.272
Total cholesterol	188.22±12.04	185.44±9.06	0.156
SBP	139.09±9.63	130.70±7.23	< 0.0001
DBP	88.09±4.41	84.32±4.59	< 0.0001
BMI	25.35±1.77	24.87±1.47	0.068

4.4. Measurement of liver functional tests

SGPT and SGOT values were found raised in T2DM with DN patients compared to T2DM without DN patients (P <0.0001 and P =0.002, respectively; (Table:-4).

Table-4:-The comparison of liver function tests of T2DM with and without DN

Variables	Patients with DN (n=45)	Patients with DN without DN (n= 154)	
SGOT	42.84±5.88	39.93±5.45	0.002
SGPT	37.94±5.56	30.78±6.23	< 0.0001

4.5. Correlation study between neutrophil-lymphocyte ratio (NLR) and others studied Variables

Significant positive correlation was observed between NLR and each of the other variables like: age (r =0.162, p =0.023), duration of illness (r =0.521, p <0.001), SBP (r= 0.338, p <0.001), DBP (r= 0.317, p <0.001), total leucocyte (r= 0.162, p= 0.023), ANC (r= 0.712, p< 0.001), serum creatine (r= 0.172, p= 0.015), SGOT (r =0.186, p=0.008), SGPT (r= 0.440, p <0.001). Similarly, NLR was negatively correlated within the following parameters: education level (r =-0.251, p< 0.001), ALC (r =-0.770, p< 0.001), and eGFR (r =-0.140, p =0.048).

Table-5:- Correlation study between Neutrophil-lymphocyte Ratio (NLR) and others Studied Variables of T2DM with and without DN

Study parameters	R	P-value
Age (years)	0.162	0.023
Gender	0.04	0.959
Duration categories	0.521	< 0.0001
Marital status	0.019	0.786
Residence	0.059	0.409
Educational level	-0.251	< 0.0001
Occupation	-0.054	0.451
Monthly income	0.057	0.422
Total leucocyte	0.162	0.022
ANC	0.712	< 0.0001
ALC	-0.770	< 0.0001
Serum urea	0.057	0.428
Serum creatinine	0.172	0.015
eGFR	-0.140	0.048
FBS	0.132	0.064
Total cholesterol	0.089	0.210
SBP	0.338	<0.0001
DBP	0.731	<0.0001
BMI	0.132	0.063
SGOT	0.186	0.008
SGPT	0.440	<0.0001

5. DISCUSSION

Several scholars that have described the association between inflammation and vascular disease showed that chronic inflammation can accelerate the development of micro and macrovascular complications in patients with diabetes. Leucocyte count is one of the useful inflammatory biomarkers in clinical practice. The neutrophil-lymphocyte ratio (NLR) in a complete blood count is studied in many non-communicable diseases as an inflammatory marker and is used to predict the prognosis of diseases such as acute myocardial infarction (MI), stroke, and heart failure (Rudiger *et al.,* 2006). In addition, NLR is useful indicator of inflammatory process progress in various diseases, including appendicitis, cardiac diseases, and cancers. Moreover, the NLR is used as an inflammation biomarker in DN (Zahorec, 2001). The key finding of this study was that the NLR values were found to be significantly higher ($p < 0.0001$) in patients who were diagnosed as albumin positive as compared with those with albumin negative. This is the first study in Ethiopia that assessed the relationship between NLR and diabetic patients with DN and without DN.

In the present study, the mean NLR among diabetic patients with albumin positive was significantly higher than NLR among those with albumin negative ($p < 0.0001$). In addition, ANC and ALC levels were also found to be significantly correlated with patients with albumin positive. A significant positive correlation was found between NLR and each of the following variable: age ($r=0.162$, $p=0.023$), duration of the disease ($r =0.521$, $p <0.001$), SBP ($r= 0.338$, $p <0.001$), DBP ($r=0.317$, $p <0.001$), TWBC ($r= 0.162$, $p= 0.023$), ANC ($r= 0.712$, $p <0.001$), serum creatine ($r =0.172$, $p =0.015$), SGOT ($r =0.186$, $p =0.008$), SGPT ($r =0.440$, $p <0.001$). On the other hand, NLR was negatively correlated with educational level ($r =-0.251$, $p <0.001$), ALC ($r =-0.770$, ($p <0.001$), and eGFR ($r =-0.140$, $p= 0.048$).

The main factor involved in the development of DN is advanced gylcation end product (AGE) activation, which cause oxidation of proteins and lipids that leads to tissue damage. Hence, in diabetic patients, these AGE activate leucocytes that secrete many kinds of cytokines and transctiption factors having a crucial role in inflammation and thereby contribute to glomerulosclerosis (Chung et al.2005; Pertynska-Marczewska *et al.,* 2004). AGE can also directly interacts with various cellular components that trigger numerous intracellular signaling pathways; elicit abnormal immune responses and leading to cellular damage (Rojas and Morale, 2004). A study done by Evans et al. also confirmed hyperglycemia-induced oxidative stress has a crucial role in the pathogenesis of DN (Evans *et al.,* 2002). In concordance with the present study, Khandare *et al.* has also shown that NLR mean values were raised in diabetic patients that diagnosed with albumin positive (2.83 ± 0.85) than in diabetic

patients with albumin negative (1.94±0.65), wich was statistically significant. The mean values of ANC and ALC were also significantly raised with DN patients (Khandare *et al.*, 2018).

Further study done in Egypt indicated that NLR levels were significantly raised in diabetic patients with micro vascular complications such as nephropathy ($P < 0.001$), neuropathy ($P = 0.025$) and retinopathy ($P < 0.001$) than in diabetic patients without any microvascular complications and healthy individuals (Moursy EY *et al.,* 2015). A study done by Azikin et al. suggested that NLR was negatively correlated with GFR. Also, different researchers suggested that NLR was positively correlated with age, WBC, duration of diseases, blood pressure, serum creatinine, SGOT, and SGPT, whereas NLR was negatively correlated with ALC and eGFR (Cuneyt *et al.,* 2016; Moawia *et al.,* 2018; Unal *et al.,* 2015).

Furthermore, study done by Azab *et al.* indicated that NLR can be used as a prognostic marker to asses severity of renal function (Azab *et al.,* 2012). Similarly, Afsar has explained that NLR was related to DN and also associated with ESRD (Afşar, 2014). Another study has proved that NLR was correlated in diabetic patients with albumin positive which indicated there was a relationship between inflammation and endothelial dysfunction in DN (Akbas *et al.,* 2014). A study done by Huang *et al.* has shown that NLR values of diabetic patients with DN (2.48 ± 0.59) was significantly higher than NLR values of diabetic patients without DN (2.20 ± 0.62) and healthy controls (1.80 ± 0.64). The mean values of ANC and ALC were also significantly raised in MD patients with DN than in DM patients without DN (Huang *et al.,* 2015).

In DN, interleukin-6 (IL-6) is a major released inflammatory mediator that has a potential of stimulating neutrophil production from progenitor cells called colony-stimulating factor (CSF) in the bone marrow. The neutrophil will adhere to the target cell, namely the endothelial cells in blood vessels, resulting in disturbed endothelial function in blood vessels (Lim and Tesch, 2012; Nakhavani *et al.,* 2015; Skikata and Makino, 2013). The increased in inflammatory mediators accelerates the muscular protein catabolism, increases positive stenosis of acute-

phase protein C-reactive protein (CRP), and suppresses protein/albumin production in addition to the loss of protein through proteinuria. This cause protein deficiency, which affect the normal hematopoiesis process, consequently will result in bone marrow dysfunction, insufficient lymphocyte production. The leptin produced by adipose tissue inhibition against T helper 2 (Th2) proliferations will also cause the lymphocyte count to decrease (Makino, 2013; Nakhavani *et al.,* 2015).

There was no significant correlation among T2DM patients with and without DN groups with relation to gender, marital status, residence, occupation, monthly income, total leucocyte, serum creatinine, serum urea, fasting blood sugar, total cholesterol, and body mass index as witnessed from the present study. However, there was a significant difference between the two groups concerning age, duration categories, education level, eGFR, blood pressure, SGOT, and SGPT. In this study, subjects with DN were older than without DN that was a statistically significant difference between the two groups (p =0.001).

Similar to the present study, many researchers concluded that older age is a high risk factor for developing DN. As age increases, patients will be at greater probability of developing kidney diseases, which are mostly reflected by proteinuria, because as age increases the immunity status of any individual will be reduced (Hintsa *et al.,* 2017). Another study conducted in Africa concluded that 95% of diabetic patients developed albuminuria within ten years of diagnosis, 35% developed the ESRD within five years of diagnosis, while 18% died of DN after 20 years of diagnosis. This study revealed that the duration of diabetes, blood pressure, and advancing age are significantly related to the DN (Noubiap *et al.*, 2015). Furthermore, the study indicated that about 20-30 percent of people with T2DM develop DN, and its incidence increases with the duration of illness. It has been reported elsewhere that up to 35% of all patients with T2DM eventually develop nephropathy after 25-30 years of suffering from diabetes (Ziyad, 2009). The DN is a chronic inflammatory disease that increases with age. As age of diabetic patients increases by one year, chance of developing DN increases by 3.7%, which means for ten years increases in the duration of illness after diagnosis, the probability of developing diabetic microvascular complication increases by 1.43 times (43%) (Hintsa *et al.,* 2017).

Education status had a significant association with DN. According to this finding more DN patients were found on illiterate individuals, followed by Primary school, College, University and above level individuals, which is reverse in patients without DN where the majority of them were categorized into University and above, followed by College, Secondary school, Primary school and illiterate. This could be attributed that participants who had high educational level have a better knowledge to modify their lifestyle and their health status by finding different alternatives for a checkup and they had good

awareness about their health status.

The presence of hypertension increases the probability of developing diabetic nephropathy. Hence, patients with DN had higher values of systolic and diastolic blood pressure than patients without DN ($p < 0.0001$). In line with the present study, the study done by Imtiaz *et al.* has suggested that non communicable diseases such as hypertension and diabetes were correlated with inflammation and reflected by NLR (Imtiaz *et al.,* 2012). Also, the studies conducted in India, Cameron, and Ethiopia support the present study finding (Noubiap *et al.,* 2015; Tesfaye, 2010; Vijay and Sk, 2012). Patients with DN had significantly low mean eGFR (eGFR 97.51±62.61) as compared to those patients without DN (GFR126.03±89.87). eGFR is one of the most specific parameters for kidney function. Hence, DN development include a pathological sequences (which start with glomerular damage, results in proteinuria, succeeded by progressive renal damage, fibrosis, inflammation, and lastly loss of functional nephrons) is known to play special role in the development and progression of DN (Gai *et al.,* 2006; Gross *et al.,* 2005 and Lim *et al.,* 2012).

In the present study, the two most important liver functional tests SGPT and SGOT mean values were significantly raised in patients with DN as compared with in patients without DN ($p <0.0001$) and and ($p =0.002$), respectively. In T2DM, hepatic glucose production and glycogenolysis are not suppressed due to the loss of the direct effect of insulin (insulin resistance) and causes hyperglycemia. In T2DM patients insulin resistance causes increased hepatic glucose production and glycogenolysis that aggravate hyperglycemic condition. Insulin resistance also cause hydrolysis of triglycerides stored in adipocytes. This two conditions (hyperglycemia and high free fatty acids) are known to up-regulate lipogenic transcription factors. Consequently, elevated levels of circulating free fatty acids in blood plasma exceeds hepatic mitochondrial oxidation system and causes excessive accumulation of triglycerides in the liver. This pathological pathway leads to a fatty liver disease known as non-alcoholic fatty liver (NAFLD) in T2DM patients. In the majority of cases, the non- alcoholic fatty liver disease causes an asymptomatic abnormality of liver enzyme levels, which include SGPT and SGOT. Therefore, SGPT and SGOT have been used as a marker of non-alcoholic fatty liver disease (Alkouri *et al.,* 2012; Khandare *et al.,* 2017).

6. CONCLUSION AND RECOMMENDATION

The result of this study has shown that there was a significant correlation between NLR and DN implying that inflammation and endothelial dysfunction could be an integral part in the pathogenesis of DN. The NLR was significantly raised in patients with T2DM having albumin positive. Therefore, NLR may be considered as a predictor and a prognostic marker of DN. In addition, NLR is could be considered as an essential and stable marker in diagnosing microvascular complications of diabetes.

This finding also suggested that there is a need to manage complications of diabetes early before it is transformed to its severe stage by the implementation of preventive and control measures. In Ethiopia, inflammatory markers have not been used to diagnose and follow-up diabetic complications. Hence, health care systems should ensure to prompt treatment of diabetes with simple and available laboratory diagnoses especially for patients with older age and longer duration of the disease.

Ministry of Health, regional health bureau, NGOs, private sector, and other responsible bodies should contribute to the prevention and control of diabetic complications. Based on the finding of the study, the investigator also would like to recommend further investigation to be undertaken using a more extensive, accurate study design and large sample size.

7. STRENGTH AND LIMITATIONS OF THE STUDY

7.1. **Strength of the Study**

Clinical parameters and laboratory measurements of T2DM patients without DN were used for comparison to investigate the effect of DN within such parameters. Correlation analysis was made to implicate inflammatory markers to type 2 DM renal complications. Comparison was made between type 2 DM patients with and without DN.

7.3. Limitations of the Study

One of the limitation of this study is that it is a cross- sectional study; any conclusive causal associations between NLR and DN was not investigated and it is difficult to draw strong conclusions.

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ANNEXES

Annex I: English Version Information sheet

ADDIS ABABA UNIVERSITY

COLLEGE OF HEALTH SCIENCES

DEPARTMENT OF MEDICAL BIOCHEMISTRY

My name is…….. .I am working as a data collector for the study entitled, "Assessment of Neutrophil-lymphocyte ratio as an inflammatory marker among Type 2 Diabetes Mellitus with diabetic nephropathy and without diabetic nephropathy patients in Bole 17 health center, Addis Ababa, Ethiopia," being conducted by Mesfin Zewude, a post graduate student of Addis Ababa, College of Health Sciences, Department of Medical Biochemistry, in partial fulfillment of the requirement for the degree of Master of Science in Medical Biochemistry. I kindly request you to lend me your attention to explain you about the study and the study participation.

1. The study /Project Title

Assessment of Neutrophil-lymphocyte ratio as an inflammatory marker among Type 2 Diabetes Mellitus with diabetic nephropathy and without diabetic nephropathy patients in Bole 17 health center, Addis Ababa, Ethiopia.

2. Purpose of the study

To assess the neutrophil-lymphocyte ratio (NLR) as an inflammatory marker among Type 2 Diabetes Mellitus with and without diabetic nephropathy patients. The finding obtained from

this study will be important for a better understanding of treating and preventing the progression of nephropathy among type 2 diabetic patients.

3. Procedure and Duration

I will be interviewing you using a questionnaire to provide me with the pertinent data that is helpful for the study. The total time it takes for interview is nearly 20-25minutes. So, I kindly request you to spare me this time for the interview.

4. Risk and benefits

The risk of participating in this study is very minimal, but it may take some minutes from your time. There is no any direct payment for your participation in this study. But, the findings from this study may be helpful in prevention of development of diabetic nephropathy among T2DM patients.

5. Confidentiality

The information you will provide us will be kept confidential. Your personal identifiers data will not be collected. The finding of the study will be generalized for the all participants, so that it will not reflect any particular information about an individual person or a household.

6. Rights

Participation in this study is fully voluntary. You have the right to declare to participate or not in this study. If you decide to participate, you have the right to withdraw from the study at any time, and this will not harm you. You will not be enforced to answer any question that you do not want to answer.

7. Contact Address

If there are any questions or inquiries regarding the study, please contact Mesfin Zewude: (+251910066805), email: mes.zewd@gmail.com

8. Declaration of Information Voluntary Consent

I have read the participant information sheet or it was read to me. I have clearly understood the purpose of the research, the procedures, the risk and benefits, issues of confidentiality, the rights of participating and the contact address for any inquiries. I have been given the opportunity to ask questions for things that may have been unclear. I was informed that I have the right to withdraw from the study at any time or not to answer any question that I do not want. Therefore, I declare my voluntary consent to participate this study with my initial signature.

Code for participant: …….. Signature of participant: ……

Name and signature of data collector: …….. Date …….

Annex II: English Version Questionnaire

I. Socio-demographic information

01. Identification number of respondents----------

1.01. Age……years

1.02. Sex 1. Male 2. Female

1.03. Marital status 1.single 2. married 3. others

1.04. Residence 1. urban 2. Rural

1.05. Educational level 1. Illiterate 2. Primary school 3. Secondary school 4.College 5.University and above

1.06. Occupation 0) employed 1) Housewife 2) merchant 3) civil servant 4) others

1.07. Monthly Income 1. ≤ 1500 ETB 2. >1500 ETB

1.08. Duration (……..years). 1). less≤ 5 2). 6-10 3). 11-15 4). 16-20 5). 21-25 6. ≥26

II. **Some clinical measurement and Laboratory Examination**

S.No.	Variable	With nephropathy (with albuminuria)	Without nephropathy (without albuminuria)
2.01	BMI		
2.02	Blood pressure	Systolic	Systolic
		Diastolic	Diastolic
2.03	WBC		
2.04	ANC		
2.05	ALC		
2.06	NLR		
2.07	Serum creatinine		
2.08	eGFR		
2.09	FBS		
2.10	Blood urea		
2.11	Total cholesterol		
2.12	SGOT		
2.13	SGPT		

Collected by…… Sign…….. Date……...

THANK YOU!!!

Annex III: Amharic Version Information Sheet

አዲስ አበባ ዩኒቨርሲቲ
ህክምናና ጤና ሳይንስ ኮሌጅ
የባዮሚስትሪ ት/ክፍል

ይህ ቃለ መጠይቅ “Assessment of Neutrophil-lymphocyte ratio as inflammatory marker for Type 2Diabetes Mellitus with and without diabetic nephropathy patients” በሚለዉ ርዕስ በቦሌ17ጤና ጣቢያ የስኳር በሽታ ታካሚዎች ክትትልና ተዛማጅ ጉዳዮች ላይ በሚደረጉ ለማጥናት የተዘጋጀነዉ።

ጤና ይስጥልኝ!..... እባላለሁ።

በአዲሰ አበባ ዩኒቨርሲቲ ህክምናና ጤና ሳይንስ ኮሌጅ የባዮ-ሚስትሪ ት/ክፍል ሁለተኛ አመት ተመራቂ ተማሪ ነኝ። እዚህ የተገኘሁት ስለ ስኳር በሽታና ተዛማጅ ጉዳዮች ላይ ለሚደረገዉ ጥናት የሚሆን መረጃ ለመሰብሰብ ነዉ። በመሆኑም እርስዎ በዚህ ጥናት እንዲሳተፉ ተመርጠዋል። ስለሆነም ለጥናቱ የሚሆኑ አንዳንድ ጥያቄዎችን እጠይቆዎታለሁ፤ከእርስዎ የሚገኘዉ መረጃ የስኳር በሽታ ክትትል በሚያደርጉት ታካሚዎች ላይ የሚከሰተዉን የኩላልት በሽታ ችግር ለመከላከል ከፍተኛ ሚና ስለሚኖረዉ ለምንጠየቅዎት ጥያቄ ትክክለኛ መረጃ እንዲሰጡን በትህትና እንጠይቃለን።ከእርስዎ የምናገኘዉን ማንኛዉንም መረጃ በሚስጢር እንይዛለን። ከዚህ ጥናት ጋር በተያያዘ በማንኛዉም ቦታና ጊዜ ስምዎ እንደማይመዘገብና እንደማይጠቀስ ልንገልፅልዎ እንወዳለን። ለጥናቱ የምናሳትፍዎ የእርስዎን ሙሉ ፈቃደኝነት ስናገኝ ብቻ ነዉ።በመጠይቁ ላለመሳተፍ ወይም በመጠይቁ ሂደት ልመልሱት የማይፈልጓቸዉን ጥያቄዎች ያለመመለስ መብትዎ የተጠበቀነዉ።

በመጠይቁ ለመሳተፍ ፈቃደኛ ነዎት?

1. አዎ ……..መጠይቁ ይቀጥላል
2. አይደለሁም……ወደ ሌላ ታካሚ ሒድ

ስለ ፈቃደኝነትዎ አመሰግናለሁ!

Annex IV. Amharic Version Questionnaires

01. የመጠይቁ ተሳታፊዎች መለያ ቁጥር.....

ክፍል 1 ኢኮኖሚያዊና ማህበራዊ መረጃዎች

1.01. እድሜ.....ዓመት

1.02. ፆታ 1). ወንድ 2). ሴት

1.03. የጋብቻ ሁኔታ 1) የላገባ/ ች 2). የገባ/ች 3). ሌላ

1.04.መኖሪያ አድራሻ 1). ከተማ 2). ገጠር

1.05.የትምህርት ደረጃ 1). የልተማረ 2). አንደኛ ደረጃ 3). ሁለተኛ ደረጃ 4). ኮሌጅ 5). ዩኒቨርሲቲ ድግሪና ከዛ በላይ

1.06. ስራ 0). ስራ የሌለዉ/ት 1). የቤት እመቤት 2). ነጋዴ 3). የመንግስት ሰራተኛ 4) ሌላ……..

1.07. የወር ገቢ 1). = <1500 ብር 2). >1500 ብር

1.08. የበሽታ ቆይታ ጊዜ…… ዓመት፤ 1). ≤ 5 2). 6-10 3). 11-15 4). 16-20 5).21-25 6) ≥26

ክፍል 2፤ የክልኒካልና ላቦራቶሪ ምርመራ ዉጤት

S.No.	Variable	With nephropathy (with albuminuria)	Without nephropathy (without albuminuria)
2.01	BMI		
2.02	Blood pressure	Systolic	Systolic
		Diastolic	Diastolic
2.03	WBC		
2.04	ANC		
2.05	ALC		
2.06	NLR		
2.07	Serum creatinine		
2.08	eGFR		
2.09	FBS		
2.10	Blood urea		
2.11	Total cholesterol		
2.12	SGOT		
2.13	SGPT		

የመረጃ ሰብሳቢዉ/ዋ ስም ……. ፊርማ ----------ቀን……. **አመሰግናለሁ!!!!**

Annex V: Laboratory procedure for venous blood sample collection

1. Label the test tubes with the client's identification number
2. After wearing rubber gloves then reassure the patient is in a comfortable position
3. Tight the tourniquet around the arm of the patient just above the bend in the elbow (tourniquet should positioned 7.5cm to 10cm above the puncture site)
4. Prepare the vacutainer tube and needle
5. Using the tip of the index finger examine the phlebotomy site
6. Disinfect the phlebotomy site by swabbing the skin in small outward circles with an alcohol swab or cotton wool soaked in isopropyl alcohol (Do not touch the prepared puncture site with your fingers after disinfecting the skin)
7. Insert the needle directly into the vein then approximately withdraw 4 or 5 ml of blood in the EDTA vacutainer tube (use pink test tube without anti-coagulant for clinical chemistry test)
8. Tell the patient open his/her clenched fist then release the tourniquet
9. Then withdraw the needle from the vein and cover the puncture site with a cotton swab
10. Then hold it (or have the subject hold) pressure at the puncture site for 3 minutes or until adequate hemostasis is visible
11. Properly discard the used materials in a safe container
12. Centrifuge the whole blood at 800–1600 × rpm for 20 minutes at room temperature when serum or plasma was required.

Annex VI. Laboratory procedure for fasting blood sugar (FBS) analysis

1. Fully insert the test strip into the meter with the contact bars end first
2. When the strip is going no further at least obtain 0.7 microliters of the blood sample
3. Hold the absorbent channel of the test strip until blood drop has filled the conformation window
4. Then check that the meter begins to count down
5. After 6 seconds read the test result
6. Document the test result
7. Then shut off the meter by removing the strip
8. Discard the used strip in a safe container
9. Finally, the test result displayed either in milligrams per deciliter (mg/dl) or in mill moles per liter (mmol/l).

Annex VII: Laboratory procedure for urine albumin analysis

1. Invert the control vial several times to mix.

2. Remove one strip from the vial and replace the cap.

3. Before testing, hold the strip against vial to observe proper reading format.

4. Place one drop level of control solution on each of the pads on the strip,

5. Start the timer, and

6. While touching the edge of the strip on an absorbent material then remove excess control solution

7. Recap the urine quality control vial

8. Document the results on the quality control sheet.

9. Repeat the above steps for level two control solution.

10. Compare the reagent areas to the corresponding color chart on the bottle label at the time specified

11. Hold the strip close to color blocks and match carefully

12. Since proper reading time is critical for optimal results protein (urine albumin); read within 60 seconds

13. Documented the test results on the quality control log, including any "out of limits" values and corrective action.

DECLARATION

I, the undersigned, hereby declare that this MSc thesis is my original work and has not been presented for a degree in any other University and that all sources of materials used for the thesis have been duly acknowledged.

Name of candidate	Signature	Date
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