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Original Article

Neutrophil-to-lymphocyte ratio in diabetic microangiopathy

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Abstract: Diabetic nephropathy (DN) and diabetic retinopathy (DR) imposes a tremendous burden on health economies, however, the exact molecular action leading to DN and DR is not yet understood, but growing evidence has emphasized the critical role of inflammation in both the pathogenesis and the progression of micro-angiopathy and macro-angiopathy in patients with diabetes. The neutrophil-to-lymphocyte ratio (NLR) was defined as a novel potential marker to determine inflammation. We aim to evaluate the relationship between DN/DR and NLR. A total of 321 patients with type 2 diabetes mellitus were included. They were divided into the following three groups by urinary albumin-to-creatinine ratio (UACR) and/or serum creatinine: normoalbuminuria group (normal): UACR<30 mg/g, microalbuminuria group (micro): UACR 30-300 mg/g, overt nephropathy group (overt): UACR >300 mg/g and/or serum creatinine >1.5 mg/dl. NLR levels were significantly higher in patients with microvascular complications than those without microvascular complications (P<0.001). Moreover, the mean NLR levels increased parallel to the severity of DN and DR. The logistic regression analysis showed that NLR was a risk factor for predicting DN and DR in type 2 diabetic patients. ROC curve analysis showed that when using a best cut-off value of 1.758 for the NLR, the sensitivity was 75.4%, the specificity was 92.5% (the ability of the NLR to predict DN risk). We recommend that the NLR values of diabetic patients be calculated as NLR is a cheap, predictive, and prognostic marker for microvascular complications of diabetes.

Keywords: Diabetic nephropathy, diabetic retinopathy, neutrophil-to-lymphocyte ratio, inflammation

Introduction

Diabetes mellitus (DM), a systemic disease characterized by vascular and neuropathic complications [1, 2], is a progressive chronic disease emerging as a global epidemic. It imposes a tremendous burden on health economies mainly because of its devastating complications. Diabetic nephropathy (DN), a common complication in patients with diabetes, is a leading cause of end stage renal failure (ESRD). Furthermore, the cardiovascular risk of diabetic patients rise progressively as DN develops and most of patients with DN die for cardiovascular events [3]. Diabetic retinopathy (DR), the most common microvascular complication of diabetes, is the major cause of acquired blindness in working-age adults [4]. So, there is an urgent need to identify new clinical biomarkers and targets for treatment to effectively prevent and slow the progression of the complications.

The exact molecular action leading to DN and DR is not yet understood, but growing evidence has emphasized the critical role of inflammation in both the pathogenesis and the progression of micro-angiopathy and macro-angiopathy in patients with diabetes, in which an altered immune system plays a decisive role in the pathogenesis of DM. These immunological alterations result in elevated circulating levels of acute-phase proteins and pro-inflammatory cytokines that play a major role in the development of chronic inflammation-induced organ dysfunction in DM [5, 6].

The count of white blood cell (WBC) is a basic but cheap, readily available, and sensitive indicator of the inflammatory status [7], particularly in cardiovascular diseases [8]. Studies have shown that neutrophilia and lymphocytopenia are independent predictors of many diseases, such as acute heart failure [9], coronary artery disease [10], and acute coronary syndromes

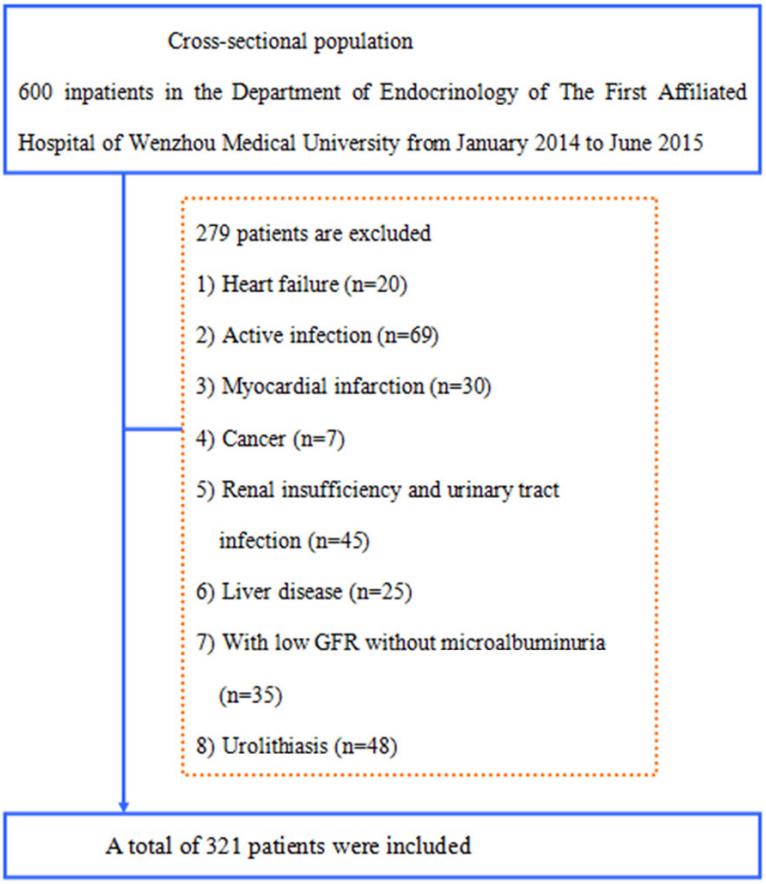

Figure 1. Study flow diagram. A total of 600 participants were enrolled initially, while 279 participants who did not meet the inclusive criteria were excluded. Finally, 321 individuals were included.

[11]. Recently, the neutrophil-lymphocyte ratio (NLR), a novel potential marker to determine inflammation, has been demonstrated to be a greater risk factor than total WBC count in the prediction of adverse outcomes in various medical conditions like cancer and cardiovascular diseases [12, 13].

However, according to our knowledge, there have been few studies evaluating the prognostic value of NLR in DR and DN. Based on this background, we aim to evaluate the relationship between diabetes complications and inflammation using NLR and estimate whether NLR can be used as a predictive and reliable marker.

Materials and methods

Study protocol

Conducted from January 2014 to June 2015 in the Department of Endocrinology of the First Affiliated Hospital of Wenzhou Medical University, Zhejiang, China, our study included 600 patients diagnosed with Diabetic nephropathy. According the exclusion criteria, finally, there were 321 subjects included. They were divided into the following three groups by urinary albumin-to-creatinine ratio (UACR) or serum creatinine: normoalbuminuria group (normal): UACR<30 mg/g, microalbuminuria group (micro): UACR 30-300 mg/g, overt nephropathy group (overt): UACR >300 mg/g and/or serum creatinine >1.5 mg/dl. All patients underwent the following procedures: history taking, physical examination, blood pressure (BP) measurement, biochemical analysis, spot urine analysis and DR assessment.

Exclusion criteria for this study included myocardial infarction, heart failure, active infection, severe tissue damage, acute massive hemorrhage, acute poisoning, cancer, AIDS, blood diseases that affect neutrophils and lymphocytes (e.g. myeloproliferative disease and leukemia), diseases that affect urinary protein excretion (e.g. nephrotic syndrome, urolithiasis, renal insufficiency and urinary tract infection) and diseases that affect the renal blood flow (renal artery stenosis, liver disease, hypovolaemia and dehydration). Patients with low GFR without microalbuminuria were also excluded from the study. No patient was reported any intake of systemic or topical steroids or anti-inflammatory drugs during the study period (**Figure 1**). Institutional ethical committee approval was obtained for the study, and informed consent was obtained from all study subjects.

Definitions

Diabetes was diagnosed based on the World Health Organization consulting criteria [14]. Retinopathy was graded using the International Clinical Diabetic Retinopathy Disease Severity

Table 1. Clinical and biochemical characteristics of the DN subjects

	Diabetes patients			
Variable	Normal (n=134)	Micro (n=74)	Overt (n=113)	*P* value
Age (years)	54.22±10.27	55.46±10.78	60.03±10.31	P<0.05b,c
Male*	72 (53.73%)	48 (64.86%)	66 (58.41%)	P>0.05
Female*	62 (46.27%)	26 (35.14%)	47 (41.59%)	P>0.05
BMI (kg/m^2)	24.20±2.77	25.80±3.88	24.89±3.47	P<0.05a
Hypertension*	65 (48.51%)	50 (67.57%)	104 (92.04%)	P<0.05a,b,c
SBP (mmHg)	136.99±18.29	141.58±19.05	156±24.87	P<0.05b,c
DBP (mmHg)	78.34±10.90	78.96±10.66	79.05±13.02	P>0.05
Smoking*	28 (20.90%)	28 (37.84%)	30 (26.55%)	P<0.05a
Drinking*	20 (14.93%)	19 (25.68%)	15 (13.27%)	P>0.05
UA (μmol/l)	298.54±72.51	326.30±86.45	368.96±87.84	P<0.05a,b,c
Albumin (g/l)	40.54±3.29	40.13±3.73	33.16±5.85	P<0.05b,c
TG (mmol/l)	1.70±0.71	1.96±0.96	1.92±0.97	P>0.05
TC (mmol/l)	4.96±1.15	4.94±1.33	5.45±1.79	P>0.05
LDL (mmol/l)	2.88±0.89	2.85±0.91	3.19±1.33	P>0.05
HbA1c	9.16±1.78	9.41±2.21	8.81±2.27	P>0.05
WBCs (109/l)	5.93±1.21	6.79±1.46	6.99±1.42	P<0.05a,b
Neutrophils (109/l)	3.03±0.77	4.04±1.14	4.47±1.09	P<0.05a,b,c
Lymphocytes (109/l)	2.26±0.54	2.13±0.60	1.83±0.54	P<0.05b,c
NLR	1.38±0.35	2.04±0.85	2.6±0.87	P<0.001a,b,c
Hemoglobin (g/l)	137.37±14.92	135.58±14.69	114.09±21.23	P<0.05b,c
DR*				
No apparent DR	66 (49.26%)	26 (35.14%)	21 (18.58%)	P<0.05b,c
Nonproliferative DR	58 (43.28%)	45 (60.81%)	65 (57.52%)	P>0.05
Proliferative DR	10 (7.46%)	3 (4.05%)	27 (23.89%)	P<0.05b,c
GFR [ml/(min 1.73 m^2)]				
GFR≥90	126 (94.03%)	65 (87.84%)	24 (21.24%)	P<0.001b,c
GFR<90	8 (5.97%)	9 (12.16%)	89 (78.76%)	P<0.001b,c

All parameters are expressed as mean ± standard deviation, unless otherwise stated. P<0.05 was accepted as the level of significance. P<0.05a: normal VS micro; P<0.05b: normal VS overt; P<0.05c: micro VS overt; *Data are expressed as number (%). SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HbA1c, glycated Hemoglobin; TG, triglycerides; TC, Total cholesterol; LDL, low-density lipoprotein; NLR, neutrophil-lymphocyte ratio; UA, uric acid; WBC, white blood cell count.

Scale [15]. Diabetic nephropathy was graded using the urinary albumin-to-creatinine ratio (UACR) or serum creatinine more than twice in 6 months.

Data collection

Medical history and a health habit inventory were performed by trained medical staff using a standardized procedure. Standing height and body weight were measured without shoes or outer clothing. Body mass index (BMI) was calculated as the ratio of weight (kg) to height (m^2). Blood pressure (BP) was measured using a noninvasive automated sphygmomanometer (OMRON, Japan) with the subjects in a quite environment and in a sitting position.

Clinical examination and data recording was conducted in the morning after an overnight fast. Fasting blood samples were collected from each subject in an antecubital vein and were used for the analysis of biochemical measurements serum samples without frozen. The biochemical measurements included albumin, creatinine (Cr), uric acid (UA), total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C), glycated hemoglobin (HbA1c). All values were measured by an automated analyzer (Abbott AxSYM) using standard

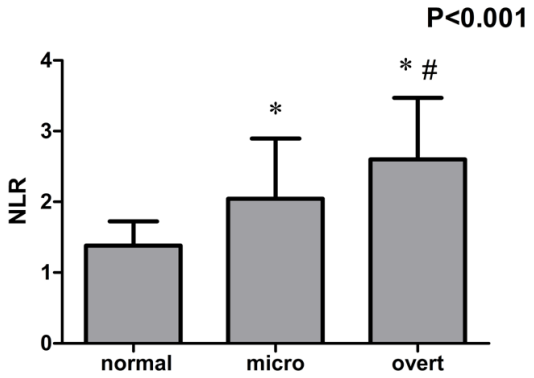

Figure 2. The mean NLR value of the DN groups. Subjects with overt nephropathy had a significantly higher NLR compared with subjects with microalbuminuria, who in turn showed a significantly higher ratio compared with subjects with normoalbuminuria. *P<0.05 for comparisons of normoalbuminuria group with microalbuminuria group; #P<0.05 for comparisons of microalbuminuria group with overt nephropathy group.

methods. NLR was calculated as a simple ratio between the absolute neutrophil and the absolute lymphocyte counts both obtained from the same automated blood sample at the admission of the study. An automated blood cell counter was used for these measurements.

Cockcroft-Gault Formula:

GFR [ml/(min 1.73 m^2)]=[(140- Age) × Weight (kg)]÷[Scr (mg/dl) × 72] (for male);

GFR [ml/(min 1.73 m^2)]=[(140- Age) × Weight (kg)] × 0.85÷[Scr (mg/dl) × 72] (for female)

Statistical analysis

Statistical analysis was performed using SPSS statistical software (SPSS for Windows, version 19.0; SPSS, Inc., Chicago, IL, USA). *P*-values <0.05 were considered statistically significant. For continuous variables with normal distributions, data were expressed as mean ± standard deviation. Categorical variables are expressed as frequencies and percentages. The Kolmogorov-Smirnov test was used to evaluate the distribution of variables. Independent-sample t test or one-way ANOVA test was used for continuous variables with normal distribution, while Kruskal-Wallis test or Mann-Whitney test was used for continuous variables without normal distribution. The chi-square test was performed for categorical variables. Logistic regression analysis was also conducted to assess relationships. The auROC analysis was performed to test the ability of the NLR to predict DN/DR risk, while Spearman correlation analysis was used to detect the correlation between NLR and urinary albumin/creatinine.

Results

Based on the inclusion criteria, a total of 321 subjects were selected for this study. They were screened based on the results of urinary albumin-to-creatinine ratio (UACR) and serum creatinine with at least two tests in 6 months. The clinical and biochemical characteristics of the subjects are shown in **Table 1**. Neutrophil counts, Age, SBP and UA were significantly higher in subjects with overt nephropathy compared with subjects with microalbuminuria or subjects with normoalbuminuria, while the lymphocyte counts, hemoglobin and albumin in overt nephropathy group were lower than that in other two groups (P<0.05). Moreover, increased probability of combined DR and GFR<90 were shown in subjects with overt nephropathy than that in other two groups (P<0.05). No significant differences in Sex, DBP, Drinking, TG, TC, LDL and HbA1c were detected between the three groups (P>0.05 for all). The mean NLR increased parallel to the severity of nephropathy. Subjects with overt nephropathy had a significantly higher NLR compared with subjects with microalbuminuria (P<0.001), who in turn showed a significantly higher ratio compared with subjects with normoalbuminuria (P<0.001) (**Figure 2**).

A logistic regression analysis was performed, using the Varying Degrees of DN/DR as the Dependent Variable and the Neutrophil-Lymphocyte Ratio as the Independent Variable, to determine the association of NLR with DN/DR (**Table 2**). For each analysis, two groups were chosen, and the group with more severe diabetic renal damage or diabetic retinopathy coded as 1, was tested against the next less severe DN/DR group, which was coded as 0. The group coded as 0 was used as reference for the analysis. In Model 1, where normoalbuminuria was coded as 0 and microalbuminuria was coded as 1, microalbuminuria showed a significant association with NLR even after adjusting for Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin,

Table 2. Logistic Analysis Using the Varying Degrees of DN/DR as the Dependent Variable and the Neutrophil-Lymphocyte Ratio as the Independent Variable

Parameter	EXP (B)	95% CI	P
DN (according to UACR)			
Model 1			
Unadjusted (normal =0, micro =1)	11.913	5.102-27.812	0.000
Adjusted for			
Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL, HbA1c, creatinine	16.188	6.174-42.445	0.000
Model 2			
Unadjusted (micro =0, overt =1)	2.29	1.519-3.452	0.000
Adjusted for			
Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL, HbA1c, creatinine			>0.05
DN (according to GFR)			
Model 3			
Unadjusted (GFR≥90=0, GFR<90=1)	3.698	2.597-5.267	0.000
Adjusted for			
Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL, HbA1c, creatinine	1.898	1.176-3.064	0.009
DR			
Model 4			
Unadjusted (No apparent =0, Nonproliferative =1)	1.68	1.214-2.325	0.002
Adjusted for			
Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL, HbA1c, creatinine			>0.05
Model 5			
Unadjusted (Nonproliferative =0, Proliferative DR=1)	1.632	1.148-2.320	0.006
Adjusted for			
Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL, HbA1c, creatinine			>0.05

SBP, systolic blood pressure; DBP, diastolic blood pressure ;BMI, body mass index;HbA1c, glycated Hemoglobin; TG, triglycerides; TC, Total cholesterol; LDL, low-density lipoprotein; NLR, neutrophil-lymphocyte ratio; UA, uric acid.

TC, LDL and HbA1c (P=0.000). Similar analysis was performed in Model 2 with microalbuminuria and overt nephropathy using microalbuminuria as the reference group. However, after adjusting for Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL and HbA1c, subjects with overt nephropathy showed no significant association with NLR (P>0.05). In Model 3, we could observe DN (according to GFR) showed a significant association with NLR even after adjusting for Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL and HbA1c (P=0.009). While subjects with DR showed no significant association with NLR (P>0.05) after adjusting for Gender, Age, SBP, DBP, Hemoglobin, BMI, Smoking, Drinking, UA, Albumin, TC, LDL and HbA1c in Model 4 and Model 5.

Figure 3 shows the ability of the NLR to predict DN (according to the UACR) risk in patients. The performance of the NLR was high, with an auROC of 0.872 (95% CI: 0.834-0.911). In the same dataset, WBCs had an auROC of 0.698 (95% CI: 0.640-0.755), a Neutrophils of 0.821 (95% CI: 0.776-0.866), a Lymphocytes of 0.664 (95% CI: 0.605-0.723), a creatinine of 0.725 (95% CI: 0.671-0.780), significantly lower than that of the NLR (all P<0.001). When using a best cut-off value of 1.758 for the NLR, the sensitivity was 75.4%, the specificity was 92.5%. As shown in **Figure 4**, the auROC analysis was performed to test the ability of the NLR to predict DN (according to the GFR) risk in patients. The auROC of NLR, Neutrophils, Lymphocytes and WBCs was 0.817 (95% CI: 0.770-0.863), 0.716 (95% CI: 0.658-0.773), 0.711 (95% CI: 0.650-0.773) and 0.605 (95% CI: 0.540-0.670), respectively. The performance of the NLR was significantly better than that of WBCs, Neutrophils and Lymphocytes (P< 0.001). **Figure 5** shows the ability of the NLR to predict DR risk in patients. The auROC of the NLR was 0.669 (95% CI: 0.606-0.732). In the same dataset, WBCs had an auROC of 0.576 (95% CI: 0.512-0.641), a Neutrophils of 0.635 (95% CI: 0.573-0.698), a Lymphocytes of 0.585 (95% CI: 0.520-0.651), a creatinine of 0.555 (95% CI: 0.491-0.619), significantly lower than

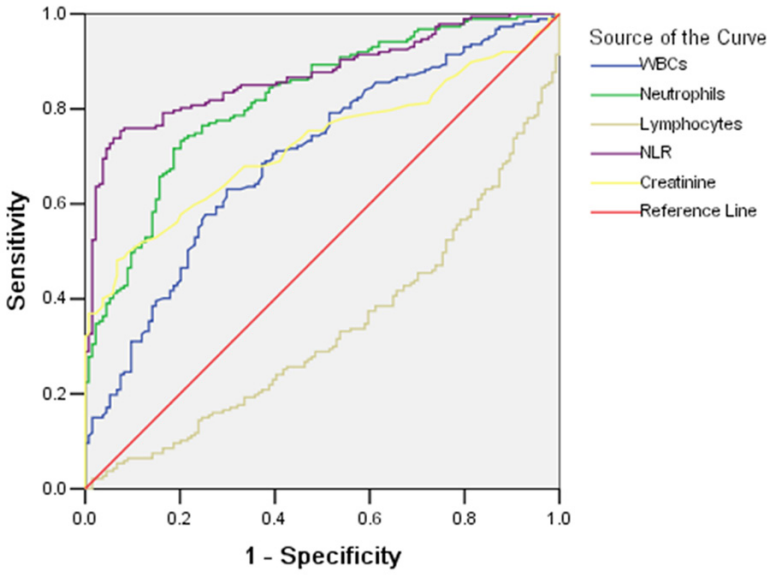

Figure 3. ROC analysis of the predictive accuracy of NLR and other models to predict DN (according to the UACR). The performance of the NLR was high, with an auROC of 0.872 (95% CI: 0.834-0.911), while WBCs had an auROC of 0.698 (95% CI: 0.640-0.755), a Neutrophils of 0.821 (95% CI: 0.776-0.866), a Lymphocytes of 0.664 (95% CI: 0.605-0.723), a creatinine of 0.725 (95% CI: 0.671-0.780), significantly lower than that of the NLR (all P<0.001).

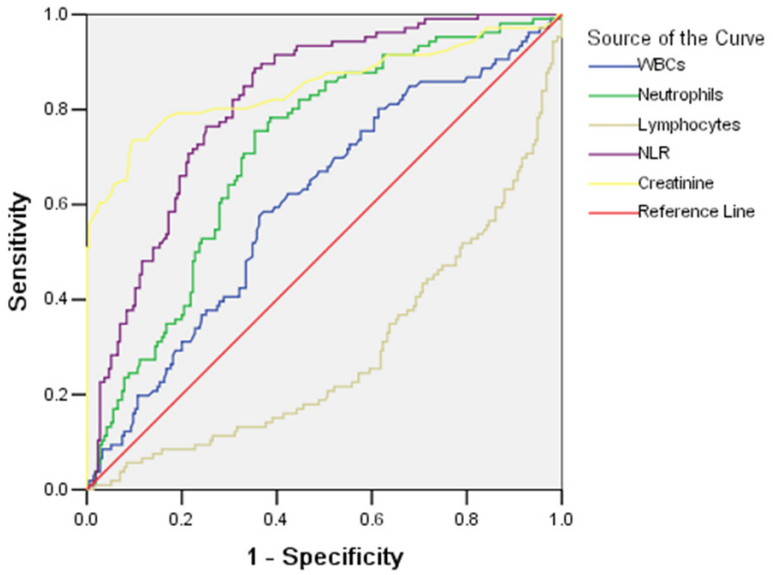

Figure 4. ROC analysis of the predictive accuracy of NLR and other models to predict DN (according to the GFR). The auROC of NLR, Neutrophils, Lymphocytes and WBCs was 0.817 (95% CI: 0.770-0.863), 0.716 (95% CI: 0.658-0.773), 0.711 (95% CI: 0.650-0.773) and 0.605 (95% CI: 0.540-0.670). The performance of the NLR was significantly better than that of WBCs, Neutrophils and Lymphocytes (P<0.001).

that of the NLR (all P<0.001). When using a best cut-off value of 1.630 for the NLR, the sensitivity was 67.8%, the specificity was 67.3%.

In diabetes patients, there was a positive correlation between NLR values and creatinine and UACR (r=0.404, P<0.001; r=0.681, P<0.001, respectively). While NLR values showed significant negative correlation with GFR (r=-0.516, P<0.001).

Discussion

In our study, we found that NLR levels were significantly higher in patients with microvascular complications than those without microvascular complications. Moreover, the mean NLR levels increased parallel to the severity of DN and DR. In addition, NLR was found to be a significant risk factor for DN and DR through logistic regression analysis. These findings are consistent with current evidence regarding the association of inflammatory markers, including neutrophil counts, with the development of diabetic microvascular and macrovascular complications [16].

Microalbuminuria was found to be one of the earliest markers for DN. However, recent studies have shown that albuminuria is a less precise predictor of overt nephropathy risk than originally thought [17]. In addition, a substantial percentage of diabetic patients develop CKD, while remaining normoalbuminuria, and reliable biomarkers are lacking in this subset of patients [18, 19]. Thus there is an increasing quest to find novel clinical biomarkers to identify individuals at risk of DN both onset and progression.

Many epidemiological studies have reported that DM is associated with chronic inflammation [20], which may promote the acceleration

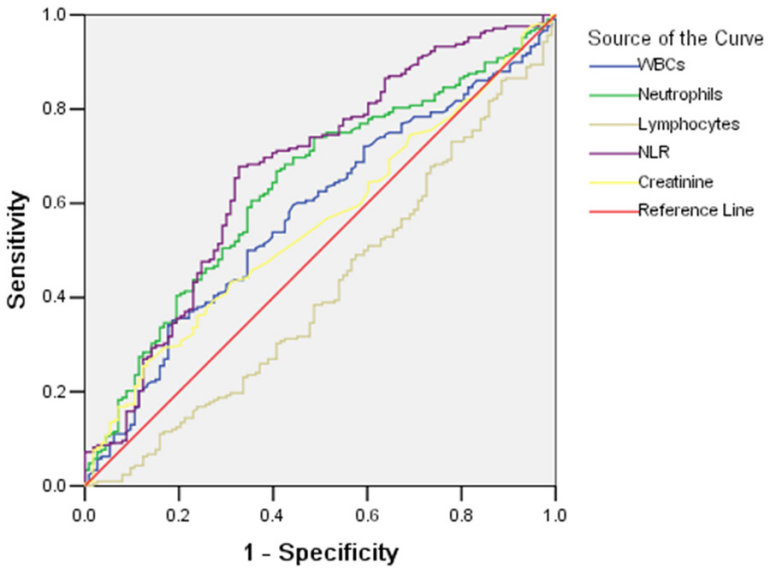

Figure 5. ROC analysis of the predictive accuracy of NLR and other models to predict DR. The auROC of the NLR was 0.669 (95% CI: 0.606-0.732). In the same dataset, WBCs had an auROC of 0.576 (95% CI: 0.512-0.641), a Neutrophils of 0.635 (95% CI: 0.573-0.698), a Lymphocytes of 0.585 (95% CI: 0.520-0.651), a creatinine of 0.555 (95% CI: 0.491-0.619), significantly lower than that of the NLR (all P<0.001).

of diabetic microangiopathy in addition to the development of macroangiopathy in diabetic patients [21]. DN and DR were common severe complications in patients with diabetes, which impose a tremendous burden on health economies, whereas the exact molecular action leading to DN and DR is not yet understood. Currently, there is increasing evidence that inflammatory processes play a considerable role in the pathogenesis of DR and DN.

Counts of white blood cells and their subtypes are known as classic inflammatory markers, with low cost and wide availability, especially in cardiovascular diseases [8]. Numerous epidemiological and clinical studies have shown leukocytosis to be an independent predictor of insulin resistance, type 2 diabetes, microvascular and macrovascular complications of diabetes, and future cardiovascular events in patients with stable angina, unstable angina, or a history of myocardial infarction [22-27]. However, DN and/or DR diagnosis based on NLR has biases. NLR was defined as a novel potential marker to determine inflammation in cardiac and noncardiac disorders [11, 28, 29]. NLR represents a combination of two markers and it is superior to other leukocyte parameters (e.g., neutrophil, lymphocyte, and total leukocyte counts) due to the stability of NLR compared with the absolute counts that could be altered by various physiological, pathological, and physical factors [10, 11]. It 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-1b and TNF-a [30].

The neutrophil-to-lymphocyte ratio (NLR) is an emerging marker for both cardiac and non-cardiac disorders. Recent studies have demonstrated the prognostic value of NLR in stable coronary artery disease, acute coronary syndromes, heart failure, as well as patients undergoing percutaneous coronary interventions (PCI) and coronary artery bypass grafting [10, 11, 28, 31-35]. However, the relationship between microvascular complications of diabetes and NLR has not been investigated so far. In our study, NLR levels were found to be higher in diabetes patients with microvascular complications than in those without microvascular complications. In addition, there was a significant relationship between DR/DN grades and NLR. This result may indicate that the severity of DR/DN and the degree of inflammation are associated with each other.

The biological mechanisms by which leukocytes and their subtypes play a role in mediating increased protein and albumin excretion and retinal injury is not fully known. Accumulating evidences have indicated that advanced glycation end products (AGEs) and its receptor, RAGE, were involved, through multiple pathways, in disturbing the rhythm of immune system of patients with diabetes [36], might be the mechanism of microangiopathy in T2DM. In addition, these immunological alterations result in elevated circulating levels of acute-phase proteins and pro-inflammatory cytokines that play a major role in the develop-

ment of chronic inflammation-induced organ dysfunction in DM [5, 6]. The increased spontaneous adherence of neutrophil to endothelial cells was also described as a possible mechanism of DN and proteinuria [37].

As we have excluded subjects with any active infection or inflammation, it is unlikely that these results are due to any infection among subjects. There are several potential limitations of our study. Firstly, analyses were based on a single measurement of WBC counts that may not reflect the relation over time. It would be interesting to measure the serial changes of WBC counts to further clarify the role of WBCs and subpopulations for development of DN and DR. Secondly, because our study is cross-sectional design, we had no direct evidence for a cause-effect relationship. The association between NLR and T2DM microvascular complication requires further investigation by the prospective studies. Besides, we were unable to compare the prognostic value of NLR with other inflammatory markers such as C-reactive protein, fibrinogen because they were not routinely obtained. However, as this is the first report on the correlation of NLR with different grades of DR and DN, we recommend that the NLR values of diabetic patients be calculated as NLR is a cheap, predictive, and prognostic marker for microvascular complications of diabetes.

In conclusion, NLR values of diabetic patients could be calculated as NLR is a cheap, predictive, and prognostic marker for microvascular complications of diabetes.

Disclosure of conflict of interest

None.

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