

Changes in Leukocyte Subpopulations with Decline in Glomerular Filtration Rate in Patients with Type 2 Diabetes

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Abstract- Recent studies suggested the role of white blood cells (WBCs) in the pathogenesis and complications of type 2 diabetes. Increased WBC counts predict mortality in patients with chronic kidney disease (CKD). In this study alterations in WBC subpopulations in diabetic patients with non-dialysis dependent CKD are investigated. This was a cross-sectional study on 376 participants, including 272 diabetic patients and 104 healthy controls. Total and differential WBC counts were compared among diabetics with CKD, diabetics without CKD and controls. Among patients with type 2 diabetes, there was no significant difference in total WBC count between those with and without CKD. Diabetic patients with CKD had higher neutrophil, monocyte and eosinophil and lower lymphocyte count compared with both diabetic patients without CKD and healthy controls. Except for monocytes, a significant association was observed between GFR and differential WBC counts, which persisted after adjustment for conventional diabetes risk factors ($R^2=0.272$, $P < 0.001$ for regression model). Neutrophil/lymphocyte ratio was the best predictor of GFR in total study population ($\beta = -1.995 \pm 0.45$, $P < 0.001$). Changes in WBC subpopulations are present even before significant alterations in total WBC count. Immune system dysfunction needs special consideration in diabetic patients with CKD.

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Introduction

Inflammation is an independent predictor of future development of diabetes in patients with impaired glucose tolerance (1). Total white blood cell (WBC), neutrophil, lymphocyte, monocyte and eosinophil counts increase as the number of components of metabolic syndrome increase in patients with type 2 diabetes mellitus (T2DM) (2,3). Major complications of diabetes, such as coronary artery disease (CAD), associate with total WBC count (4,5). Leukocyte count also has prognostic relevance in these patients; five-year survival rate is significantly better in diabetic patients with normal lymphocyte counts compared to those with relatively low counts (6).

Leukocytes are leading players in the mechanism of atherosclerosis (7,8) and alterations of their counts in cardiovascular disease have been subject to a vast

number of studies (4,5,9). However, little evidence exists about similar changes in other diabetic complications. There is a well-recognized association between renal and cardiac complications in patients with T2DM (10). Persistent inflammation is a consistent finding in diabetic nephropathy and infiltration of leukocytes has been observed at each stage of diabetes-induced renal injury (11,12). Total and differential WBC counts are associated with the degree of albuminuria in patients with T2DM (13). Interestingly, up to 50% of diabetic patients with chronic kidney disease (CKD) experience progressive deterioration of renal function without developing significant proteinuria (14), implying that different rules may govern CKD versus albuminuria. Patients with end stage renal disease (ESRD) experience significant immune dysfunction (15). Increased neutrophils, together with reduced lymphocytes, are independent predictors of mortality in

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patients on hemodialysis (16). Nevertheless, alteration in WBC count at earlier stages of CKD has already been less clarified.

This study aimed to investigate WBC differential counts in diabetic patients with CKD and compare it with diabetic and non-diabetic subjects with normal kidney functions.

Materials and Methods

Participants and study design

We performed a cross-sectional study on a group of 376 participants, including 272 patients with T2DM and 104 healthy controls. Diabetes was diagnosed according to American Diabetes Association criteria (17). Poor-control diabetes was defined as HbA1C more than 7% (17). Patients with poor control T2DM were selected from an outpatient diabetes clinic (affiliated with Vali-Asr Hospital of Tehran University of Medical Sciences) and blood samples were taken at the first visit. Controls were randomly selected from those who were diagnosed as benign solitary thyroid nodule in outpatient thyroid clinic of the same hospital.

Exclusion criteria were smoking, dialysis, glomerulonephritis, alanine aminotransferase (AST) >30 U/L and/or alanine aminotransferase (ALT) >40 U/L, hypo/hyperthyroidism, diffuse/multimodular goiter, thyroid cancer or other known proliferative disease, history of radiation therapy, known hematologic disease, acute infection, type 1 diabetes, congestive heart failure, stroke or myocardial infarction in previous six months, diabetic ketoacidosis and non-ketonic hyperosmolar diabetes, autoimmune disease, hormone replacement therapy, oral contraceptive pills (OCP) use, pregnancy and hospital admission in previous six months. All controls had fasting blood sugar (FBS) <100 mg/dl, and benign solitary thyroid nodule were diagnosed according to thyroid function tests, ultrasonography, and fine-needle aspiration. Considering the effects of seasonality on eosinophil count, recruitment continued from May 2010 to May 2012. Demographic and anthropometric data including age, sex, height and weight were recorded, as well as the duration of diabetes in the diabetic group. Body mass index (BMI; Kg/m²) was calculated according to the Quetelet formula. Blood pressure was measured in sitting a position and re-measured twice after five minutes and averaged. A thorough investigation of diabetic complications was carried out in patients with diabetes. Ischemic heart disease (IHD) was defined as previously known coronary artery disease, positive exercise stress test or

at-rest electrocardiographic changes. Diagnosis of diabetic retinopathy was made by an expert ophthalmologist based on chart review results and ophthalmologic funduscopy examination. Clinical examination assessed diabetic neuropathy. All participants gave written informed consent before participating in this study. Local ethics review committee of Tehran University of Medical Sciences approved the study protocol. This study complied with the principles of the declaration of Helsinki.

Blood and urine samples

Fasting blood samples were taken after 12 hours overnight fasting. Fresh blood was used for complete blood count (CBC) test and for the measurement of FBS, HbA1C, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), creatinine, urea, uric acid, ALT, AST and erythrocyte sedimentation rate (ESR). Glucose measurements were carried out using glucose oxidase method (intra-assay coefficient of variance [CV] 2.1%, inter-assay CV 2.6%). High-pressure liquid chromatography determined HbA1C. Total cholesterol, HDL-C, LDL-C, and triglycerides levels were determined using direct enzymatic methods (Parsazmoon, Karaj, Iran). Creatinine was measured using calibrated Jaffe method (Parsazmoon, Karaj, Iran, intra-assay CV=3.3%). Urea was measured using colorimetric assay (Parsazmoon, Karaj, Iran). Analyzes of serum ALT and AST were performed using enzymatic photometry by IFCC (International Federation of Clinical Chemistry and Laboratory Medicine) method (ALT intra-assay CV=3.7%, AST intra-assay CV=2.5%). Uric acid was measured by calorimetric method (intra-assay CV=1.27%). Westergren method determined ESR.

Complete blood count measurements were performed by an automatic cell counter machine (XT-1800i model, Sysmex, Japan). Lymphocyte, neutrophil, monocyte and eosinophil counts were calculated using total WBC count and differential percentages. Patients were instructed in timed 24-hour urine collection for measurement of urinary protein excretion. Urine protein was measured by immunoturbidometry (intra-assay CV=2.1%). GFR was calculated using MDRD formula (18). Diabetic patients with GFR <60 ml/min (corresponding to stages 3, 4 and 5 of non-dialysis-dependent CKD according to national kidney foundation guideline) were considered as CKD group, while diabetic patients with GFR ≥ 60 ml/min were considered as non-CKD group (19).

Statistical analysis

Continuous variables are presented as Mean \pm Standard Error of Mean (SEM). Categorical variables are presented as number and percent. *Chi-square* test, independent samples *t*-test and one-way analysis of variance (ANOVA) were used for group comparisons as indicated. As the distribution of proteinuria was positively skewed, its log-transformed values were employed for analysis. The association between GFR and other parameters were first analyzed by univariate linear regression analysis, with GFR as outcome variable. Significant variables were selected for multivariate backward linear regression analysis. Alpha = 0.05 for variable entry and alpha = 0.1 for variable removal were considered as significance

limitations in regression analysis. *P*-value < 0.05 was considered as statistically significant for group's comparisons. Statistical package for social science (SPSS for windows, version 19; Chicago, IL) program was used for data analysis.

Results

Baseline characteristics of study population are presented in Table 1. Diabetic patients with CKD were older and had longer duration of diabetes, higher systolic blood pressure, urea, creatinine, uric acid and triglycerides/HDL ratio and lower BMI and hemoglobin in comparison with non-CKD diabetics (Table 1).

Table 1. Primary characteristics of study population

	Control (n=104)	Non-CKD Diabetes (n=190)	CKD Diabetes (n=82)	P-value
Age (years)	45.55 \pm 1.06	53.59 \pm 0.72 †	63.56 \pm 1.09 *†	<0.001
Female n (%)	72 (69.2)	119 (62.6)	53 (64.6)	0.54
Duration of diabetes (years)	—	10.6 \pm 0.5	13.1 \pm 0.8 *	<0.05
BMI (Kg/m ²)	22.16 \pm 0.66	28.64 \pm 0.59 †	24.40 \pm 0.72 *	<0.001
Systolic Blood Pressure (mmHg)	122.8 \pm 1.5	124.4 \pm 1.2	132.3 \pm 2.4 *†	<0.001
Diastolic Blood Pressure (mmHg)	78.4 \pm 0.8	77.6 \pm 0.7	78.9 \pm 1.2	0.53
Fasting Blood Sugar (mg/dl)	93.5 \pm 1.1	236.5 \pm 5.7 †	228 \pm 10.7 †	<0.001
HbA1C (%)	4.8 \pm 0.6	10.5 \pm 0.2 †	10.7 \pm 0.4 †	<0.05
HbA1C (mmol/mol)	28.93 \pm 6.56	91.18 \pm 2.18 †	93.36 \pm 4.37 †	<0.05
Triglycerides (mg/dl)	118.5 \pm 5.7	167.7 \pm 6.4 †	187.4 \pm 11 †	<0.001
Total cholesterol (mg/dl)	196.2 \pm 3.8	185.2 \pm 3.9	188.6 \pm 6.3	0.22
LDL-C (mg/dl)	112.7 \pm 3.4	99.9 \pm 2.4 †	104.3 \pm 4.4 †	<0.001
HDL-C (mg/dl)	52.3 \pm 1.2	44.8 \pm 1.2 †	41 \pm 2.4 †	<0.05
Triglycerides/HDL	2.42 \pm 0.16	4.19 \pm 0.22 †	5.59 \pm 0.58 *†	<0.001
Uric acid (mg/dl)	4.7 \pm 0.2	4.3 \pm 0.2	5.5 \pm 0.4 *†	<0.005
Urea (mg/dl)	28.8 \pm 0.8	34.8 \pm 1.3 †	52.2 \pm 2.2 *†	<0.001
Creatinine (mg/dl)	0.9 \pm 0.01	0.88 \pm 0.01	1.36 \pm 0.04 *†	<0.001
GFR (ml/min)	85.95 \pm 1.96	89.62 \pm 1.55	47.26 \pm .95 *†	<0.001
Hemoglobin (g/dl)	13.4 \pm 0.1	13.1 \pm 0.1	12.3 \pm 0.2 *†	<0.001
Platelet (10 ⁹ /l)	250490 \pm 6529	230408 \pm 4374 †	232738 \pm 7429 †	<0.05
ESR (mm/hr)	12.8 \pm 2.1	23.6 \pm 1.7 †	29.5 \pm 2.6 †	<0.001
AST (U/L)	21.1 \pm 1.0	19.08 \pm 0.9	18.1 \pm 1.0	0.36
ALT (U/L)	20.9 \pm 1.3	23.3 \pm 1.5	18.9 \pm 1.4	0.21
Proteinuria (mg/day)	90.82 \pm 44.16	506.27 \pm 148.92 †	733.46 \pm 239.85 †	<0.01
Ischemic heart disease (%)	—	26.2	40.3	<0.05
Retinopathy (%)	—	39	54.8	<0.05
Neuropathy (%)	—	57.6	54.2	0.68
Glucose-lowering drug (%)				
Oral hypoglycemic drug	—	82.5	74.8	<0.01
Insulin	—	7.9	17.6	
Oral hypoglycemic drug + insulin	—	9.6	7.6	

CKD, chronic kidney disease; BMI, body mass index; HbA1C, hemoglobin A1C; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; GFR, glomerular filtration rate; ESR, erythrocyte sedimentation rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

* Significantly different from "Non-CKD Diabetes" group.

† Significantly different from "Control" group

Table 2 represents total and differential WBC counts in three study groups. Patients with diabetes had higher total WBC count compared to controls, irrespective of

their kidney function status (*P*-value < 0.05). There was no significant difference in total WBC count between two groups of diabetic patients (6640 \pm 113 in non-CKD

vs. 6781 ± 205 in CKD group; P -value=0.51). Neutrophil, monocyte, and eosinophil counts were higher, and lymphocyte count was lower in diabetic patients with CKD compared with their non-CKD diabetic

counterparts (Table 2). Differential counts were not significantly different between non-CKD diabetic patients and controls, except for monocyte count (P -value<0.001).

Table 2. Total and differential WBC count of subjects in study groups

	Control (n=104)	Non-CKD Diabetes (n=190)	CKD Diabetes (n=82)	P-value
WBC count ($10^6/l$)	6350 ± 159	$6640 \pm 113 \uparrow$	$6781 \pm 205 \uparrow$	<0.05
Neutrophil count ($10^6/l$)	3578 ± 111	3529 ± 74	$3826 \pm 151 * \uparrow$	<0.05
Lymphocyte count ($10^6/l$)	2348 ± 69	2415 ± 55	$2183 \pm 88 * \uparrow$	<0.01
Monocyte count ($10^6/l$)	244 ± 21	$456 \pm 13 \uparrow$	$502 \pm 25 * \uparrow$	<0.001
Eosinophil count ($10^6/l$)	164 ± 10	160 ± 8	$209 \pm 22 * \uparrow$	<0.05

CKD, chronic kidney disease; WBC, white blood cell

* Significantly different from "Non-CKD Diabetes" group

† Significantly different from "Control" group

To investigate the association between WBC differentials and GFR, we used stepwise regression modeling. In univariate analysis, significant predictors of GFR included: duration of diabetes, systolic blood pressure, triglycerides, HDL-C, triglycerides/HDL ratio, proteinuria, neutrophil, lymphocyte, monocyte and eosinophil counts (Table 3).

No association was found between total WBC count and GFR (P -value = 0.28). In a multivariate model, duration of diabetes, systolic blood pressure, triglycerides/HDL ratio, neutrophil, lymphocyte and

eosinophil counts remained significant ($R^2 = 0.272$, $P < 0.001$ for the final model).

We also investigated neutrophil/lymphocyte ratio as a predictor of GFR. When neutrophil/lymphocyte ratio was introduced to the fitted model, WBC differentials were no more significant predictors of GFR. The new model consisted of neutrophil/lymphocyte ratio ($\beta = -1.995 \pm 0.45$, $P < 0.001$), systolic blood pressure ($P < 0.05$), duration of diabetes ($P < 0.05$), Triglycerides/HDL ratio ($P < 0.01$). Figure 1 represents the association between GFR and neutrophil/lymphocyte ratio.

Table 3. Univariate and multivariate linear regression analysis

	Univariate analysis			Multivariate analysis	
	Beta-coefficient	P-value	R	Beta-coefficient	P-value
Sex	0.39 ± 2.54	0.87	0.008	—	—
Duration of diabetes	$-0.43 \pm 0.19 *$	< 0.05	0.136	$-0.49 \pm 0.27 *$	<0.1
BMI	-0.29 ± 0.28	0.30	0.083	—	—
SBP	$-0.23 \pm 0.07 *$	< 0.005	0.194	$-0.30 \pm 0.10 *$	<0.005
DBP	-0.04 ± 0.13	0.74	0.020	—	—
FBS	0.008 ± 0.013	0.53	0.034	—	—
HbA1C	1.12 ± 0.65	0.08	0.119	—	—
Triglycerides	$-0.035 \pm 0.016 *$	< 0.05	0.131	—	NS
Cholesterol	-0.009 ± 0.026	0.71	0.021	—	—
LDL-C	-0.058 ± 0.038	0.13	0.089	—	—
HDL-C	$0.173 \pm 0.075 *$	< 0.05	0.136	—	NS
Triglycerides/HDL	$-1.472 \pm 0.388 *$	< 0.001	0.224	$-1.25 \pm 0.51 *$	<0.05
Proteinuria	$-5.59 \pm 2.49 *$	< 0.05	0.181	—	NS
WBC	-0.001 ± 0.001	0.28	0.058	—	—
Neutrophil	$-0.004 \pm 0.001 *$	< 0.005	0.174	$-0.005 \pm 0.002 *$	<0.05
Lymphocyte	$0.004 \pm 0.002 *$	< 0.05	0.131	$0.006 \pm 0.003 *$	<0.05
Monocyte	$-0.019 \pm 0.006 *$	< 0.005	0.178	—	NS
Eosinophil	$-0.021 \pm 0.011 *$	0.05	0.116	$-0.032 \pm 0.014 *$	<0.05

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA1C, hemoglobin A1C; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; WBC, white blood cell

* Significant variables in linear regression analysis

NS: not accepted as significant variable in multivariate stepwise analysis

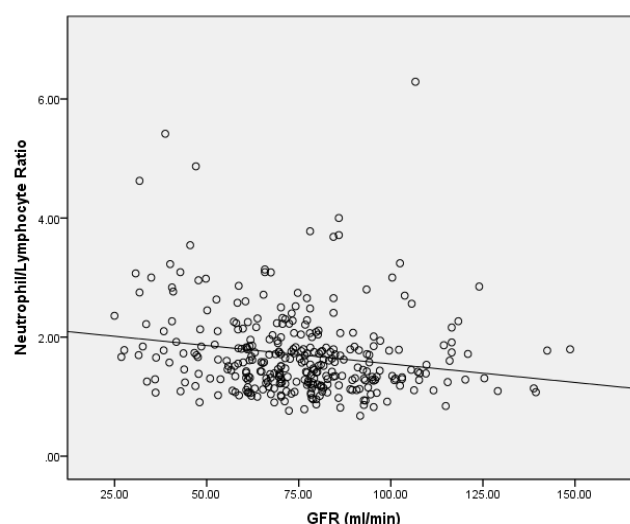


Figure 1. The association between GFR and Neutrophil/lymphocyte ratio

Discussion

We observed that diabetic patients with CKD had different total, and differential WBC counts compared with both diabetic patients without CKD and healthy controls. Patients with declined GFR were found to have increased neutrophil, monocyte and eosinophil and decreased lymphocyte counts. Even after adjustment for conventional risk factors, GFR and WBC differentials were found to be significantly associated, except for monocyte count. Current findings suggest that alterations in leukocyte count in patients with declined glomerular filtration function occur much earlier than what was previously thought. By excluding dialysis-dependent patients, we ruled out contribution of dialysis membranes or dialysate in these immunological changes as some previous studies had suggested (20,21). Total WBC count has been reported as an inflammatory marker in patients with CKD (16,22). However, we observed that in early and moderate stages of CKD, with unchanged total WBCs, differential counts are significant predictors of renal glomerular filtration function in diabetic patients. This implies that leukocyte subpopulations may have already changed when total WBC count does not reflect significant alterations.

Inflammation is an important player, and not an innocent bystander, in the pathogenesis of insulin resistance and diabetes (23). Diabetic nephropathy was not considered an inflammatory process in the past, but paradigms have shifted in recent years (11). WBC count provides a rough estimate of the degree of activation of the immune system (24). Changed WBC counts in

diabetic patients with CKD cannot be solely explained by the inflammatory nature of diabetes, as intensive alterations were not observed in differential counts in non-CKD diabetic group. In fact, the underlying low-grade inflammation in patients with T2DM seems to be aggravated in those with declined GFR. Immune system dysfunction is more evident in later stages of CKD and manifests in both innate and adaptive immune system dysfunction in patients with ESRD (15). Two leading causes of death in this group of patients is infectious and cardiovascular diseases (25,26), both associated with immune system role-playing. Alterations in leukocyte subpopulations in the early stages of CKD may also explain the increased susceptibility of these patients to cardiovascular diseases (22).

Present results are consistent with previous reports of immunological changes in patients with impaired renal function. Chung *et al.*, indicated that total and differential WBC counts change in paralleled with the degree of albuminuria in patients with diabetic nephropathy (13). They suggested that circulating leukocytes contribute to the development and progression of nephropathy in patients with T2DM. Fukui *et al.*, showed a similar correlation between peripheral eosinophil count and albumin excretion rate in men with T2DM (27). It was also reported that increased neutrophil count and reduced lymphocyte count are independent predictors of mortality in hemodialysis patients (16). Leukocyte counts are among the recognized risk markers at early stages of diabetic nephropathy and CKD (28,29). Cardiovascular risk increases very early on in the evolution of CKD (at a

GFR of about 75 ml/min); an association that linearly continues with decline in renal function (30). Even small reduction in renal function, as well as minor levels of albuminuria, can predict the development of cardiovascular disease and mortality (22).

Increased neutrophil/lymphocyte ratio has been consistently reported as a novel immunological marker in a variety of diseases. Neutrophil/lymphocyte ratio was found to be increased in patients with non-alcoholic steatohepatitis and advanced liver fibrosis (31). In acute myocardial infarction, larger infarction size is associated with higher neutrophil count and increased monocyte count 12 hours after onset of infarction (32). Increased neutrophil/lymphocyte ratio is also an independent predictor of mortality rates in patients with non-ST-segment elevation myocardial infarction and heart failure (33,34). Increased apoptosis and a fall of T helper, cytotoxic T and regulatory T lymphocytes, as well as B lymphocytes, were also observed after cerebrovascular accident (35). Current finding of a strong association between neutrophil/lymphocyte ratio and GFR gives evidence in this area. Although the underlying mechanism of WBC count alterations is possibly dissimilar in these different conditions, a shared pattern of combined neutrophilia and lymphopenia can be observed in these stressful states.

Several limitations of this study merit consideration. The cross-sectional design does not allow us to determine the temporal sequence of immunological changes and GFR changes in patients with CKD; hence the direction of the causal association cannot be determined. Present study population was limited to patients with T2DM and our findings cannot be generalized to patients with other etiologies of CKD. By including both diabetic patients without CKD and healthy controls, we could determine the relative roles of diabetes and CKD on differential WBC counts. Alteration in differential WBC counts in early stages of CKD was the main finding of this study and explained another aspect of this mystifying multi-systemic disease.

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