Lymphocytes ≥ 2.9 (10^9/L) in Newly Diagnosed Diabetes Are A Predictor of Future CVD Events*

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[Abstract] Objective: Atherosclerosis is considered a chronic inflammatory condition. The immune system is a key mediator in the initiation and progression of atherosclerosis. In a previous study, we found that the immune system was activated in diabetes and that total white blood cell (WBC) counts were elevated significantly in diabetic patients. To investigate whether WBC subtype counts in newly diagnosed diabetes are risk factors for future cardiovascular disease (CVD) events, we conducted a prospective population-based cohort study. **Methods:** A total of 1498 newly diagnosed diabetic patients aged 40 to 70 years old were followed up for three years. Participants with previous CVD history and abnormal WBC counts were excluded. CVD events were recorded during follow-up. **Results:** We found that the baseline lymphocyte counts were independently associated with cardiovascular events during follow-up, with the Exp (β) (95% CI) at 1.749 (1.084–2.821). Lymphocyte count ≥2.9 (10^9/L) was significantly associated with the development of CVD (HR, 2.29; 95% CI, 1.12–4.67). The corresponding incidence of CVD per 1000 person-year for the lymphocyte count ≤2.8 (10^9/L) and lymphocyte count ≥2.9 (10^9/L) groups were 11.26 and 26.38, respectively. **Conclusion:** We concluded that even in a normal range, higher lymphocyte levels may result in a significantly higher CVD risk among diabetic patients. Lymphocyte count ≥2.9 (10^9/L) is an independent predictor of developing future CVD events.
Key words: lymphocytes; risk; cardiovascular events; diabetes

Diabetes is a major risk factor of cardiovascular disease (CVD), and about 65% of patients with diabetes militus die from CVD complications[1]. Atherosclerosis, the main pathophysiological condition causing CVD, is now considered a chronic inflammatory condition. It is well known that the immune system is a key mediator in the initiation and progression of atherosclerosis[2]. Diabetes is a chronic state of low-grade inflammation with a general activation of the innate immune system[3]. Our results have been consistent with this as we have found that white blood cell (WBC), neutrophil and lymphocyte levels are all elevated in diabetes patients compared with non-diabetes patients[4]. Since there exists immune system activation and inflammation in diabetes, and immune system is related to atherosclerosis, it is worth exploring the relationship of WBC subtypes with the incidence of CVD events in diabetes. Investigating the association of WBC subtypes in newly diagnosed diabetes with the risk of CVD events is a better choice because this will help avoid biases from varied durations of diabetes and CVD courses. In this study, we conducted a prospective study including 1073 newly diagnosed diabetes patients aged 40 to 70 years old from the Chongming District of Shanghai. These participants were followed up for 3.25 years and the CVD event information was recorded accordingly.

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*This work was supported by the National Key R&D Program of China (No. 2016YFC0901200 and No. 2016YFC0901203), Shanghai Pujiang Program (No. 2019PJD033), the Shanghai Science and Technology Commission (No. 15411953200, No. 10411956600 and No. 14ZR1427400), the National Clinical Research Center for Metabolic Diseases (No. 2013BAI09B13), the National Key New Drug Creation and Manufacturing Program of Ministry of Science and Technology (No. 2012ZX09303006-001).

1 MATERIALS AND METHODS

1.1 Study Population

This study was part of the REACTION study[5, 6]. The participants in this study were from the Chongming District of Shanghai, China. Geographically, Chongming Island is located in the

northeast of Shanghai, across a branch of Yangzi River and has developed rapidly in the past 30 years into a microcosm of China's economic development. The population of Chongming is less inclined to move and is more suitable for follow-up studies of metabolic diseases. The Chengqiao Town is the biggest town within Chongming and has the biggest population. It is the seat of local district government and represents the development of the entire Chongming.

In 2011, the number of residents aged 40–70 years in Chongming was 359 600, almost half of the total population. We selected our targeted study population in Chengqiao town, which is a typical newly urbanized town and may best represent the development and economic status of the entire Chongming. Of 45 876 residents aged 40–70 years in the whole Chengqiao town, we randomly selected 10 060 subjects to conduct our study using a stratified cluster sampling method. All eligible individuals within each of the selected communities/streets were sampled. In the recruiting process, all of the inhabitants were invited by telephone or door-to-door visit to participate in this study. Informed consent was obtained from all participants and the study was approved by the Institutional Review Board of Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine.

The missing anthropometry was an exclusion criterion. Other exclusion criteria were acute or chronic inflammatory diseases, neoplasm, and autoimmune diseases, based on medical history. At baseline in 2011, a total of 10 060 subjects were recruited, among which 9930 had the full required information. Of the initial 9930 individuals, 8944 participants without DM history underwent a 75 g oral glucose tolerance test (OGTT) and 1498 were subsequently diagnosed with diabetes. At the end of three years' follow-up, 338 subjects with newly diagnosed diabetes did not attend the study, and the remaining 1160 patients in the cohort were followed up. Finally, after excluding 9 participants with previous CVD history and 78 participants with abnormal WBC counts, 1073 newly diagnosed diabetes patients were analyzed in this study (fig. 1 and table 1).

1.2 Study End Points

Cardiovascular events were documented as including cardiovascular death, non-fatal myocardial infarction or non-fatal stroke. Documented hospital data, electrocardiogram, and imaging data of head computed tomography (CT) scan or magnetic resonance imaging (MRI) scan at onset were collected to validate the disease. Information on cardiovascular deaths was obtained from the official death certificates of the Chongming district.

1.3 Blood Indices Measurement

WBC subtype counts including neutrophils, lymphocytes, eosinophils, basophils and monocytes were detected automatically by a blood cell analyzer (Beckman-Coulter LH750, USA). Plasma glucose was measured during a 75 g OGTT, and diabetes was defined with a fasting plasma glucose (FPG) level ≥7.0 mmol/L and/or a 2 h post-challenge glucose (2 hPG) level ≥11.1 mmol/L. Plasma glucose was detected using the glucose oxidase method. The Hemoglobin

Table 1 Baseline characteristics of subjects with and without CVD events

Characteristics	All	CVD events (+)	CVD events (–)	*P* value
n	1073	41	1032	
Age (years)	58.49±7.13	60.92±7.43	58.39±7.11	0.026
Male, *n* (%)	416 (42.87)	16 (39.0)	400 (38.8)	0.973
BMI (kg/m^2)	25.77±3.44	26.44±3.71	25.74±3.43	0.202
WHR	0.90±0.06	0.92±0.06	0.90±0.06	0.048
SBP (mmHg)	138.10±17.93	142.63±18.66	137.92±17.88	0.098
DBP (mmHg)	82.91±9.50	83.09±9.85	82.90±9.50	0.903
HbA1c	6.49±1.18	6.75±1.39	6.48±1.17	0.141
FBG (mmol/L)	7.43±1.96	7.70±2.08	7.42±1.96	0.368
PBG (mmol/L)	13.20±4.40	13.50±4.58	13.18±4.39	0.653
HDL-C (mmol/L)	1.22±0.32	1.17±0.31	1.22±0.32	0.353
LDL-C (mmol/L)	2.76±0.81	2.81±0.80	2.76±0.81	0.722
TC (mmol/L)	4.94±1.07	4.91±1.28	4.94±1.06	0.876
TG (mmol/L)	1.72 (1.21, 2.55)	1.59 (1.07, 2.15)	1.74 (1.23, 2.57)	0.554
WBC (10^9/L)	6.31±1.28	6.51±1.59	6.31±1.26	0.308
Lymphocytes (10^9/L)	2.13±0.60	2.34±0.69	2.12±0.60	0.022
Neutrophils (10^9/L)	3.71±1.00	3.71±1.11	3.71±1.00	0.685
Hypertension, *n* (%)	697 (65.0)	17 (41.5)	433 (42.5)	0.896
Anti-hypertension therapy, *n* (%)	224 (20.9)	11 (26.8)	213 (20.6)	0.339
Smoking, *n* (%)	183 (17.1)	9 (22.0)	174 (16.9)	0.396
Drinking, *n* (%)	255 (23.8)	7 (17.1)	248 (24.0)	0.305

BMI, body mass index; WHR, waist-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; PBG, post-challenge blood glucose; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; TC, total serum cholesterol; TG, triglycerides

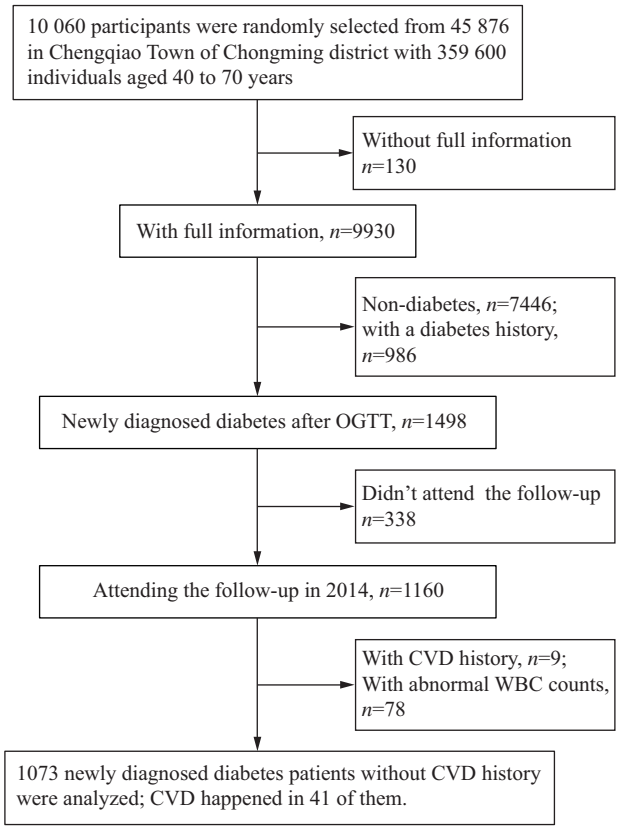

Fig. 1 Participant flow diagram
OGGT: oral glucose tolerance test; CVD: cardiovascular disease; WBC: white blood cell

Capillary Collection System (Bio-Rad Laboratories, USA) was used to collect finger capillary blood samples from each participant according to the manufacturer's instructions.

Triglycerides (TG), total serum cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) levels were measured on an automatic analyzer (Hitachi 7080; Japan). Anthropometric measurements were conducted by trained nurses or postgraduates. Blood pressure was measured three times from the right arm of each individual in a sitting position after having rested for 30 min. Three measurements were taken at 5 min intervals. Systolic blood pressure (SBP) and diastolic BP (DBP) were the mean value of the last two of the three measurements. Medical history, smoking and drinking information were gathered with a questionnaire.

1.4 Data Analysis

For database management and statistical analysis, we used SPSS 25 software (SPSS Inc., USA). Data are presented as mean±SD, median (interquartile range) or number (percent). An independent sample *t* test was used to compare the differences between groups at baseline. A log-rank test was used to compare the cumulative incidence of CVD between groups, with a Kaplan-Meier survival function to show the time to events. Unadjusted and adjusted hazard ratios (HRs) for cardiovascular events according to different lymphocyte counts were estimated using Cox proportional hazards models. Potential confounders including age, gender, waist-hip ratio (WHR), current smoking and drinking, LDL-C, HDL-C, HbA1c, and hypertension were adjusted in the Cox regression models. *P* values <0.05 were considered statistically significant.

2 RESULTS

2.1 Lymphocyte Counts and Cardiovascular Events

At the end of the follow-up, 41 CVD events occurred. As shown in fig. 2, the baseline lymphocyte counts were significantly higher in subjects who developed cardiovascular events than in those who didn't have CVD [2.34±0.69 (10^9/L) *vs.* 2.12±0.60 (10^9/L), $P<0.05$]. Multivariate logistic regression analysis showed that lymphocyte counts were independently associated with cardiovascular events during follow-up. Exp (β) (95%CI) was 1.749 (1.084–2.821) ($P<0.05$, table 2). WBC counts, neutrophils, eosinophils, basophils and monocytes were all not independently related to CVD events during follow-up (data not shown).

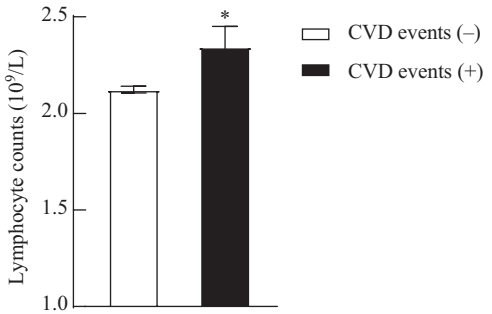

Fig. 2 Lymphocyte counts according to CVD events status
The baseline lymphocyte counts were significantly higher in subjects who developed cardiovascular events during the follow-up (*$P<0.05$). CVD: cardiovascular disease

Table 2 Logistic regression analysis showing variables that are independently associated with cardiovascular events

Independent variables	β	Exp (β) (95% CI)	*P* value
Age	0.055	1.056 (1.006–1.109)	0.027
Lymphocyte count	0.559	1.749 (1.084–2.821)	0.022

The variables entered in the analysis also included sex, SBP, WHR, LDL-C, HDL-C, HbA1c, smoking, hypertention history, and anti-hypertension therapy which were all excluded from the model.

2.2 Baseline Characteristics According to Different Lymphocyte Counts

X-Tile (Yale University, USA) was used to find the appropriate cut-point in lymphocyte counts for cardiovascular events[7]. According to the X-Tile

analysis, lymphocyte counts were divided into two groups, lymphocyte count ≤2.8 (10^9/L) and lymphocyte count ≥2.9 (10^9/L) group. The baseline characteristics of 1073 newly diagnosed diabetes patients are shown in table 3 according to different lymphocyte counts. Participants with lymphocyte counts ≥2.9 (10^9/L) were more likely to receive anti-hypertension therapy. Moreover, we compared the baseline characteristics of the diabetes participants who participated in the follow-up with those who were lost to follow-up, and found no significant differences in glucose levels, blood lipid profile, SBP and DBP between the two groups (data not shown).

2.3 Lymphocyte Counts Categories and CVD

During the follow-up, 41 newly diagnosed diabetes patients (3.82%) experienced a first CVD event, and the corresponding incidence of CVD per 1000 persons per year for the lymphocyte count ≤2.8 (10^9/L) and lymphocyte count ≥2.9 (10^9/L) groups were 11.26 and 26.38, respectively. A Kaplan-Meier survival curve for CVD events according to different lymphocyte counts is shown in fig. 3. Table 4 displays the HRs and 95% CIs for cardiovascular events by different lymphocyte counts. By taking lymphocyte count ≤2.8 (10^9/L) as the reference, lymphocyte count ≥2.9 (10^9/L) was significantly associated with the development of CVD both in the crude model (HR, 2.34; 95% CI, 1.15–4.77) and in the adjusted model (HR, 2.29; 95% CI, 1.12–4.67).

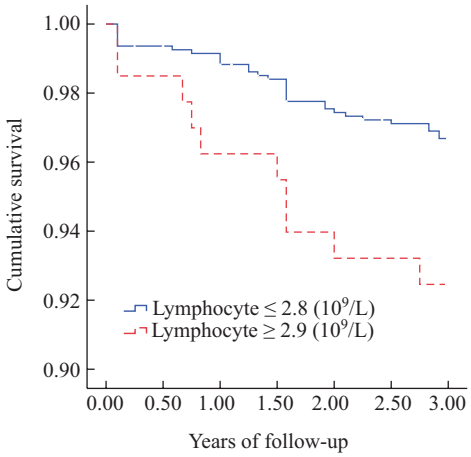

Fig. 3 Kaplan-Meier survival curve for CVD events according to lymphocyte category
Lymphocyte ≥ 2.9 (10^9/L) was significantly associated with CVD events. CVD: cardiovascular disease

3 DISCUSSION

Diabetes is one of the major risk factors of CVD.

Table 3 Baseline characteristics of subjects with different lymphocyte counts

Characteristics	All	Lymphocyte count ≤2.8 (10^9/L)	Lymphocyte count ≥2.9 (10^9/L)	*P* value
n	1073	940	133	
Age (years)	58.49±7.13	58.42±7.15	58.96±7.02	0.414
Male, *n* (%)	416 (42.87)	360 (38.30)	56 (42.1)	0.344
BMI (kg/m^2)	25.77±3.44	25.70±3.43	26.21±3.49	0.115
WHR	0.90±0.06	0.90±0.06	0.91±0.07	0.145
SBP (mmHg)	138.10±17.93	137.96±17.68	139.06±19.60	0.507
DBP (mmHg)	82.91±9.50	83.00±9.44	82.26±9.97	0.400
HbA1c	6.49±1.18	6.50±1.19	6.41±1.10	0.405
FBG (mmol/l)	7.43±1.96	7.44±1.98	7.35±1.85	0.614
PBG (mmol/l)	13.20±4.40	13.28±4.43	12.59±4.12	0.092
HDL-C (mmol/L)	1.22±0.32	1.21±0.32	1.25±0.35	0.253
LDL-C (mmol/L)	2.76±0.81	2.75±0.80	2.84±0.82	0.259
TC (mmol/L)	4.94±1.07	4.93±1.06	4.98±1.12	0.670
TG (mmol/L)	1.72 (1.21, 2.55)	1.74 (1.22, 2.60)	1.61 (1.19, 2.35)	0.166
Hypertension, *n* (%)	697 (65.0)	608 (64.7)	89 (66.9)	0.505
Anti-hypertension therapy, *n* (%)	224 (20.9)	183 (19.5)	41 (30.8)	0.003
Smoking, *n* (%)	183 (17.1)	154 (16.4)	29 (21.8)	0.091
Drinking, *n* (%)	255 (23.8)	223 (23.7)	32 (24.1)	0.942

Data are mean±SD or number (percent) or median (interquartile range). BMI, body mass index; WHR, waist-hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; PBG, post-challenge blood glucose; HDL-C, high density lipoprotein; LDL-C, low density lipoprotein; TC, total serum cholesterol; TG, triglycerides

Table 4 Adjusted HRs and 95% CIs for cardiovascular events according to lymphocyte category

Lymphocyte count	*n*/total	Cases/1000 person-year	Crude HR (95% CI)	Adjusted HR (95% CI)	
				Model 1	Model 2
Lymphocyte ≤2.8 (10^9/L)	31/940	11.26	1	1	1
Lymphocyte ≥2.9 (10^9/L)	10/133	26.38	2.34* (1.15–4.77)	2.29* (1.12–4.67)	2.24* (1.06–4.72)

Model 1 adjusted for age and sex.
Model 2 further adjusted for age, sex, WHR, LDL-C, HDL-C, HbA1c, smoking, hypertension-history, and anti-hypertension therapy.
*P<0.05

Studies indicated that diabetes increases the risk of CVD two to three folds in men and four to six folds in women[8]. The most common underlying cause of CVD, such as myocardial infarction or stroke, is atherosclerosis[9, 10]. ASCVD are the leading causes of morbidity and mortality for individuals with diabetes and are the greatest contributor to the direct and indirect costs of diabetes[11]. In addition to increased LDL-C levels and albuminuria, other CVD risk factors in diabetes include increased levels of fibrinogen, C-reactive protein, and leukocytosis[8]. Inflammation and immunity are involved in the development of ASCVD. In one of our previous studies, we found that the immune system was activated and that the total WBC counts and the lymphocyte counts were elevated significantly in diabetes[4]. Chronic inflammation and activated immune system in diabetic patients make these patients more susceptible to cardiovascular events. Atherosclerosis is known to be a chronic inflammatory disease, and the innate and adaptive immune system can both play crucial roles in the pathogenesis of ASCVD[9]. The innate immune system is the first line of defense in the body. Adaptive immune responses are initiated by the innate immune system. Adaptive immunity includes the humoral mechanism, which is executed by B lymphocytes and cell-mediated mechanisms that are executed by T lymphocytes[9]. Cell-mediated mechanisms are more important for atherosclerosis since they regulate the magnitude of the pro-inflammatory atherogenic response. T cells affect the stability of the atherosclerotic lesion and the propensity for thrombus formation[12]. Activated T lymphocytes are involved in the whole process of coronary arteriosclerosis[13]. Early atherosclerosis is particularly influenced by activated $CD4^+$ T cells[14]. Th9 cells and Th17 cells are reported to affect the progression of coronary atherosclerosis[12]. Stimulation of inflammatory mechanisms in ASCVD is not limited to the micro-enviroment of the plaque, and also involves circulating cell populations. In this study, we found that lymphocyte counts were increased significantly in diabetic subjects who experienced first cardiovascular events during follow-up, and that increased lymphocyte counts were independently associated with the occurrence of cardiovascular events. Our results proved that the immune system was involved in the pathogenesis of cardiovascular events in recently diagnosed diabetes.

We further discovered that lymphocyte count ≥2.9 (10^9/L) was significantly associated with the development of CVD events. Lymphocyte count ≥2.9 (10^9/L) is an independent predictor of future CVD events in patients without previous cardiovascular event history in newly diagnosed diabetes. Finding the cut-off point of lymphocyte levels to predict the future risk of CVD is very important. This may provide guiding information for the prevention and treatment of cardiovascular disease patients with diabetes. Monitoring the levels of lymphocytes in patients with diabetes is easy to carry out and can help identify an important group of diabetic patients who are at a higher risk of ischemic events. This can thereby allow aggressive modification of cardiovascular risk factors and initiate further investigations and management.

This study has some advantages. First, our analysis was stratified by lymphocyte count ≥2.9 (10^9/L) to evaluate CVD risk in diabetes patients. Second, we selected newly diagnosed diabetes patients without previous CVD as our study subjects, which excluded diabetes duration and CVD history as major confounders in identifying risk factors of future CVD events. Our study also has some limitations, including the follow-up duration of three years with CVD information recorded. Three years may be relatively short, and more time may be needed to assess the true CVD risks. Future data with continuous follow-up of these participants are warranted to evaluate longer-term health implications. More studies are needed to research the role of lymphocytes in the pathogenesis of ASCVD.

4 CONCLUSIONS

Overall, this study confirms that the immune system plays a crucial role in the pathogenesis of ASCVD, and that higher lymphocyte levels may result in a significantly higher CVD risk among diabetes. Lymphocyte count ≥2.9 (10^9/L) is an independent predictor of developing future CVD events. Furthermore, diabetes patients with lymphocyte count ≥2.9 (10^9/L), even within normal range, need aggressive modification of cardiovascular risk factors.

Conflict of Interest Statement

The authors have no conflict of interest.

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(Received Dec. 30, 2020; accepted Aug. 28, 2021)