Absolute Counts of Peripheral Lymphocyte Subsets as Potential Immune Impairment Markers in Patients With Breast Cancer

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Abstract

Purpose This study was to evaluate clinic value of absolute counts of lymphocyte subsets (ACL) as potential blood biomarkers in progression and prognosis in breast cancer (BC) patients.

Methods A total of 237 BC patients and 55 age-matched female normal healthy donors (normal cantrals, NCs) were enrolled in this study. The absolute counts (AC) and percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B and NK cells were determined by flow cytometry. The clinicopathological parameters influencing disease progression were determined by binary logistic regression. The progression-free survival (PFS) was evaluated by Kaplan-Meier. Univariable and multivariable analyses were performed using log-rank test and proportional hazard regression models, respectively.

Results Compared with NCs, the ACL in BC patients decreased significantly, while the percentages of lymphocytes showed no change. Of them, AC of $CD3^+CD4^+$ cells was closely related to clinical stages. The ACL, especially $CD3^+CD4^+$ cells, were affected by different treatments. Analysis of logistic regression showed that the cut-off value of $CD3^+CD4^+$ cells $\geq$ 451 cells/μL was the favorable prognostic factor. Multivariate analysis of prognostic factors of PFS showed $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were independent factors for predicting PFS.

Conclusions The AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells in BC patients were impaired obviously and can be as potential susceptive indications to evaluate the patient's immune states. The higher level of AC of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells contributed to longer PFS and favorable outcome, and could help to adopt appropriate treatment strategies in clinic.

Introduction

Breast cancer (BC) has become one of the main causes of cancer death in women all over the world. According to the latest statistics in 2018, patients with BC in 185 countries and regions in the world increase by 2.089 million every year, with 627000 deaths [1]. In recent years, the incidence rate of BC is increasing and getting younger, but BC is a type of cancer with a good prognosis [2]. At present, the main treatments of BC are surgery, chemotherapy, endocrine, and other comprehensive therapy. For patients with early and mid-term BC, postoperative adjuvant chemotherapy can improve the local control rate, and reduce the recurrence rate of local and regional lymph nodes [3].

It has been found that the recurrence and metastasis of tumors are closely related to the decline of immune function, which leads to the tumor immune escape [4]. The classical lymphocyte subsets include $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and nature killer (NK) cells,which are crucial in regulating immunity and specific killing of tumor cells [5]. Although their compositions are simple, the roles in immune responses are diverse, encompassing both innate and adaptive immunity, cell and humoral immunity simultaneously. $CD3^+CD4^+$ cells can activate other lymphocyte subsets by releasing cytokines and suppress tumor development by directly killing tumor cells expressing adequate levels of major

histocompatibility complex (MHC) class II molecules [6]. $CD3^+CD8^+$ cells, a kind of cytotoxic cells, are able to recognize tumor cells expressing MHC class I molecules presented by antigen presenting cells and produce IFN-γ, perforin, granzyme B for targeting and killing tumor cells [7]. B cells play an important role in humoral immunity, which can not only present antigen to T cells, participate in the immune response of T cells, but also can recognize different antigen epitopes, secrete immunoglobulin and participate in the humoral immune response [8]. NK cells are indispensable effector cells in the innate immune system, and they are the first line of defense for host immune defense against cancer and pathogens [9]. Therefore, the analysis of peripheral lymphocyte subsets is the most significant and convenient way to assess the functions of immune including the percentages of lymphocytes (PL) and the absolute count of lymphocytes (ACL). Especially, the ratio of $CD4^+/CD8^+$ is an indicator of the balance of the immune system, which is maintained by the proportion and the number of peripheral blood lymphocytes [10].

Traditionally, the ACL was measured by dual-platform technology by which the percentages of lymphocyte subsets measured by flow cytometry was combined with the absolute lymphocyte count measured by an automatic hematology analyzer,which produced obvious errors among different laboratories[11–14]. Here, we adopted the single-platform technology to carry out the detection entirely on the flow cytometer, which significantly improved the accuracy of the analysis. Published studies showed that lymphopenia, or low peripheral blood lymphocyte count, may predict higher mortality and increase the risk of recurrence after primary surgery and neoadjuvant therapy, whereas higher absolute lymphocyte counts predict lower mortality from early-stage triple-negative BC [15, 16]. But, these studies only analyzed the total number of lymphocytes, and did not further analyze the influence of the ACL. However, the correlations of prognosis and the ACL in patients with BC remain unclear.

The aim of this study was to evaluate the clinic value of PL and ACL in BC progression and prognosis, and analyze the importance and susceptivity of ACL served as biomarkers of immune impairment.

Materials And Methods

Clinical data

All the subjects were given informed consent in accordance with the Declaration of Helsinki. This study was approved by the hospital ethics committee (TYLL2017[K]002) and registered at the Chinese Clinic Trial Registry (ChiCTR-IOR-17014139). A total of 237 BC patients and 55 age-matched female normal healthy donors [normal controls (NCs)] were enrolled in First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. The cohort of 237 patients were all women with a median age of 64 years (range from 36 to 80 years), postmenopausal patients were 208 cases, premenopausal patients were 29 cases. NCs were all females with a median age of 64 years (range from 35 to 80 years), including 7 premenopausal females, 48 postmenopausal females. No significant differences in age and menstrual status of the two groups ($P > 0.05$).

Inclusion criteria

BC patients

Total 237 patients with BC were included in this study from January 2018 to October 2020, and were eligible for the following conditions:(1) Pathologically ascertained diagnosis of malignant BC, and disease stages were determined according to the tumor-node-metastasis (TNM) classification scheme recommended by the Union for International Cancer Control; (2) There was at least one measurable lesion according to the Response Evaluation Criteria in Solid Tumor(RECIST), version 1.1[17]; (3) Without other malignant tumors; (4) All participants had a baseline Eastern Cooperative Oncology Group(ECOG) performance-status score 0 or 1, and the expected survival ≥ 6 months; (5) All patients enrolled should have complete clinical and laboratory data including PL and ACL; (6) No severe hypertension, diabetes, coronary heart disease, infectious diseases, hematopoietic system and immune system diseases and so on.

NCs

Subjects who served as NCs were inquired about their physical condition, usage of medicines, smoking, and alcohol consumption. The NCs with normal blood routine examination, liver functions (AST and ALT), renal functions (SCr), blood glucose levels, and without tumors, were considered normal.

Exclusion criteria

BC

(1) The diagnosis of breast malignancy was uncertain; (2) With other malignant tumors; (3) personal data and clinical case data cannot be traced; (4) Patients with acute infection, hematological disorders, autoimmune diseases, pregnancy or lactation; (5) Do not accord with medical advice.

NCs

Excluded healthy physical examinees with breast disease, immune system disease, metabolic disease, acute infection, or tumors.

Treatments

(1) Surgery: There were 83 patients received surgery alone, including mastectomy or lumpectomy with sentinel node biopsy or full axillary dissection; (2) Surgery combined with chemotherapy (called chemotherapy): 82 patients were treated with chemotherapy after surgery, including neoadjuvant/adjuvant chemotherapy within one year of diagnosis. The treatment regimens employed were paclitaxel/epirubicin/cyclophosphamide/docetaxel (CE/TAC); (3) Surgery combined with endocrinotherapy (called endocrinotherapy): 72 patients received endocrinotherapy after surgery. The endocrine therapy for premenopausal BC patients who with estrogen/progesterone receptor (ER/PR) positive was tamoxifen, while for postmenopausal patients who with ER/PR positive was letrozole.

Efficacy evaluation

Based on RECIST, version 1.1[17], the efficacy evaluation was divided into complete response (CR), partial response (PR), stable disease (SD) and progress disease (PD). CR + PR were considered to be effective, while SD + PD were considered ineffective.

The main reagents and instruments

The assay of lymphocyte subsets was performed by using a lyse/no-wash procedure based on asingle-platform technique by ten-color flow cytometry (BD FACS Canto II: U6573380-00541). The main reagents were BD Multitest IMK kit (Catalog NO: 662965) containing (BD MultitestCD3 FITC/CD8PE/CD45PerCP/CD4APC and BD Multitest CD3 FITC/$CD16^{+}CD56^{+}$PE/CD45PerCP/CD19APC), BD Multitest IMK kit lysing solution (Catalog NO: 91-1087); The EDTA bloodcollecting tubes and trucount tubes (Catalog NO: 340334) were also from BD Biosciences.

Sample collection

Two milliliters of fresh whole blood were drawn from NCs and patients respectively and were stored in the EDTA-anticoagulant blood collecting tubes.

Cellular staining and analyzing

The whole blood of the 292 participants in this study were collected between January 2018 and October 2020 and performed by flow cytometry. The manipulation was referred to BD operating instruction. in brief, for each sample, two trucount tubes were labeled with letters A and B to distinguish them from each other; added 20μL of BD Multitest CD3/CD4/CD8/CD45 and CD3/$CD16^{+}CD56^{+}$/CD45/CD19 reagents into the bottom of each tube A and B, respectively; then added 50μL of well-mixed whole blood into the bottom of every tube; finally, added 450μL of lysing solution into every tube, mixed and incubated for 15 min in dark at room temperature for analysis.

Statistics

The normality of statistical data was evaluated by the Shapiro-Wilk test. Using two independent sample *t*-test to analyze the differences in PL and ACL between patients with BC and NCs. The differences among three or more groups of continuous numerical variables with normal distribution were analyzed by one-way ANOVA (Bonferroni or Tamhane's test). Progression-free survival (PFS) was defined as the time from the date of enrollment to disease progression, recurrence, or death, and the follow-up deadline was October 31, 2020. Categorical variables were analyzed using the chi-squared test. Factors affecting disease progression were analyzed by binary logistic regression. Calculating survivals were with the method of Kaplan-Meier. Univariate and multivariate analyses were performed to assess the relationship between clinicopathologic parameters and PFS, for which the Log-Rank test and proportional hazard regression model were used, respectively. Variables with a *P*-value < 0.05 by a univariable analysis were entered for multivariable analysis. Odds ratios (ORs) were reported with 95% confidence intervals (CIs). Values of $P < 0.05$ were considered statistically significant. Data were analyzed by SPSS 25.0(IBM Corporation). Figures were prepared using Graph Pad Prism version 9.0 software (San Diego, USA).

Result

Analysis of lymphocyte subsets between patients and NCs

The principle of ACL detected by the single-platform was that the known total number of fluorescent microbeads were used as the standard internal parameters and fluorescent-labeled antibodies added into the trucount tubes, then applied acquisition and analysis software in the flow cytometry to calculate data.

$$\left(\text{cells}/\mu\text{L} = \frac{\text{acquired cells} \times \text{totle beads}}{\text{acquired beads} \times \text{volume of sample}} \times 100\%\right)$$

Using the method, we primitively compared both the PL and AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells in all patients with BC and NCs. There was no difference in percentages between the two groups ($P > 0.05$, Fig. 1a). But the AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B and NK cells were significantly decreased in patients with BC ($P < 0.001$, Fig. 1b). The results suggested that it's ACL but not PL decreased in patients with BC. To our knowledge, PL represents the proportion or composition of each subsets, indicating the development and differentiation of lymphocytes, while ACL demonstrates the exact number of peripheral lymphocyte subsets, indicating the proliferation of lymphocyte progenitor. The results showed that the proliferation ability of lymphocytes was impaired distinctly.

In addition, compared with NCs,the PL in patients with BC at stage Ⅰ-Ⅱ and Ⅲ-Ⅳ showed no significant difference($P > 0.05$, Fig. 1c, e), but significant differences were observed in the ACL ($P < 0.001$, Fig. 1d, f). The results suggested that significant decrease of ACL was one of important characteristics of immune impairment or one sign of immunodepletion.

ACL in patients with different clinical stages

There was no significant difference in PL of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B and NK cells among patients at different clinical stages (Fig. 2f). Compared with the AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B and NK cells in patients at different clinical stages, we discovered that all of them had varying degrees of decline (Fig. 2). The AC of $CD3^+$ in patients at stage IV was lower than patients at stage I and II(P 0.001), but patients at stage III had no significant difference compared with patients at stage I and II (Fig. 2a). With the progression of clinical stages, the decline of AC of $CD4^+$ was particularly significant in BC patients. Compared with patients at stage I, II and III, the AC of $CD4^+$ in patients at stage IV decreased most strikingly (P 0.001), then followed by stage III (P 0.01), stage II (P 0.01), and stage I (P 0.001). However, there was no obvious difference of AC between stage I and stage II in patients (Fig. 2b). Compared to patients at stage I, the AC of $CD8^+$ was decreased in patients at stage II ($P < 0.05$) and stage III-IV ($P < 0.01$) (Fig. 2c). Compared with patients at different stages, the variation of B cells AC was only observed in patients at stage I and IV ($P < 0.01$) (Fig. 2d). Meanwhile, the difference in NK cell AC was only found in patients at stage II and IV ($P < 0.001$) (Fig. 2e). In a word, our data showed that the decrease

of $CD4^+$ and $CD8^+$ AC was closely related to clinical stages, which indicated that it declined upon the exacerbations of BC.

ACL between CR + PR and SD + PD group

To further study the relationship of ACL and efficacy, we divided the patients into two groups according to curative effect, one was effective group (CR + PR), and the other was ineffective group(SD + PD). Independent sample *t*-test revealed that AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells in CR + PR group were significantly higher than that in SD + PD group ($P < 0.001$, Fig. 3a, b, d, e; $P < 0.01$, Fig. 3c). In brief, the results indicated that ACL, especially AC of $CD3^+CD4^+$ was closely related to the curative effect, the higher the ACL, the better the efficiency.

ACL in different treatments

To further study the relationship of ACL and different treatments, we divided the patients in effective(CR + PR) and ineffective group(SD + PD) into surgery, chemotherapy and endorinotherapy according to curative ways. The common characteristics of effective and ineffective groups were that AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells were all the highest treated by endocrinotherapy, then followed by surgery and chemotherapy (Fig. 4). The differences were that mean numbers of ACL of every treatment in effective group were higher than that in ineffective group. Strikingly, the mean value of AC of $CD3^+CD4^+$ showed the most obvious difference with 518 cells/uL in effective group and 393 cells/uL in ineffective group (Fig. 4b), suggesting AC of $CD3^+CD4^+$ was closely related to the efficacy, and may be served as potent blood biomarker to evaluate the prognosis.

Effect of ACL on the progression of BC

In order to ascertain whether ACL can influence the progression of BC (freedom from any events as follows: other newly diagnosed tumors; distant organ metastasis; concurrent infection), we further conducted binary logistic regression analysis to determine the influencing factors of disease progression. From the forest plots of subgroups analysis for progression, we could see AC of $CD3^+CD4^+$ cells (95% confidence interval 0.015-0.202, $P < 0.001$), distant metastasis (95% confidence interval 1.628-28.296, $P = 0.009$), clinical stages (95% confidence interval 1.252-48.192, $P = 0.028$), pathological category (95% confidence interval 0.015-0.469, $P = 0.005$), tumor size (95% confidence interval 0.027-0.601, $P = 0.009$), menopause (95% confidence interval 2.159-86.12, $P = 0.005$) could affect the progression of disease(Fig.5). Of them, median AC of $CD3^+CD4^+ \geq 451$cells/μL, tumor size≥2cm, and pathological category (invasive lobular carcinoma) helpfully contributed to favorable prognosis, which showed in the picture were on the left side of the line of OR = 1. On the contrary, distant metastasis, clinical stages, menopause led to unfavorable prognosis. However, age, family history, age of menarche, differentiation, lymphatic metastasis, vessel carcinoma embolus, treatment, ACL of $CD3^+CD8^+$, B cells, and NK cells were not the factors of BC progression ($P > 0.05$).

Relationship between AC of $CD3^+CD4^+$ and clinicopathologic parameters of BC

As can be seen from the figure above, a high AC of $CD3^+CD4^+$ cells is beneficial to the prognosis. But, the correlation between $CD3^+CD4^+$ cells and clinicopathologic parameters of BC patients is unclear. In 237 patients enrolled, 209(88.2%) were invasive lobular carcinomas (ILC), 28(11.8%) were invasive ductal carcinomas (IDC). Patients with stage I-II and III-IV were 89(37.6%), 148(63.4%), respectively. 76.4% of the patients were medium/low differentiation, 66.7% had lymph node metastasis, 49.4% had distant metastasis, 48.9% had family history, 84.0% had a previous history, such as cyclomastopathy, diabetes, hypertension. The median (cut-off value) AC of $CD3^+CD4^+$ cells were 451 cells/μL. According to the cut-off value 451 cells/μL, the patients were divided into two groups□one was $CD3^+CD4^+$ cells < 451 cells/μL, there were 118 cases, the other was $CD3^+CD4^+$cells ≥ 451 cells/μL, there were 119 cases. Further analysis showed that $CD3^+CD4^+$ level was not correlated with age, age of menarche, menopause, tumor size, and differentiation degree of BC patients ($P > 0.05$), but significantly correlated with family history, previous history, pathological category, clinical stages, lymphatic metastasis, distant metastases, vessel carcinoma embolus, and treatments ($P < 0.05$) (Table 1).

Table 1

Relationship between AC of $CD3^+CD4^+$ and clinicopathologic parameters of BC

Characteristics	Overall, n(%)	AC of $CD3^+CD4^+$		χ^2	*P*-value
		< 451 cells/μL	≥ 451 cells/μL		
Age	113(47.7%)	60	50	3.072	0.08
≥ 64					
< 64	124(52.3%)	55	69		
Family history	116(48.9%)	68	48	7.089	**0.008**
Yes	121(51.1%)	50	71		
No					
Previous history				4.393	**0.036**
Yes	199(84.0%)	105	94		
No	38(16.0%)	13	25		
Age of menarche	121(51.1%)	56	65	1.217	0.270
< 12	116(48.9%)	62	54		
≥ 12					
Menopause				1.031	0.310
Yes	208(87.8%)	101	107		
No	29(12.2%)	17	12		
Tumor size				1.016	0.602
< 2cm	61(25.7%)	27	34		
=2-5cm	127(53.6%)	66	61		
> 5cm	49(20.7%)	25	24		
Pathological category				10.215	**0.001**
IDC	28(11.8%)	6	22		
ILC	209(88.2%)	112	97		
Differentiation				0.777	0.378
High	56(23.6%)	25	31		
Medium/low	181(76.4%)	93	88		

Bold value represents that *P* value was significant

Characteristics	Overall, n(%)	AC of $CD3^+CD4^+$		χ^2	*P*-value
		< 451 cells/μL	≥ 451 cells/μL		
Clinical stages				57.692	**< 0.001**
I + II	89(37.6%)	16	73		
III + IV	148(63.4%)	102	46	34.566	**< 0.001**
Lymph node metastasis					
Yes	158(66.7%)	100	58		
No	79(33.3%)	18	61		
Distant metastasis				48.305	**< 0.001**
Yes	117(49.4%)	85	32		
No	120(50.6%)	33	87		
Vessel carcinoma embolus				28.040	**< 0.001**
Yes	59(21.6%)	47	12		
No	178(78.4%)	71	107		
Treatments				22.069	**< 0.001**
Surgery	83(35.0%)	42	41		
Surgery + chemotherapy	82(34.6%)	55	27		
Surgery + endocrinotherapy	72(30.4%)	21	51		
Bold value represents that *P* value was significant					

ACL affecting progression-free survival

Moreover, kaplan-Meier survival and multivariate Cox regression model were used to investigate whether ACL could affect PFS. Table 2 showed the results of univariate analysis and multivariate analysis of prognostic factors of PFS. Univariate analysis suggested that 14 clinicopathological parameters contributed to be important predictors of PFS, including AC of $CD3^+CD4^+$ (cut-off value 451 cells/μL vs ≥451 cells/μL, $P < 0.001$)(Fig. 6a), AC of $CD3^+CD8^+$ (cut-off value 324 cells/μL vs ≥324 cells/μL, $P = 0.001$) (Fig. 6b), AC of B cells (cut-off value 155 cells/μL vs ≥155 cells/μL, $P < 0.001$)(Fig. 6c), AC of NK cells (cut-off value 162 cells/μL vs ≥162 cells/μL, $P < 0.001$) (Fig. 6d), family history(yes vs no, P 0.001), previous history(yes vs no, $P = 0.001$), smoke(yes vs no, $P = 0.043$), age of menarche(12 vs ≥12, $P = 0.022$) , clinical stages (I-II vs III-IV, P 0.001) (Fig. 6e) , tumor size (2cm vs ≥2cm, $P = 0.011$) (Fig. 6f), pathological category (ILC vs IDC, $P = 0.022$), lymphatic metastasis (yes vs no, $P < 0.001$), distant

metastasis (yes vs no, $P < 0.001$) (Fig. 6g), vessel carcinoma embolus(yes vs no, $P < 0.001$) (Fig. 6h). Multivariate analysis revealed that clinical stages (*OR* = 5.706, 95% confidence interval 1.884-17.277, $P = 0.002$) and distant metastasis (*OR* = 1.929, 95% confidence interval 1.122-3.318, $P = 0.018$) were the unfavorable prognostic factor, whereas AC of $CD3^+CD4^+$ (*OR* = 0.435, 95% confidence interval 0.269-0.703, $P = 0.001$) and AC of $CD3^+CD8^+$ (*OR* = 0.435, 95% confidence interval 0.414-0.896, $P = 0.012$) were the favorable prognostic factors. Kaplan–Meier survival curve of PFS and the cut-off value were shown in Fig.6. The PFS of patients with AC of $CD3^+CD4^+ \geq 451$ cells/μL were longer than that of $CD3^+CD4^+$ 451 cells/μL. Meanwhile, The patients with AC of $CD3^+CD8^+ \geq 324$ cells/μL had longer PFS than that of $CD3^+CD8^+$ 324 cells/μL. Conversely, patients with later clinical stages or distant metastasis predicted poor prognosis. Surprisingly, AC of $CD3^+$ and different treatments had no effect on PFS (Fig. 5i, j).

Table 2
Univariate and multivariate analysis of prognostic factors of PFS

Characteristics		Univariate		Multivariate	
		Log-rank χ^2	*P*-value	*OR*(95%*CI*)	*P*-value
Age	< 64	0.318	0.573	- - -	
	≥ 64				
Family history	No Yes	15.503	**< 0.001**	1.115(0.766–1.622)	0.57
Previous history	No Yes	10.976	**0.001**	1.107(0.572–2.143)	0.763
Smoke	No Yes	4.079	**0.043**	0.765(0.485–1.208)	0.25
Drink	No Yes	0.046	0.830	- - -	
Age of menarche	< 12 ≥ 12	5.214	**0.022**	1.044(0.725–1.504)	0.817
Menopause	No Yes	1.135	0.287	- - -	
Tumor size	< 2cm ≥ 2cm	6.694	**0.011**	1.003(0.631–1.595)	0.99
Pathological category	ILC IDC	9.251	**0.002**	0.577(0.261–1.276)	0.174
ER	Negative Positive	1.755	0.185	- - -	
PR	Negative Positive	0.921	0.337	- - -	

Bold value represents that *P* value was significant

Characteristics		Univariate		Multivariate	
		Log-rank χ^2	*P*-value	*OR*(95%*CI*)	*P*-value
Her2	Negative	0.001	0.979	-	
	Positive			-	
Differentiation	High	1.322	0.250	-	
	Medium/low			-	
Clinical stages	I + II	111.916	**< 0.001**	5.706(1.884–17.277)	**0.002**
	III + IV				
Lymphatic metastasis	No	80.679	**< 0.001**	1.224(0.450–3.330)	0.692
	Yes				
Distant metastasis	No	115.315	**< 0.001**	1.929(1.122–3.318)	**0.018**
	Yes				
Vessel carcinoma embolus	No	62.756	**< 0.001**	1.425(0.953–2.131)	0.085
	Yes				
Treatment	Surgery	1.927	0.382	-	
	Surgery + chemotherapy			-	
	Surgery + endocrinotherapy				
AC of $CD3^+$	< 924	0.032	0.858	-	
	≥ 924			-	
AC of $CD3^+CD4^+$	< 451	93.634	**< 0.001**	0.435(0.269–0.703)	**0.001**
	≥ 451				
AC of $CD3^+CD4^+$	< 324	12.045	**0.001**	0.609(0.414–0.896)	**0.012**
	≥ 324				
AC of B cells	< 155	16.644	**< 0.001**	0.948(0.641–1.401)	0.788
	≥ 155				

Bold value represents that *P* value was significant

Characteristics		Univariate		Multivariate	
		Log-rank χ^2	*P*-value	*OR*(95%*CI*)	*P*-value
AC of NK cells	< 162 ≥ 162	23.792	**< 0.001**	1.011(0.671–1.522)	0.96
Bold value represents that *P* value was significant					

Discussion

The study showed that it was much more convenient and accurate to apply the single-platform flow cytometry to analyze the PL and ACL in BC patients. Currently, the PL are mostly used to evaluate the immune function of tumor patients in clinical, but the detection of ACL are relatively insufficient, which may lead to serious impairment of the immune function of cancer patients being ignored.

The immune system normally protects our body from the invasion of pathogens and malignant tumors, mainly including cellular immunity and humoral immunity. Studies have shown that cellular immunity plays a major role in the process of anti-tumor immunity [18–19]. $CD3^+$ cells mainly consists of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells, $CD3^+CD4^+$ cells play an immunomodulation role, which can assist B cells to produce antibodies and meanwhile secrete cytokines to enhance the $CD3^+CD8^+$ cells to kill tumors [20]. Thus, $CD3^+CD4^+$ cells can target tumor cells in various ways, either directly by eliminating tumor cells through cytolytic mechanisms or indirectly by modulating the tumor microenvironment [21]. The increase in the number of $CD3^+CD4^+$ cells indicates that the immune response is improved and the anti-tumor activity is strengthened [22]. $CD3^+CD8^+$ cells can be classified into cytotoxic T lymphocytes (CTLs) and inhibitory cells. CTLs are the preferred tool to target tumors, as they detect extracellular antigens that are presented by MHC class I molecules expressed by all tumor cell types [23]. $CD19^+$, as a surface marker of B cells, is mainly involved in the humoral immunity, and the level of its expression can measure the strength of the humoral immunity [24]. $CD16^+CD56^+$ is a specific marker of NK cells which are a group of cells with special properties and do not have antigen-recognition receptors on the surface [25]. NK cells are the first activated immune cells in anti-tumor immunity and important cytotoxic cells in the innate immunity, which can be activated and exert their effector functions without antigenic stimulation [26]. The decreased number of NK cells indicates that the immune system is less capable of monitoring, killing, and clearing tumor cells [27]. Hence, the detection of the ACL in tumor patients is of great significance for understanding the changes of patients' condition and prognosis [28].

The research suggested that ACL could be served as a potential peripheral blood immune impairment markers in patients with BC to monitor the immune function, and predict the prognosis and therapeutic effect. It is the first time to report the clinic value of peripheral ACL in BC, though some research suggested that total number of peripheral blood lymphocytes was associated to the prognosis and

therapeutic effect [13, 14, 15, 29, 30]. Firstly, just as our study showed, there was no significant difference in the percentages of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells between NCs and BC patients. However, the AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells decreased markedly in patients with BC compared to NCs. Consequently, the study highlighted the necessity and importance of detecting ACL in BC patients beside the percentages, which may help us to understand the immunologic injury, analyze the clinical condition, and predict the curative effect of patients [31]. Secondly, we found that $CD3^+CD4^+$ cells was close negative correlation to clinical stage and decreased upon the development and deterioration of BC. Compared with BC patients at stage I and II, the AC of $CD3^+CD4^+$ in patients at stage III and IV decreased more strikingly. Compared to patients at stage I, the AC of $CD3^+CD8^+$ were decreased in patients at stage II, stage III, and stage IV too. Thirdly, our study also revealed that the different treatments could affect AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B and NK cells, chemotherapy significantly impaired the ACL, especially AC of $CD3^+CD4^+$ due to its cytotoxicity. Interestingly, ACL were positively correlated with curative effect, in CR and PR patients, AC of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, B, and NK cells were significantly higher, but lower in SD + PD group. The data suggested that no matter what the curative effect was, chemotherapy could decrease the ACL, especially AC of $CD3^+CD4^+$ cells. Finally, the results reveal that patients with AC of $CD3^+CD4^+ \geq 451$ cells/μL or AC of $CD3^+CD8^+ \geq 324$ cells/L indicated a longer PFS, which was consistent with our previous study [32]. Therefore, more attention should be paid to AC of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in anti-tumor immunity.

The increased numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells signify a good prognosis in BC, which is consistent with some previous study [33–34]. $CD3^+CD4^+$ cells can promote the activation of $CD8^+$CTLs, boost the effector and memory function of CTLs, and reduce the immunosuppression of CTLs,which helps T cells to amplify their response to tumor-associated antigens without generating an autoimmune response[35]. Additionally, antigen-specific contacting with $CD3^+CD4^+$ cells enables dendritic cells to optimize antigen presentation and deliver specific cytokines and co-stimulation signals to $CD3^+CD8^+$ cells, so as to promote their cloning, amplification, and differentiation into effector or memory T cells [36]. In brief, the ACL in BC patients were low, indicating that the immune function of patients was impaired. The patient's impaired immune system could not indeed keep tumor from progressing [37]. Therefore, it is essential to enhance the immune function of patients and improve their anti-tumor ability during the clinical therapy. $CD3^+CD4^+$ cells contribute to initiating a gene expression program of $CD3^+CD8^+$ cells via multiple molecular mechanisms to enhance the function of $CD8^+$CTLs and overcome the obstacle of anti-tumor immunity [38].

Consequently, the ACL not only reflects the immune status of the body, but also plays a pivotal role in the prediction of disease prognosis and the curative effect [31], therefore we should pay more attention to the change of ACL in clinic, so as to provide more reference value for predicting the patient's condition and clinical treatment.

Conclusion

The ACL and PL have different clinical significance, it was ACL, not PL appeared significant impairment in BC patients, and correlated to progression of tumor, PFS, efficacy, it is necessary and pivotal to detect ACL in clinic. ACL can be as potential peripheral blood biomarkers to monitor the immune impairment, and predict the prognosis and therapeutic effect.

Abbreviations

BC Breast cancer

MHC Major histocompatibility complex

PL Percentages of lymphocyte subsets

ACL Absolute counts of lymphocyte subsets

AC Absolute counts

CTLs Cytotoxic T lymphocytes

NCs Normal controls

NK Nature killer cell

PFS Progression-free survival

ER Estrogen receptor

PR Progesterone receptor

Her2 Human epidermal growth factor receptor-2

CR Complete response

PR Partial response

SD Stable disease

PD Progress disease

ILC Invasive lobular carcinoma

IDC Invasive ductal carcinomas

Declarations

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Conflicts of interest

All authors declare that they have no conflicts of interests.

Availability of data and materials

All data generated and analyzed during this study are available from the corresponding author in response to reasonable requests.

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Author contributions

Jianchun Yu: Conceptualization, Methodology. Aqing Liu, Ying Xia, Wentao Li: Writing - Original Draft, Formal analysis, Writing - Review & Editing. Guan Zhang, Yunhe Liu, Songshan Ye, Zhi-jie Ruo Zhao, Yanjie Yang: Data Curation, Validation. Yingjie Jia, Yongtie Guo, Xu Liu, Huayu Chen: Resources. All authors read and approved the final manuscript.

Ethical approval

This article does not contain any studies with animals performed by any of the authors. All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki Declaration and the clinical trial was approved by the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine ethics committee: (TYLL2017 [K] 002) and registered at Chinese Clinic Trial Registry (ChiCTR-IOR-17014139).

Consent for publication

Not applicable.

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Figures

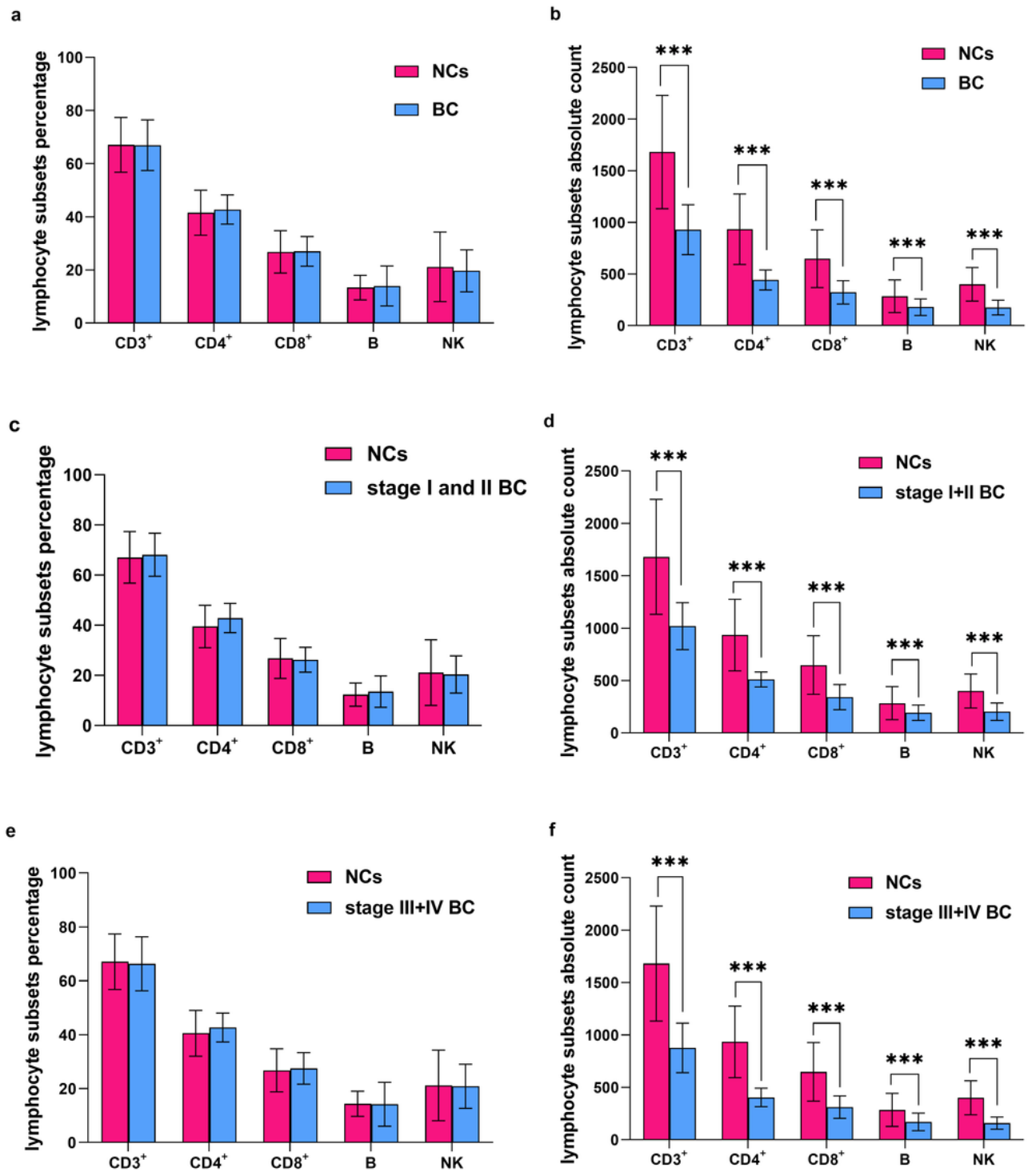

Figure 1

Comparison of percentages and absolute counts of lymphocyte subsets between BC patients and NCs. (a) showed that there were no significant differences in the percentages between the two groups ($P > 0.05$). (b) revealed that absolute counts of CD3+, CD3+CD4+, CD3+CD8+, B, and NK cells were decreased significantly in BC patients ($P < 0.001$).(c) and (e) showed that BC patients at different stages had no significant differences in the percentages of lymphocyte compared to NCs ($P > 0.05$).(d) and (f)

demonstrated that □-□ and □-□ stages of BC patients had lower absolute counts of lymphocyte subsets compared to NCs, and much lower in patients with III-IV stages compared with NCs(P < 0.001). (*** represents P < 0.001)

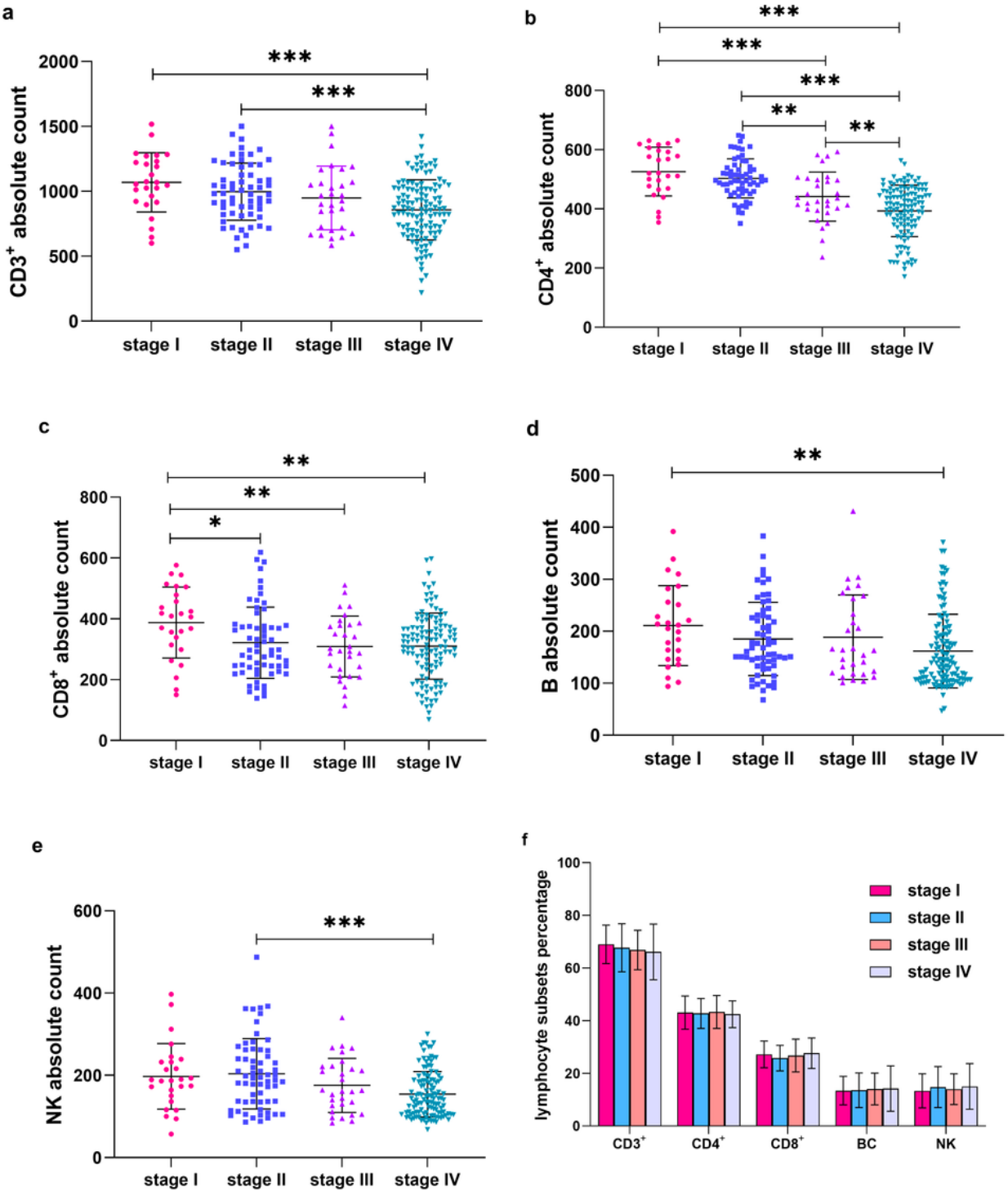

Figure 2

The comparison of AC in patients with different clinical stages. AC of CD3+ (a), CD3+CD4+ (b), CD3+CD8+ (c), B (d), and NK cells (e) in patients at different stages. (f) PL of CD3+, CD3+CD4+,

CD3+CD8+, B and NK cells among different clinical stages, showing no significant difference. (*represents P < 0.05, ** represents P < 0.01, *** represents P < 0.001)

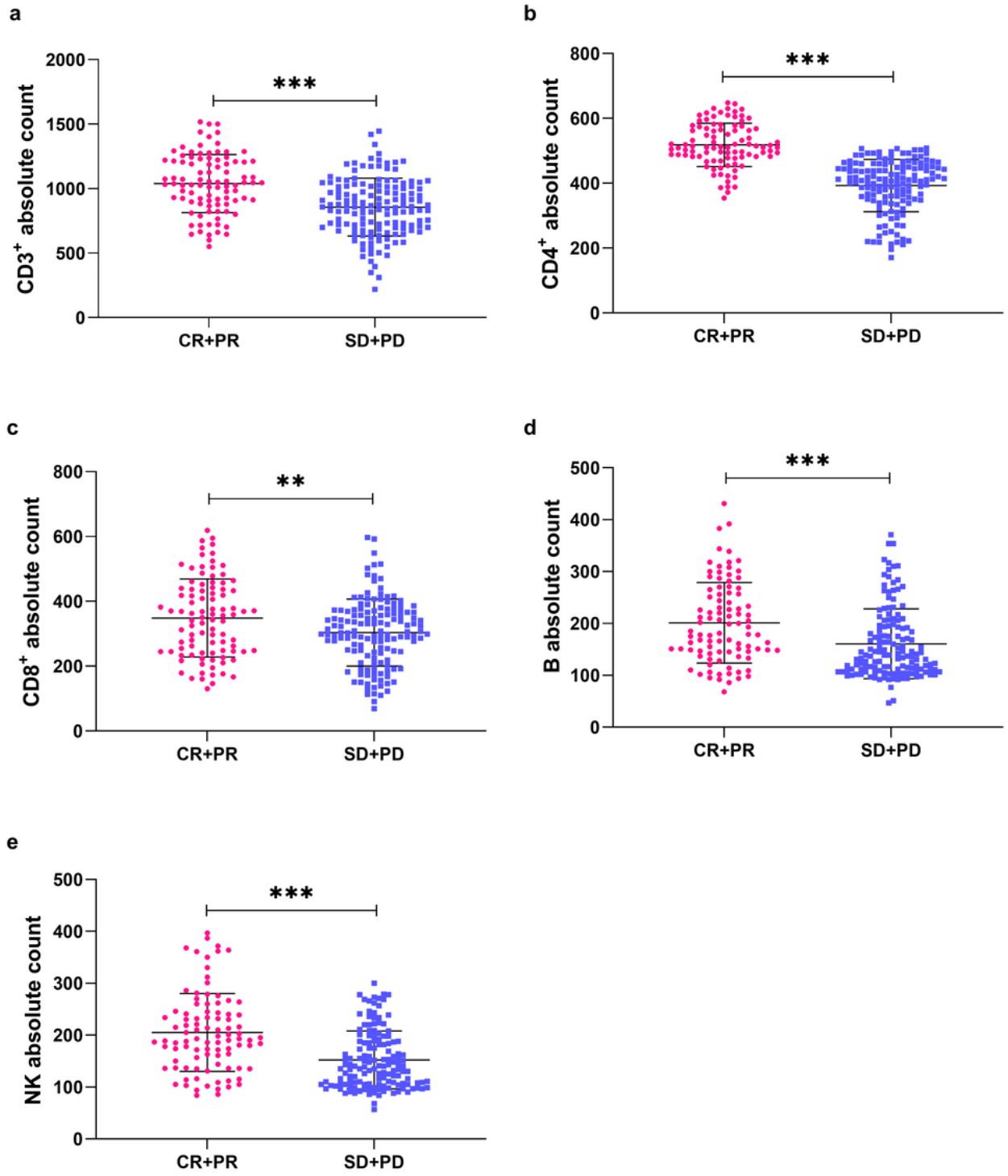

Figure 3

The comparison of AC between CR + PR and SD + PD. AC of CD3+ (a), CD3+CD4+ (b), CD3+CD8+ (c), B (d), and NK cells (e) in CR + PR group and SD + PD group (** represents P < 0.01, *** represents P < 0.001)

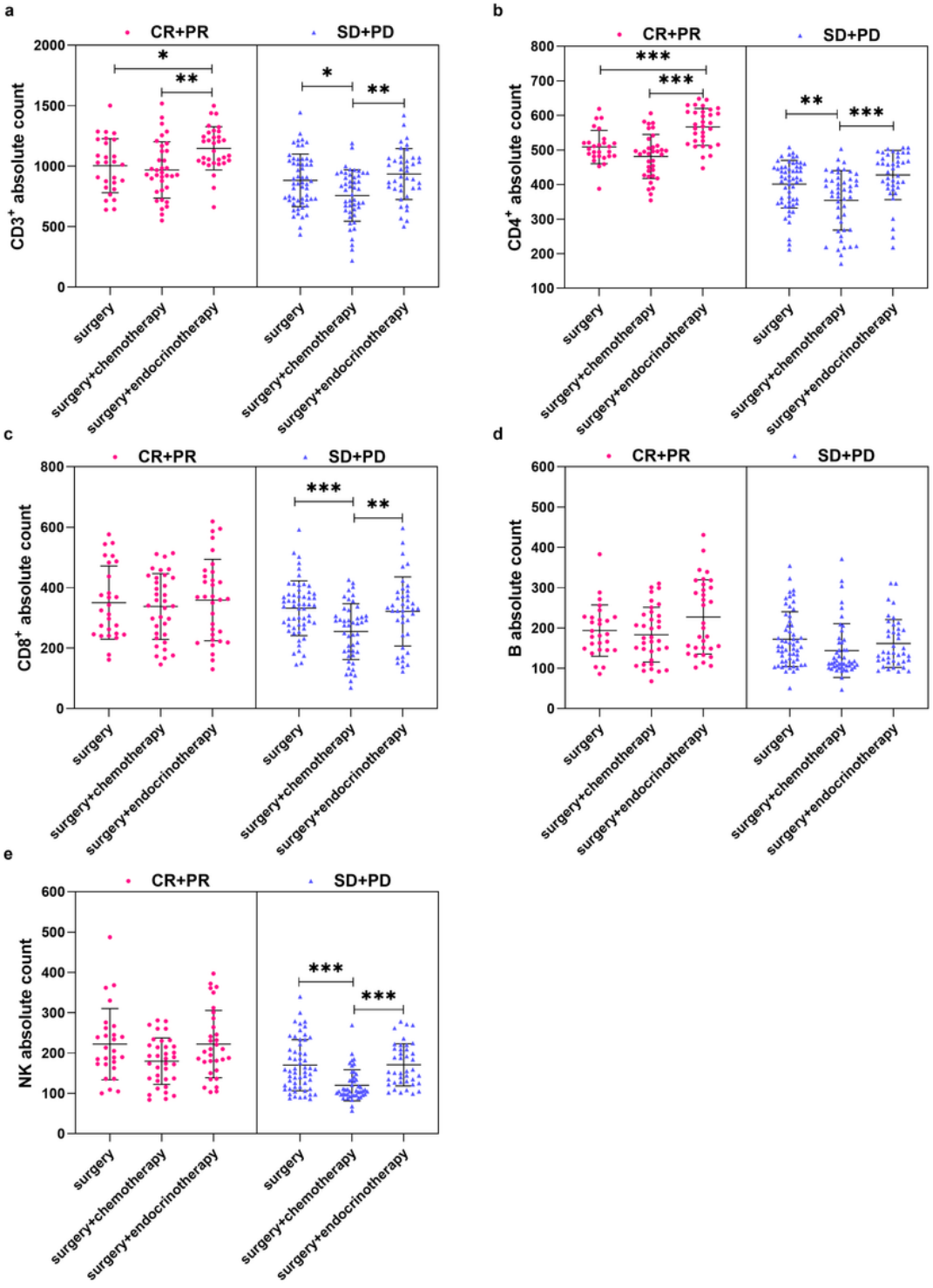

Figure 4

Effect of different treatments on AC. AC of CD3+ (a), CD3+CD4+ (b), CD3+CD8+ (c), B cells (d), NK (e) cells in CR+PR and SD+PD group. (*represents P □ 0.05, ** represents P < 0.01,*** represents P < 0.001)

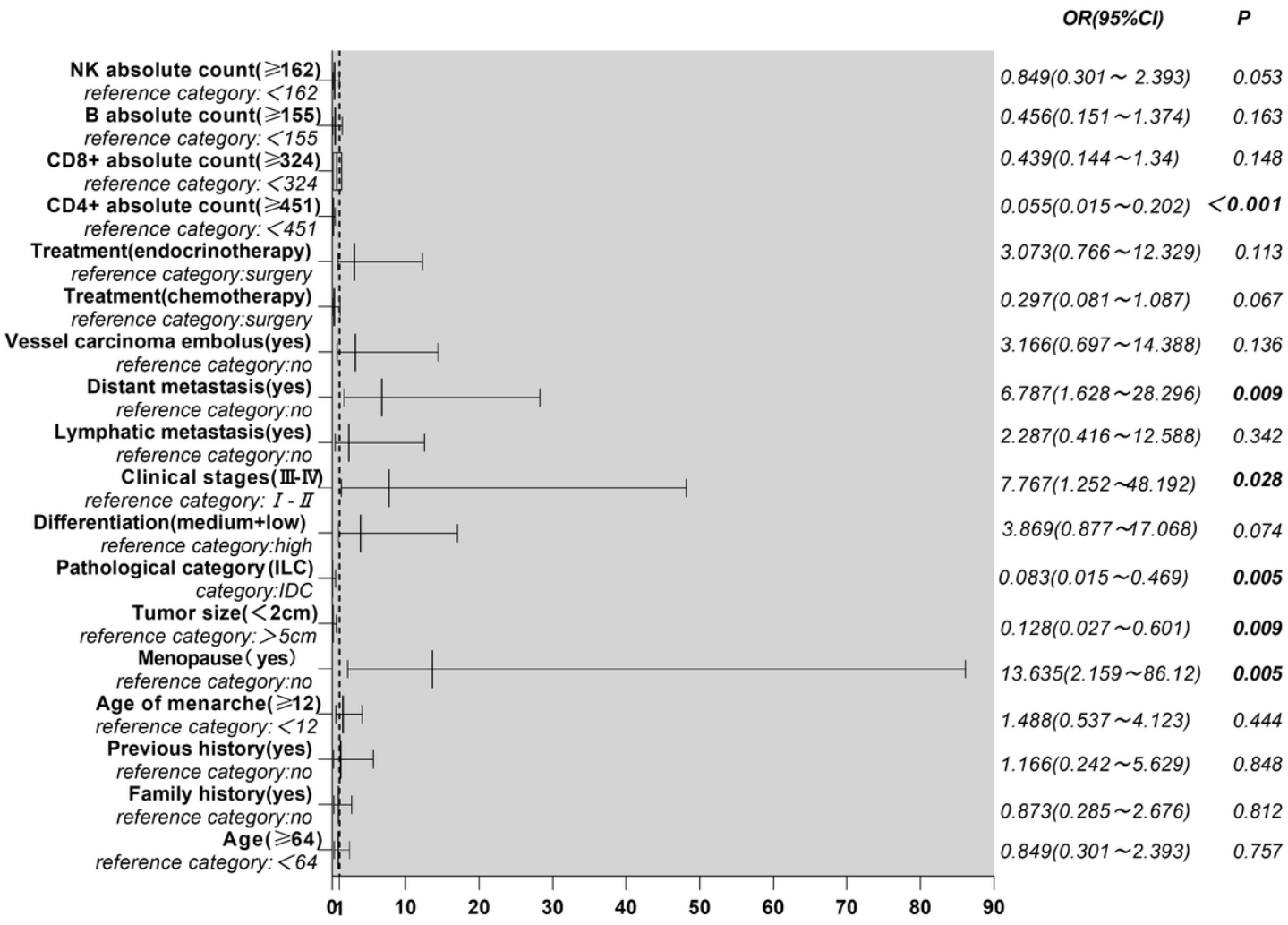

Figure 5

The forest plots of factors affected the progression of the disease. OR > 1 indicates variable is considered a risk factor, OR< 1 represents variables is a protective factor. Bold value represents that P value was significant

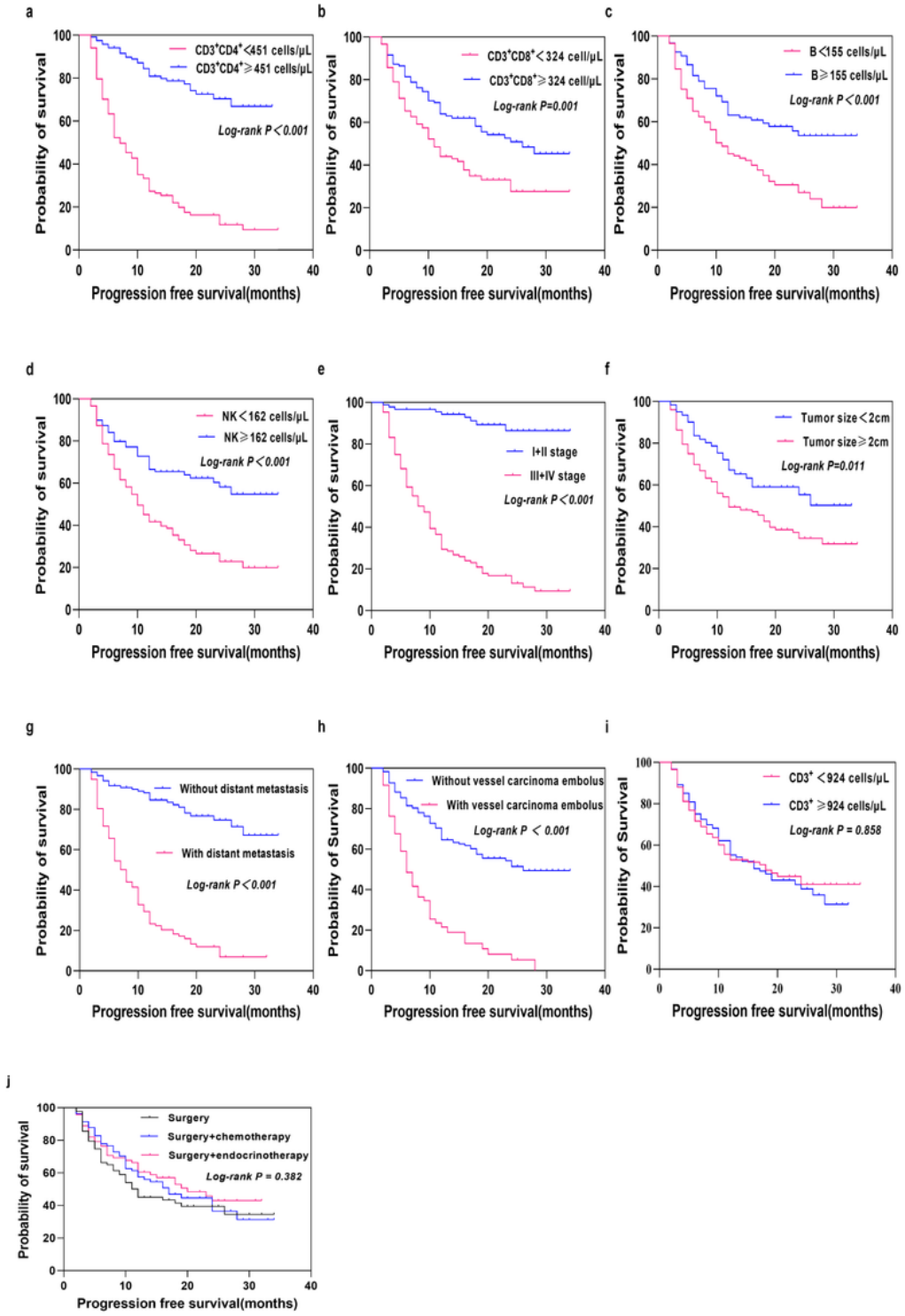

Figure 6

Progression-free survival curves of BC patients. a, b, c, d, e, f, g, h, i, j represents the AC of CD3+CD4+, CD3+CD8+, B and NK cells, different clinical stages, tumor size, with or without distant metastasis, with or without vessel carcinoma embolus, CD3+, and different treatments on PFS, respectively