ORIGINAL RESEARCH

Beneficial Effect of *Toxoplasma gondii* Infection on the Prognosis of Breast Cancer Was Modified by Cytokines

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Background: Animal experiments have shown the anticancer activity of *Toxoplasma gondii* (*T. gondii*), but its effect on the prognosis of cancer patients is unclear. Thus, the present study aimed to investigate the prognostic role of anti-*T. gondii* IgG in breast cancer patients and the modification effect of cytokines.

Methods: A total of 1121 breast cancer patients were recruited between 2008 and 2018 and followed up until December 31, 2021. Anti-*T. gondii* IgG and cytokines were measured using an enzyme-linked immunosorbent assay (ELISA) kit and a multiplex assay platform. Endpoints were overall survival (OS) and progression-free survival (PFS). Survival and multiplicative interaction analyses were performed using multivariate Cox regression models.

Results: According to the cutoff value of optical density (OD=0.111), 900 (80.29%) and 221 (19.71%) patients were divided into two groups: low or high anti-*T. gondii* IgG. Compared to patients with a low anti-*T. gondii* IgG level, the adjusted hazard ratios (HRs) of OS and PFS for patients with high anti-*T. gondii* IgG levels were 0.60 (95% confidence interval (CI): 0.37–0.99) and 0.67 (0.46–0.98), respectively. These associations were profound among patients with a high cytokine score (HR=0.29, 95% CI: 0.10–0.82 for OS; HR=0.30, 95% CI: 0.13–0.69 for PFS), accompanied by a significant interaction between the level of anti-*T. gondii* IgG and the cytokine score ($P_{interaction}$=0.019 for PFS); interleukin-17 (IL-17) and interleukin-9 (IL-9) were the main contributors to the interaction.

Conclusion: Anti-*T. gondii* IgG was found to be beneficial to breast cancer survival, especially in women with systematic inflammation and high IL-17 or IL-9 levels, suggesting the potential of *T. gondii* as a prognostic marker and a novel immunotherapy approach for cancer patients.

Keywords: *Toxoplasma gondii*, breast cancer, prognosis, cytokines

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan that infects approximately one-third of the global population.[1] *T. gondii* can be acquired through food or water contaminated with the parasite or by vertical transmission from mother to child.[2] Typically, *T. gondii* infection is asymptomatic or self-limiting but can cause severe toxoplasmosis in immunocompromised hosts and severe damage to the fetus during pregnancy.[3,4] Interestingly, *T. gondii* possesses anticancer activity, as shown in animal models, by secreting cancer-associated mucin-type O-glycans, rhoptry proteins (ROPs), and dense granule antigens (GRAs) that activate the host antigen-presenting system to inhibit tumor growth.[5,6] After efficiently promoting activation of cellular immunity,[6] it also inhibits progression of various cancers in animal models, such as melanoma,[7] ovarian cancer,[8] pancreatic cancer,[9] and breast cancer.[10,11] A recent study revealed that *T. gondii* DNA is present in malignant breast tissues of humans.[12] Several studies have shown a higher prevalence of *T. gondii* infection in cancer patients than in healthy controls, including breast cancer patients,[13–15] but whether infection by *T. gondii* might affect the prognosis of cancer patients has yet to be explored.[16]

In addition, *T. gondii* infection has been shown to modify cytokine levels among breast cancer patients,[17] which are associated with prognosis,[18] suggesting that *T. gondii* infection might affect survival in breast cancer by regulating cytokine-related signaling pathways. Therefore, we investigated the impact of *T. gondii* infection on the survival of breast cancer patients using Guangzhou Breast Cancer Study (GZBCS) data and further explored whether the association is modified by cytokines.

Materials and Methods

Study Population and Serum Sample Collection

Patients with breast cancer identified by pathology were recruited from the First and Second Affiliated Hospitals of Sun Yat-sen University in Guangzhou, China, from October 2008 to January 2018.[19] Serum samples from 1775 patients were collected at diagnosis and stored at −80 °C. Next, we excluded patients for whom any of the following information was missing: clinical stage (N=243), estrogen receptor (ER) status (N=144), progesterone receptor (PR) status (N=147), human epidermal growth receptor (HER2) status (N=207), and follow-up (N=108). After excluding patients due to poor serum quality (N=204), we obtained an analytic sample size of 1121 cases. Written informed consent was obtained from all participants. This study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the School of Public Health at Sun Yat-sen University (Institutional Review Board approval number: 2012-8).

Data Collection

Baseline information was obtained through a structured questionnaire by trained investigators. The following information was collected: demographic characteristics, reproductive history, menstrual history, lifestyles, and family history of cancer. Clinical and pathological characteristics, such as tumor size, nodal status, and clinical stage, were collected from medical records. The status of ER, PR, and HER2 was determined based on immunohistochemistry and the definitions of these biomarkers, as described previously.[20]

Follow-Up

Follow-up was conducted at least every 3 months during the first year after diagnosis and every 6 months in the second and third years. Thereafter, the patients were followed up annually. The median follow-up time was 63.91 months. Follow-up information, including recurrence, metastasis, and death, was obtained through phone calls and outpatient visits. The primary and secondary endpoints of this study were OS and PFS, respectively, as described previously.[19] Survival statuses were censored at the latest follow-up date or December 31, 2021.

Serological Assay

Anti-*T. gondii* IgG in serum samples was detected using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Haitai Biological Pharmaceuticals Co., Ltd, Zhuhai, China), which is a routine method for *T. gondii* infection screening and diagnosis in China.[21] The assays were performed according to the manufacturer's instructions. The kit's positive, negative, and critical controls were placed on each test plate; optical density (OD) values were measured at 450 nm using an automatic microplate reader (800 TS, BIO-TEK, Winooski, Vermont, USA). The results were considered positive if the OD was equal to or greater than the threshold value (determined by the mean of three critical controls in each test). Total IgG was measured using an immunoglobulin kit (CUSABIO, Wuhan, China) according to the manufacturer's instructions. All samples were tested in duplicate.

Cytokine Measurement

A total of 794 serum samples were randomly selected to detect cytokines. A total of 27 cytokines were measured using the Bio-Plex Pro Human Cytokine 27-plex assay (Bio-Rad, M500KCAF0Y)[22] and Luminex 200 platform (Luminex Corporation, Austin, TX, USA). The assays included a series of known concentrations to generate standard curves

constructed by Bio-Plex Manager 6.0 (Bio-Rad Laboratories). For samples with values below the assay's lower limit of detection, values equal to half of the lower limit of detection were used.[23]

Statistical Analysis

To determine potential confounders, we estimated associations of clinicopathological and demographic factors with breast cancer survival. Next, X-tile software version 3.6.1 (Yale University, New Haven, CT, USA) was applied to obtain the optimal OD cutoff value for dividing the patients into low, middle, or high anti-*T. gondii* IgG level groups.[24] Then, Cox proportional hazards regression models were used to evaluate the association between anti-*T. gondii* IgG levels and prognosis.

Cytokines were analyzed separately and used as a composite indicator based on a previously developed cytokine score.[25] Briefly, after log-transforming and standardizing the values of all cytokines, a Cox proportional hazards regression model was constructed to test the association between OS (dependent variable) and all cytokines (independent variables) while considering the regression coefficients as weights. The processed cytokine levels mentioned above were weighted by these regression coefficients and summed for a total cytokine score (formula: $\sum_{CK=1}^{27} Bi \frac{\log(CK_i)}{SD((CK_i)}$, where B_i is the regression coefficient and CK is the cytokine). The cytokine score was input into a Cox regression model using restricted cubic splines to estimate the hazard ratios (HRs) and 95% confidence intervals (CIs). The cytokine score was divided into two groups (low and high) according to the restricted cubic spline results.

Furthermore, stratified analyses were performed to assess whether associations between anti-*T. gondii* IgG and breast cancer prognosis are modified by the cytokine score, individual cytokines, or other clinical characteristics. Multiplicative interaction for survival was estimated by the product terms in the model.

Additionally, mediation analyses were performed to assess whether the association between anti-*T. gondii* IgG and breast cancer survival is mediated by individual cytokines and cytokine scores. Sensitivity analysis was conducted excluding patients without cytokine data (N=327). All statistical tests were two-tailed, with *P*<0.05 indicating significance. All statistical analyses were conducted using R software version 4.1.3.

Results

Demographic and Clinicopathological Characteristics and Their Associations with Breast Cancer Prognosis

At the time of diagnosis, the mean age of the patients was 49.35±10.88 years, and 59.3% of them were premenopausal (Table 1). Most of the patients were ER positive (76.8%), and >75% were diagnosed with early-stage cancer (stage I/II: 78.5%) (Table 1). Age at diagnosis, menopausal status, ER status, PR status, Ki-67, tumor size, nodal status, and clinical stage were significantly associated with breast cancer survival (Table 1).

Association of Anti-*T. gondii* IgG with Breast Cancer Survival

According to the ELISA kit's criteria, 69 (6.16%) of the patients were seropositive for anti-*T. gondii* IgG. After adjustment for total IgG and potential prognostic factors of breast cancer, patients seropositive for anti-*T. gondii* IgG exhibited a significantly decreased risk of progression compared to seronegative patients (HR=0.41, 95% CI: 0.18–0.92; Table 2). Based on the direction of these HRs and the small sample size of the seropositive patients, we used X-tile software to obtain an optimal cutoff dividing anti-*T. gondii* IgG OD into three levels. As shown in Table 2, compared to patients with anti-*T. gondii* IgG OD<0.020, those (anti-*T. gondii* IgG OD>0.111) had a significantly decreased risk of death (HR=0.48, 95% CI: 0.24–0.93) and a nonsignificantly decreased risk of progression (HR=0.65, 95% CI: 0.38–1.11); a trend of dose–response association between anti-*T. gondii* IgG and OS was also observed (*P*=0.024). When the two lower levels of anti-*T. gondii* IgG were combined as the reference level, a higher level of anti-*T. gondii* IgG was associated with a significantly decreased risk of death or progression (HR=0.60, 95% CI: 0.37–0.99 for OS; HR= 0.67, 95% CI: 0.46–0.98 for PFS; Table 2). In contrast, no significant associations between anti-*T. gondii* IgG and clinicopathological characteristics were observed in addition to age at diagnosis (Table 3). Patients with a high anti-*T. gondii* IgG level were more likely to be younger than those with a low anti-*T. gondii* IgG level (Table 3). Stratified analyses

Table 1 Demographic and Clinicopathologic Characteristics and the Associations with Breast Cancer Prognosis

Characteristics	Total (%)	Overall Survival		Progression-Free Survival	
		Events	HR (95% CI)	Events	HR (95% CI)
Age at diagnosis (yr)					
≤40	244 (21.8)	30	1.00 (reference)	53	1.00 (reference)
41–60	714 (63.7)	86	1.00 (0.66, 1.52)	138	0.90 (0.65, 1.23)
>60	163 (14.5)	34	**1.71 (1.05, 2.80)**	50	1.41 (0.96, 2.07)
Education status					
Below Junior school	451 (43.6)	66	1.00 (reference)	106	1.00 (reference)
Senior high school	317 (30.6)	36	0.73 (0.48, 1.09)	62	0.79 (0.58, 1.09)
College or above	267 (25.8)	29	0.73 (0.47, 1.12)	47	0.73 (0.52, 1.04)
Marital status[a]					
Unmarried	91 (8.3)	18	1.00 (reference)	27	1.00 (reference)
Married	1007 (91.7)	122	0.67 (0.41, 1.10)	201	0.72 (0.48, 1.07)
Age at menarche (yr)					
≤12	136 (12.5)	20	1.00 (reference)	31	1.00 (reference)
>12	952 (87.5)	119	0.84 (0.52, 1.34)	198	0.89 (0.61, 1.30)
Menopausal status					
Premenopausal	643 (59.3)	74	1.00 (reference)	124	1.00 (reference)
Postmenopausal	442 (40.7)	69	**1.43 (1.03, 1.99)**	108	**1.33 (1.03, 1.72)**
Parity					
0	62 (5.7)	11	1.00 (reference)	17	1.00 (reference)
≥1	1029 (94.3)	128	0.73 (0.39, 1.35)	211	0.76 (0.47, 1.25)
Breast feeding					
Absent	79 (8.0)	7	1.00 (reference)	11	1.00 (reference)
Present	908 (92.0)	117	1.53 (0.71, 3.28)	193	1.60 (0.87, 2.93)
Family history of breast cancer					
Absent	1024 (94.2)	139	1.00 (reference)	219	1.00 (reference)
Present	63 (5.8)	4	0.47 (0.17, 1.27)	11	0.79 (0.43, 1.45)
BMI (kg/m^2)					
<18.5	68 (6.3)	12	1.32 (0.72, 2.42)	17	1.16 (0.70, 1.92)
18.5–23.9	663 (61.1)	84	1.00 (reference)	136	1.00 (reference)
≥24.0	354 (32.6)	46	1.06 (0.74, 1.51)	**77**	1.08 (0.82, 1.43)
ER status					
Negative	260 (23.2)	45	1.00 (reference)	70	1.00 (reference)
Positive	861 (76.8)	105	**0.64 (0.45, 0.91)**	171	**0.68 (0.51, 0.89)**
PR status					
Negative	365 (32.6)	67	1.00 (reference)	102	1.00 (reference)
Positive	756 (67.4)	83	**0.54 (0.39, 0.74)**	139	**0.58 (0.45, 0.75)**
HER2					
Negative	650 (58.0)	85	1.00 (reference)	138	1.00 (reference)
Positive/equivocal	471 (42.0)	65	1.32 (0.96, 1.83)	103	1.26 (0.97, 1.62)
Triple-negative breast cancer					
No	990 (90.7)	128	1.00 (reference)	205	1.00 (reference)
Yes	102 (9.3)	16	1.16 (0.69, 1.96)	24	1.08 (0.71, 1.66)
Ki67 index					
<14%+	246 (23.9)	25	1.00 (reference)	40	1.00 (reference)
≥14%+	783 (76.1)	107	1.51 (0.97, 2.33)	175	**1.54 (1.10, 2.18)**
Tumor size (cm)					
≤2.0	472 (42.3)	37	1.00 (reference)	64	1.00 (reference)
>2.0	644 (57.7)	111	**2.51 (1.73, 3.65)**	174	**2.35 (1.76, 3.13)**

(*Continued*)

Table 1 (Continued).

Characteristics	Total (%)	Overall Survival		Progression-Free Survival	
		Events	**HR (95% CI)**	**Events**	**HR (95% CI)**
Nodal status					
Negative	623 (55.9)	49	1.00 (reference)	93	1.00 (reference)
Positive	491 (44.1)	97	**2.87 (2.03, 4.05)**	142	**2.26 (174, 2.93)**
Clinical stage					
I	319 (28.5)	17	1.00 (reference)	35	1.00 (reference)
II	567 (50.6)	49	**1.80 (1.03, 3.12)**	93	**1.66 (1.13, 2.45)**
III	180 (16.1)	50	**6.84 (3.94, 11.87)**	73	**5.09 (3.40, 7.62)**
IV	55 (4.9)	34	**24.27 (13.44, 43.84)**	40	**16.15 (10.15, 25.67)**

Notes: Bold character indicates statistically significant result. [a]Unmarried status included never married, separated, divorced, and widowed; married status included living as married.
Abbreviations: BMI, body mass index; CI, confidence interval; HR, hazard ratio.

were performed to explore whether demographic or clinicopathological characteristics modify the effect of anti-*T. gondii* IgG on prognosis. As shown in Table S1, no significant differential associations were detected among different ages, ER, PR, and HER2 statuses, or clinical stages.

Associations of Cytokines with Prognosis of Breast Cancer

As shown in Figure 1, none of the cytokines were associated with OS. However, in multivariate Cox regression analyses, TNF-α was associated with poor breast cancer PFS (HR=1.60, 95% CI: 1.13–2.26; Figure 2). As the composite measure of multiple cytokines, the continuous cytokine score was associated with prognosis in breast cancer. With regard to OS, risk of death increased slightly until the cytokine score of approximately −12.89 sharply increased to the highest level, which was accompanied by a significant association between the cytokine score and OS ($P<0.001$; P nonlinear=0.648; Figure 3a). A similar significant pattern was observed for PFS ($P<0.001$; P nonlinear=0.385; Figure 3b). Based on the results of restricted cubic splines of the cytokine score, we categorized the cytokine score into two levels using a cutoff point of −12.89. After adjusting for potential prognostic factors, patients whose cytokine score was >−12.89 exhibited a significantly increased risk of death compared to those with a score ≤−12.89 (HR=1.56, 95% CI: 1.01–2.43; Table 2).

Interaction Between Anti-*T. gondii* IgG and Cytokines and Prognosis of Breast Cancer

The protective effect of a high anti-*T. gondii* IgG level on breast cancer PFS was more evident in patients with a cytokine score >−12.89 (HR=0.30, 95% CI: 0.13–0.69) than in those with a cytokine score ≤−12.89 (HR=0.96, 95% CI: 0.51–1.80), a significant interaction ($P=0.019$, Table 4). Moreover, the negative association between high anti-*T. gondii* IgG and risk of death (HR=0.29, 95% CI=0.10–0.82) was observed among patients with a cytokine score >−12.89 but not among those with a cytokine score ≤−12.89 (HR=0.85, 95% CI 0.35–2.04; Table 4), though the interaction was not significant.

To determine the cytokines contributing maximally to the interaction, we performed stratified analyses according to the median value of cytokines. As shown in Table 5, a high anti-*T. gondii* IgG level significantly improved PFS among breast cancer patients with high IL-17 (HR=0.40, 95% CI=0.19–0.84) but not among those with low IL-17 (HR=1.09, 95% CI=0.64–1.86); this interaction was significant ($P=0.032$). A high anti-*T. gondii* IgG level also significantly reduced risk of death among patients with high IL-17 (HR=0.25, 95% CI: 0.08–0.72) but not among those with low IL-17 (HR=1.15, 95% CI=0.59–2.26); this interaction was also significant ($P=0.027$; Table 5). Similarly, the protective effect of high anti-*T. gondii* IgG on PFS was observed among patients with high IL-9 (HR=0.37, 95% CI: 0.17–0.81) but not among those with low IL-9 (HR=1.08, 95% CI: 0.63–1.84), and this interaction was significant ($P=0.032$; Table 5). Other cytokines did not significantly interact with anti-*T. gondii* IgG levels with respect to breast cancer prognosis (data not shown). In addition, no significant mediation by cytokines was detected in the association between anti-*T. gondii* IgG level and survival (Tables S2 and S3).

Table 2 Univariate and Multivariate Cox Regression for Anti-*T. gondii* IgG and Cytokine Score with Breast Cancer OS and PFS

Variables	Total	Overall Survival				Progression-Free Survival			
		Events	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]	Events	HR (95% CI)[a]	HR (95% CI)[b]	HR (95% CI)[c]
Anti-*T. gondii* IgG (ELISA)									
Negative	1052	145	1.00 (reference)	1.00 (reference)	1.00 (reference)	231	1.00 (reference)	1.00 (reference)	1.00 (reference)
Positive	69	5	0.51 (0.21, 1.24)	0.44 (0.16, 1.21)	0.46 (0.17, 1.26)	10	0.62 (0.33, 1.17)	**0.39 (0.17, 0.89)**	**0.41 (0.18, 0.92)**
Anti-*T. gondii* IgG (X-tile)									
< 0.020	120	21	1.00 (reference)	1.00 (reference)	1.00 (reference)	27	1.00 (reference)	1.00 (reference)	1.00 (reference)
0.020 ~ 0.111	780	105	0.63 (0.39, 1.00)	0.76 (0.45, 1.28)	0.76 (0.45, 1.29)	173	0.83 (0.56, 1.25)	0.97 (0.62, 1.51)	0.96 (0.62, 1.50)
> 0.111	221	24	**0.51 (0.28, 0.91)**	**0.48 (0.25, 0.94)**	**0.48 (0.24, 0.93)**	41	0.68 (0.42, 1.11)	0.64 (0.38, 1.10)	0.65 (0.38, 1.11)
P for trend			0.032	0.025	0.024		0.113	0.056	0.065
Anti-*T. gondii* IgG (X-tile)									
Low	900	126	1.00 (reference)	1.00 (reference)	1.00 (reference)	200	1.00 (reference)	1.00 (reference)	1.00 (reference)
High	221	24	0.76 (0.49, 1.17)	**0.61 (0.38, 1.00)[d]**	**0.60 (0.37, 0.99)**	41	0.80 (0.57, 1.12)	**0.66 (0.46, 0.96)**	**0.67 (0.46, 0.98)**
Cytokine score[e]									
≤ −12.89	397	36	1.00 (reference)	1.00 (reference)	1.00 (reference)	65	1.00 (reference)	1.00 (reference)	1.00 (reference)
> −12.89	397	67	**1.88 (1.25, 2.82)**	**1.56 (1.01, 2.43)**	**1.56 (1.01, 2.43)**	98	**1.56 (1.14, 2.14)**	1.40 (1.00, 1.96)	1.40 (1.00, 1.96)

Notes: Bold character indicates statistically significant result. [a]The univariate COX model. [b]Adjusted for age at diagnosis (continuous variable), ER status, PR status, HER2 status, Ki67 index, tumor size, nodal status, and clinical stage (early stage VS advanced stage). [c]Adjusted for age at diagnosis (continuous variable), ER status, PR status, HER2 status, Ki67 index, tumor size, nodal status, clinical stage (early stage VS advanced stage), and total IgG. [d]*P* = 0.049. [e]A total of 794 serum samples were randomly selected to detect cytokines.

Table 3 Relationships Between Anti-*T. gondii* IgG and Clinicopathologic Characteristics of Breast Cancer

Characteristics	Anti-*T. gondii* IgG		P*
	Low (%)	High (%)	
Age at diagnosis			**0.006**
≤40	210 (23.3)	34 (15.4)	
41–59	553 (61.4)	161 (72.9)	
≥60	137 (15.2)	26 (11.8)	
Menopausal status			0.093
Premenopausal	527 (60.5)	116 (54.2)	
Postmenopausal	344 (39.5)	98 (45.8)	
Family history of breast cancer			0.240
No	826 (94.6)	198 (92.5)	
Yes	47 (5.4)	16 (7.5)	
BMI (kg/m^2)			0.801
<18.5	57 (6.5)	11 (5.3)	
18.5–23.9	534 (61.0)	129 (61.7)	
≥24.0	285 (32.5)	69 (33.0)	
ER			0.963
Negative	209 (23.2)	51 (23.1)	
Positive	691 (76.8)	170 (76.9)	
PR			0.995
Negative	293 (32.6)	72 (32.6)	
Positive	607 (67.4)	149 (67.4)	
HER2			0.982
Negative	522 (58.0)	128 (57.9)	
Positive/equivocal	378 (42.0)	93 (42.1)	
Triple-negative breast cancer			0.791
No	797 (90.8)	193 (90.2)	
Yes	81 (9.2)	21 (9.8)	
Ki-67			0.603
< 14%	201 (24.2)	45 (22.5)	
≥14%	628 (75.8)	155 (77.5)	
Tumor size (cm)			0.885
≤2.0	378 (42.2)	94 (42.7)	
>2.0	518 (57.8)	126 (57.3)	
Lymph nodes status			0.360
Negative	506 (56.6)	117 (53.2)	
Positive	388 (43.4)	103 (46.8)	
Clinical stage			0.219
I/II	718 (79.8)	168 (76.0)	
III	182 (20.2)	53 (24.0)	

Note: *Chi-square test; Bold character indicate statistically significant result.

Discussion

The present hospital-based cohort study reports that a high anti-*T. gondii* IgG level is associated with improved breast cancer OS and PFS, independent of multiple well-known prognostic factors. The protective effect of anti-*T. gondii* IgG was evident among patients with a high cytokine score, accompanied by a significant interaction (between anti-*T. gondii* IgG and the cytokine score) for breast cancer PFS. Furthermore, IL-17 and IL-9 were identified as the main contributors to this interaction.
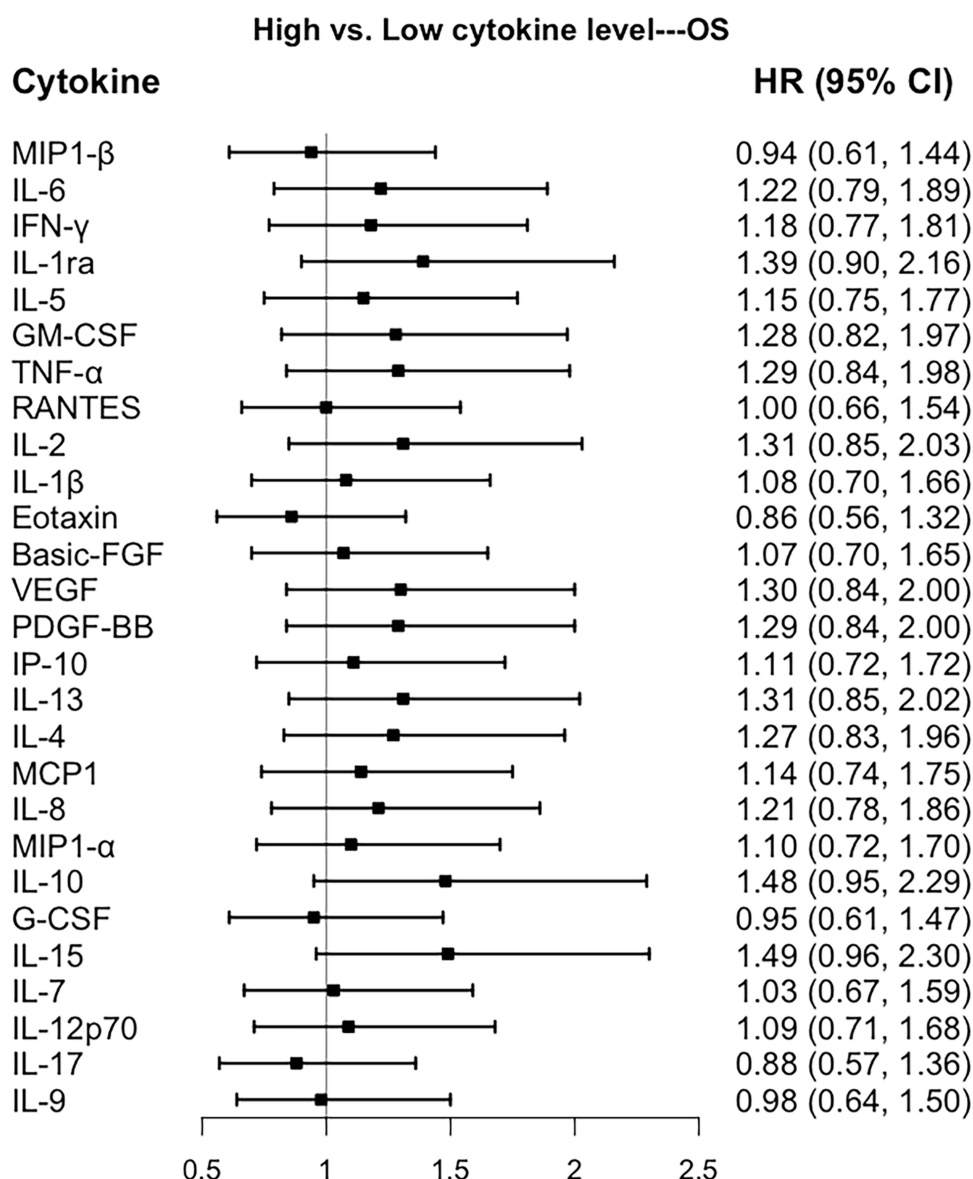
High vs. Low cytokine level---OS

Cytokine		HR (95% CI)
MIP1-β		0.94 (0.61, 1.44)
IL-6		1.22 (0.79, 1.89)
IFN-γ		1.18 (0.77, 1.81)
IL-1ra		1.39 (0.90, 2.16)
IL-5		1.15 (0.75, 1.77)
GM-CSF		1.28 (0.82, 1.97)
TNF-α		1.29 (0.84, 1.98)
RANTES		1.00 (0.66, 1.54)
IL-2		1.31 (0.85, 2.03)
IL-1β		1.08 (0.70, 1.66)
Eotaxin		0.86 (0.56, 1.32)
Basic-FGF		1.07 (0.70, 1.65)
VEGF		1.30 (0.84, 2.00)
PDGF-BB		1.29 (0.84, 2.00)
IP-10		1.11 (0.72, 1.72)
IL-13		1.31 (0.85, 2.02)
IL-4		1.27 (0.83, 1.96)
MCP1		1.14 (0.74, 1.75)
IL-8		1.21 (0.78, 1.86)
MIP1-α		1.10 (0.72, 1.70)
IL-10		1.48 (0.95, 2.29)
G-CSF		0.95 (0.61, 1.47)
IL-15		1.49 (0.96, 2.30)
IL-7		1.03 (0.67, 1.59)
IL-12p70		1.09 (0.71, 1.68)
IL-17		0.88 (0.57, 1.36)
IL-9		0.98 (0.64, 1.50)

0.5   1   1.5   2   2.5

Figure 1 Forest plot of the associations between individual cytokines with overall survival in a multivariable model adjusted with age at diagnosis (continuous variable), ER status, PR status, HER2 status, Ki67 index, tumor size, nodal status, and clinical stage (early stage VS advanced stage). All cytokines were modeled as binary variables according to the median value of the cytokines.

In agreement with the protective effects of *T. gondii* in tumor-bearing mouse models, we observed beneficial effects in breast cancer patients, indicating great potential for application of *T. gondii* in tumor therapy.[6] Recently, Xu et al confirmed the antitumor effect of *T. gondii* on breast cancer by injecting artificial attenuated strains into mice inoculated with 4T1 cells (murine triple-negative breast cancer cells).[11] The mechanisms underlying such effects include hypoxia, avascular necrosis, and anti-angiogenesis.[26] Moreover, the parasite can remain viable in the form of tissue cysts throughout the life of the host, maintaining long-lasting effects.[27] Therefore, *T. gondii* may persistently play a protective role in cancer patients and improve their prognosis, especially for those with recurrence-prone and refractory types, such as invasive papillary carcinoma.[28,29] Notably, a previous study showed the rate of *T. gondii* infection to be higher in cancer patients than in healthy controls, indicating that infection by *T. gondii* is a possible risk factor for cancer.[30] However, these were retrospective studies, and a reverse causality could not be ruled out.[13]

Inflammation is a hallmark of cancer that contributes to angiogenesis and activation of the epithelial-mesenchymal transition (EMT) by supplying several cytokines.[31] In the current study, we observed a harmful effect of higher levels of
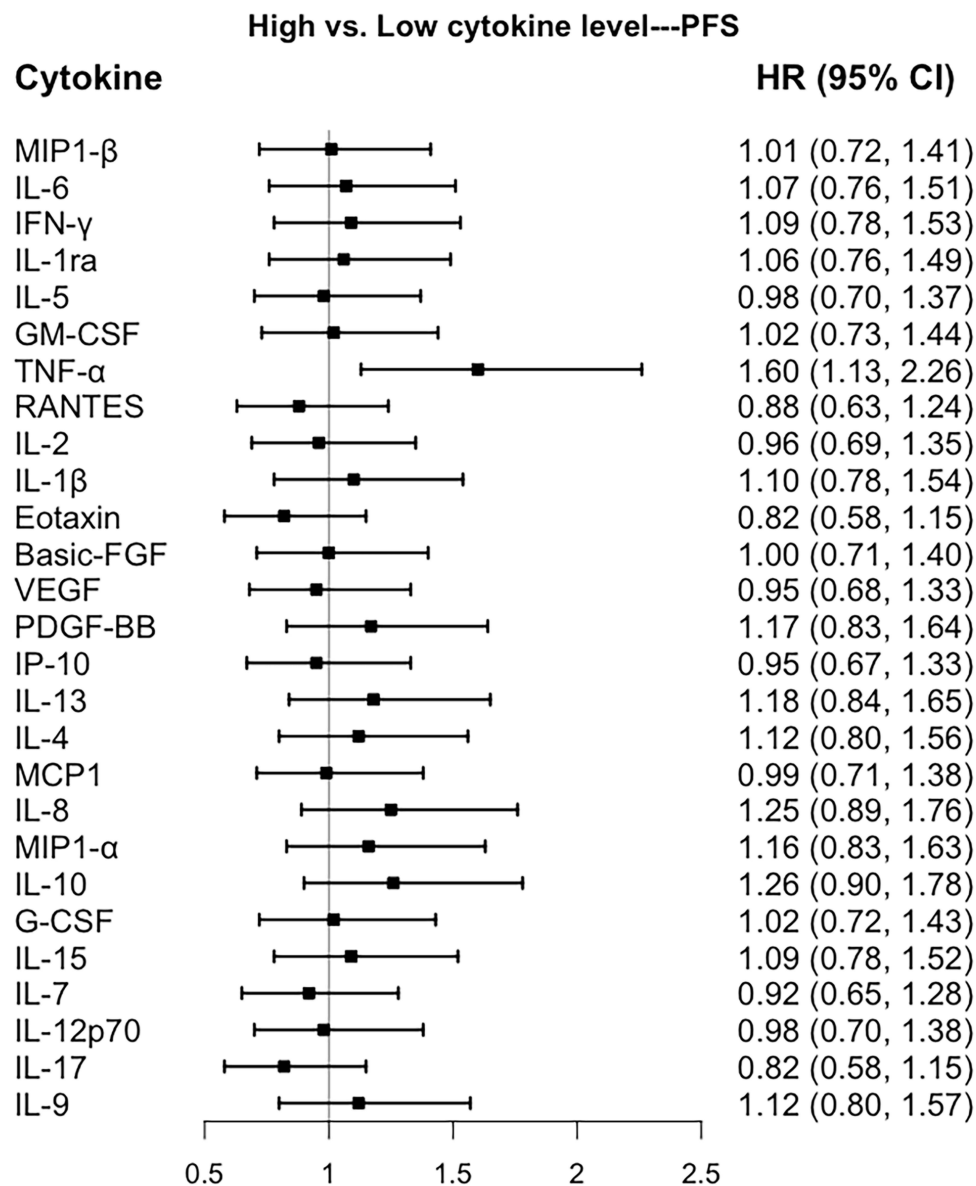
High vs. Low cytokine level---PFS

Cytokine	HR (95% CI)
MIP1-β	1.01 (0.72, 1.41)
IL-6	1.07 (0.76, 1.51)
IFN-γ	1.09 (0.78, 1.53)
IL-1ra	1.06 (0.76, 1.49)
IL-5	0.98 (0.70, 1.37)
GM-CSF	1.02 (0.73, 1.44)
TNF-α	1.60 (1.13, 2.26)
RANTES	0.88 (0.63, 1.24)
IL-2	0.96 (0.69, 1.35)
IL-1β	1.10 (0.78, 1.54)
Eotaxin	0.82 (0.58, 1.15)
Basic-FGF	1.00 (0.71, 1.40)
VEGF	0.95 (0.68, 1.33)
PDGF-BB	1.17 (0.83, 1.64)
IP-10	0.95 (0.67, 1.33)
IL-13	1.18 (0.84, 1.65)
IL-4	1.12 (0.80, 1.56)
MCP1	0.99 (0.71, 1.38)
IL-8	1.25 (0.89, 1.76)
MIP1-α	1.16 (0.83, 1.63)
IL-10	1.26 (0.90, 1.78)
G-CSF	1.02 (0.72, 1.43)
IL-15	1.09 (0.78, 1.52)
IL-7	0.92 (0.65, 1.28)
IL-12p70	0.98 (0.70, 1.38)
IL-17	0.82 (0.58, 1.15)
IL-9	1.12 (0.80, 1.57)

Figure 2 Forest plot of the associations between individual cytokines with progression-free survival in a multivariable model adjusted with age at diagnosis (continuous variable), ER status, PR status, HER2 status, Ki67 index, tumor size, nodal status, and clinical stage (early stage VS advanced stage). All cytokines were modeled as binary variables according to the median value of the cytokines.

TNF-α on PFS in breast cancer, consistent with previous results.[32] For example, Kim et al reported that a high level of TNF-α at baseline is associated with a significantly high risk of disease progression.[33] As a composite measure of individual cytokine values, the cytokine score showed a significant association with breast cancer prognosis in our study. The direction of this association was consistent with that of individual cytokines and breast cancer prognosis, with its magnitude being slightly greater than that of individual cytokines. These results are in accordance with previous studies, in which circulating systemic inflammatory markers were found to be poor prognosis indicators in breast cancer patients.[34,35]

Furthermore, we observed marked protective effects of anti-*T. gondii* IgG on breast cancer prognosis among women with a higher cytokine score than those with a lower score, a significant interaction (mainly contributed by IL-17 and IL-9). Patients with a high cytokine score might experience systemic inflammation, which induces angiogenesis,[36] a well-established driver of breast cancer aggressiveness and poor prognosis,[37] yet *T. gondii* infection inhibits angiogenesis.[11,38] Therefore, patients with a higher cytokine score might have better prognosis because tumor neovascularization in these
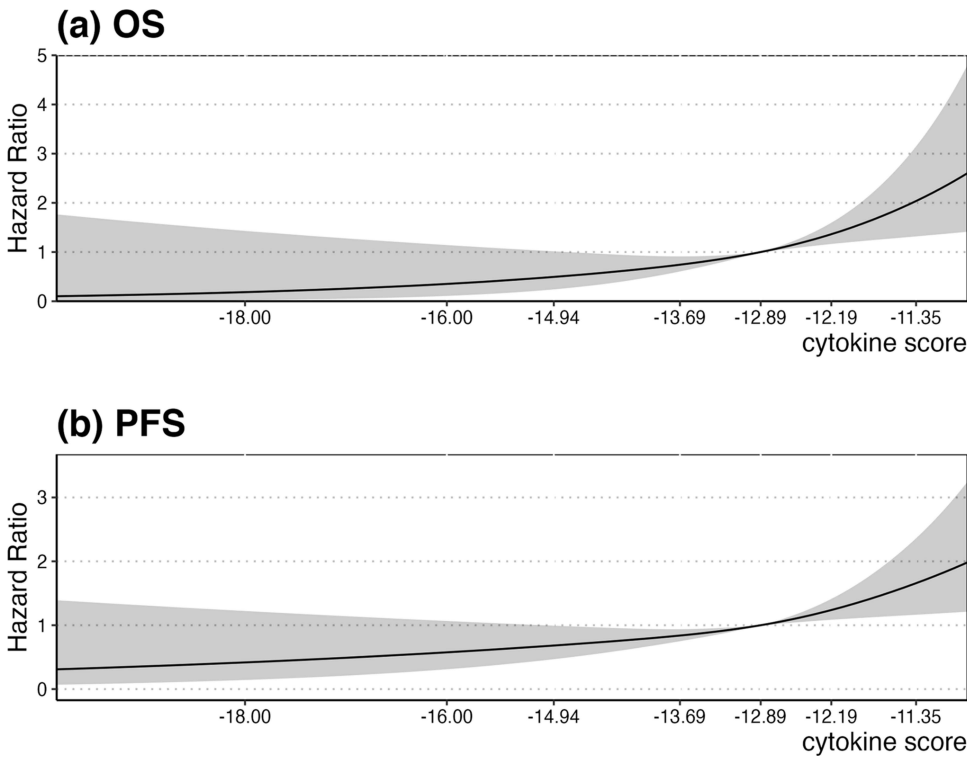
(a) OS

(b) PFS

Figure 3 Restricted cubic splines of cytokine score with breast cancer (**a**) OS and (**b**) PFS. Solid black lines were hazard ratios, with gray area showing 95% CIs derived from restricted cubic spline regressions with three knots.

patients is more effectively inhibited by *T. gondii* than in patients with a lower cytokine score; IL-17 and IL-9 promote angiogenesis and are major contributors to the interaction.[39,40] However, the precise mechanisms underlying the interaction have yet to be explored.

Nevertheless, the present study has some limitations. First, only patients with serum samples were included, making it difficult to avoid selection bias. However, the distribution of the subjects' clinicopathological characteristics in the present study was similar to that from the same cohort as reported previously.[19] Moreover, the seroprevalence of anti-*T. gondii* IgG (69/1121, 6.16%) in this study was in line with that found in a previous study (1/56, 1.79%).[41] Second, the modification effect of cytokines was assessed among only a subset of 794 individuals, indicating that the results might not be generalizable to all study subjects. Nonetheless, the protective effects of anti-*T. gondii* IgG in this subset of patients was significant among all patients (Table S4). In addition, the patients with cytokine data were randomly selected from the whole study population, suggesting that the modification effect may be maintained in all patients. Although we

Table 4 Association Between Anti-*T. gondii* IgG Level and the Prognosis of Breast Cancer Stratified by Cytokine Score

Cytokine Score	Anti-*T. gondii* IgG Level	Total	Overall Survival			Progression-Free Survival		
			Events	HR (95% CI)[a]	HR (95% CI)[b]	Events	HR (95% CI)[a]	HR (95% CI)[b]
≤ −12.89	Low	326	29	1.00 (reference)	1.00 (reference)	51	1.00 (reference)	1.00 (reference)
	High	71	7	1.16 (0.51, 2.65)	0.85 (0.35, 2.04)	14	1.22 (0.68, 2.21)	0.96 (0.51, 1.80)
> −12.89	Low	320	59	1.00 (reference)	1.00 (reference)	86	1.00 (reference)	1.00 (reference)
	High	77	8	0.50 (0.24, 1.05)	**0.29 (0.10, 0.82)**	12	**0.51 (0.28, 0.94)**	**0.30 (0.13, 0.69)**
P for interaction				0.151	0.095		**0.036**	**0.019**

Notes: Bold character indicates statistically significant result. [a]Univariate analysis. [b]Adjusted for age at diagnosis (continuous variable), ER status, PR status, HER2 status, Ki67 index, tumor size, nodal status, and clinical stage (early stage VS advanced stage).

Table 5 Association Between Anti-*T. gondii* IgG Level and the Prognosis of Breast Cancer Stratified by IL-17 and IL-9

Cytokines	Anti-*T.gondii* IgG Level	Total	Overall Survival			Progression-Free Survival		
			Events	HR (95% CI)[a]	HR (95% CI)[b]	Events	HR (95% CI)[a]	HR (95% CI)[b]
IL-17								
Low	Low	322	46	1.00 (reference)	1.00 (reference)	72	1.00 (reference)	1.00 (reference)
	High	76	11	0.97 (0.50, 1.88)	1.15 (0.59, 2.26)	18	1.00 (0.60, 1.68)	1.09 (0.64, 1.86)
High	Low	324	42	1.00 (reference)	1.00 (reference)	65	1.00 (reference)	1.00 (reference)
	High	72	4	0.40 (0.14, 1.12)	**0.25 (0.08, 0.72)**	8	0.51 (0.24, 1.06)	**0.40 (0.19, 0.84)**
P for interaction				0.170	**0.027**		0.146	**0.032**
IL-9								
Low	Low	320	45	1.00 (reference)	1.00 (reference)	63	1.00 (reference)	1.00 (reference)
	High	77	10	0.91 (0.46, 1.81)	0.73 (0.35, 1.49)	19	1.24 (0.74, 2.07)	1.08 (0.63, 1.84)
High	Low	326	43	1.00 (reference)	1.00 (reference)	74	1.00 (reference)	1.00 (reference)
	High	71	5	0.49 (0.19, 1.24)	0.47 (0.18, 1.19)	7	**0.38 (0.18, 0.83)**	**0.37 (0.17, 0.81)**
P for interaction				0.286	0.525		**0.012**	**0.032**

Notes: Bold character indicates statistically significant result. All cytokines were modeled as binary variables according to the median value of the cytokines. [a]Univariate COX regression. [b]Adjusted for age at diagnosis (continuous variable), ER status, PR status, HER2 status, tumor size, nodal status, and clinical stage (early stage VS advanced stage).

did not collect information on treatment, which is crucial for prognosis, we adjusted clinicopathological characteristics to partially control for the confounding effect of treatment.

Conclusions

In conclusion, the current results suggest a protective effect of anti-*T. gondii* IgG on prognosis in breast cancer, and this effect is modified by serum cytokine levels, particularly IL-17 and IL-9. The current study demonstrates that *T. gondii* infection confers a beneficial effect on the prognosis of breast cancer patients, which has only been found in mice thus far, indicating that attenuated *T. gondii* has significant potential as a novel immunotherapy for the treatment of breast cancer.[42] However, additional studies are required to elucidate the exact mechanisms.

Data Sharing Statement

The datasets used and analyzed during this study were available from the corresponding author on request.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of the School of Public Health at Sun Yat-sen University (number of Institutional Review Board approval: 2012-8) and performed in accordance with the Declaration of Helsinki. The written informed consents were obtained from all the participants.

Consent for Publication

Written informed consent for publication was obtained from all participants.

Author Contributions

All authors contributed to study design, data collection, statistical analysis and interpretation, visualization, drafting or revising the article; have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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