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PRECLINICAL STUDY

β_2-Adrenergic receptor expression is associated with biomarkers of tumor immunity and predicts poor prognosis in estrogen receptor-negative breast cancer

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Abstract

Purpose Antitumor immunity plays an important role in the progression of breast cancer. β_2-adrenergic receptor (β_2AR) was found to regulate the antitumor immune response and breast cancer progression in preclinical studies. To understand the clinical role of β_2AR in cancer progression, we investigated the clinicopathological and prognostic significance of β_2AR expression in invasive breast cancer.

Methods β_2AR levels in breast tumors were evaluated by immunohistochemistry in a well-characterized patient cohort with long-term follow-up ($n=278$). We evaluated the relationship of β_2AR expression to patient survival and clinicopathological factors, including immune biomarkers such as tumor-infiltrating lymphocytes (TILs) and programmed death ligand 1 (PD-L1) expression. Breast cancer-specific survival was compared between high- and low-β_2AR expression groups.

Results Although β_2AR was not related to clinicopathological factors across the whole cohort, high β_2AR was significantly related to PD-L1 negativity in estrogen receptor (ER)-negative patients. Tumors with high β_2AR tended to have low TIL grade, and high β_2AR was an independent prognostic factor for reduced survival in ER-negative patients.

Conclusions β_2AR is an independent poor prognostic factor in ER-negative breast cancer. The findings suggest that tumor β_2AR regulates immune checkpoint activity, which may have therapeutic implications for patients with ER-negative breast cancer.

Keywords Invasive breast cancer · ER-negative · β_2-Adrenergic receptor · Tumor-infiltrating lymphocytes · PD-L1 · Immune checkpoint

Abbreviations

β_2AR	β_2-Adrenergic receptor
CI	Confidence interval

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CSS	Breast cancer-specific survival
ER	Estrogen receptor
HER2	Human epidermal growth factor 2
HR	Hazard ratio
PD-L1	Programmed death ligand 1
PgR	Progesterone receptor
TIL	Tumor-infiltrating lymphocyte

Introduction

Although recent improvements in treatment have increased the survival of patients with invasive breast cancer, recurrence and metastasis occur in approximately 20% of breast cancer patients [1, 2]. Characterization of factors that contribute to tumor progression will help identify promising candidates for the next generation of molecular targeted therapy [3–5].

Signaling through α- and β-adrenergic receptors mediates the effects of the sympathetic nervous system on physiology, including the regulation of stress responses [6]. In preclinical studies, activation of β-adrenergic receptors (βARs) by the endogenous ligands norepinephrine and epinephrine has been shown to drive cancer progression [7, 8] by increasing tumor cell invasion and metastasis [9–11]. Use of drugs that block βAR signaling (beta-blockers) for non-cancer indications such as hypertension was associated with reduced metastasis and recurrence in breast cancer patients [12–14], providing clinical evidence that βAR signaling regulates cancer progression.

β_2AR, one of the βAR subtypes, is highly expressed in a variety of cancers, including breast cancer [15, 16]. Preclinical studies have identified plausible molecular and cellular mechanisms for cancer progression, including dysregulation of the DNA repair system [17], enhanced epithelial–mesenchymal transition [10, 11, 18], and impaired antitumor immunity [19–21]. Although β_2AR is expressed in breast cancer samples from patients [16], it is unknown whether tumor β_2AR levels are related to survival or immune outcomes. Recent studies suggest that tumor-infiltrating lymphocyte (TIL) grade is considered an important prognostic factor and a predictive factor for treatment with programmed death ligand 1 (PD-L1) inhibitor [22, 23]. To address this question, the relationship between β_2AR expression—as revealed by immunostaining—and clinicopathological factors, including TIL grade, PD-L1 expression, and patient outcomes, was assessed in a well-characterized cohort of patients with invasive breast cancer.

Materials and methods

Patient characteristics

A total of 278 breast cancer patients who underwent breast surgery at Saitama Cancer Center in Japan in 2000 and 2001 were included in the study. The median age was 54 years (range, 25–87 years). All patients underwent breast surgery without neoadjuvant treatment. A total of 216 patients (77.7%) had breast-conserving surgery, and 139 patients (50.0%) underwent axillary lymph node dissection. A total of 158 patients (56.8%) reported being in the postmenopausal stage at the initial surgery. In total, 9.4% of patients had pathological grade T3 or T4 tumors, and 46.8% of patients had a pathological lymph node metastasis-positive status. Assessment of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor 2 (HER2) was described in previous studies [24]. This study was approved by the Institutional Review Board of the Saitama Cancer Center (reference number 533).

Evaluation of tumor immunity-related biomarkers

TILs were assessed in 4-μm sections from formalin-fixed specimens stained with hematoxylin and eosin. Using an optical microscope with × 200 and × 400 magnification, an investigator specializing in breast pathology (MK) evaluated the density of stromal TILs based on the International Working Group guideline [22, 25]. Less than 10% area was defined as low TIL grade and ≥ 10% as high TIL grade.

PD-L1 expression was assessed by immunohistochemistry using rabbit monoclonal anti-PD-L1 clone SP142 (Spring Biosience, USA) diluted 1:50. Immunohistochemistry for PD-L1 was performed by manual procedures. For antigen retrieval, deparaffinized sections were immersed in 10 mmol/L citrate buffer (pH 6.0) and boiled for 10 min in a microwave oven. Sections were incubated for 1 h with anti-PD-L1 antibody followed by 30 min incubation with secondary antibody (EnVision+ Peroxidase System; DAKO, Glostrup, Denmark). Immune cells in the breast cancer served as an internal control, and immune cells in tonsil served as an external control. Staining was assessed by an investigator specializing in breast pathology (MK) according to the evaluation method initially established for urothelial cancer [26]. Tumors with ≥ 1% cancer cells with cytoplasmic and/or membrane PD-L1 staining were defined as PD-L1 positive.

β_2AR immunohistochemistry

β_2AR expression was assessed by immunohistochemistry using a rabbit anti-β_2AR antibody (Abcam, UK). Using manual procedures dewaxed sections were incubated in high-pH antigen retrieval reagent for 10 min at 97 °C and incubated with the primary antibody diluted 1:100 for 60 min at room temperature, followed by secondary antibody (EnVision+ Peroxidase System; DAKO, Glostrup, Denmark). β_2AR protein expression was assessed by cytoplasmic staining of the cancer cells in the full-face

slides from the 278 cases. Internal controls were provided by sweat gland cells and normal mammary ductal cells in normal tissue adjacent to breast tumor that showed strong positive staining and weak staining, respectively. Lung adenocarcinoma cells provided an external positive control, consistent with our previous studies [27–30]. For stratification of β_2AR expression, the Allred score system was used because of marked intra-tumoral heterogeneous expressions in proportion and intensity in cancer tissue [31]. Allred score system provides a summed score for proportion (0, 0%; 1, 0–1%; 2, 1–10%; 3, 10–33%; 4, 33–66%; 5, > 66%) and intensity (0, negative; 1, weak; 2, moderate; 3, strong) and was assessed by one pathologist (MK). Staining in a subset of samples was independently validated by SK. The cohort of 278 samples was divided into high- and low-β_2AR expression groups based on a cutoff score of 6.

Statistical analysis

SPSS statistical software v24.0 (IBM, Armonk, NY, USA) was used for the statistical analyses. The chi-square and Fisher's exact tests were used to evaluate the relationships between β_2AR expression and several clinicopathological factors, including PD-L1 expression, TIL grade, tumor size, lymph node status, ER, PgR, and HER2 expression. Breast cancer-specific survival (CSS) was used to assess the prognostic utility of β_2AR expression. CSS was determined as the time from initial breast surgery to death caused by breast cancer. For univariate and multivariate assessment of survival, clinicopathological factors, including β_2AR expression, hazard ratios (HRs), and 95% confidence intervals (CIs), were assessed using the Cox proportional hazards regression model.

Results

Prognostic utility of β_2AR expression in ER-negative breast cancer

In the total cohort of 278 patients, 235 patients (84.5%) had low β_2AR expression, and 43 patients (15.5%) had high β_2AR expression (Fig. 1). Across the entire cohort, CSS did not significantly differ between patients with high-β_2AR-expressing tumors and patients with low-β_2AR-expressing tumors (HR = 1.64; 95% CI 0.91–2.98; $p = 0.10$) (Fig. 2), and β_2AR expression was not associated with clinicopathological characteristics that predicted survival (Supplementary Table 1).

However, among patients with ER-negative cancer, those with high-β_2AR tumors had significantly lower CSS than those with low-β_2AR tumors (HR = 2.53; 95% CI 1.15–5.58; $p = 0.021$) (Fig. 3). In contrast, among patients with ER-positive cancer, β_2AR expression was not a prognostic factor (HR = 0.96; 95% CI 0.37–2.49; $p = 0.93$) (Fig. 3). The frequency of high β_2AR expression was similar in ER-positive tumors (14.6%) and ER-negative tumors (17.4%).

To identify additional factors associated with survival in ER-negative breast cancer, we used univariate analysis with a Cox proportional hazards regression model. In addition to high β_2AR expression, low TIL grade (HR = 2.42; 95% CI 1.10–5.31; $p = 0.028$) and positive nodal status (HR = 3.57; 95% CI 1.58–8.07; $p = 0.0023$) predicted reduced survival (Table 1). Multivariate analysis showed that β_2AR expression was a poor independent prognostic factor in patients with ER-negative breast cancer after controlling for other prognostic factors (HR = 3.12; 95% CI 1.38–7.02; $p = 0.0061$) (Table 1).

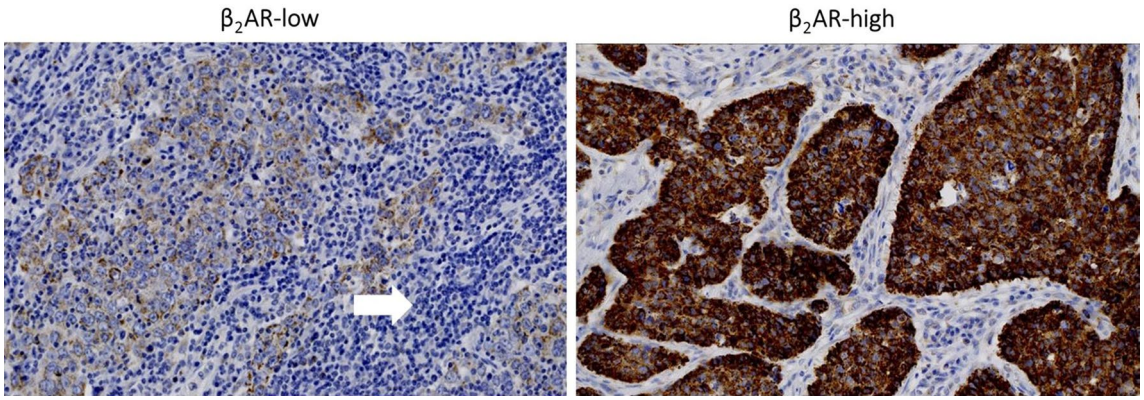

Fig. 1 β_2-adrenergic receptor (β_2AR) expression in breast cancer. Representative images of weak staining for β2AR (β2AR-low) and strong cytoplasmic staining of β2AR in cancer cells (β_2AR-high). The arrow indicates tumor-infiltrating lymphocytes (TILs)

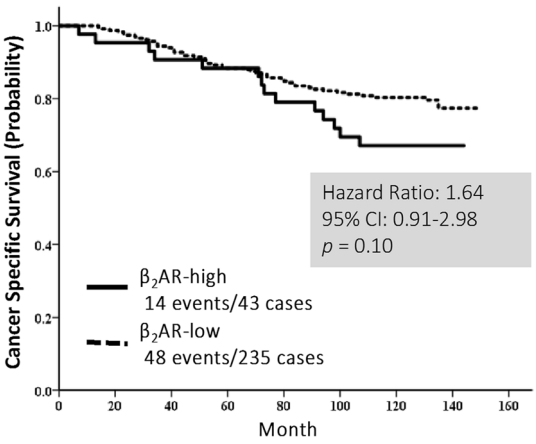

Fig. 2 Breast cancer-specific survival (CSS) stratified by β_2-adrenergic receptor (β_2AR) expression. Survival curves of breast cancer patients after diagnosis stratified by β_2AR expression

Clinicopathological significance of β_2AR expression in ER-negative breast cancer

To obtain insight into how β_2AR might be linked to survival, we investigated which factors were associated with β_2AR staining in ER-negative tumors. Across the ER-negative cohort, expression of β_2AR was not significantly associated with tumor size, nodal status, histological grade, and HER2 status (Table 2).

In terms of the relationships between β_2AR expression and tumor immunity-related biomarkers, β_2AR-high tumors were more likely to have low TIL grade than high TIL grade (66.7% vs. 33.3%. respectively) (Fig. 1), although the difference did not reach statistical significance (Table 2). In ER-negative tumors, high β_2AR expression was significantly associated with PD-L1 negativity ($p = 0.033$); all PD-L1-positive patients had low β_2AR expression (Fig. 4).

Discussion

The current study has shown that β_2AR is a poor independent prognostic factor in ER-negative breast cancer. High β_2AR was associated with changes in immune biomarkers, including PD-L1 negativity and reduced TILs. The immune system affects all phases of tumor growth from initiation to progression and dissemination [32], as well as treatment response [33]. Previous mechanistic studies found that β_2AR signaling regulates anticancer immunity by reducing the proliferation and activation of cytotoxic CD8 T cells [21, 34, 35]. β_2AR signaling also regulates tumor infiltration by macrophages [36, 37], skewing macrophages toward an M2 phenotype and impairing antigen presentation by dendritic cells [38]. The findings presented here raise the possibility that β_2AR regulation of anticancer immunity may have effects on clinical outcomes, especially in ER-negative breast cancer.

It will be important to determine why β_2AR expression predicts poor outcome in ER-negative tumors but not in ER-positive tumors. Consistent with the findings presented here, previous studies support the hypothesis that ER-negative cancer is sensitive to modulation by β_2AR. Pharmacologically blocking β_2AR using the cardiac drug propranolol (which also blocks β_1AR) at the time of cancer diagnosis was

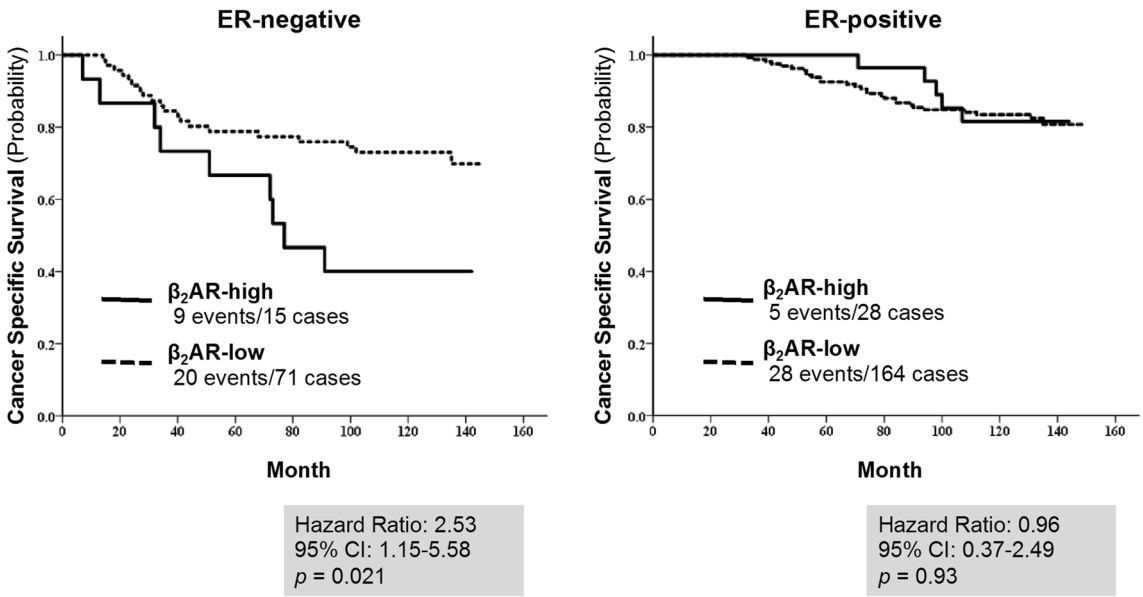

Fig. 3 Cumulative breast cancer-specific survival (CSS) of estrogen receptor (ER)-negative and ER-positive breast cancer patients stratified by β_2-adrenergic receptor (β_2AR) expression

Table 1 Relationship of clinicopathological factors, including β_2AR, to survival in patients with ER-negative tumors

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
β_2AR expression						
Low	Reference			Reference		
High	2.53	1.15–5.58	0.021	3.12	1.38–7.02	0.0061
PD-L1						
Negative	Reference			–		
Positive	0.42	0.13–1.38	0.15	–		
TIL grade						
High (≥ 10%)	Reference			Reference		
Low (< 10%)	2.42	1.10–5.31	0.028	1.80	0.81–4.02	0.15
HER2						
Negative	Reference			–		
Positive	1.36	0.65–2.85	0.42	–		
Tumor size						
T1 and T2	Reference			–		
T3 and T4	1.58	0.55–4.54	0.40	–		
Nodal status						
Negative	Reference			Reference		
Positive	3.57	1.58–8.07	0.0023	3.83	1.65–8.88	0.0018
Histological grade						
Grades 1, 2	Reference			–		
Grade 3	1.06	0.37–3.04	0.92	–		

β_2AR β_2-adrenergic receptor, *ER* estrogen receptor, *HR* hazard ratio, *CI* confidence interval, *PD-L1* programmed death ligand 1, *TIL* tumor-infiltrating lymphocyte, *HER2* human epidermal growth factor 2

associated with reduced metastasis and improved survival in triple-negative breast cancer patients, but not in ER-positive cancer patients [12, 14]. As we have shown that there is no difference between β_2AR expression in ER-positive and ER-negative breast cancer (approximately 15% in each subtype), the findings suggest that β_2AR signaling is differentially coupled to progression in ER-negative cancer. Recent studies found that the prognostic significance of TIL grade is opposite in ER-positive and ER-negative breast cancer: high TIL grade predicted improved outcome and treatment response in ER-negative cancer but predicted poor outcome in ER-positive cancer [22, 39–41]. In future research, it will be important to determine the mechanisms for the effects of TILs on outcome in different breast cancer subtypes and the role of β_2AR signaling. Metabolic pathways influence cancer progression and the antitumor immune response and are regulated by adrenergic signaling, suggesting one possible mechanism [42, 43]. It will also be important to determine how the reduction in TILs in β_2AR-high tumors impacts patient response to checkpoint inhibitors, as CD8-positive T lymphocytes are associated with therapeutic efficacy of the immune checkpoint inhibitors [44].

The finding that β_2AR-high tumors are negative for PD-L1 has therapeutic implications for ER-negative breast cancer patients. PD-L1 is found on antigen-presenting cells and tumor cells [45] where it binds to PD-1 on T lymphocytes to limit the cytotoxic T cell response [32, 46]. PD-L1 expression is a diagnostic biomarker that has been used to determine treatment with atezolizumab, a PD-L1 inhibitor, in triple-negative breast cancer patients [45]. To the extent that PD-L1 limits anticancer immunity, PD-L1 negativity is unlikely to contribute to reduced survival in ER-positive and β_2AR-high tumors. However, the absence of PD-L1 in β_2AR-high tumors may predict a poor response to immunotherapy including checkpoint inhibitors in ER-negative patients. This raises the possibility that pharmacologically blocking β_2AR signaling may enhance the response to immunotherapy, particularly in ER-negative patients. Consistent with this hypothesis, treatment with the beta-blocker propranolol increased TIL grade and improved the response to an immune checkpoint inhibitor in a mouse model of triple-negative breast cancer [21, 34]. These findings suggest that evaluation of a beta-blocker in combination with immune checkpoint inhibitors is warranted in future clinical trials of ER-negative breast cancer.

In conclusion, β_2AR expression is associated with survival outcomes and PD-L1 expression in ER-negative breast cancer. These findings indicate that β_2AR may control the tumor microenvironment, including antitumor immunity, to promote progression and metastasis of ER-negative breast

Table 2 Relationship between β_2AR expression and clinicopathological factors in ER-negative breast cancer

Factors	β_2AR			Significance
	Low	High	Total	*p* Value
PD-L1				
Positive	18 (100.0%)	0 (0.0%)	18	0.033
Negative	53 (77.9%)	15 (22.1%)	68	
TIL grade				
High (≥ 10%)	38 (88.4%)	5 (11.6%)	43	0.26
Low (< 10%)	33 (76.7%)	10 (23.3%)	43	
HER2				
Positive	26 (83.9%)	5 (16.1%)	31	1.00
Negative	45 (81.8%)	10 (18.2%)	55	
Tumor size				
T1 and T2	64 (83.1%)	13 (16.9%)	77	0.65
T3 and T4	7 (77.8%)	2 (22.2%)	9	
Nodal status				
Positive	36 (85.7%)	6 (14.3%)	42	0.57
Negative	35 (79.5%)	9 (20.5%)	44	
Histological grade				
Grade 3	60 (80.0%)	15 (20.0%)	75	0.20
Grades 1, 2	11 (100.0%)	0 (0.0%)	11	

β_2AR β_2-adrenergic receptor, *ER* estrogen receptor, *PD-L1* programmed death ligand 1, *TIL* tumor-infiltrating lymphocyte, *HER2* human epidermal growth factor 2

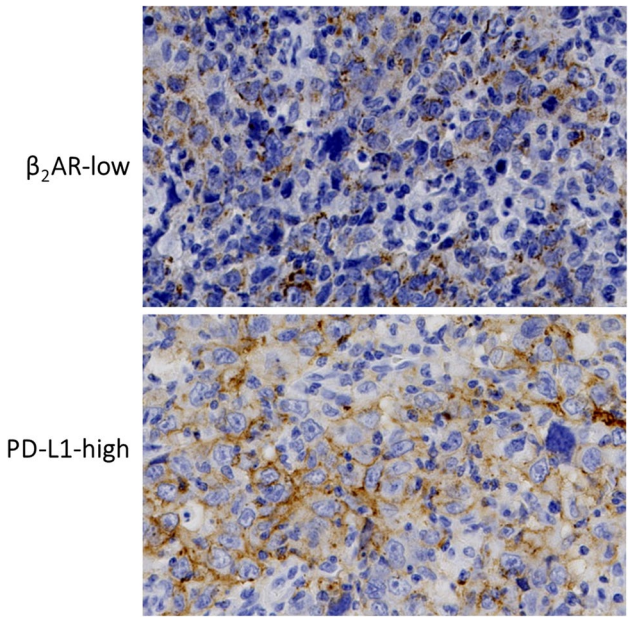

Fig. 4 β_2-adrenergic receptor (β_2AR) and programmed death ligand 1 (PD-L1) expression in an estrogen receptor (ER)-negative breast cancer. Representative case showing positive PD-L1 expression found in β_2AR-low tumors

cancer. Additional functional studies of β_2AR using beta-blockers will be necessary to determine how β_2AR activity controls antitumor immune reactions and immune checkpoint systems in ER-negative breast cancer.

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Compliance with ethical standards

Conflict of interest TY has received research grants from Ono Pharmaceutical Co., Ltd., CHUGAI Pharmaceutical Co., Ltd., and Memolead CO. KI has received a speaker honorarium from Eisai Co., Ltd., CHUGAI Pharmaceutical Co., Ltd., Pfizer Inc. KI has received research grants from Novartis Pharma K.K., Pfizer Inc., CHUGAI Pharmaceutical Co., Ltd., Daiichi Sankyo Co., Ltd., PAREXEL/Puma Biotechnology, Merck Sharp & Dohme Ltd., Bayer Yakuhin, Ltd., Eli Lilly and Company, and Eisai Co., Ltd. EKS is a member of the scientific advisory board of Cygnal Therapeutics. MK received a speaker honorarium CHUGAI Pharmaceutical Co., Ltd., Taiho Pharmaceutical Co., Ltd. KS has received research grants from CHUGAI Pharmaceutical Co., Ltd., and Ono Pharmaceutical Co., Ltd. The other authors declare that they have no conflicts of interest.

Research involving human and animal participants All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from the participants included in the study.

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