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ORIGINAL PAPER

Phosphorylated HER3 and FITC-labeled trastuzumab immunohistochemistry in patients with HER2-positive breast cancer treated with adjuvant trastuzumab

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Abstract
The development of trastuzumab has significantly improved the prognosis of HER2-positive breast cancer. However, disease recurs in some patients with HER2-positive breast cancer. A new strategy for treating HER2-positive breast cancer is necessary. Although several studies have reported that HER3 is a prognostic factor for HER2-positive breast cancers, phosphorylated HER3 (pHER3) has not been well studied. There has been no survival analysis including immunohistochemistry with trastuzumab as the primary antibody. We analyzed immunohistochemistry using anti-pHER3 antibody and FITC-labeled trastuzumab (FITC-tra). Of 78 patients enrolled in the study, we could evaluate the immunohistochemistry for pHER3 in 71 cases and that for FITC-tra in 72 cases. Sixteen cases were positive for pHER3 (16/71, 22.5%), and 19 positive for FITC-tra (19/72, 26.4%). Kaplan–Meier analysis showed a significant association of pHER3 positivity ($p=0.011$) but not HER3 positivity or FITC-tra positivity with disease-free survival. Therefore, immunohistochemical evaluation of pHER3 in HER2-positive breast cancer may provide a useful biomarker. An expanded study of pHER3 involving standardization of the pHER3 test to be encouraged.

Keywords Phosphorylated HER3 · HER3 · HER2 · Trastuzumab · Breast cancer

Introduction

Human epidermal growth factor receptor 2 (HER2) overexpressing breast cancer comprises 15–30% of cases of invasive breast cancer [1, 2]. HER2 overexpression has been regarded as a negative prognostic factor. Trastuzumab has improved the mortality rate of HER2-positive breast cancer [3, 4], but disease still recurs in about 20% of patients with HER2-positive breast cancer [4].

Several mechanisms of resistance to trastuzumab therapy have been reported. Expression of p95HER2 [5], a truncated form of the HER2 receptor, tyrosine kinase activation [6], abnormalities in signal transduction downstream of HER2, such as the loss of phosphatase and tensin homolog [7], or continuous activation of PI3K/Akt [8, 9] and the L755S acquisition mutation in HER2 [10] have been reported as factors conferring resistance to trastuzumab. Our previous study revealed that phosphorylated HER2, p53, and the HercepTest score were prognostic factors for disease-free survival in patients with HER2-overexpressing breast cancer who were treated with adjuvant trastuzumab therapy [11].

HER3 overexpression in breast cancers has been frequently reported as a marker of aggressive disease [12–14]. However, Cha et al. studied the expression of HER3 and phosphorylated HER3 (pHER3) by immunohistochemistry in lapatinib-plus-capecitabine-treated HER2-positive breast cancers [15], and reported that HER3 and pHER3 were not significant prognostic factors in that situation. No study of pHER3 expression in HER2-positive breast cancer treated with standard trastuzumab therapy has been reported. We hypothesized that an immunohistochemistry study using trastuzumab itself might be a good method to identify trastuzumab sensitivity. The aim of this study was to elucidate the significance of pHER3 expression and of HER2 expression measured by trastuzumab itself in HER2-positive breast cancer treated with standard adjuvant trastuzumab therapy.

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Materials and methods

This retrospective study was approved by the local ethics committee (the Institutional Review Board of Kawasaki Medical School Ethics Committee, approval #1084) and conducted in accordance with institutional guidelines.

From 2005 to 2010, 87 cases of HER2-positive breast cancer, as defined by HercepTest 3 + or HercepTest 2 + and FISH positive, were surgically treated and received 1-year adjuvant trastuzumab therapy for in Kawasaki Medical School Hospital. Of these, five cases did not receive either anthracyclin or taxane because the patients were older and/or had poor performance status; these were excluded from this study. Two cases of metachronous bilateral breast cancer and two cases with complications of other cancers (tongue cancer and gastric cancer) were also excluded.

We constructed a tissue microarray with 78 cases using the KIN-2 system (Azumaya, Tokyo, Japan) and a 2-mm needle. Four-micrometer sections were cut from the tissue microarrays (TMAs). After dewaxing and hydration, they were placed in a hot bath of Target Retrieval Solution, pH 9.0 (Dako, Glostrup, Denmark), at 95C for 40 min. The sections were incubated overnight with primary antibodies anti-HER3 (DAK-H3-IC, Dako) at 1:100 and anti-pHER3 (Tyr 1289) (21D3, Cell Signaling, Danvers, MA, USA) at 1:500. Then, signals were detected using the CSA II system (Dako) for HER3 and Envision plus (Dako) for pHER3. The chromogen used was 3,3′-diaminobenzidine tetrachloride, and the sections were counterstained with hematoxylin. Trastuzumab (Herceptin, Chugai Pharmaceutical Co., Ltd. Tokyo, Japan) was dialyzed using a Slide-A-Lyzer Mini Dialysis Unit (Thermo Scientific, Waltham, MA, USA) and labeled with FITC Antibody Labeling Kit (Thermo Scientific) to produce FITC-trastuzumab (FITC-tra). Deparaffinized slide was treated with Target Retrieval Solution, pH 9.0 (Dako), at 95 °C for 40 min, then, incubated with FITC-tra (1μ g/ml) for two hours at room temperature, washed with PBS, then counter stained by DAPI. HER3 expression was scored as 0 to 3 + according to the 2013 ASCO/CAP HER2 guidelines [16]. Any membranous staining was regarded as indicating positive for pHER3, and FITC-tra staining.

Statistical analyses were performed using IBM SPSS Statistics for Windows (version 25; IBM Corp., Armonk, NY). $P < 0.05$ was considered significant.

Results

Clinicopathological information

Sufficient tumor tissue for analysis was not available in 6/78 cases, because of the invasive cancer was small or because of tissue detachment from the TMA slides. We could evaluate the immunohistochemistry for HER3 and FITC-tra in 72 cases. Because of one additional case of tissue detachment, we could evaluate pHER3 in 71 cases. The patients' ages ranged from 24 to 87 years (median 55). The follow-up period ranged from 4.1 to 89.0 months (median 40.1). Disease recurred in seven cases (9.7%), and no patient died. The histology was invasive carcinoma, NST in 71 cases and invasive lobular carcinoma in one case. Thirty cases (41.7%) were estrogen receptor (ER)-positive, and 19 cases (26.4%) were progesterone receptor (PgR)-positive. Thirty-two cases (44.4%) were histological grade two, while 40 cases were grade three (55.6%). Breast-conserving surgery was performed in 24 cases (33.3%) and modified radical mastectomy in 48 cases (66.7%). Anthracyclin was the only chemotherapy drug used in 44 cases (61.1%), taxane was the only chemotherapy drug in five cases (6.9%), and both anthracyclin and taxane were used in 23 cases (31.9%). ER- and/or PgR-positive cases were additionally administrated adjuvant endocrine therapy (tamoxifen with or without luteinizing hormone-releasing hormone agonist or aromatase inhibitor) after chemotherapy.

Immunohistochemistry

The results of immunohistochemically indicated that HER3 was expressed (1 + to 3+) in 68 cases (68/72, 94.4%): four cases were negative (0), 22 were 1+, 25 were 2 + and 21 were 3+. pHER3 expression was noted in 16 cases (16/71, 22.5%). FITC-tra staining was positive in 19 cases (19/72, 26.4%). Representative figures are shown in Fig. 1.The clinicopathological analyses are shown in Table 1. HER3 expression inversely correlated with pT ($p = 0.006$). No significant correlation was observed between HER3 expression and the clinicopathological factors pN, stage, histological grade, ER, PgR, HercepTest, ly, v, and Ki-67 index. No significant correlation was observed between pHER3 expression, FITC-tra positivity and any clinicopathological factor. Fisher's extract test for pHER3 and FITC-tra was not significant ($p = 0.75$) (Table 2).

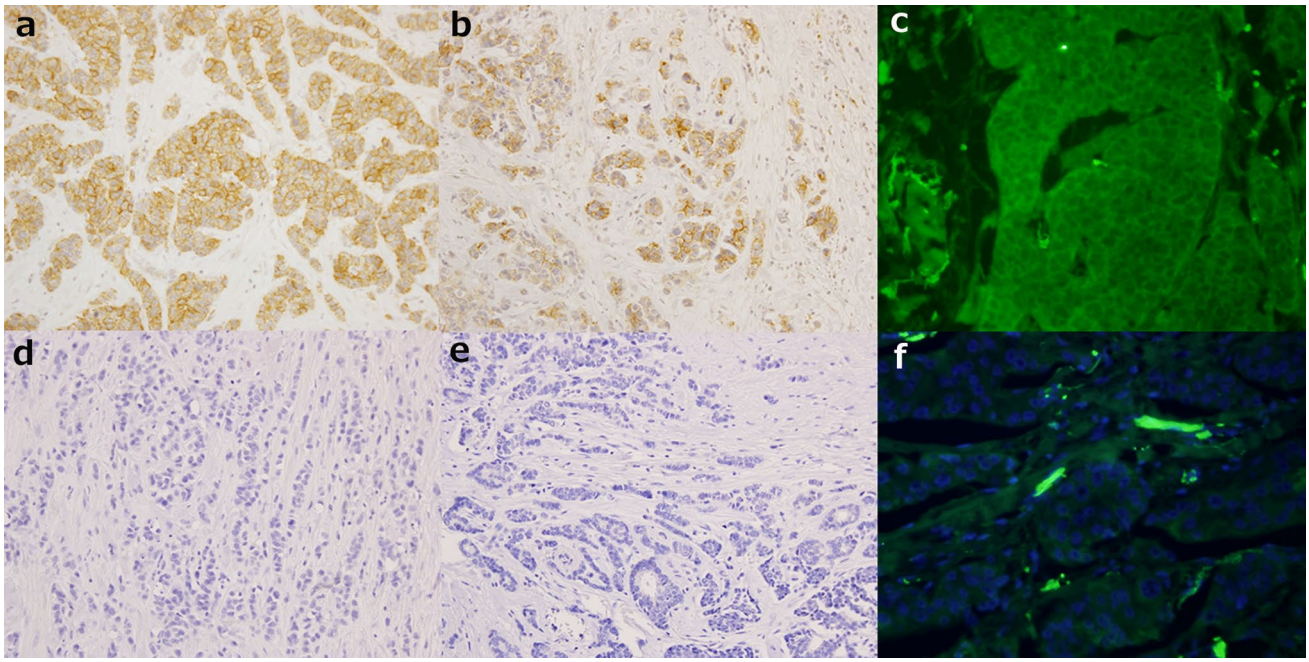

Fig. 1 Immunohistochemistry for HER3 (**a, d**), pHER3 (**b, e**) and FITC-tra (**c, f**). An invasive ductal carcinoma consisting of irregular shaped nests showing diffuse membranous staining for HER3 (**a**). Negative staining for HER3 in trabecular shaped invasive ductal carcinoma (**d**). An invasive ductal carcinoma of scirrhous type showing patchy but distinct membranous staining for pHER3 (**b**). Negative staining for pHER3 in trabecular and tubular shaped invasive ductal carcinoma (**e**). An invasive ductal carcinoma of solid type showing diffuse membranous staining for FITC-tra (**c**). Negative staining for FITC-tra. Focal non-specific reaction in stroma cells is present. However, the cancer cells don't express green FITC signal (blue signal is of DAPI) (**f**)

Prognostic analyses

Kaplan–Meier analysis of disease-free survival by pHER3 positivity showed prognostic significance ($p = 0.011$) (Fig. 2), but that for HER3 positivity did not ($p = 0.371$ for 0 vs. any positive, $p = 0.224$ for 0 to 1 + vs. 2 + to 3+, $p = 0.615$ for 0 to 2 + vs. 3+) (Fig. 3). There was no prognostic significance for FITC-tra staining ($p = 0.804$) (Fig. 4), pT ($p = 0.411$), pN ($p = 0.999$), stage ($p = 0.781$), histologic grade ($p = 0.425$), ER ($p = 0.960$), PgR ($p = 0.791$), HercepTest ($p = 0.117$), or Ki-67 index ($p = 0.967$). The Cox model of multivariable disease-free survival that included pT, pN, stage, histologic grade, HercepTest and pHER3 indicated that HercepTest ($p = 0.021$) and pHER3 ($p = 0.016$) were independent prognostic factors (Table 3).

Discussion

HER3 binds heregulin [17] and NRG-2 [18] as ligands. HER3 can heterodimerize with other HER family members, and the HER2–HER3 dimer is the most active of these heterodimers [19], and activates multiple pathways including MAPK and PI3K/Akt pathways [20]. EGF-like ligands can also bind the HER2–HER3 dimer [21, 22].

HER3 overexpression has been reported to be an indicator of poor prognosis for HER2-overexpressing breast cancers [12–14]. In the present study, all patients with HER3-negative (score 0) disease survived without recurrence, but this was not statistically different from those with HER3-positive disease ($p = 0.371$). Pan-HER3 expression might not as crucial as expression of pHER3.

We showed that pHER3 was a significant prognostic factor for disease-free survival ($p = 0.011$). Phosphorylation of HER3 provides binding sites for PI3K, Shc, and other proteins [23, 24], and increases activation of the PI3K/Akt and Ras/Raf/MAPK pathways [25, 26]. Immnohistochemical detection of expression of pHER3 could be a good prognostic marker for HER2-positive breast cancer treated with standard adjuvant trastuzumab therapy.

Pertuzumab is known to suppress the activity of both HER3 and HER2 [27]. Patients given pertuzumab plus docetaxel showed significantly better rate of pathological confirmed complete response than those given trastuzumab plus docetaxel [28]. The Adjuvant Pertuzumab and Herceptin IN Initial TherapY in Breast Cancer (APHINITY) study showed that pertuzumab significantly improved the 3-year invasive-disease-free survival rate in patients with node-positive disease (92.0% vs. 90.2%, $p = 0.02$), although its effect was not significant in patients with node-negative disease (97.5% vs.

Table 1 Association between clinicopathological factors and immunohistochemistry for HER3, pHER3, and FITC-tra

	HER3				*p* value	pHER3		*p* value	FITC-tra		*p* value
	0	1+	2+	3+		Negative	Positive		Negative	Positive	
Age											
<50	0	9	9	3	0.115	17	4	0.731	15	6	0.777
≤ 50	4	13	16	18		38	12		38	13	
pT											
1–2	2	18	25	20	0.006*	49	15	0.812	47	18	0.667
3–4	2	4	0	1		6	1		6	1	
pN											
0	1	15	14	13	0.416	33	10	0.464	31	12	0.790
1–3	3	7	11	8		22	6		22	7	
pStage											
1–2	1	18	19	18	0.058	44	12	0.155	41	15	1.00
3	3	4	6	3		11	4		12	4	
Histologic score											
2	1	12	11	8	0.601	22	9	0.395	23	9	0.794
3	3	10	14	13		32	7		30	10	
ER											
Negative	2	12	16	12	0.899	33	9	0.474	29	13	0.417
Positive	2	10	9	9		22	7		24	6	
PgR											
Negative	3	15	19	16	0.923	40	13	0.193	38	15	0.763
Positive	1	7	6	5		15	3		15	4	
HercepTest											
2+	0	8	5	4	0.523	14	4	0.844	14	4	0.764
3+	3	14	20	17		41	12		39	15	
ly											
Negative	1	8	10	12	0.432	24	7	0.682	21	10	0.420
Positive	3	14	15	9		31	9		32	9	
v											
Negative	3	20	21	17	0.757	47	13	0.839	45	16	1.000
Positive	1	2	4	4		8	3		8	3	
Ki-67 index											
≤ 14	0	4	3	3	0.786	8	2	0.902	7	3	0.717
>14	4	18	22	18		47	14		46	16	

*$p < 0.05$

Table 2 Correlation of pHER3 and FITC-tra immunostaining

	FITC-tra		*P* value
	Negative	Positive	
pHER3 negative (0)	41	14	0.75
Positive (1 + to 3+)	11	5	

98.4%, $p = 0.64$) [29]. The advantage of pertuzumab over trastuzumab might be at least partly because of its effect on HER3. Poziotinib is a pan-HER inhibitor, for which the results of a phase II trial in breast cancer have been reported [30]. An assessment of pHER3 expression in such a clinical trial using pertuzumab or poziotinib would be interesting. Monoclonal antibodies against HER3, such as RG7116 [31] or LJM716 [32], are under development. This new antibody therapy against HER3 is promising for trastuzumab-resistant HER2-postive breast cancer.

We anticipated that immunohistochemistry using trastuzumab itself as the primary antibody might be useful. However, we failed to find any significant prognostic value for FITC-tra staining. Glazyrin et al. reported that immunohistochemistry using unmodified trastuzumab with a human-to-human blocking reagent and immunohistochemistry using biotinylated trastuzumab showed a lower rate of reactivity than the HercepTest (12.3%, 12.9% vs 20.9%) [33]. We thought that a more sensitive method would be useful, and tested the FITC-tra staining method. However,

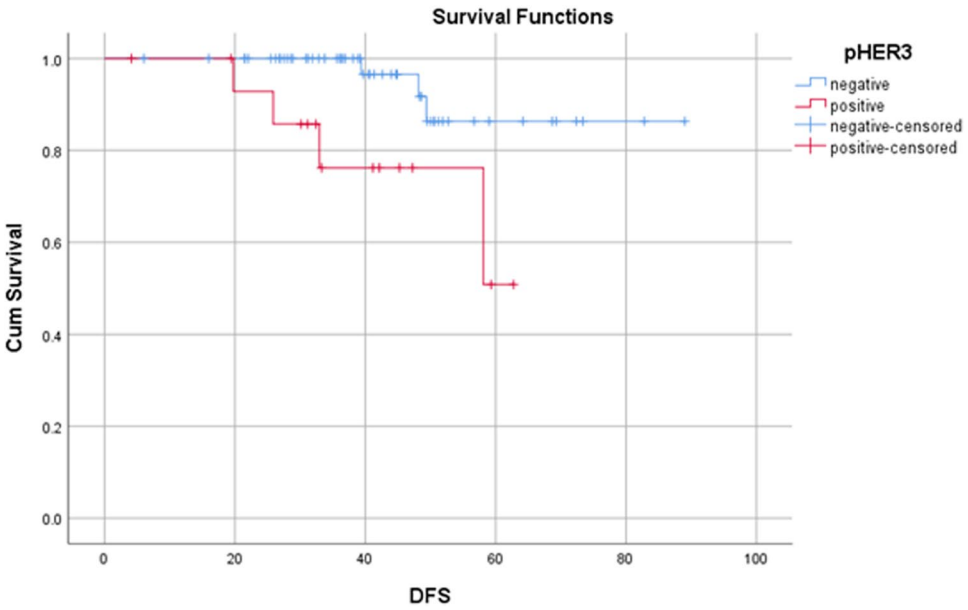

Fig. 2 Kaplan–Meier disease-free survival analysis for pHER3. Patients with pHER3-positive disease showing significantly worse disease-free survival than those with pHER3-negative disease. *P* value is 0.011

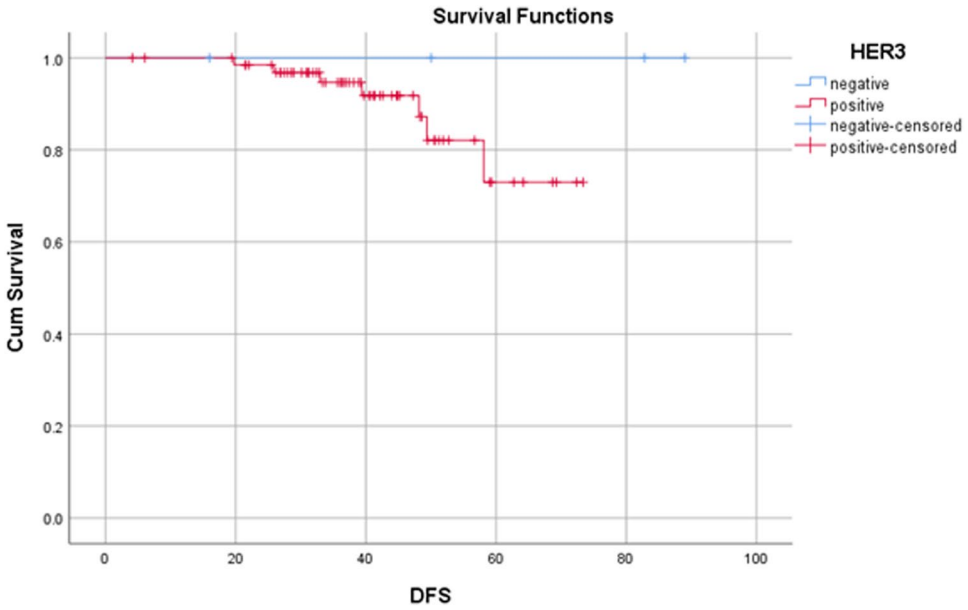

Fig. 3 Kaplan–Meier disease-free survival analysis for HER3. All patients with HER3-negative (score 0) disease survived without recurrence, but this was not statistically different from those with HER3-positive disease. *P* value is 0.371

in our study only 19 cases (19/72, 26.4%) were positive for FITC-tra, even using a lower cutoff than for the HercepTest (all cases were HercepTest 2 + or 3+). Insufficient labelling efficiency of FITC to trastuzumab could be the major reason for this low sensitivity. It is possible that our unsophisticated staining method might be improved using a modification of Glazyrin's methods.

One of the limitations of this study is retrospective study. Pertuzumab efficiency for patients with HER2 and pHER3-positive disease has not been investigated. A prospective study for pertuzumab and pHER3 is warranted. This study was done with the patients with post-operative adjuvant trastuzumab and chemotherapy. Pure correlation between pHER3 or FITC-tra staining status and response for trastuzumab is difficult to estimate. A study using cultured breast cancer cells could solve this problem.

This study suggested a potential benefit of immunohistochemical evaluation of pHER3 expression in HER2-positive breast cancer. The undertaking of an expanded study including standardization of pHER3 testing methods is to be encouraged.

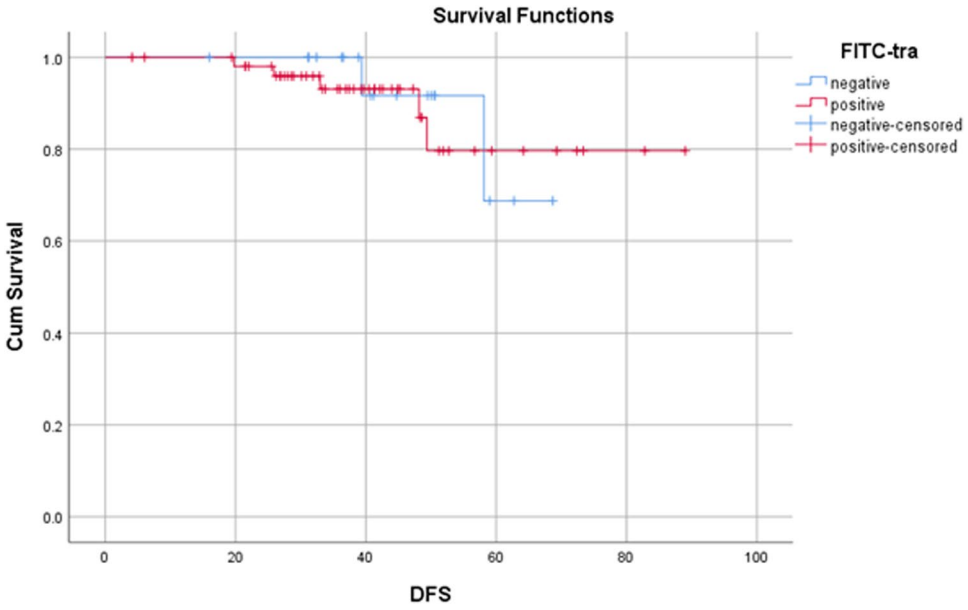

Fig. 4 Kaplan–Meier disease-free survival analysis for FITC-tra. The two curves are crossing, no apparent tendency was found. *P* value is 0.804

Table 3 Cox multivariate regression analyses of selected variables in relation to disease-free survival

Variable	*P* value	Relative risk (95% confidence interval)
pT (1–2 vs. 3)	0.987	22020940.2 (0.000)
pN (0 vs. 1–3)	0.481	2.382 (0.213–26.60)
Stage (1–2 vs. 3)	0.054	27.99 (0.949–825.7)
Histologic grade (2 vs. 3)	0.304	2.599 (0.420–16.08)
HercepTest (2 + vs. 3+)	0.021*	0.087 (0.011–0.693)
pHER3 (0 vs. any positive)	0.016*	13.9 (1.63–118.6)

*$p < 0.05$

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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