



# The prognostic importance of PD-L1, PTEN, PHH3, and Ki-67 expressions in invasive breast carcinoma

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## SUMMARY

**OBJECTIVE:** The aim of this study was to investigate the relationship of PD-L1, PTEN, PHH3, and Ki-67 immunohistochemical stain expressions with prognostic clinicopathological parameters in breast cancer.

**METHODS:** Lumpectomy and mastectomy materials from 85 patients operated at the Department of Pathology, Bolu Abant İzzet Baysal University, Faculty of Medicine between 2014 and 2019 were retrospectively reviewed. PD-L1, PTEN, PHH3, and Ki-67 expressions were examined. Immunohistochemical staining results were compared with clinicopathological parameters and found to be associated with prognosis.

**RESULTS:** A statistically significant correlation was found between PD-L1 and large tumor size, high histological grade, multifocality, and lymphovascular invasion. A statistically significant correlation was found between the loss of PTEN and large tumor size and histological grade. There was a statistically significant correlation between PHH3 and advanced age, large tumor size, and high histological grade. A statistically significant correlation was found between Ki-67 and large tumor size, high histological grade, and lymphovascular invasion.

**CONCLUSION:** PD-L1, PTEN, PHH3, and Ki-67 are regarded as potential biomarkers that can be used to predict the prognosis of breast cancer and to develop targeted therapies.

**KEYWORDS:** Carcinoma, ductal. Breast. Immunohistochemistry. Immune checkpoint inhibitors. Ki-67 antigen.

## INTRODUCTION

Breast carcinoma is the most common cancer in women<sup>1</sup>. The presence of tumor-infiltrating lymphocytes and immune response markers are good prognostic features in breast tumors<sup>2</sup>. Programmed cell death 1 (PD-1) is a surface membrane antigen expressed by various cells of the immune system, including T lymphocytes<sup>3</sup>. Programmed cell death ligand 1 (PD-L1) is expressed in breast tumors and tumor-infiltrating lymphocytes, but not in healthy breast tissue<sup>4</sup>. PTEN is a novel tumor suppressor gene located on chromosome band 10q23. The main function of PTEN is to inhibit the PI3K/AKT pathway, which plays a role in cell proliferation and cell survival<sup>5</sup>. Loss of PTEN function plays a role in the progression of several cancers<sup>6</sup>.

Many prognostic factors in breast cancer are directly or indirectly related to proliferation. One of the best known methods for immunohistochemical measurement of proliferation is Ki-67<sup>7</sup>. Studies have revealed that Ki-67 correlates strongly with biomarkers such as hormone receptor status, HER2 status, tumor staging, and axillary lymph node status<sup>8</sup>. Phosphohistone H3 (PHH3) has long been known as a marker of cellular proliferation in various cancers<sup>9</sup>.

The aim of this study was to investigate the correlation of the expressions in PD-L1, PTEN, PHH3, and Ki-67 immunohistochemical stains with prognostic clinicopathological parameters in breast cancer.

## METHODS

### Case selection

Lumpectomy and mastectomy materials from 85 patients (with invasive ductal carcinoma and lobular carcinoma) treated and operated at the Department of Pathology, Bolu Abant İzzet Baysal University, Faculty of Medicine between 2014 and 2019 were included in the study. The blocks that best reflected the tumor and were most suitable for immunohistochemical examination were selected.

### Immunohistochemical staining

Thin sections at a thickness of 4 microns were taken from the formalin-fixed, paraffin-embedded blocks. The sections were stained with primary antibodies PD-L1, PTEN, PHH3, and Ki-67 according to the manufacturers' instructions using a Leica

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Bond-Max fully automated immunohistochemistry machine. Staining was performed using a standard compact polymer technology kit for immunohistochemistry.

Sections were stained with PD-L1 antibody (Cell Signaling Technology, clone: E1L3N; diluted 1:200). Placental sections were used as positive controls for PD-L1. Membranous and cytoplasmic staining were considered positive<sup>3</sup>. The total percentage of positive cells and their staining intensities (0–3) were determined. The modified Histo-score (H-score) was used [PD-L1 staining intensity (0–3) × percentage of positive cells, between 0 and 300]. PD-L1 expression was dichotomized into two groups, using a cutoff score of 100 (H-score 0–99: negative/low expression, and 100–300: positive expression)<sup>4</sup>.

Sections were stained with anti-PTEN antibody (Dako, Monoclonal Mouse anti-PTEN, clone 6H2.1; diluted 1:40). Colon tumor sections were used as positive controls for PTEN. Both cytoplasmic and nuclear staining were observed with PTEN. The staining intensity for PTEN was scored 0–3<sup>10</sup>. The percentage of positively stained tumor cells was calculated. The total PTEN index was calculated using the following formula: H score = (Percentage of positive cells) × (Immunostaining intensity). The H-score ranged from 0 to 300, and case with a score of less than 90 was considered PTEN loss<sup>11</sup>.

Sections were stained with PHH3 (Cell Marque Corp. Rocklin, rabbit polyclonal antibody; diluted 1:200). Tonsil sections were used as positive controls. The percentage of positively stained tumor cells in the region with the highest PHH3 concentration was determined<sup>12</sup>.

Ki-67 (Dako Autostainer/Autostainer Plus, monoclonal Mouse Anti-Human Ki-67 antigen clone MIB-1) was applied to formalin-fixed tissues. In determining the score, the entire section taken from the invasive border of the tumor was examined, and at least 1000 cells with the highest staining in the 40x objective were evaluated and averaged<sup>13</sup>. The percentage of tumor cells that showed only nuclear staining with Ki-67 determined the Ki-67 score<sup>13</sup>.

## Statistical analysis

To analyze the data, descriptive statistics were given with frequency, percentage, mean, and standard deviation. The Mann-Whitney U test was performed to investigate whether prognostic factor measures in the study differed according to immunohistochemical staining positivity. When evaluating PD-L1, PHH3, Ki-67, and PTEN positivity, the Mann-Whitney U test was used because the number in the positive groups was small and the measurements did not have a parametric distribution. Chi-square analysis and Fisher's exact test were applied to examine the relationships between the proportional values

of prognostic factors according to PD-L1, PHH3, Ki-67, and PTEN positivity status. The SPSS 25.0 program was used for statistical analysis ( $p < 0.05$ ).

## RESULTS

### Clinicopathological features

The study covered 85 cases of invasive breast carcinoma. Of the patients aged between 35 and 88 years (mean  $58.33 \pm 12.50$  years), 82 (96.4%) were female and 3 (3.6%) were male. Of the cases diagnosed with breast carcinoma, 77 (90.5%) had invasive ductal carcinoma and 8 (9.5%) had invasive lobular carcinoma. According to tumor size, patients were divided into two groups:  $< 2$  cm in 34 (40%) patients and  $\geq 2$  cm in 51 (60%) patients.

Nottingham histological grade was found: 12.9% in grade 1, 56.5% in grade 2, and 30.6% in grade 3. Tumor metastasis to the axillary lymph node was detected: 1–3 lymph nodes in 17 (20.0%), 4–9 lymph nodes in 15 (17.6%), and 10 or more lymph nodes in 13 (15.3%) patients. Of the patients, 30% were found with stage 1, 31.8% with stage 2, 20.2% with stage 3, and 17.9% with stage 4.

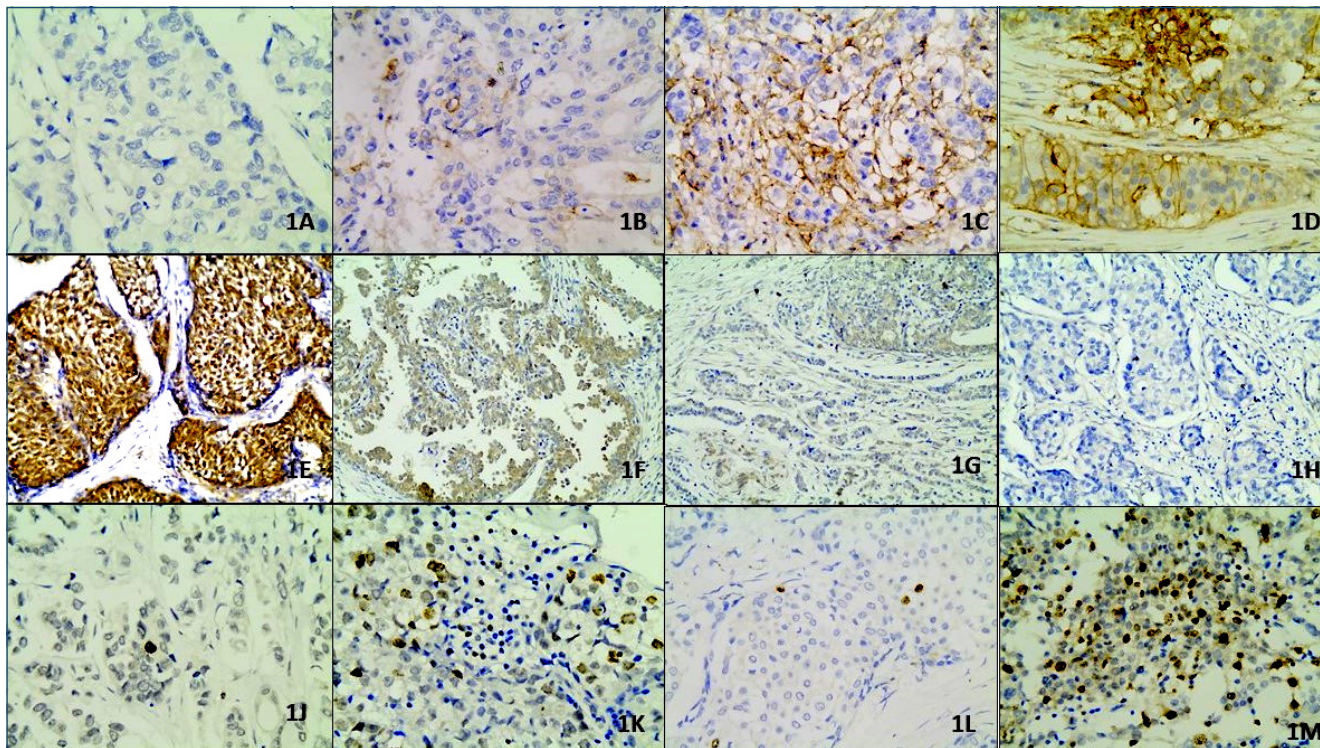
### Immunohistochemical staining with clinicopathological parameters

#### *PD-L1 expression and its relationship to clinicopathological factors*

Table 1 shows the relationship between PD-L1 and prognostic factors. In sections prepared from invasive breast cancer blocks, 21 (24.7%) of 85 patients expressed PD-L1. Tumor cells and/or lymphocytes showed membranous staining in 13 (15.3%) cases, cytoplasmic staining in 3 (3.5%) cases, and cytoplasmic and membranous staining in 21 (24.7%) cases (Figures 1A–D); 48 (56.5%) cases were scored as skor 0, 20 (23.5%) as skor 1, 12 (14.1%) as skor 2, and 5 (5.9%) as skor 3. No significant association was found between PD-L1 expression and age, gender, tumor location, surgical procedure of patients, lymph node metastasis, perineural invasion, stage, and HER2 score.

#### *PTEN expression and its relationship with clinicopathological factors*

The relationship between PTEN-1 and prognostic factors is given in Table 1. Loss of PTEN expression was observed in 42 (49.4%) of 85 cases with invasive breast carcinoma. It was found that there was no staining in 14 (16.4%) cases, weak staining in 24 (28.2%) cases, moderate staining in 30 (35.2%) cases, and strong staining in 17 (20%) cases (Figures 1E–H).



**Figure 1.** (A) Negative expression of PD-L1 in tumor cells, immune peroxidase, 400x. (B) Mild expression of PD-L1 in tumor cells, immune peroxidase, 400x. (C) Moderate expression of PD-L1 in tumor cells, immune peroxidase, 400x. (D) Severe expression of PD-L1 in tumor cells, immune peroxidase, 400x. (E) Intense expression of PTEN in tumor cells, immune peroxidase, 400x. (F) Moderate-intense expression of PTEN in tumor cells, immune peroxidase, 400x. (G) Mild-intense expression of PTEN in tumor cells, immune peroxidase, 400x. (H) Negative expression of PTEN in tumor cells, immune peroxidase, 400x. (J) Low nuclear PHH3 expression in tumor cells, immune peroxidase, 400x. (K) High nuclear PHH3 expression in tumor cells, immune peroxidase, 400x. (L) Low nuclear Ki-67 expression in tumor cells, immune peroxidase, 400x. (M) High nuclear Ki-67 expression in tumor cells, immune peroxidase, 400x.

No significant correlation was found between loss of PTEN expression and age, gender, histological type, lymphovascular invasion, perineural invasion, histological grade, stage, ER, PR, HER2 score, and Ki-67 expression.

#### *PHH3 expression and its relationship with clinicopathological factors*

Table 2 shows the relationship between PHH-3 and prognostic factors. PHH3 stained positive in 36 (42.3%) cases and negative in 49 (47.7%) cases (Figures 1J and K). It was observed that the PHH3 group did not differ by pathological T, N, M grading and staging ( $p=0.06$ ;  $p=0.12$ ;  $p=0.51$ ;  $p=0.35$ , respectively). No significant correlation was found between loss of PHH3 expression and gender, tumor localization, lymphovascular invasion, HER2 score, and stage.

#### *Ki-67 expression and its relationship with clinicopathological factors*

The relationship between PHH-3 and prognostic factors is shown in Table 2. Ki-67 expression was  $<20\%$  in 57 (67%) cases and

$\geq 20\%$  in 28 (33%) cases (Figures 1L and M). The values of the Ki-67 group differed by tumor size, which was larger than 2 cm ( $p=0.01$ ). Patient's age, gender, tumor localization, histological type of tumor, and the number of metastatic lymph nodes did not differ in Ki-67 groups below or above 20%.

## DISCUSSION

The increase in the incidence of breast cancer in recent years has led to studies on the development of new methods of diagnosis and treatment<sup>14</sup>. Many studies have shown the expression of PD-L1 in both tumor cells and TIL in breast carcinoma. In studies, PD-L1 expression in all breast cancer subtypes varies from 0 to 83%, and most studies show PD-L1 expression below 50%<sup>15</sup>. In this study, we identified PD-L1 positivity in 21 (24%) of 85 breast cancer patients and found that it was associated with poor prognostic parameters such as high histological grade, large tumor size, ER and PR negativity, and high Ki-67 expression. Thus, we obtained similar results to recent studies<sup>14,16,17</sup>.



**Table 1.** PD-L1 and PTEN by prognostic factors.

Prognostic factors		PD-L1				p	PTEN				p-value
		Negative		Positive			<90		≥90		
		n	%	N	%		n	%	n	%	
Age	<40	5	7.8%	2	9.5%	0.67	4	9.5%	3	7.0%	0.60
	≥40	59	92.2%	19	90.5%		38	90.5%	40	93.0%	
Gender	Female	62	96.9%	20	95.2%	0.73	42	100.0%	40	93.0%	0.08
	Male	2	3.1%	1	4.8%		0	0.0%	3	7.0%	
Tumor Localization	Right	31	48.4%	13	61.9%	0.01*	25	59.5%	19	44.2%	0.11
	Left	33	51.6%	8	38.1%		17	40.5%	24	55.8%	
Tumor size (cm)	<2	27	42.2%	7	33.3%	0.04*	14	33.3%	20	46.5%	0.03*
	≥2	37	57.8%	14	66.7%		28	66.7%	23	53.5%	
Histological grade	1	10	15.6%	1	4.8%	0.03*	7	16.7%	4	9.3%	0.03*
	2	37	57.8%	11	52.4%		25	59.5%	23	53.5%	
	3	17	26.6%	9	42.9%		10	23.8%	16	37.2%	
Lymphovascular invasion	None	38	59.4%	9	42.9%	0.01*	22	52.38%	25	58.1%	0.13
	Yes	26	40.6%	12	57.1%		20	47.62%	18	41.9%	
Perineural invasion	None	50	78.1%	16	76.2%	0.56	32	76.19%	34	79.07%	0.21
	Yes	14	21.9%	5	23.8%		10	23.81%	9	20.93%	
Pathologic T	T1	29	45.3%	7	33.3%	0.06	15	35.71%	21	48.84%	0.24
	T2	27	42.2%	13	61.9%		23	54.76%	17	39.53%	
	T3	3	4.7%	1	4.8%		2	4.76%	2	4.65%	
	T4	5	7.8%	0	0.0%		2	4.76%	3	6.98%	
Pathologic N	N0	3	9.4%	0	0.0%	0.12	1	4.55%	2	9.09%	0.13
	N1	5	15.6%	2	16.7%		1	4.55%	6	27.27%	
	N2	15	46.9%	8	66.7%		13	59.09%	10	45.45%	
	N3	9	28.1%	2	16.7%		7	31.82%	4	18.18%	
Pathologic M	M0	57	89.1%	21	100.0%	0.03*	38	90.48%	40	93.02%	0.11
	M1	7	10.9%	0	0.0%		4	9.52%	3	6.98%	
Stage	1A	20	31.3%	5	23.8%	0.14	12	28.57%	13	30.23%	0.25
	1B	1	1.6%	0	0.0%		0	0.00%	1	2.33%	
	2A	12	18.8%	5	23.8%		9	21.43%	8	18.60%	
	2B	9	14.1%	1	4.8%		4	9.52%	6	13.95%	
	3A	7	10.9%	7	33.3%		7	16.67%	7	16.28%	
	3B	2	3.1%	0	0.0%		0	0.00%	2	4.65%	
	3C	10	15.6%	3	14.3%		9	21.43%	4	9.30%	
	4	3	4.7%	0	0.0%		1	2.38%	2	4.65%	
ER	Negative	7	10.9%	5	23.8%	0.03*	9	15.00%	3	12.00%	0.08
	Positive	57	89.1%	16	76.2%		51	85.00%	22	88.00%	
PR	Negative	11	17.2%	9	42.9%	0.01*	14	23.30%	6	24.00%	0.21
	Positive	53	82.8%	12	57.1%		46	76.70%	19	76.00%	
HER2	0	32	50.0%	8	38.1%	0.16	17	40.48%	23	53.49%	0.13
	1	12	18.8%	5	23.8%		10	23.81%	7	16.28%	
	2	10	15.6%	4	19.0%		9	21.43%	5	11.63%	
	3	10	15.6%	4	19.0%		6	14.29%	8	18.60%	

\*Statistical significance at the p<0.05 level.

In a study by Ming Li et al., PD-L1 was found to be positive in 38.6% of patients, and PD-L1 positivity was significantly associated with high histological grade, recurrence, and distant metastasis<sup>17</sup>. Hazem et al. identified PD-L1 positivity in 34% of cases and found that PD-L1 expression was associated with

large tumor size, histological grade, HER2 positivity, and ER and PR negativity<sup>16</sup>. In this study, similar to the literature, PD-L1 expression increased with the increasing histological grade of the tumor<sup>4,18</sup>. Moreover, in agreement with the literature, PD-L1 expression was found to be higher in ER- and PR-negative cases<sup>18</sup>.

Table 2. PHH3 and Ki-67 by prognostic factors.

Prognostic factors		PHH3					Ki-67				
		<2		≥2		p	<20%		≥20%		p
		n	%	n	%		n	%	n	%	
Tumor size (cm)	<2	23	46.9%	11	30.6%	0.01*	28	49.1%	6	21.4%	0.01*
	≥2	26	53.1%	25	69.4%		29	50.9%	22	78.6%	
Histological grade	1	10	20.4%	1	2.8%	0.02*	1	1.8%	0	0.0%	0.01*
	2	26	53.1%	22	61.1%		11	19.3%	0	0.0%	
	3	13	26.5%	13	36.1%		35	61.4%	13	46.4%	
Lymphovascular invasion	None	29	59.2%	18	50.0%	0.58	37	64.9%	10	35.7%	0.01*
	Yes	20	40.8%	18	50.0%		20	35.1%	18	64.3%	
Perineural invasion	None	39	79.6%	27	75.0%	0.01*	43	75.4%	23	82.1%	0.01*
	Yes	10	20.4%	9	25.0%		14	24.6%	5	17.9%	
Pathologic T	T1	24	49.0%	12	33.3%	0.05	29	50.9%	7	25.0%	0.05
	T2	20	40.8%	20	55.6%		23	40.4%	17	60.7%	
	T3	2	4.1%	2	5.6%		3	5.3%	1	3.6%	
	T4	3	6.1%	2	5.6%		2	3.5%	3	10.7%	
Pathologic N	N0	2	8.0%	1	5.3%	0.36	1	3.7%	2	11.8%	0.28
	N1	3	12.0%	4	21.1%		4	14.8%	3	17.6%	
	N2	15	60.0%	8	42.1%		14	51.9%	9	52.9%	
	N3	5	20.0%	6	31.6%		8	29.6%	3	17.6%	
Pathologic M	M0	45	91.8%	33	91.7%	0.51	53	93.0%	25	89.3%	0.30
	M1	4	8.2%	3	8.3%		4	7.0%	3	10.7%	
Stage	1A	16	32.7%	9	25.0%	0.35	21	36.8%	4	14.3%	0.38
	1B	1	2.0%	0	0.0%		1	1.8%	0	0.0%	
	2A	9	18.4%	8	22.2%		10	17.5%	7	25.0%	
	2B	6	12.2%	4	11.1%		6	10.5%	4	14.3%	
	3A	6	12.2%	8	22.2%		7	12.3%	7	25.0%	
	3B	2	4.1%	0	0.0%		1	1.8%	1	3.6%	
	3C	7	14.3%	6	16.7%		9	15.8%	4	14.3%	
	4	2	4.1%	1	2.8%		2	3.5%	1	3.6%	
ER	Negative	4	8.2%	8	22.2%	0.02*	4	7.0%	8	28.6%	0.01*
	Positive	45	91.8%	28	77.8%		53	93.0%	20	71.4%	
PR	Negative	9	18.4%	11	30.6%	0.01*	8	14.0%	12	42.9%	0.01*
	Positive	40	81.6%	25	69.4%		49	86.0%	16	57.1%	
HER2	0	9	18.4%	8	22.2%	0.05	31	54.4%	9	32.1%	0.07
	1	7	14.3%	7	19.4%		12	21.1%	5	17.9%	
	2	8	16.3%	6	16.7%		10	17.5%	4	14.3%	
	3	9	18.4%	8	22.2%		4	7.0%	10	35.7%	

\*Statistical significance at the p<0.05 level.

Recent studies have shown that PTEN has a regulatory role in breast carcinoma and may be a predictive and prognostic marker<sup>19</sup>. Shaham et al. found that the loss of PTEN was significantly associated with large tumor size, high grade, and triple-negative breast cancer<sup>11</sup>. In our study of 85 patients, the loss of PTEN was observed in 49.4% of patients. This result from our study was evaluated in accordance with the loss of PTEN expression reported in the literature as 8–86%<sup>11,20</sup>. In this study, the loss of PTEN expression was associated with large tumor size and low histological grade. The low histological grade was probably due to the fact that only a small number of cases were studied. Although a higher loss of PTEN expression was observed in the group with LN metastases in this study, this was statistically insignificant.

PHH3 expression has been shown to be stronger than classic prognostic factors such as axillary lymph node status, tumor size, histological grade, and ER and PR negativity<sup>12,21</sup>. Ivar et al. showed that low PHH3 expression is associated with an excellent prognosis. PHH3 was associated with age, tumor size, grade, tubular formation, nuclear atypia, mitotic activity index, and ER and PR negativity<sup>12</sup>. In this study, high PHH3 expression was associated with advanced age, large tumor size, high histological grade, ER and PR negativity, and high Ki-67 expression. This result suggests that nuclear PHH3 expression may be a poor prognostic factor for breast cancer patients.

The percentage of cells staining positive for Ki-67 has been used as a measure of proliferation and as a prognostic factor<sup>22</sup>. Wiesner et al. found that Ki-67 correlated strongly with other biomarkers such as negative ER and PR, HER2 status, tumor

staging, and histological grade<sup>8</sup>. In this study, high Ki-67 expression was found to be associated with large tumor size, high histological grade, lymphovascular invasion, and ER and PR negativity.

Our study has several limitations. Our cases were few in number, and some of the blocks had technical difficulties, such as failure to obtain optimal staining during immunohistochemical staining.

## CONCLUSION

As a result of this study, expressions of PD-L1, PHH3, Ki-67, and loss of PTEN were found to be associated with poor prognostic factors. We believe that these biomarkers, in addition to being used for prognosis in breast cancer, can be used for therapeutic purposes and may contribute to the development of appropriate immunotherapies through a better understanding of the immunological basis of the tumor.

## AUTHORS' CONTRIBUTIONS

**SED:** Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **EHI:** Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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