RESEARCH ARTICLE

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Association between PIK3CA Mutations in Blood and Tumor-Infiltrating Lymphocytes in Peruvian Breast Cancer Patients

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Abstract

Objective: To evaluate the relationship between circulating tumor DNA (ctDNA) presence and tumor features including tumor-infiltrating lymphocyte (TIL) levels in Peruvian breast cancer patients. **Materials and Methods:** This was a prospective study conducted at the Instituto Nacional de Enfemedades Neoplasicas, Peru. We evaluated level of TIL and PIK3CA mutations in ctDNA. Clinical characteristics, including outcome data, were collected from the patient file. Survival was calculated from the date of blood sample drawn to the event time. Data collected were analyzed using SPSS software version 25. **Results:** We analyzed plasma samples from 183 breast cancer patients. most cases were of Luminal-B (44.8%) phenotype and stage II (41.5%), and median stromal TIL was 30%. PIK3CA mutation in ctDNA was detected in 35% cases (most with E545K) and was associated with lower TIL level (p=0.04). PIK3CA in ctDNA tended to be associated with advanced stages (p=0.09) in the whole series and with higher recurrence rates (p=0.053) in the non-metastatic setting. Patients with presence of PIK3CA in ctDNA tended to have shorter survival (p=0.083). **Conclusion:** Presence of PIK3CA mutation in ctDNA was frequently found in our Peruvian breast cancer series, was associated with lower TIL levels and tended to predict poor outcomes.

Keywords: ctDNA- Breast cancer- PIK3CA- Lymphocytes

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Introduction

Breast cancer is the most common worldwide cancer in women, and it is the leading cause of cancer-related death in women. As we are entering the era of personalized medicine, much attention has been paid to identifying predictive biomarkers for early progression and response to therapies targeting a tumor's genetic background (Andre et al., 2019).

PIK3CA-activating mutations, oncogene encoding the p110-alpha component of the phosphoinositide 3-kinase (PIK3CA), has been extensively studied in breast cancer tumor samples and is present in approximately 30- 40% of all breast cancers. A previous publication of our group indicated that PIK3CA mutation rates in the Peruvian population are similar to other populations (Castaneda et al., 2014). Between 80 to 90% of PIK3CA mutations are located within two exons (exon 9: E545K and E542K and exon 20: H1047R and H1047L). The presence of mutation appears to predict a lower response to drugs targeting hormone (Castaneda et al., 2010) and HER2 receptors (Baselga et al., 2014; Loibl et al., 2016). There has been extensive research in drugs targeting PIK3CA pathway in breast cancer and they have been demonstrated to increase activity of antihormone therapy (Saura et al., 2019), reverse endocrine resistance (Baselga et al., 2018; Baselga

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et al., 2017; Moynahan et al., 2017), and also appears to increase activity of systemic therapy in triple-negative breast cancer (Kim et al., 2017). Recently, presence of PIK3CA tumor mutations has been demonstrated to predict response to the alpha-specific PIK3CA inhibitor Alpelisib in hormone receptor-positive HER2-negative patients (Andre et al., 2019).

Circulating tumor-derived DNA (ctDNA) is composed of small fragments of nucleic acid, which is derived from necrosis, apoptosis, and secretions of tumor cells that can be detected in the plasma of patients with cancer, and recent reports suggest that its presence and its level can predict survival as well as response to treatment (Bettegowda et al., 2014; Garcia-Murillas et al., 2019; Olsson et al., 2015). Identification of PIK3CA tumor mutations in plasma (ctDNA)(Garcia-Murillas et al., 2015; Hrebien et al., 2019; Kaisaki et al., 2016) can predict benefit to Alpelisib and Buparlisib in breast cancer (José Baselga et al., 2017; André et al., 2019), give the category of M0i stage in the AJCC 8th edition (Giuliano et al., 2017) and is currently included in precision medicine programs.

The level of tumor-infiltrating lymphocytes (TIL) has been demonstrated to be associated with longer survival and higher response to both chemotherapy and checkpoint inhibitors in different breast cancer subtypes (Galvez et al., 2018; Giuliano et al., 2017; Salgado et al., 2015).

Although the presence and amount of ctDNA are influenced by the extent of the disease, recent publications describe that tumor features can also influence it (García-Saenz et al., 2017; Higgins et al., 2012; Kodahl et al., 2018; Zhou et al., 2019). However, there is no information about the effect of TIL level over ctDNA presence.

In this study, we prospectively collected plasma samples to explore the detection of hotspot PIK3CA mutations and compare it with tumor features including TIL levels and outcomes of Peruvian breast cancer patients.

Materials and Methods

Patient selection

This was a single-institution prospective study of women who had a confirmed histological diagnosis of invasive breast cancer, and had a plasma sample taken at any surgical procedure and preserved at the biobank from the Instituto Nacional de Enfermedades Neoplasicas. A total of 183 patients were available for the period May 2016 to May 2018 (n=63 in 2016, n=96 in 2017, and n=24 in 2018). Informed consent was obtained from all patients before enrolment and biobanking of their samples.

Paired paraffin sample that was taken in a closest date to the plasma sample was identified and requested from the Institute pathology archive. Haematoxylin & eosin slides from the pathology archive were prospectively evaluated by experienced pathologists to record routine histopathological data and TIL levels in accordance with international recommendation (JS & HG) (Salgado et al., 2015; WHO Classification of Tumours Editorial Board, 2019).

DNA extraction and digital PCR (dPCR)

We determined E542K, E545K, and H1047R mutations in 183 preserved plasma samples. Circulating cell-free DNA (ccfDNA) or circulating tumor DNA (ctDNA) was extracted from 0.2–1 ml plasma samples using affinity-based binding to magnetic beads according to the manufacturer's instructions (Maxwell® RSC ccfDNA Plasma Kit, Promega). For the dPCR, 9 µL of template DNA was mixed with 1 µL of 20x TaqMan® Assay primer/ probe mix and 10 µL of 2x QuantStudioTM 3D Digital PCR Master Mix (Life Technologies) according to the manufacturer's instructions. Fifteen µL aliquots of the PCR solutions were then loaded into QuantStudioTM 3D Digital PCR 20 K chips, and the PCR reaction was performed. The thermal cycler protocol was as follows: 10 min at 96 C, 39 cycles at 60 C for 2 min, 98 C for 30 s, and 60 C for 1 min. Custom TaqMan primers and probes were designed for the three PIK3CA mutations (PIK3CA 760: c.1624 G (VIC) >A (FAM), PIK3CA 763: c.1633 G (VIC) >A (FAM), PIK3CA 775: c.3140 A (VIC) >G (FAM), ThermoFisher scientific). All samples were analyzed in a single assay for each mutation. The data were analyzed with the QuantStudioTM3D AnalysisSuiteTM v1.1.3 (Life Technologies, Carlsbad, CA) for mutation search and quantification of the DNA copies in the plasma.

Statistical analysis

Relationships between PIK3CA mutational status in ctDNA and clinicopathological characteristics were detected using the Chi-square or Fisher exact tests. Median copy number and allele fraction was compared with clinical features using the Mann-Whitney-test. Survival rates were calculated from blood drawn to recurrence or last follow-up/ death date, and the analyses was done with Mann Whitney U test for median time comparison and Kaplan Meier survival curves. A p-value less than 0.05 was considered statistically significant. Statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) for Windows version 10.0 (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological features

Plasma samples from 183 breast cancer patients were analyzed in total, most were of Ductal histology (90.2%), Luminal-B (45%) and Luminal-A (15%) phenotype, and stage-II (Table 1).

TIL was evaluated in 160 cases; median percentage was 30% (1-90%) and higher levels were associated with non-luminal phenotype (p=0.001). The median time from evaluated paraffin tumor tissue to blood sampling was 5 days (0- 47.9 months).

PIK3CA mutation analysis in plasma

Blood sample was collected before any chemotherapy in the no-metastatic-breast-cancer setting in 77.6% cases, after neoadjuvant chemotherapy in 7.7% cases and in the metastatic setting in 14.8% cases.

In total, detection of at least one mutation was found in 64/183 (34.9%) plasma samples. Mutation of PIK3CA

Table 1. Clinicopathological Features and Survival Influence

Features	N=183	%	OS-3years (%)	P
Median age	54 (25-88)			0.405
<54 years	89	48.6	83.1	
≥54 years	94	51.4	78.7	
Histology				0.444
Ductal-NST	165	90.2	80.6	
Mucinous	8	4.4	75.0	
Lobular	5	2.7	100.0	
Others	5	2.7	80.0	
Phenotypic Subtype				0.005
Luminal-A	28	15.3	85.7	
Luminal-B	82	44.8	89.0	
HER2-enriched	20	10.9	85.0	
Triple-Negative	53	28.9	63.5	
Stage				<0.001
I	5	2.7	100.0	
II	76	41.5	93.4	
III	71	38.9	84.5	
IV/ Recurrent	31	16.9	35.5	
Stromal TIL (n=160)				0.578
<30%	72	45.0	83.3	
≥30%	88	55.0	85.2	
Recurrence in early disease (n=152)				<0.001
No	135	88.8	94.1	
Yes	17	11.2	52.9	
Presence of ctDNA PIK3CA Mutation				0.083
No	119	65.0	79.8	
Yes	64	35.0	73.4	
Presence of ctDNA PIK3CA Mutation in metastatic disease				0.01
No	16	51.6	43.8	
Yes	15	48.4	20.0	

ctDNA, circulating tumor-derived DNA; NST, no specific type; OS, overall survival; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

Table 2. Clinicopathological Features Associated with ctDNA Detection

Features	ctDNA Negative		ctDNA Positive		P
	(n=119)	%	(n= 64)	%	
Age					0.156
<54	58	61.7	36	38.3	
≥54	61	68.5	28	31.5	
Histology					0.909
Ductal-NST	106	64.3	59	35.7	
Mucinous	5	62.5	3	37.5	
Lobular	4	80.0	1	20.0	
Others	4	80.0	1	20.0	
Phenotypic Subtype					0.323
Luminal A	15	53.6	13	46.4	
Luminal B	57	69.5	25	30.5	
HER2-enriched	15	75.0	5	25.0	
Triple-Negative	32	60.4	21	39.6	
Stromal TIL (n=160)					0.038
<30%	42	58.3	30	41.7	
≥30%	65	73.9	23	26.1	
Stage					0.326
I	4	80.0	1	20.0	
II	50	65.8	26	34.2	
III	49	69.0	22	31.0	
IV/ Recurrent	16	51.6	15	48.4	
Recurrence in early disease (n=152)					0.053
No	95	70.4	40	29.6	
Yes	8	47.1	9	52.9	

ctDNA, circulating tumor-derived DNA; NST, no specific type; TIL, tumor-infiltrating lymphocyte; TNBC, triple-negative breast cancer.

3140 A>G (p.H1047R), 1633G>A (p.E545K) and 1624G>A (p.E542K) in ctDNA were found in 10.9, 19.1 and 7.1%, respectively. Four cases had two mutations (E545K- H1047R in two cases and E545K- E542K in two cases).

Median copy number (n=64) and mutant allele fraction (n=43) for E542K was 1.024 (0.284-4.190 cop/μL) and 5.09%, for E545K was 2.680 (0.135-9.684 cop/μL) and 3.84%; and for H1047R was 0.798 (0.125-32.864 cop/μL) and 0.94%, respectively.

Presence of ctDNA mutations was associated with TIL level of <30% (p=0.038), but not to phenotype subtype (Luminal A 46.4%, Luminal B 30.5%, HER2 enriched 25% & TNBC 39.6%, p=0.323). There was a trend of higher rates of positive ctDNA in stage IV-recurrent disease (48.4% vs 32.2%, p=0.086) (Table 2).

Presence of ctDNA and recurrence in early breast cancer

The analysis of the 152 non-metastatic cases at the drawn time found a total of 17 recurrence events during the follow-up, and 9 of them were found in cases with presence of ctDNA (p=0.053). Lesion images of three cases are described in Figure 1. Median time between blood draw and clinical recurrence was 10.3 months (3.7-29.8 months) for cases with presence of ctDNA and 13.5 months (4.7 – 26.3) (p=0.963) for those without. There was a numerical higher median copy number (3.36 copies/mL (n= 9) vs 1.72 (n= 40); p=0.339) and allele fraction (8.44% (n= 6) vs 3.84% (n= 30); p=0.137) among those who developed or not clinical recurrences in the subset of cases with positive ctDNA, however, they did not achieve statistical significance. High or low (below the median) copy number (p=0.694) or allelic fraction (p=0.755) was not associated with TIL levels.

Survival analysis

The median follow-up from the plasma drawn was 29.3 months (range 1.4– 44.4 months). Shorter survival was associated with TNBC phenotype (p=0.005) and advanced stages (p<0.001) (Figure 2, 3). Presence of ctDNA tended to be associated with shorter survival (73.4 vs 79.8% at 3 years, p=0.083) in the whole series (Figure 4) and the association achieved significance in the advanced disease group (stage IV/Recurrent disease at the blood drawn time group, n= 31) (20.0 vs 43.8% at 3 years, p=0.010).

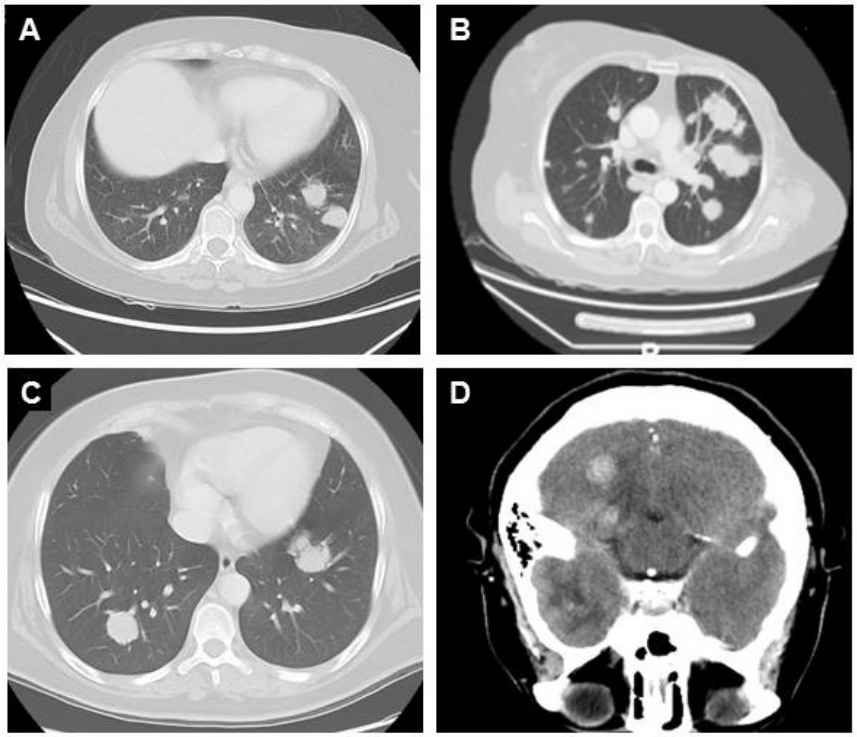

Figure 1. Lesion Images of Three Early Breast Cancer Patients with Presence of ctDNA and Recurrence. (A) Lung metastases were diagnosed after 20 months of detection of ctDNA E545K mutation in a 59 years old woman with stage III metaplastic triple-negative breast cancer (TNBC). After liquid biopsy, the patient underwent neoadjuvant doxorubicin, paclitaxel, mastectomy, and adjuvant capecitabine and radiation. (B) Lung metastases were diagnosed after 3 months of detection of ctDNA E542K mutation in a 74 years old woman with stage III ductal grade-3 TNBC. Before liquid biopsy, the patient completed neoadjuvant doxorubicin, paclitaxel, mastectomy, and adjuvant radiation. (C, D) Lung and brain metastases were diagnosed after 30 months of ctDNA E545K mutation in a 46 woman with stage I grade-3 ductal TNBC. After liquid biopsy patient underwent tumorectomy/sentinel node biopsy, adjuvant doxorubicin, paclitaxel, and radiation.

Similarly, the analysis of the 152 non metastatic cases at the drawn time found that recurrences were associated with shorter overall survival (p<0.001), however, ctDNA was not associated with shorter disease survival (p=0.13) nor overall survival in this subgroup (p=0.8).

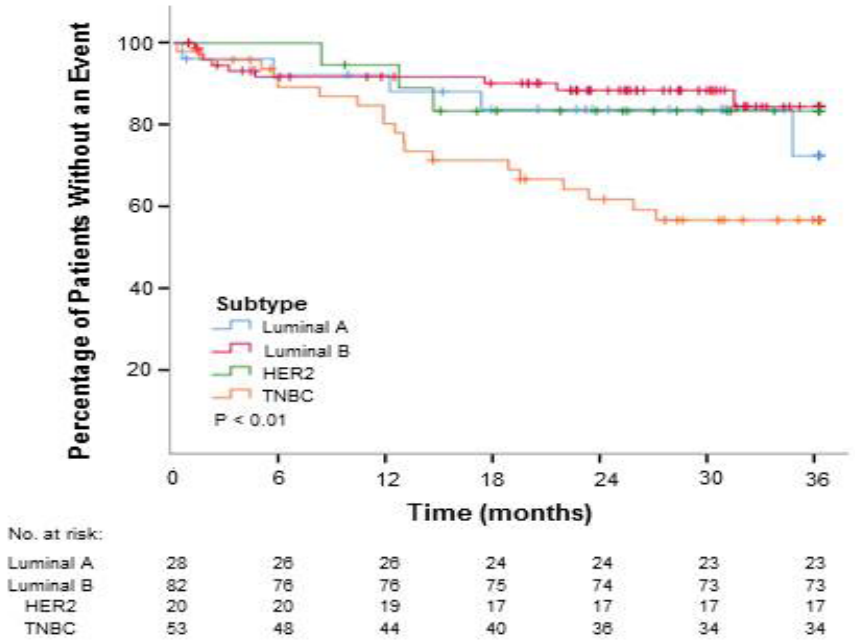

Figure 2. Kaplan-Meier Curve for Overall Survival by Subtype

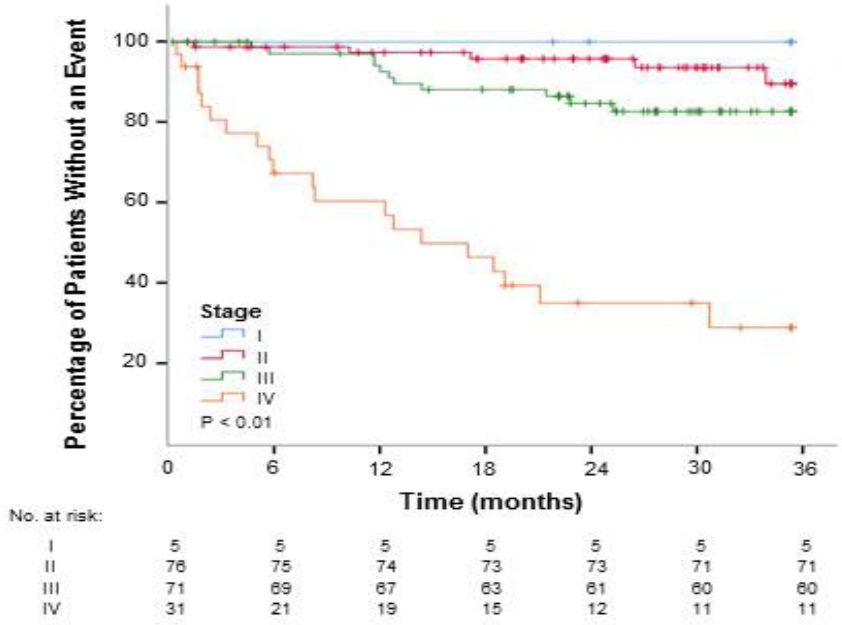

Figure 3. Kaplan-Meier Curve for Overall Survival by Stage

Discussion

PIK3CA hotspot mutations in plasma was detected in 35% of the cases which is in the previously reported range (25-40%) (Garcia-Murillas et al., 2019; Olsson et al., 2015). The detection of PIK3CA mutation in plasma was associated with lower TIL level and tends to predict shorter survival.

TILs are lymphocytes that have penetrated tumor stroma, and laboratory studies indicate that they can detect tumor cells and control their spread (Dieci et al., 2021). Different studies have described that higher TIL level is associated with longer survival (Galvez et al., 2018; Giuliano et al., 2017; Salgado et al., 2015). Our remarkable finding of an inverse relationship between TIL level and presence of PIK3CA mutation in plasma could indicate that impaired immune activity against the tumor allows the release of ctDNA into the blood.

Presence of PIK3CA mutation in ctDNA has previously been identified as a marker of shorter survival in advanced breast cancer (Olsson et al., 2015) and has been described to predict clinical recurrence more than ten months earlier than images in early breast cancer series (Garcia-Murillas et al., 2019). We confirmed the negative prognosis value of ctDNA in the advanced disease group and the trend in the whole group. We also found that presence of ctDNA tends to predict recurrence in our non- metastatic cases. Additionally, the analysis of mutant allele fraction and

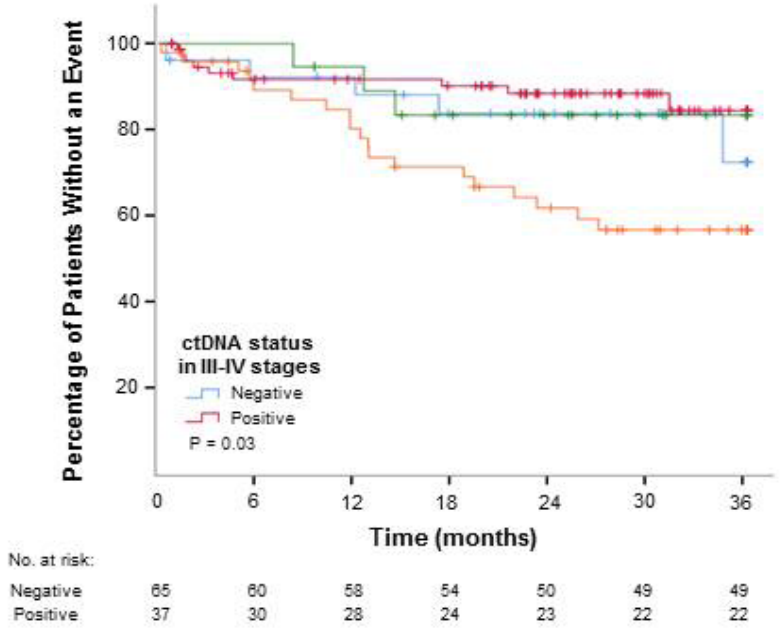

Figure 4. Kaplan-Meier Curve for Overall Survival by ctDNA Status

mutant copy number in plasma found numerically higher median values in cases who develop clinical recurrence during follow- up as has been reported by other series (Oshiro et al., 2015).

Presence of PIK3CA mutations in ctDNA tended to be associated with advanced clinical stage in our series and confirms that a positive liquid biopsy correlates with disease extension as previously described (García-Saenz et al., 2017; Higgins et al., 2012; Kodahl et al., 2018; Zhou et al., 2019).

A weakness of this study is that our population is not homogeneous and the sample size is small, however, evaluation and analysis have been developed in a homogeneous pattern as it is a single institution. Overall, our results describe a successful experience in implementing digital PCR methodology for evaluating ctDNA in a South-American Cancer Center. As precision medicine is incorporating into the routine treatment of breast cancer, the introduction of liquid biopsy into our high-volume facility is expected to improve patient outcomes and give more tools for a better decision of local breast cancer tumor boards.

In conclusion, Detection of hotspot PIK3CA mutations in ctDNA tend to be associated with shorter survival and is related to lower TIL levels in the tissue tumor.

Author Contribution Statement

CAC and HLG conceived the study, CAC and MC designed, and supervised the study, analyzed and interpreted the participants' data, then wrote the manuscript, JMC, JD, MDLC, JA, MAP, KR, HF, EP, HLG, JM and HG, collected and interpreted the participants' data, JS, HG, NS and LAB collected and analyzed the participants' data. All authors interpreted the participants' data, then revised and edited the manuscript. All authors read, edited and approved the final manuscript.

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Ethics approval and consent to participate

All procedures conducted in the study were under the ethical standards of the institutional review board of the of Instituto Nacional de Enfermedades Neoplasicas (approval no. INEN-16-30). The study was processed under the ethical standards of the Helsinki declaration. Informed consent for this study was obtained from all patients.

Conflict of interest

The authors declared no potential conflicts of interest.

Data avail ability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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