Intraoperative prediction of non-sentinel lymph node metastases in breast cancer using cytokeratin 19 mRNA copy number: A retrospective analysis

HELOÏSE PINA[1], JULIA SALLERON[2], PAULINE GILSON[1,3], MARIE HUSSON[1], MARIE ROUYER[1], AGNES LEROUX[1], PHILIPPE RAUCH[4], FREDERIC MARCHAL[3,4], MATHILDE KÄPPELI[3], JEAN-LOUIS MERLIN[1,3] and ALEXANDRE HARLÉ[1,3]

Départements de [1]Biopathologie, [2]Biostatistique, Institut de Cancérologie de Lorraine, F-54519 Vandœuvre-lès-Nancy; [3]Université de Lorraine, CNRS UMR 7039 CRAN; [4]Département de Chirurgie, Institut de Cancérologie de Lorraine, F-54519 Vandœuvre-lès-Nancy, France

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Abstract. One-step nucleic acid amplification (OSNA) is a molecular procedure used intraoperatively for the detection of sentinel lymph node (SLN) metastases. The aim of the present study was to define a cut-off of cytokeratin (CK)19 mRNA copy number predictive of positive completion axillary lymph node dissection (ALND). The OSNA procedure was employed for SLN analysis in 812 patients with T1-T2 N0 breast cancer. A total of 197 patients with SLN metastases were retrospectively analyzed. A total of 40 patients (20%) had non-SLN metastases. Receiver operating characteristics curve analysis established a cut-off of 5,000 CK19 mRNA copy number with 75% sensitivity and 72% specificity. The positive and negative predictive values were 40.5 and 92%, respectively. Multivariate analysis showed that this cut-off and tumor localization in the outer or lower-outer quadrant of the breast were significantly associated with non-SNL involvement (P<0.001 and P=0.025, respectively). The findings of the present study support the conventional cut-off of 5,000 copies for intraoperative decision to perform ALND, whereas ALND can safely be avoided in patients with tumor located outside the outer or lower-outer quadrant of the breast if the CK19 mRNA copy number is <5,000.

Introduction

Breast cancer is the most common malignancy in women, accounting for 627,000 deaths worldwide in 2018 (1). Lymph node involvement is one of the most important prognostic factors in breast cancer (2). Axillary lymph node dissection (ALND) significantly reduces recurrence and improves regional control and nodal staging, which is important for the selection of adjuvant therapy (3) and prognostic evaluation (4). However, ALND is associated with various adverse side effects, such as lymphedema, numbness, chronic pain, seroma or infection, and its impact on survival and recurrence is subject to controversy (5,6).

Sentinel lymph node biopsy (SLNB) is a common procedure used to detect the presence of metastatic cells and to decide whether ALND is required. SLNB may also help with breast cancer staging (7). Histological examination of step section or serial section slides of SLNs is the most widely used method. One-step nucleic acid amplification (OSNA; Sysmex Corporation) is an alternative loop-mediated isothermal amplification (LAMP)-based semi-quantitative assay that quantifies copies of cytokeratin (CK)19 mRNA, which is expressed in most breast cancer cells (8). The determination of CK19 mRNA copy number can predict the presence of micro- or macro-metastases in the SNL (9). Several studies have shown that OSNA is more sensitive and objective compared with histological examination (10). It is also a cost-effective strategy (11,12) that is widely used in Europe and Japan (13,14).

OSNA is an intraoperative procedure; therefore, ALND can be performed during the same surgery, thereby avoiding a second surgery. The OSNA procedure also makes it possible to commence adjuvant treatment earlier (15). It is also important to identify patients in whom ALND can safely be avoided, without increasing the risk of recurrence (16,17).

The aim of this retrospective study was to determine whether CK19 mRNA copy number in the SNL could predict positivity of ALND.

Materials and methods

Study population. A total of 812 patients with early-stage invasive breast cancer underwent breast surgery, SNL biopsy and OSNA analysis between January 2010 and August 2014 at the Institut

Correspondence to: Professor Alexandre Harlé, Département de Biopathologie, Institut de Cancérologie de Lorraine, 6 Avenue de Bourgogne, CS 30519, F-54519 Vandœuvre-lès-Nancy, France
E-mail: a.harle@nancy.unicancer.fr

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de Cancérologie de Lorraine (ICL; Vandoeuvre-lès-Nancy, France). Surgery and OSNA procedures were decided for all patients with clinically or ultrasonographically node-negative cT1-2 breast cancer. All patients provided informed oral consent and a signed a non-opposition form and the study was approved by the Ethics Committee of the ICL (CAV-2009-osna).

The exclusion criteria were as follows: i) Patients who had received neoadjuvant treatment, had undergone previous ipsilateral breast or axillary surgery, had cT3-T4 tumors, and clinically or ultrasonographically positive axilla confirmed by fine-needle aspiration biopsy; ii) a total of 20 patients were excluded due to positive inhibition status (+I), corresponding to values greater than the highest point of the calibration curve, making the determination of exact number of copies not possible; and iii) a total of 49 patients were also excluded as one central slice of their SLNs had been investigated by histology, potentially decreasing the number of copies of CK19 mRNA detected. Data collected from each patient are listed in Table I. Data for the current study were obtained from the prospective breast cancer database at the ICL. All data were anonymized prior to analysis to protect patient confidentiality.

SLN biopsy procedure. SLNs were localized using the isotope method, alone or combined with the dye procedure. The isotope method consisted of ^{99m}Tc-labeled rhenium sulfur (Amersham; Cytiva) periareolar injection the day before surgery, followed by lymphoscintigraphy 1-3 h later. The dye procedure consisted of 2 ml of patent blue dye (Guerbet) administered by subareolar injection at surgery. SLNs were identified using a hand-held gamma-probe (Europrobe 3; Euromedical Instruments), isolated, and perinodal fat was removed. All suspicious lymph nodes identified during surgery were sent for analysis. Data on the SLNs included their color (blue or not), localization, signal intensity and size.

Lymph nodes with a weight of >0.6 g were subdivided into two or more samples and processed separately, as recommended by the manufacturer of the OSNA assay (Sysmex Corporation). A maximum of 4 samples were assessed per run, for a total running time of 15-60 min for 1-4 samples, respectively.

Histopathology. Each non-SNL was measured, cut longitudinally into 2-mm sections, fixed in formalin for 8 h at room temperature and embedded in paraffin. The sections were then prepared for hematoxylin and eosin staining.

Breast tumors were examined by hematoxylin and eosin staining. CK19 (clone RCK 108; cat. no. M0888; Agilent Technologies, Inc.), hormonal receptors, including estrogen receptor (ER; clone SP1; cat. no. 790-4325) and progesterone receptor (PR; clone 1E2; cat. no. 790-4296; Ventana Medical Systems, Inc.; Roche Diagnostics), HER2 (clone 4B5; cat. no. 790-4493; Ventana Medical Systems, Inc.; Roche Diagnostics) and Ki-67 (clone MIB-1; cat. no. M7240; Agilent Technologies, Inc.) expression were determined using immunohistochemistry. All assays were automated using Benchmark (Roche Diagnostics) according to the manufacturer's protocols. Histopathological categories were defined according to the sixth edition of the TNM classification (18).

OSNA analysis and mRNA CK19 copy determination. The OSNA assay was processed as previously described using the OSNA BC System (Sysmex Corporation) (9). Briefly, whole SLNs were homogenized in 4 ml Lynorhag lysis buffer (Sysmex Corporation). The homogenate was centrifuged at 10,000 x g for 1 min at room temperature and directly used as a template for amplification. CK19 mRNA detection was assessed using reverse transcription-LAMP with the RD-100i analyzer (Sysmex Corporation).

Results for each sample were presented on the RD-100i instrument in qualitative categories along with the CK19 mRNA copy number/μl. The (-), (+), (++) and (+I) symbols were used by the OSNA instrument to indicate copy numbers of <250, 250-5,000, >5,000 and greater than the highest point of the standard curve, respectively.

According to the cut-off levels defined by Tsujimoto *et al* (9), a copy number between 250 and 5,000 copies/μl (+) was considered as predictive of the presence of SLN micrometastases in the analyzed lymph node, and a copy number >5,000/μl (++) was considered as predictive of the presence of SLN macrometastases. A copy number <250 copies/μl was considered as predictive of the absence of tumor cells.

The number of copies was then estimated using the number of copies measured in a 1/10 dilution of the sample. The node total copy number was estimated by adding CK19 mRNA copies of each piece of the sample, in nodes weighing >0.6 g. Tubes containing more than one node for the same patient were excluded from the analysis. Only the SLN with the highest number of copies was considered for each patient.

Statistical analysis. Statistical analysis was performed using SAS software version 9.4 (SAS Institute Inc.). The significance level was set at 0.05. Qualitative variables are described as number and percentage, and quantitative variables as mean ± standard deviation, or median and interquartile range (IQR), according to the normality test (Kolmogorov-Smirnov test). Predictive factors of positive ALND were investigated using bivariate logistic regression and the results are expressed as ORs and 95% CIs. The log-linearity assumption of the logistic model was checked by categorizing each variable in 10 groups (corresponding to deciles) and by examining the plots of the logit of observed percentages of positive ALND in each class. Quantitative variables were transformed into binary variables if the log-linearity assumption was violated, using the threshold maximizing sensitivity and specificity (Youden index). All variables with a P-value <0.10 in bivariate logistic regression were included in a multivariate logistic regression model with backward selection at P=0.10. The results of the final multivariate model are presented as adjusted ORs (95% CIs). The stability of the selected model was investigated using the bootstrap resampling method (19).

Results

Study population. Among the 812 patients who underwent OSNA analysis, 246 patients had at least one positive SLN. Among these, a total of 197 patients with positive OSNA analysis were included in this retrospective study (Fig. 1). A comparison of the characteristics of included (n=197) vs. excluded (n=615) patients is presented in Table I. Patient and disease characteristics are summarized in Table II. Patients with SLN micro- or macrometastases as determined by OSNA

Table I. Comparison of clinicopathological characteristics between the included and the excluded patients.

Characteristics	All patients (n=812), n (%)	Included (n=197), n (%)	Excluded (n=615), n (%)	P-value
Age, years (mean ± SD)	60±11	59±11	61±12	0.264
Body mass index ≥30 kg/m^2	170 (20.9)	42 (21.3)	128 (20.8)	0.879
Tumor size ≥13 mm	377 (46.4)	111 (56.3)	266 (43.2)	0.001
Bloom-Richardson histological grade				
1	229 (29.6)	59 (30.3)	170 (29.4)	0.787
2	397 (51.3)	102 (52.3)	295 (50.9)	
3	148 (19.1)	34 (17.4)	114 (19.7)	
Tumor localization				
Outer or lower-outer quadrant	165 (20.7)	35 (17.8)	130 (21.6)	0.245
Other	633 (79.3)	162 (82.2)	471 (78.4)	
Histological type				
Ductal	598 (73.6)	153 (77.7)	445 (72.4)	0.070
Lobular	76 (9.4)	21 (10.7)	55 (8.9)	
Other	138 (17.0)	23 (11.7)	115 (18.7)	
Positive ER status	742 (91.4)	183 (92.9)	559 (90.9)	0.384
Positive PR status	635 (78.2)	156 (79.2)	479 (77.9)	0.700
Positive ER and/or PR status	751 (92.5)	183 (92.3)	568 (92.5)	0.804
Positive HER2 receptor status	52 (6.4)	13 (6.6)	39 (6.3)	0.898
Triple-negative breast cancer	47 (5.8)	12 (6.1)	35 (5.7)	0.834
Sentinel lymph nodes removed, median (range)	3 (2-4)	2 (2-4)	3 (2-4)	0.081

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor.

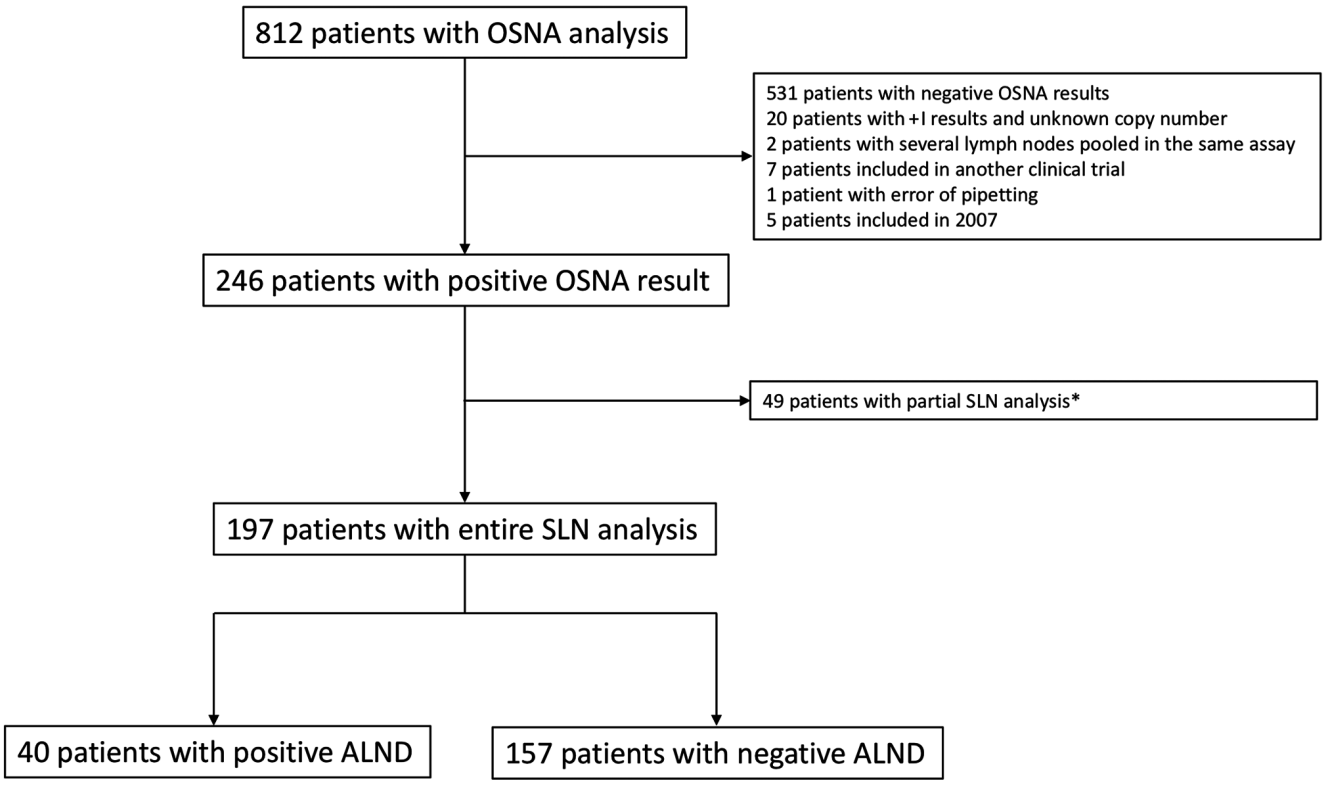

Figure 1. Study workflow diagram. Among the 812 patients who underwent OSNA analysis, 246 patients had at least one positive SLN. Among these, a total of 197 patients with positive OSNA analysis were included in this retrospective study. OSNA, one-step nucleic acid amplification; SLN, sentinel lymph node; ALND, axillary lymph node dissection. *, patients were excluded if the lymph node was analyzed only partially.

Table II. Clinicopathological characteristics for the 197 patients and according to the positivity of ALND.

Characteristics	All patients (n=197), n (%)	Negative ALND (n=157), n (%)	Positive ALND (n=40), n (%)	P-value
Age, years (mean ± SD)	59±11	60±11	58±13	0.296
Body mass index ≥30 kg/m^2	42 (21.3)	29 (18.5)	13 (32.5)	0.053
Tumor size ≥13 mm[a]	111 (56.3)	82 (52.2)	29 (72.5)	0.021
Bloom-Richardson histological grade				0.548
1	59 (30.3)	49 (31.6)	10 (25.0)	
2	102 (52.3)	78 (50.3)	24 (60.0)	
3	34 (17.4)	28 (18.1)	6 (15.0)	
Tumor localization				0.023
Outer or lower-outer quadrant	35 (17.8)	23 (14.6)	12 (30.0)	
Other	162 (82.2)	134 (85.4)	28 (70)	
Histological type				
Ductal	153 (77.7)	118 (75.2)	35 (87.5)	0.144
Lobular	21 (10.7)	20 (12.7)	1 (2.5)	
Other	23 (11.7)	19 (12.1)	4 (10.0)	
Positive ER status	183 (92.9)	145 (92.4)	38 (95.0)	0.739
Positive PR status	156 (79.2)	121 (77.1)	35 (87.5)	0.147
Positive ER and/or PR status	183 (92.3)	145 (92.4)	38 (95.0)	0.739
Positive HER2 receptor status	13 (6.6)	11 (7.0)	2 (5.0)	1
Triple-negative breast cancer	12 (6.1)	10 (6.4)	2 (5.0)	1
SLNs removed, median (range)	2 (2-4)	2 (2-3)	2.5 (2-4)	0.38
Positive SLNs, median (range)	1 (1-1)	1 (1-1)	1 (1-2)	0.23
1	147 (74.8)	121 (77.1)	26 (65.0)	
2	39 (19.4)	30 (19.1)	9 (22.5)	
3	10 (5.2)	6 (3.8)	4 (10.0)	
4	1 (0.6)	0	1 (2.5)	
SLN maximal copy number/μl, median (range)	2,100 (540-38,000)	1,300 (530-6,900)	60,830 (5,000-695,000)	<0.001
≥4,700[a]	77 (39.1)	46 (29.3)	31 (77.5)	<0.001
≥5,000	74 (37.6)	44 (28.0)	30 (75.0)	<0.001

[a]Threshold value maximizing the sensitivity and the specificity (Youden index). ALND, axillary lymph node dissection; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; SLN, sentinel lymph node.

underwent ALND. Patients with a negative OSNA result did not undergo further ALND.

OSNA analysis. The median number of SLNs removed was 2 (IQR, 2-4). A total of 123 patients (62%) had SLN micrometastases, while 74 had SLN macrometastases (38%). The median number of ALNs removed was 15 (IQR, 13-19.5) with a median of 2 (IQR, 1-3) positive ALNs. A total of 40 patients (20%) had non-SNL metastases. The patient characteristics according to lymph node status are presented in Table II. A tumor size >13 mm localized in the outer quadrant (OQ) or lower-outer quadrant (LOQ) of the breast was more frequent in the group with positive ALND. Two or more positive SLNs were found in all patients with positive ALND. The CK19 mRNA copy number was also higher in the positive ALND group [median, 60,830 (IQR, 5,000-695,000) vs. 1,300 (IQR, 530-6,900)]. The threshold of 4,700 CK19 mRNA copies was found to be the optimal cut-off for distinguishing patients with vs. those without non-SNLs. The value of 5,000 CK19 mRNA copies commonly used to differentiate micrometastases and macrometastases was retained, since only 3 patients had values between 4,700 and 5,000. A total of 30 patients of the group with positive ALND (75%) had SLN macrometastases vs. only 28% in the group with negative ALND, corresponding to a specificity of 72% (113/157). The positive predictive value was 40.5% (30/74) and the negative predictive value (NPV) was 92% (113/123). The factors predictive of positive ALND are presented in Table III, whereas a comparison of the OSNA cut-off of the present study with other alternatives from the literature for prediction of non-SLN metastasis is presented in Table IV.

By multivariate analysis, two parameters remained significantly associated with positive ALND, namely SLN macrometastases (OR=8.07, 95% CI: 3.58-18.23) and tumor localization in the OQ [centered around the 3 o'clock position (left breast) or 9 o'clock position (right breast)] or LOQ

Table III. Predictive factors for positive axillary lymph node dissection by bivariate and multivariate logistic regression analysis.

	Bivariate analyses		Multivariate analysis	
Factors	OR (95% CI)	P-value	OR (95% CI)	P-value
Age (per 1-year increase)	0.98 (0.95-1.01)	0.295		
Body mass index ≥30 kg/m^2	2.12 (0.98-4.61)	0.056		
Tumor size >13 mm	2.41 (1.13-5.16)	0.023		
Bloom-Richardson histological grade		0.551		
1	1.0 (reference)			
2	1.51 (0.66-3.42)			
3	1.05 (0.34-3.20)			
Tumor localization in the outer or lower-outer quadrant	2.50 (1.11-5.60)	0.026	2.84 (1.14-7.05)	0.025
Ductal carcinoma	2.31 (0.85-6.32)	0.102		
Copy number ≥5,000/μl	7.70 (3.48-17.08)	<0.001	8.07 (3.58-18.23)	<0.001
Positive ER status	1.57 (0.34-7.33)	0.564		
Positive PR status	2.08 (0.76-5.71)	0.154		
Positive ER or PR status	1.57 (0.34-7.38)	0.564		
Positive HER2 status	0.70 (0.15-3.29)	0.650		
Triple-negative breast cancer	0.77 (0.16-3.68)	0.747		

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

(OR=2.84; 95% CI: 1.14-7.05). The AUC was 0.77 (95% CI: 0.69-0.85). Considering that 20% of the patients had non-SLN metastases in our population (40/197), the estimated probability from the multivariate model was 36% for patients with SLN macrometastases and tumor outside of the OQ or LOQ, and 61% for patients with SLN macrometastases and tumor localization within the OQ or LOQ. The estimated probability of positive ALND for patients with micrometastases was 6% in case of tumors located outside of the OQ or LOQ and 16% in case of tumors within the OQ or LOQ.

Discussion

Intraoperative SLN evaluation has limited ability to detect metastases due to the partial evaluation of the node (10,11). SLN histopathological examination is thus performed post-operatively in several centers. Since 2007, the OSNA assay has been used as an objective, simple and automated tool for the intraoperative assessment of whole SLNs. The high concordance of OSNA with histological techniques has been shown in several studies (20-23), as has its high sensitivity and specificity (24). OSNA avoids sampling errors and second-stage surgeries due to false-negative results, without increasing operative time, except in breast-conserving surgery (25).

However, the need for ALND in SLN-positive early breast cancer remains controversial. ALND is a possible cause of morbidity, incurs greater costs and is associated with lower quality of life (26). Furthermore, the selection of adjuvant therapy currently relies more on the characteristics of the primary tumor rather than on the number of affected lymph nodes. Several studies have shown poorer prognosis and a higher recurrence rate in cases with SLN micrometastases without adjuvant therapy (6,27,28). In line with the findings of the IBCSG 23-01 trial (5), the American College of Surgeons Oncology Group (ACOSOG) Z0011 randomized trial (16) stated that ALND could be avoided in patients with T1-T2 N0 breast cancer and 1-2 SLN metastases undergoing breast-conserving surgery and receiving adjuvant whole-breast irradiation and adjuvant systemic therapy. In the AMAROS trial (17), the authors demonstrated the non-inferiority of axillary radiotherapy vs. ALND in patients with SLN micro- or macrometastases.

Due to certain limitations in these trials, including a high rate of loss to follow-up (18.6% in the ACOSOG study), and imbalances in several prognostic characteristics between groups (29,16), several trials are still ongoing to confirm these results (30). The 2015 National Comprehensive Cancer Network guidelines (14) already stated that ALND was not necessary in the population of the ACOSOG trial. Current recommendations in France indicate that ALND is necessary if SLN macro-metastases are present, whereas multidisciplinary discussion is recommended in case of micrometastases (31).

The use of objective tools capable of predicting non-SLN axillary involvement could therefore be useful, at least for patients who do not meet the Z0011 criteria, or who were underrepresented in that trial (for example, patients with invasive lobular carcinoma, estrogen receptor-negative status, or age <50 years). An optimal negative cut-off would also help to identify patients who would not benefit from ALND, given that it can safely be omitted if SLN is negative (32).

Many available prediction models for positive ALND are based on factors that cannot be determined preoperatively and are therefore not clinically relevant. The OSNA procedure can be performed during surgery and is an independent predictive factor of potential further axillary metastasis progression, with a good diagnostic capacity [area under the receiver operating characteristics curve (AUC) = 0.77 in the present study].

Table IV. Comparison of our OSNA cut-off with other alternatives from literature for prediction of non-SLN metastasis.

Study (Refs.)	Number of patients	Threshold (copies/μl)	Se	Sp	PPV	NPV	FN, n (%)	FP, n (%)	Method	AUC	Problems
Present study	812 patients 197 OSNA+	5,000	75.0	72.0	40.5	91.9	10 (8.1)	44 (59.5)	Maximal copy number	0.77	
Peg *et al* (37)	697 patients OSNA+	15,000	76.7	55.2	41.1	85.5	14.7%		TTL	0.709	T1-T3 breast tumors.
Deambrogio *et al* (38)	1,080 patients 194 OSNA+	7,700	78	57	50	83	15 (17.4)	54 (50)			T1-T3 breast tumors. 46 patients with OSNA+ analysis did not undergo further surgery.
Heilmann *et al* (39)	143 patients 39 OSNA+	7,900	91	61							T1-T3 breast tumors. Part of the lymph node analyzed by histology.
Terrenato *et al* (20)	1,140 patients 318 OSNA+	2,150	94.9	51.4	46.5	95.8	5 (4.2)	107 (53.5)	TTL	0.765	No description of +I case management. Lack of representation of cancers other than ductal or lobular carcinomas.
Nabais *et al* (40)	598 patients 58 OSNA+	190,000	73.3	74.4		88.9			TTL	0.805	T1-T3 breast tumors.
Banerjee *et al* (41)	170 patients 49 OSNA+	1,400									T1-T3 breast tumors. 50% of the lymph node analyzed by OSNA.
Espinosa-Bravo *et al* (42)	306 patients 108 OSNA+	120,000	47	85.3	56	80			TTL		
Buglioni *et al* (43)	709 patients 179 OSNA+	2,000									50% of the lymph node analyzed by OSNA.

Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; FN, false-negative; FP, false-positive; AUC, area under receiver operating characteristics curve; TTL, total tumor load; OSNA, one-step nucleic acid amplification.

Predictive cut-offs of CK19 mRNA copy number have already been investigated in other studies (20,37-43). Some authors (43) considered the maximal copy number, whereas others (20,37,40,42) considered the total tumor load (TTL), defined by the number of CK19 mRNA copies in all positive SLNs. TTL could be considered as more representative of the tumor cell load, but is linked to the number of SLNs analyzed during the procedure, which depends on the highly variable standard practices in each center. This variability in practices may explain the differences between the cut-offs across published studies.

In some studies (39,41), SLN sections were used for histological evaluation. Our supplementary analysis including the 49 patients with histological analysis of SLN sections confirmed that this practice may lead to possible underestimation of CK19 mRNA total copies. This analysis yielded a cut-off of 3,500 copies [AUC=0.741 (95% CI: 0.657-0.825), data not shown]. A false-negative result would prevent some

patients from undergoing ALND. The undervaluation of copy number could also explain the varying cut-offs reported in published studies (40,41). There is potential for bias in our study due to the exclusion of 20 +I cases the total copy number of which was not available (33) and because of the 49 cases with histological analysis of the central section of the SLN. Some disadvantages of the OSNA assay must also be considered, such as te inability to conduct further histological analysis.

Shimazu *et al* (34) proposed an intraoperative nomogram based on tumor size and TTL, but their NPV and AUC were lower compared with those in the present study. Furthermore, a central section was removed for histological examination in one institution.

In our cohort of patients, when the copy number was <5,000, 113 patients had no further axillary involvement (92%) and only 10 patients (8%) had positive ALND. These results indicate that ALND can safely be avoided when the tumor is localized outside of the OQ or LOQ of the breast, and the copy number is <5,000. A total of 30 ALNDs were positive when CK19 mRNA was >5,000 copies (41%). These results support the concept that ALND must be considered in this case, particularly when the tumor is in the OQ. We believe that the high cut-offs described by Heilmann *et al* (39), Deambrogio *et al* (38) or Peg *et al* (37) may result in a very high false-negative rate. We herein confirmed that ALND can safely be avoided in patients with tumors in the other quadrants if the CK19 mRNA copy number is <5,000. These results are almost in line with previous published studies (20,38), and the copy threshold for OSNA was confirmed based on a large cohort. The present study may also help to overcome certain drawbacks of previous studies (39,41), such as partial evaluation of the node.

Predictive thresholds for non-SLN positivity should be assessed in other cancers, such as cervical cancer, in which pelvic lymphadenectomy results are negative in >80% of cases (35). The OSNA assay may also contribute to prognostic evaluation (36).

In conclusion, a cut-off of 5,000 copies for CK19 mRNA combined with tumor localization may represent an intraoperative objective and useful tool for predicting further non-SLN axillary involvement and the need for completion ALND in patients with breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization, JLM, PR and FM; methodology, AH and JLM; validation and interpretation of raw data, AH, FM and JLM; formal analysis, JS; data collection, MK, MR, MH, PG and HP; resources, PR, FM and JLM; data curation, MK and AH; original manuscript draft preparation, HP; manuscript review and editing, AH; visualization, HP and AH; supervision, AH; project administration, JLM, AL and AH. All the authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

All patients provided informed oral consent and a signed a non-opposition form and the study was approved by the Ethics Committee of the ICL (CAV-2009-osna).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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