

Intraoperative prediction of the two axillary lymph node macrometastases threshold in patients with breast cancer using a one-step nucleic acid cytokeratin-19 amplification assay

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Abstract. The aim of the present study was to assess the sensitivity, specificity and practicality of using a one-step nucleic acid amplification (OSNA) assay during breast cancer staging surgery to predict and discriminate between at least 2 involved nodes and more than 2 involved nodes and facilitate the decision to provide axillary conservation in the presence of a low total axillary node tumour burden. A total of 700 consecutive patients, not treated with neo-adjuvant chemotherapy, received intraoperative sentinel lymph node (SLN) analysis using OSNA for cT1-T3 cN0 invasive breast cancer. Patients with at least one macrometastasis on whole-node SLN analysis underwent axillary lymph node dissection (ALND). The total tumour load (TTL) of the macrometastatic SLN sample was compared with the non-sentinel lymph node (NSLN) status of the ALND specimen using routine histological assessment. In total, 122/683 patients (17.9%) were found to have an OSNA TTL indicative of macrometastasis. In addition, 45/122 (37%) patients had NSLN metastases on ALND with a total positive lymph node burden exceeding the American College of Surgeons Oncology Group Z0011 trial threshold of two macrometastatic nodes. The TTL negative predictive value was 0.975 [95% confidence interval (CI), 0.962-0.988]. The area under the curve for the receiver operating characteristic curve was 0.86 (95% CI, 0.81-0.91), indicating that SLN TTL was associated with the prediction (and partitioning) of total axillary disease burden. OSNA identifies a TTL threshold value where, in the presence of involved SLNs, ALND may

be avoided. This technique offers objective confidence in adopting conservative management of the axilla in patients with SLN macrometastases.

Introduction

Sentinel lymph node biopsy (SLNB) has replaced complete axillary lymph node dissection (ALND) as the current standard of care for axillary node staging in patients with clinically node-negative breast cancer (1). When the sentinel nodes are found to be free from metastatic disease, no further axillary treatment is recommended (1). The role of ALND, for patients with positive sentinel lymph nodes, is currently being redefined. Research has indicated that patients with sentinel lymph node (SLN) micrometastases treated with SLNB alone have similar disease-free and overall survival to those receiving ALND (2). This has also been observed for stage T1-2 disease with ≤ 2 macro-metastatic SLNs treated with breast conservation surgery, whole-breast radiotherapy and adjuvant systemic therapy (3). Therefore, ALND may be over-treatment for patients with early breast cancer and a low burden of axillary node involvement.

A reliable intraoperative technique that predicts non-sentinel lymph node (NSLN) involvement would offer selective and more conservative treatment of the axilla in a single surgical procedure. This would avoid unnecessary surgery and its associated morbidity while providing benefits to the patient, conserving resources and complying with emerging clinical practice guidelines (4).

SLN assessment with one-step nucleic acid amplification (OSNA) provides an intraoperative molecular-based objective whole-node assessment of SLN disease burden that is independent of the size or number of lymph nodes tested (5). For these reasons, OSNA has greater potential to predict NSLN involvement than routine histopathology assessment, which cannot offer timely intraoperative SLN evaluation, remains subjective, categorical, and is exposed to sampling errors with sub-total node assessment (6,7). OSNA amplifies cytokeratin-19 (CK19) mRNA in SLN samples, typically providing a quantitative measurement of metastatic disease burden in 35 min. The total

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CK19 mRNA copy number of a SLN biopsy (total tumour load, TTL) may predict NSLN involvement and axillary node disease burden, facilitating the decision to proceed with, or avoid, complete ALND.

The present study compared SLN OSNA TTL with NSLN involvement following ALND, and assessed the sensitivity and specificity of TTL, and patient and tumour characteristics to predict NSLN metastatic burden. The present findings have generated a selective treatment protocol for the conservative management of the axilla in patients with early breast cancer that may be used in the pre- and intraoperative setting.

Patients and methods

Patients. The present study was a retrospective, single-centre cohort study of patients diagnosed with breast cancer from symptomatic and screening services. A total of 700 consecutive patients (681 females and 3 males; mean age, 62 years; range, 23–93 years) treated between December 2012 and August 2015 at the Royal Hallamshire Hospital (Sheffield, UK) who underwent a successful SLN biopsy with OSNA assessment were studied. The patients had primary invasive cT1–3 breast carcinoma, a clinically negative axilla, normal pre-operative axillary ultrasound, or benign ultrasound-guided axillary node biopsy, and were medically fit for general anaesthetic and axillary treatment. Patients with prior neo-adjuvant chemotherapy, ipsilateral axillary surgery, recurrent disease or extensive ductal carcinoma *in situ* (DCIS) without invasion were excluded. Subset analysis was performed for patients who had metastatic axillary disease identified by OSNA and subsequent ALND. The following parameters were recorded: Age, tumour size and grade, multifocality, histological subtype, type of surgery, oestrogen receptor status, human epidermal growth factor receptor 2 (HER2) status, Ki67, the presence of lymphovascular invasion, total number of SLNs and NSLNs, the number of positive and negative SLNs and NSLNs, and ratio of the number of positive SLNs to the total number of removed SLNs and NSLNs following ALND. CK19 mRNA copy number (copies/ μ l) in each SLN were recorded. CK19 expression was not routinely tested on pre-operative biopsy specimens. For statistical analysis, when ≥ 2 SLNs were involved, the combined value of CK19 mRNA copies was calculated. The TTL was defined as the total CK19 mRNA copy number in the positive SLNs (with units of copies/ μ l). The TTL of the macrometastatic SLN sample was compared with the total lymph node status and NSLN status of ALND, following routine histological assessment with haematoxylin and eosin staining (8).

SLN identification. SLNs were identified using a standard protocol of combination radiopharmaceutical and blue dye, as described by Mansel *et al* (9). 99m Tc-labelled albumin nanocolloid (Nanocoll[®]; GE Healthcare Life Sciences, Little Chalfont, UK) was injected intradermally (0.1–0.5 ml) at a single periareolar site corresponding to the tumour quadrant; 40 MBq the day before surgery or 20 MBq on the day of surgery. Patent Blue V dye (Laboratoire Guebet, Aulnay-sous-Bois, France), 2 ml undiluted, was injected subdermally at a single periareolar site corresponding to the tumour quadrant immediately prior to surgery. Under general anaesthetic, SLNs were identified and removed prior to breast tumour excision, and sent on ice to

the Pathology Department. No more than two nodes were sent for assessment by OSNA. Any additional SLNs were sent for routine fixation, hematoxylin and eosin staining (8) and delayed reporting. Therapeutic local excision, therapeutic mastoplasty or mastectomy was performed as part of the planned breast cancer treatment. Each SLN, trimmed of fat, was weighed and recorded. SLNs weighing < 50 mg were too small to be processed by OSNA, and were therefore diverted to routine histological assessment. SLNs weighing > 600 mg were divided into two or more pieces and processed separately, and the results combined.

OSNA. The OSNA assay (Sysmex Europe GmbH, Norderstedt, Germany) was performed according to the manufacturer's protocols, as described by Tsujimoto *et al* (5). Each SLN was homogenized in 4 ml homogenizing buffer on ice. The lysate was centrifuged to remove fat, cellular debris and other contaminants and the mRNA containing supernatant was extracted and diluted. A 2- μ l aliquot of the buffered lymph node lysate was used for automated real-time amplification of CK19 mRNA via reverse transcription loop-mediated isothermal amplification with a ready-to-use reagent kit on the RD-100i (Sysmex Europe GmbH). The rate of amplification was measured spectrophotometrically and the CK19 copy number was calculated by comparison to a standard curve. Based on the number of CK19 mRNA copies/ μ l, the result was assessed in accordance with the cut-off levels determined in a study by Tsujimoto *et al* (5), with macrometastasis (OSNA ++) defined as $> 5,000$ copies/ μ l of CK19 mRNA, micrometastasis (OSNA +) as 250–5,000 copies/ μ l and non-metastasis (OSNA -) as < 250 copies/ μ l. The OSNA results were immediately communicated by telephone to the surgeon within 45 min of sample receipt. Patients with at least one macrometastasis on intraoperative OSNA analysis underwent levels I, II and III ALND. Between December 2012 and June 2013, a positive OSNA result for one or two nodes with micro-metastases also triggered an immediate ALND. In June 2013, the departmental protocol was amended to recommend the removal of two further nodes for routine histological processing, with a delayed ALND if these returned macrometastatic involvement.

The remaining lymph nodes not involved in the OSNA test were processed according to the UK Breast Cancer pathology protocol (8). Lymph nodes < 5 mm were bisected whereas larger nodes were sliced at 3-mm intervals and single sections assessed using haematoxylin and eosin staining. Immunohistochemical staining was not used for evaluation of NSLNs.

Preoperative assessment of axilla. Patients underwent axillary ultrasonography (US) at the time of breast assessment, or soon after the diagnosis of breast carcinoma. Those with abnormal lymph node morphology according to local protocol (cortical thickening 2–3 mm, focal bulge, rounded shape, partial or complete loss of fatty hilum, non-hilar blood flow, or partial/complete replacement of node with mass) underwent US-guided lymph node biopsy. Patients with confirmed invasive disease on lymph node biopsy proceeded to neo-adjuvant therapy or ALND without SLNB.

Patient data were anonymised, and collected retrospectively, without influence on patient therapy. The ethical considerations of the present study were approved by the

Clinical Effectiveness Unit of the Sheffield Teaching Hospitals NHS Trust (Sheffield, UK).

Statistical analysis. Distributions of continuous variables were determined by visual inspection of frequency-distribution plots; variables were summarised as the mean and confidence interval (after transformation if required), or median and interquartile range, as appropriate. Tests for association between categorical variables were determined using the Chi-square and Fisher's exact tests. $P < 0.05$ was considered to indicate a statistically significant difference. Receiver operating characteristic (ROC) analyses were performed to compute the area under the curve (AUC) to estimate concordance. The statistical analysis was conducted using SAS software, version 9.4 (SAS Institute, Inc., Cary, NC, USA) for Windows 7.

Results

Clinical cohort. A total of 1,315 patients were diagnosed with breast carcinoma between December 2012 and August 2015 at the institution (Fig. 1). Of these, 700 consecutive patients received OSNA during surgery for a preoperatively identified cT1-T3 cN0 breast carcinoma. Sixteen cases were excluded from the present study; 15 patients had extensive DCIS only and 1 patient had received incomplete neo-adjuvant therapy. There were 681 female patients and 3 male cases (mean age, 62 years; range, 23-93 years) in the remaining study cohort. Of the cases, 9 were bilateral.

In contrast to the American College of Surgeons Oncology Group (ACOSOG) Z0011 study (3), all the patients in our unit underwent axillary US prior to breast surgery. Those with abnormal lymph node morphology underwent immediate US-guided biopsy, and those with confirmed metastatic disease were recommended to undergo ALND without SLNB/OSNA, which was the protocol at the time of the present study. These assessments removed 14.5% (191/1315) of our total new diagnosis patient cohort from analysis. These patients did not receive further assessment to explore the role of conservative management of the axilla as part of their treatment.

Clinicopathological characteristics. The mean number of nodes harvested for OSNA per patient was 1.94, with a total of 1,356 SLNs assessed. A total of 123/684 patients (17.9%) were found to have OSNA CK19 mRNA copy numbers indicative of macro-metastasis and all but 1 patient underwent ALND. In total, 45/122 (37%) patients had NSLN metastases on ALND with a total positive lymph node burden exceeding the ACOSOG Z0011 threshold of two macro-metastatic nodes (Fig. 2).

The distribution of tumour sizes within the cohort displayed the expected log-normal distribution for total and sub-group distribution of size by node involvement. There were 143 tumours with a diameter ≤ 10 mm, and none were associated with >2 macrometastases in ALND. TTL was the only clinicopathological variable significantly associated with risk of NSLN involvement and three or more positive nodes ($P < 0.0001$). Tumour size ($P = 0.14$), tumour grade ($P = 0.84$), oestrogen receptor status ($P = 0.09$), HER2 ($P = 1.00$) and lymphovascular invasion ($P = 0.30$) were not significantly associated with the risk of NSLN involvement and three or more positive nodes (data not shown).

TTL, axillary lymph node burden and prediction. Sensitivity and specificity of OSNA TTL vs. NSLN status using the protocol thresholds of $TTL \geq 250$, ≥ 5000 and $\geq 15,000$ copies/ μ l are detailed in Table I. Diagnostic accuracy of TTL, as demonstrated by ROC AUC, was 0.86 (Fig. 3).

In the present cohort, 11.4% (13/114) of the grade 1 tumours had evidence of SLN or NSLN metastases, compared with 19.9% (70/351) of the grade 2 and 19.4% (42/216) of the grade 3 tumours. The maximum total axillary burden for any grade 1 tumour was four nodes for an 18-mm tumour.

SLNB TTL vs. total lymph node involvement for the entire cohort, and for the subset of patients undergoing breast conserving surgery (BCS), was plotted (Figs. 4 and 5, respectively). The distribution of NSLN metastases was partitioned vertically by the point separation of data found at 15,000 copies/ μ l and horizontally by the two lymph node involvement threshold by which the ACOSOG Z0011 trial would not recommend ALND (3). BCS was performed in 499/700 (71.2%) patients and they represented a surrogate sub-group for comparison with the ACOSOG Z0011 (3) trial. However, the present group had broader inclusion criteria and was selection-modified by preoperative axillary US filtering. In total, 66/499 patients (13.2%) had ALND, 27 (41%) with NSLN involvement and 39 (59%) without NSLN involvement. In total, 20/23 (87%) patients with $TTL > 15,000$ copies/ μ l of CK19 mRNA had more than two involved NSLNs on ALND. Sensitivity, specificity, positive predictive value and negative predictive value of SLNB TTL vs. NSLN status using the threshold of $TTL \geq 15,000$ copies/ μ l for the subset of patients undergoing BCS are detailed in Table I. For patients with a SLNB TTL $< 15,000$ copies/ μ l and who had ≤ 2 nodes involved, the NPV was 0.983. According to Z0011 criteria, 35/499 (7.0%) patients could be regarded as being over-treated with a 3/499 (0.6%) false-negative rate (potential under-treatment).

Discussion

SLNB remains the standard of care for staging breast carcinoma in the clinically uninvolved axilla (1). Research has suggested that ALND may be safely omitted in patients with a low burden of axillary disease (3,10,11), particularly with micrometastatic disease only (12), but also where only one or two nodes with macrometastases are identified (3). Identifying patients who should proceed with ALND has been attempted with nomograms (13) and prediction models (7) with varying degrees of accuracy. Several studies have reported methods to predict ≥ 4 lymph node metastases (14-17). However, the majority of these prediction models were constructed using predictors derived from breast resection specimen pathology, such as lymphovascular invasion, and have not been widely adopted as they fail to confidently facilitate one-stage intraoperative decision making about the role of ALND. A small number of studies have reported on the prediction of NSLN metastases in SLN-positive patients, including the use of SLN OSNA TTL (7,18,19).

OSNA was formally approved by the UK National Institute of Clinical Excellence and included in routine surgical practice in 2013 (20). OSNA is at least equally cost effective as routine histology; however, it has substantial patient benefits (21,22). It remains the only intraoperative diagnostic test recommended

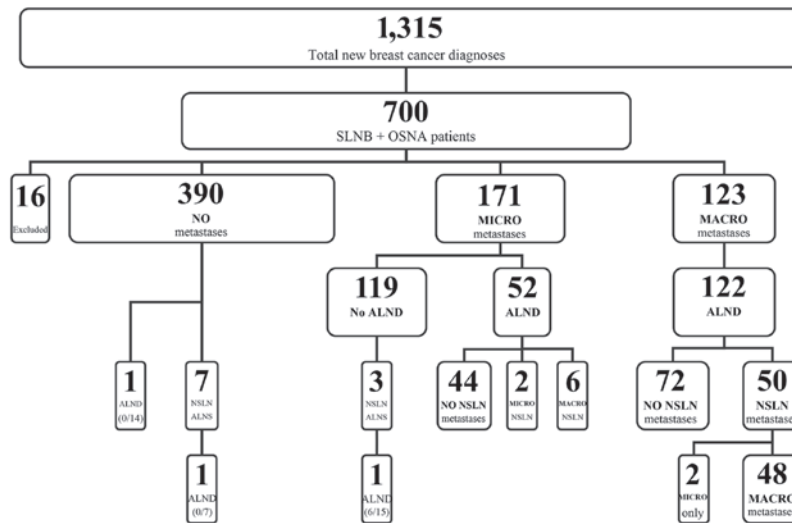


Figure 1. Cohort distribution flow chart. SLNB, sentinel lymph node biopsy; OSNA, one-step nucleic acid amplification; ALND, axillary lymph node dissection; NSLN, non-sentinel lymph node.

for whole-node analysis for detecting SLN metastases during breast surgery in individuals with early invasive breast cancer.

As a categorical diagnostic tool, OSNA alone is insufficient to determine which patients with macro-metastatic nodal disease may be spared ALND. The present study revealed that 60.7% of the cohort with macrometastatic lymph nodes on SLN OSNA had no further nodal involvement on ALND. Indeed, only 7.9% (54/683) of the entire cohort had more than two nodes with macrometastases. However, the continuous exponential quantification of TTL extends its utility considerably as a predictive tool (7,23-25). Stratification of CK19 mRNA copy numbers facilitates intraoperative decision-making for management of the axilla. In the present cohort of patients, the ROC AUC of 0.86 for two node TTL indicated that SLN TTL represents a good association with the presence of NSLN metastases. However, developing a model that predicts more than two node involvement and NSLN involvement is challenged by the small numbers of patients who satisfy this subset. In total, 21% of patients in the ALND arm of the Z0011 trial had additional positive nodes (3) whereas this occurred in 37% of the present cohort, despite our routine use of preoperative axillary US assessment. This observation may reflect a cohort of patients with more favourable disease in the ACOSOG Z0011 study, and/or higher sensitivity of lower disease burden detected with OSNA. Furthermore, the present cohort included 21/683 (3%) patients with pT3 tumours and the overall mean tumour size was 20 mm compared to 16 mm in Z0011 (3). The larger proportion of patients with additional NSLN node involvement in the present cohort may have contributed to the generation of an ROC AUC >0.8. Identifying any predictive markers with statistical significance was not possible using traditional clinicopathological factors in the present study. When lymphovascular invasion was reported in the resection specimen, it was twice as likely that NSLN metastases would be present than otherwise. If lymphovascular invasion was reported as being absent, the likelihood of NSLN metastases was 30% less. These findings, however, did not translate into statistical significance for predicting the risk of additional NSLN disease.

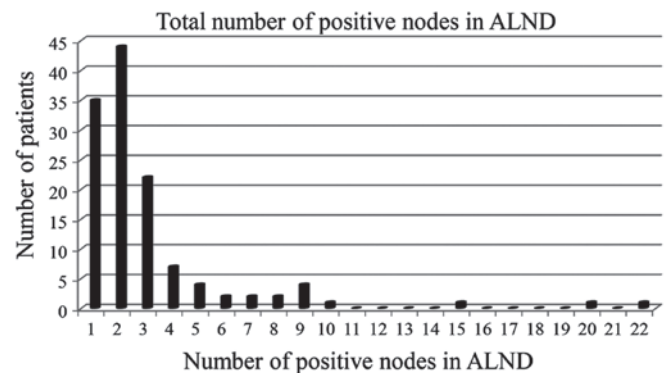


Figure 2. Distribution of the total number of positive nodes in an ALND for 122 patients with macrometastases. ALND, axillary lymph node dissection.

A study by Kubota *et al* (18) reported a single-centre standardised method for SLN detection that used indocyanine green; whereas, the types of injection and the use of radioisotope, with or without dye, varied according to institutional practice in a multi-centre study by Piñero-Madrona *et al* (19). The present single-centre dual technique of SLN detection is uniform and standardised (9). Differences in the methods of SLN detection, in particular OSNA TTL quantification restricted to two nodes in the present study, may account for some of the differences observed in ROC AUC values and in the association of NSLN positivity with other putative predictive variables. The mean number of SLNs removed in the studies by Kubota *et al* (18) and Piñero-Madrona *et al* (19) are not reported for comparison. An average SLN harvest >2.0 may suggest some persistence in retrieval, diluting the residual pool of NSLN with the removal of some nodes that would otherwise be accounted for in a subsequent ALND analysis. This would impact on how the relationship between SLN TTL burden and associated NSLN positivity is interpreted and compared in practice.

The present study identified an extension of the TTL threshold for conservative management of the axilla beyond 5,000 to 15,000 copies/ μ l and confirmed the findings of

Table I. Sensitivities and specificities of OSNA TTL of cytokeratin-19 mRNA copy numbers.

A, OSNA by NSLN using TTL cut-off of 250^a copies/ μ l for all the patients

OSNA	NSLN			Sensitivity ^b	Specificity ^b	PPV ^b	NPV ^b
	+	-	Total				
+	58	231	289	0.906 (0.835-0.978)	0.627 (0.589-0.665)	0.201 (0.155-0.247)	0.985 (0.973-0.997)
-	6	388	394				
Total	64	619	683				

B, OSNA by NSLN using TTL cut-off of 5,000^c copies/ μ l for all patients

OSNA	NSLN			Sensitivity ^b	Specificity ^b	PPV ^b	NPV ^b
	+	-	Total				
+	50	72	122	0.781 (0.680-0.883)	0.884 (0.858-0.909)	0.410 (0.323-0.497)	0.975 (0.962-0.988)
-	14	547	561				
Total	64	619	683				

C, OSNA by NSLN using TTL cut-off of 15,000^c copies/ μ l for all patients

OSNA	NSLN			Sensitivity ^b	Specificity ^b	PPV ^b	NPV ^b
	+	-	Total				
+	44	58	102	0.815 (0.711-0.918)	0.908 (0.885-0.930)	0.431 (0.335-0.528)	0.983 (0.972-0.992)
-	10	571	581				
Total	54	629	683				

D, OSNA by NSLN using TTL cut-off of 15,000^c copies/ μ l for patients undergoing breast conserving surgery

OSNA	NSLN			Sensitivity ^b	Specificity ^b	PPV ^b	NPV ^b
	+	-	Total				
+	20	35	55	0.870 (0.732-1.000)	0.927 (0.903-0.950)	0.364 (0.237-0.491)	0.993 (0.986-1.000)
-	3	441	444				
Total	23	476	499				

^aIndicates micrometastasis; ^bdata are presented as value (95% confidence interval); ^cindicates macrometastasis. For NSLN, + indicates present and - indicates absent. For OSNA, + indicates TTL cut-off reached or exceeded and - indicates below TTL cut-off. OSNA, one-step nucleic acid amplification; TTL, total tumour load; NSLN, non-sentinel lymph node; PPV, positive predictive value; NPV, negative predictive value.

Peg *et al* (7). In the present study, 70.0% (14/20) of patients with a SLN TTL between 5,000 and 15,000 copies/ μ l had no further metastatic disease identified on ALND. Those patients with a SLN TTL \geq 15,000 copies/ μ l continue to attract the recommendation to proceed with ALND. In the present cohort, 43.1% (44/102) of these patients (44/683, 6.4% of total cohort) had more than two involved nodes in total. The remaining 56.9% (58/683, 8.5% of total cohort) had only one or two positive nodes, and could be regarded as having their axilla over-treated. In those patients who underwent BCS, 20/23 (87%) patients who had more than two involved NSLNs on ALND had a SLNB TTL >15,000 copies/ μ l. The 3/23 (13%) patients who had more than

two nodes involved on ALND following TTL <15,000 copies/ μ l on OSNA was markedly less than the 23% of patients estimated to have had residual axillary disease in the non-ALND arm of Z0011, which reported a 0.9 and 1.5% regional recurrence rate at 5 and 10 years, respectively, for SLN positive disease without ALND (3,26). Our new recommended threshold for no ALND of TTL <15,000 copies/ μ l of CK19 mRNA is also markedly less than the recently adjusted threshold demonstrated by Peg *et al* (27) that correlates with disease-free, local recurrence-free and overall survival. They defined a TTL threshold of 25,000 CK19 mRNA copies/ μ l for a low-risk group below, and a high-risk group above, this threshold (27).

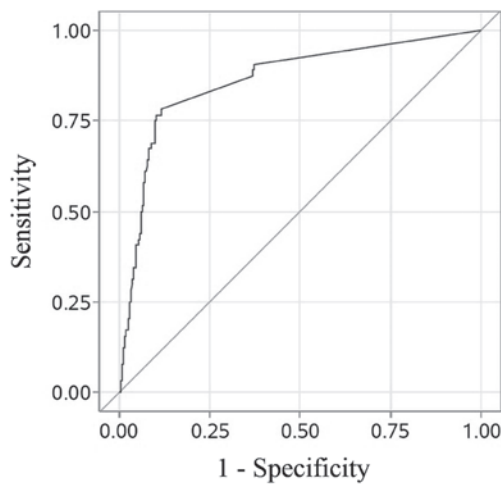


Figure 3. Receiver operating characteristic curve demonstrating diagnostic accuracy. Area under the curve, 0.8618 (95% confidence interval, 0.8119-0.9118).

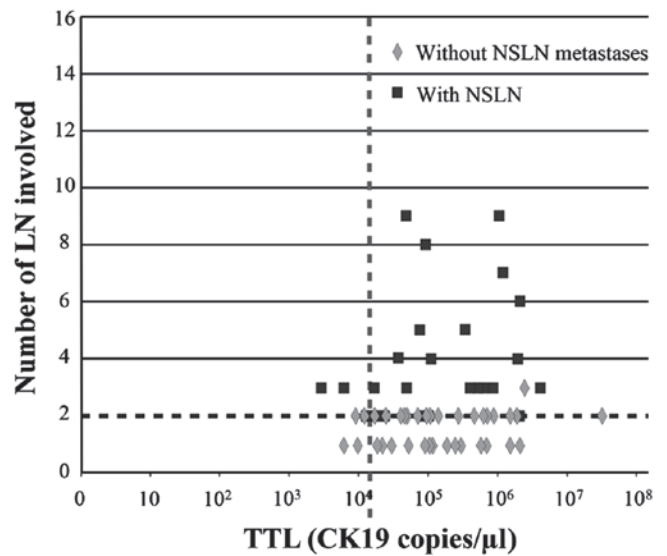


Figure 5. Sentinel lymph node biopsy TTL vs. total lymph node involvement for patients undergoing breast conservation surgery. TTL, total tumour load (of CK19 mRNA copy numbers); LN, lymph node; NSLN, non-sentinel lymph node; CK19, cytokeratin-19.

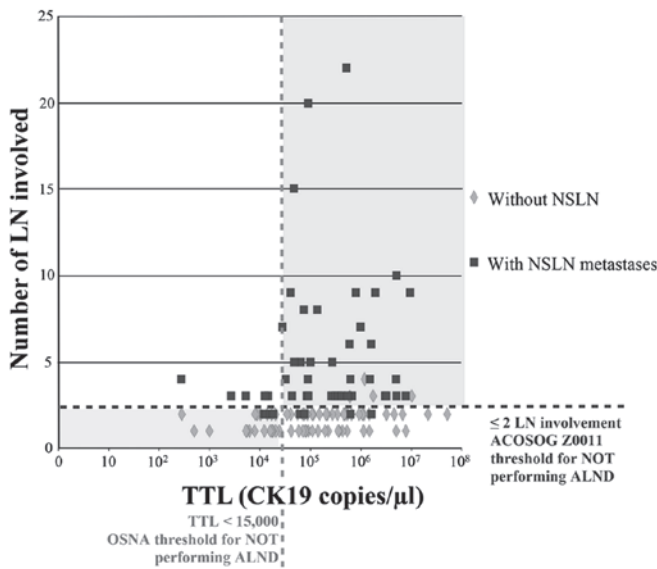


Figure 4. Sentinel lymph node biopsy TTL vs. total lymph node involvement. TTL, total tumour load (of CK19 mRNA copy numbers); LN, lymph node; NSLN, non-sentinel lymph node; ACOSOG Z0011, American College of Surgeons Oncology Group (Alliance) ACOSOG Z0011 randomized trial (3,26); ALND, axillary lymph node dissection; OSNA, one-step nucleic acid cytokeatin-19 amplification assay; CK19, cytokeratin-19.

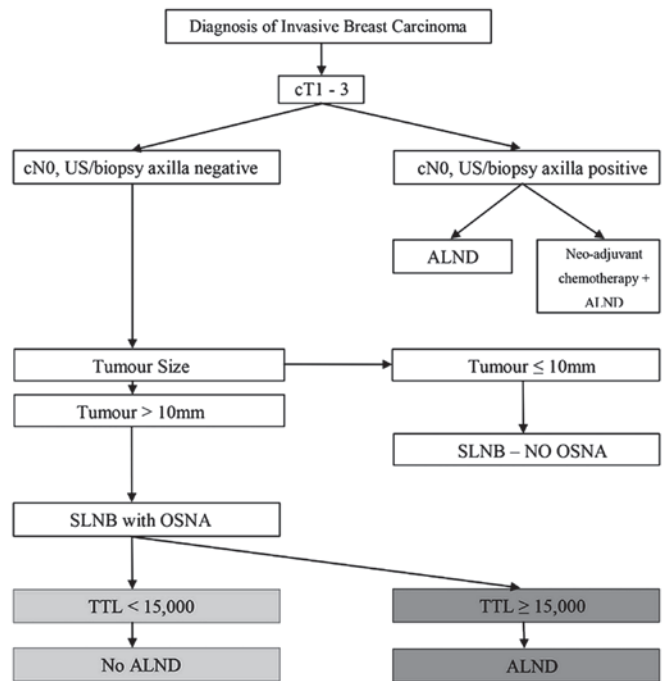


Figure 6. Protocol for management of the clinically uninvolved axilla using OSNA and TTL. OSNA, one-step nucleic acid amplification; TTL, total tumour load (of cytokeatin-19 mRNA copy numbers); cT1-T3 and cN0, TNM classification; US, ultrasound (of axilla); ALND, axillary lymph node dissection; SLNB, sentinel lymph node biopsy.

ALND is not recommended for patients when the SLNB is deemed negative (OSNA TTL <250 copies/ μ l) or interpreted as micrometastatic disease only (OSNA TTL between 250 and 5,000 copies/ μ l) [25.0% (171/683) in the present cohort] (2). Only 2.3% (4/171) of these patients had more than two involved nodes. We would recommend that patients with a tumour of <10 mm diameter receive standard SLNB without OSNA. Only 5.6% (8/143) of these patients had evidence of metastatic axillary disease, none had more than two involved nodes and the rate of involvement was less than the reported 9% false-negative rate for a two-node assessment (28).

False-negative SLN assessment by OSNA is very low (1.4%) and also well within the two SLNB false-negative rate of 9.0% (29). We suggest that pre-SLNB testing of the tumour

biopsy specimen with CK19 immunostaining is unnecessary or restricted to defining an inclusion role for OSNA in uncommon circumstances where absent or minimal CK19 mRNA expression may be suspected, such as metaplastic or high Ki67 scoring tumours, inversely reflecting the aggressive nature of the tumour (29,30). Much higher rates of CK19 negative tumours, up to 20%, have been reported; however, these are related to assessments of CK19 immunostaining of protein rather than

CK19 mRNA levels (30-32). A study by Pegolo *et al* (33), reported no correlation between CK19 protein expression and CK19 mRNA levels within primary breast cancer or the associated metastatic lymph node. In their study, CK19 mRNA was detected in all cases by OSNA. Similarly, a study by Fujisue *et al* (29), reported that the incidence of CK19 negative tumours was 12.3% with immunostaining; however, CK19 mRNA expression was absent in only 1.4% of the cases (29). These observations have allowed us to generate a protocol for the management of the axilla in our practice (Fig. 6).

The ACOSOG Z0011 trial demonstrated non-inferiority of no ALND for patients with T1 or T2 primary breast lesions and one or two positive axillary lymph nodes having BCS, whole-breast radiotherapy and adjuvant systemic therapy (3). The findings and recommendations of the Z0011 trial have attracted notable controversy and review. These issues have been extensively discussed (34), recently updated (26) and remain defensible in clinical practice. Regardless of controversy, it is clear that not all patients with a positive SLN require an ALND if there is minimal risk of axillary recurrence or compromise of their overall survival. In the present study, 92.1% of patients had two or less positive nodes on ALND. Axillary recurrence risk is low, even with a reported false-negative SLNB rate varying between 6.7 and 14.8% (1,9,35,36). The reported rates of isolated axillary recurrence in SLN negative disease are ~0.6% at 3 years and 1.1% at 5 years (37), similar to 0.9 and 1.5% reported at 5 and 10 years, respectively, for SLN-positive disease without ALND in the Z0011 trial (3,26). It appears illogical to remain preoccupied with the requirement to perform ALND on patients with cT1-T2 disease and a clinically negative axilla when local recurrence is 2.5-11 fold more likely to occur in the fully irradiated breast than the partially irradiated ipsilateral axilla (26). These rates of axillary recurrence are markedly less than the rates of ALND morbidity affecting up to 36% of patients with lymphoedema (13-19.1%), paraesthesia (31-37.7%), pain (21.1%) and decreased mobility (11.3%) (38,39).

In the present study, the results of testing two nodes have provided a robust threshold for clinical decision-making. Four-node testing would potentially alter the management of just 8.5% (58/683) of the present cohort. Used in combination with TTL, categorical four-node status may facilitate the intraoperative decision to perform ALND along traditional lines, bridging and translating the utility of SLNB TTL in existing models of prognosis and in guiding adjuvant treatment. Enhancement in prediction and treatment guidance is expected with extended genomic biomarker analyses of the OSNA sample homogenate (40).

The observation that 92.1% of the present total cohort had two or less nodes involved with macrometastatic disease and 63% of the present patients with positive SLNs did not have additional node metastases supports the move toward selective conservative axillary management. The present study did not demonstrate any benefit in performing ALND on patients with an OSNA TTL $\geq 15,000$ copies/ μ l. However, while there are no studies that confirm otherwise, it remains practical to identify the ACOSOG two-node macrometastases threshold for guiding clinical practice, informing systemic treatment and axillary radiotherapy field planning, and to putatively improve local disease control in the axilla.

The present study confirmed the thresholds and utility of including OSNA TTL in intraoperative ALND decision modelling that predict and discriminate between ≤ 2 involved nodes and >2 involved nodes. The present study identified, and confirmed, an extended threshold of OSNA TTL that may independently predict when ALND may be avoided, facilitating adoption of the emerging acceptance and recommendations for selective conservative management of the node positive axilla.

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