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Nuno Miguel Gomes Barbosa de Sousa

Total Tumor Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients

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Doutora Bárbara Neves Peleteiro

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Total Tumor Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTE TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.	☐
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Faculdade de Medicina da Universidade do Porto, 07/10/2022

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Total Tumor Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients

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Keywords

Breast cancer; Sentinel node; OSNA; Total Tumor Load; Non-sentinel node; Axillary lymph node dissection

Author contributions

NS: Data collection, analysis and interpretation, statistical analysis, manuscript preparation and editing.

BP: Statistical analysis and manuscript review.

JLF: Study design, surgical procedures, data collection, manuscript review and supervision.

Abstract

The Total Tumor Load (TTL) concept has been demonstrated to accurately predict the status of the non-sentinel lymph nodes (NSLN) in breast cancer patients. In 2019, our center implemented the TTL cut-off of 30,000 CK19 mRNA copies/μL as sole criterion for deciding on performing ALND.

This retrospective, unicentric, study analyzed 87 cT1-3N0 breast cancer patients treated consecutively in a period of two years and aimed to evaluate the performance of this criterion. Secondary objectives included the comparison of the criterion versus our previous Clinical Decision Rule (CDR) versus ACOSOG Z0011 criteria for avoiding an ALND in proportion of patients spared an ALND and in proportion of patients left with a surgically untreated metastasized axilla. An interim analysis revealed new TTL cut-offs for deciding on performing an ALND.

The 30,000 CK19 mRNA copies/μL criterion yielded an area under the ROC Curve (AUC) of 0.849, a false positive (FP) rate of 30.1% and a positive predictive value (PPV) of 38.9%. The 30,000 CK19 mRNA copies/μL criterion spared 58.6% of the patients an ALND versus 41.4% with CDR versus 73.6% with Z0011 and left 0.0% patients with a surgically untreated metastasized axilla versus 21.4% with CDR versus 42.9% with Z0011. The new TTL cut-off of 260,000 CK19 mRNA copies/μL for deciding on an ALND yielded an AUC of 0.753, a FP rate of 13.7% and a PPV of 47.4%. This new criterion spared 78.2% of the study sample an ALND and left 35.7% of metastasized axillae surgically untreated.

This study emphasizes the need to find a new balance between locoregional control and the morbidity associated with Berg levels I+II axillary lymph node dissection.

1. Introduction

Axillary lymph node status remains an important determinant of prognosis in patients with invasive breast cancer.

Sentinel lymph node (SLN) biopsy has gradually replaced axillary lymph node dissection (ALND) as the preferred method for nodal staging in the clinically node-negative early stage breast cancer patient [1,2]. In current state of the art, a patient with a negative SLN can forego an ALND [3,4]. Also, the presence of isolated tumor cells in the SLN does not warrant an ALND [5].

Non-sentinel lymph node (NSLN) metastasis can be present in 13-24% of the patients with a SLN micrometastasis and in 45-79% of the patients with a SLN macrometastasis [6].

The IBCSG 23-01 trial showed that no-ALND was non-inferior to performing ALND in terms of disease-free survival and overall survival in patients with SLN micrometastasis [7].

If accurate prediction of NSLN status can be achieved, many patients can be spared an ALND, thus avoiding the well-known comorbidities of this procedure and securing a better quality of life [8,9]. Therefore, numerous nomograms and clinical decision rules based on patient, tumor and node characteristics have been developed in an attempt to predict NSLN status [10,11].

The ACOSOG Z0011 trial results identified a group of patients who were able to avoid an ALND without a significant 10-year impact on their outcomes [12]. However, the group was based on restrictive criteria. For example, the conclusions do not apply to total mastectomy patients, and in some centers, total mastectomy patients can outnumber breast-conserving surgery patients. Some studies also challenge the methodology of this trial [13,14].

So far, consensus is still not reached on how to proceed after detection of a SLN macrometastasis. Current focus is on developing a reliable and simple tool for prediction of NSLN status, which could be widely applied to most of the breast cancer population.

The One Step Nucleic Acid Amplification (OSNA) assay is a fast, reproducible, semi-quantitative, automated and definite method of analyzing the whole of the

SLN [15]. The Total Tumor Load (TTL) concept has been demonstrated to accurately predict NSLN involvement [16,17]. An analysis of a national multicenter cohort of breast cancer patients, suggested that a TTL cut-off of 30,000 CK19 mRNA copies/μL was able to adequately predict NSLN involvement in 54.8% of the positive SLN patients with an area under the receiver operating characteristics curve (AUC) of 0.636, regardless of the type of surgery performed [11].

The main objective of this study was to evaluate the performance of the 30,000 CK19 mRNA copies/μL criterion for deciding on an ALND when applied to a cohort of consecutive patients for a period of 2 years.

Secondary objectives included comparison of the 30,000 CK19 mRNA copies/μL criterion with the previous Breast Center Clinical Decision Rule (CDR) [10] and Z0011 criteria for avoiding ALND [12] in the proportion of patients spared an ALND and the proportion of NSLN positive patients not submitted to an ALND.

In an interim analysis, additional TTL cut-offs for predicting NSLN involvement were searched with emphasis on specificity.

2. Methods

2.1. Study type and population

The present study is observational, retrospective and unicentric. We studied patients treated between July 1st 2019 to June 30th 2021, at the Breast Center of CHUSJ, Porto, Portugal. Data from patients with a cT1-3 cN0 tumor whose SLN was processed via OSNA assay and the TTL calculated were collected. Patients submitted to neoadjuvant systemic treatment, patients who had previous ipsilateral breast carcinoma, and patients with a TTL equal to 0 CK19 mRNA copies/μL were excluded. Total study sample consisted of 87 patients. Figure 1 illustrates a flowchart of patients included.

2.2. Variables

Variables collected were age at diagnosis, follow-up time, tumor size, grade and histological type, multifocality, lymphovascular and perineural invasion, estrogen and progesterone receptor status, HER2 status, surgical treatment, number of biopsied SLN, number of positive SLN, TTL, number of excised NSLN during ALND, number of positive NSLN, adjuvant treatments, locoregional recurrence, distant metastases and vital status.

2.3. Sentinel lymph node biopsy

SLN were identified using a combined technique, that mingles peritumoral injection of indocyanine green [18] with subareolar injection of patent blue V dye [19,20]. All blue, fluorescent or clinically suspicious nodes were considered SLN. The SLN were processed via the OSNA assay after the SLN biopsy procedure and the TTL was obtained.

2.4. Total Tumor Load interpretation

TTL represents the sum of the number of CK19 mRNA copies/μL of every positive SLN. TTL results were assessed in line with the original cut-offs established by *Tsujimoto et al*: non-metastasis/isolated tumor cells as below 250 CK19 mRNA copies/μL; micrometastasis as 250 – 5,000 CK19 mRNA copies/μL; macrometastasis as above 5,000 CK19 mRNA copies/μL [15]. Previous Breast Center Clinical Decision Rule (CDR) [10] for deciding on performing an ALND

applied to all patients with metastasized SLN, even if it contained isolated tumor cells as assessed by conventional pathology. Due to the secondary objective of comparing the 30,000 CK19 mRNA copies/μL criterion with the previous CDR, all patients with a TTL equal to 0 CK19 mRNA copies/μL were excluded and patients with a TTL more than 0 CK19 mRNA copies/μL were included (Figure 1). Patients with a TTL between 1 and 249 CK19 mRNA copies/μL were considered as having isolated tumor cells in their SLN.

2.5. Surgery

Patients were submitted to breast conserving surgery or total mastectomy according to tumor characteristics, multidisciplinary team meeting decision and discussion between surgeon and patient. Patients with a TTL of 30,000 CK19 mRNA copies/μL or below did not undergo ALND. Patients with a TTL of more than 30,000 CK19 mRNA copies/μL underwent ALND (Berg levels I+II) [21]. ALND product was sent to pathology for histological analysis.

2.6. Adjuvant treatments

Adjuvant treatments included endocrine therapy, chemotherapy and radiotherapy. A patient was considered to have received endocrine therapy if drug prescription and successive renewals were registered in the clinical records. A patient was considered to have received chemotherapy (including anti-HER2 therapy) if completion of all chemotherapy cycles as defined by a medical oncologist was documented in the clinical records. A patient was considered to have received adjuvant radiotherapy if completion of a whole breast irradiation course was documented in the clinical records.

2.7. Follow-up

All patients were assessed at the Breast Center out-patient clinic every 6 months. Patients were submitted to clinical examination, breast and axillary ultrasound (US), mammography, serum tumor markers, trunk CT scan and bone scan, according to institutional follow-up protocol. Regional node recurrence was mainly assessed by clinical examination and axillary US. Suspicious nodes were submitted to US guided needle biopsy (cytology or histology were both accepted).

2.8. Tumor type

Molecular subtype was obtained by assessing estrogen and progesterone receptor and HER2 status. Classification was defined in line with St Gallen's International Expert Consensus Conference [31].

2.9 *NSLN status*

In patients with a TTL above 30,000 CK19 mRNA copies/μL, NSLN status was given by histological analysis of the ALND product and by follow-up, because the surgeon did not perform a complete three-level ALND. In patients with a TTL of 30,000 CK19 mRNA copies/μL or below, NSLN status was given by follow-up and by histological analysis of an ALND product, in case clinical axillary recurrence occurred and the patient underwent an ALND.

2.10. Statistics

A database containing patient, tumor and node characteristics was created and all statistical analyses were performed using IBM SPSS Statistics v27.0, Chicago, USA. Frequencies were used for qualitative variables and medians for quantitative variables. A receiver operating characteristic curve (ROC) analysis was performed and the area under the curve (AUC) obtained for evaluation of the TTL discriminative power of NSLN status in our study sample. The AUC was also obtained for the 30,000 CK19 mRNA copies/μL criterion discriminative power of NSLN status. In an interim analysis, ROC analysis was used to search for additional TTL cut-offs in our study sample, and their AUC obtained. Finally, sensitivity, specificity, positive and negative predictive values and accuracy were calculated for the 30,000 CK19 mRNA copies/μL criterion and for the additional TTL cut-offs obtained.

2.11. Ethics

The study was approved by the conjoint Ethics Committee of University Hospital Center of São João and Faculty of Medicine of Porto University – project number 187/21.

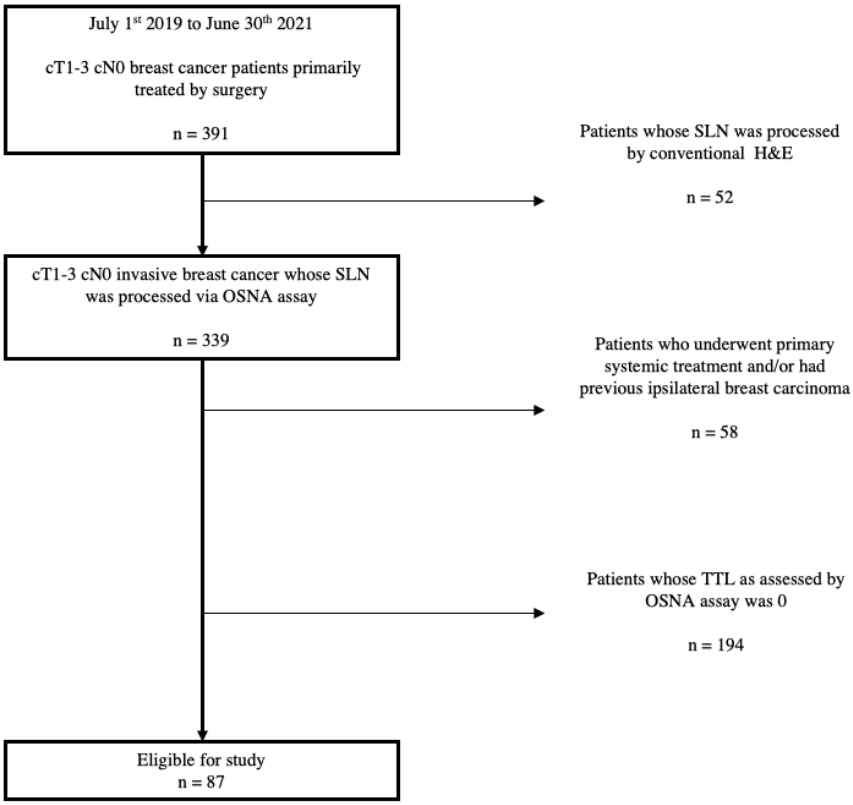

Figure 1 - Patients included in the study. Abbreviations: H&E -Hematoxylin and Eosin.

3. Results

Total study sample included 87 patients (Figure 1). The characteristics of the study sample are synthetized in Table 1. The majority of the study sample (95.4%) are estrogen receptor-positive tumors. Median follow-up time was 21 months (range: 7 – 36 months). All but one patient had a follow-up equal to or more than 12 months. The patient died at 7 months of follow-up.

The TTL as a tool for distinguishing between NSLN status in our study sample yielded an AUC of 0.841 (Figure 2). Median TTL was 10,000 CK19 mRNA copies/μL (range 210-4,040,000) in the study sample (Table 2). Only one patient had isolated tumor cells (TTL of 210 CK19 mRNA copies/μL) in the SLN. 36 patients had micrometastasis in the SLN and 50 patients had macrometastasis in the SLN.

Of the 87 patients included, 36 had a TTL above 30,000 CK19 mRNA copies/μL and were submitted to ALND. A total of 51 patients had a TTL of 30,000 CK19

mRNA copies/μL or below and did not perform ALND (Tables 1 and 2). Statistically significant differences were found between patients with a TTL of 30,000 CK19 mRNA copies/μL or below and patients with a TTL above 30,000 CK19 mRNA copies/μL regarding tumor size, tumor grade and presence of lymphovascular invasion (Table 1). Patients with a TTL less than or equal to 30,000 CK19 mRNA copies/μL also had significantly longer follow-up time (Table 1). Statistically significant differences between these two groups were also found regarding the number of SLN excised during the biopsy procedure, the number of SLN metastasized and the total number of lymph-nodes metastasized. The group with 30,000 CK19 mRNA copies/μL or below had a greater number of SLN excised and the more than 30,000 CK19 mRNA copies/μL group had more SLN and total lymph-nodes metastasized (Table 2).

Of the 36 patients submitted to ALND, 14 had metastasized NSLN, whereas 22 performed ALND and all their NSLN were negative. No statistically significant differences were found regarding the number of NSLN removed during the ALND procedure or the TTL in the 14 NSLN positive patients versus the 22 NSLN negative patients (Table 2).

None of the 87 patients developed locoregional recurrence or distant metastases during follow-up.

Statistically significant differences were found between the 14 NSLN positive patients and all the other 73 NSLN negative patients regarding the tumor size by pathology workup ($p=0.031$), the number of SLN metastasized ($p<0.001$), the number of NSLN removed ($p<0.001$), the number of NSLN metastasized ($p<0.001$) and the TTL ($p<0.001$).

The 30,000 CK19 mRNA copies/μL criterion yielded a sensitivity (SN) of 100.0%, a false negative rate (FN) of 0.0%, a specificity (SP) of 69.9%, a false positive rate (FP) of 30.1%, a positive predictive value (PPV) of 38.9% and a negative predictive value (NPV) of 100.0%. Accuracy was 74.7% and the AUC was 0.849 (Table 3).

Due to the unexpectedly high FP rate (30.1%), a decision was made to search for additional TTL cut-offs in our study sample that sacrificed sensitivity but gained

in specificity. The receiver operating characteristics curve (ROC) analysis revealed an interval of TTL values with the same sensitivity, but increasing specificity. Their rounded-up values were 140,000, 150,000, 200,000 and 260,000 CK19 mRNA copies/μL (Figure 3). All had a sensitivity of 64.3% and a false negative rate of 35.7%. Specificities were 79.4%, 83.6%, 84.9% and 86.3% and false positive rates were 20.5%, 16.4%, 15.1% and 13.7% respectively. Their PPV were 37.5%, 42.9%, 45.0% and 47.4%, respectively. The AUC were 0.719, 0.739, 0.746 and 0.753, respectively (Table 3).

Of the 87 patients in our study sample, the 30,000 CK19 mRNA copies/μL criterion spared 51 patients (58.6%) an ALND. The previous CDR [15] and the Z0011 eligibility criteria [19], had they been applied, would spare 36 (41.4%) and 64 (73.6%) patients an ALND, respectively. The new TTL cut-offs spared a growing number of ALND until a maximum of 68 (78.2%) patients, obtained with the 260,000 CK19 mRNA copies/μL cut-off (Table 3). Of the 14 patients with a metastasized axilla, the 30,000 CK19 mRNA copies/μL criterion left 0 patients with a surgically untreated axilla versus 3 (21.4%) and 6 (42.9%) patients, had the previous CDR and Z0011 criteria been applied, respectively. All of the new TTL cut-offs left the same 5 (35.7%) patients with a surgically untreated metastasized axilla (Table 3).

Of these 5 patients, two were cT1 and three were cT2. While two had 1 positive SLN, the other three had 2 positive SLN. A total of 4 were treated with lumpectomy and 1 with total mastectomy. All were treated with whole breast irradiation and hormone therapy. This means that of these 5 patients, four fulfilled the Z0011 eligibility criteria for avoiding ALND [19]. The only patient not fulfilling the eligibility criteria was due to total mastectomy performed. Also, four had Luminal HER2- tumors and one had a Luminal HER2+ tumor. While four had 2 or less positive NSLN, one had 4 positive NSLN, leading to two pN1 patients and three pN2. A total of 2 patients received adjuvant chemotherapy, one of whom was the patient with 4 metastasized NSLN (see supplementary material).

Tables 1 and 2 were repeated using the 260,000 CK19 mRNA copies/μL as cut-off point. Statistically significant differences were observed between the below or equal to 260,000 CK19 mRNA copies/μL group and the more than 260,000 CK19

mRNA copies/μL group regarding tumor size in mm by pathology workup and presence of lymphovascular invasion (see supplementary material). The more than 260,000 CK19 mRNA copies/μL group also had a statistically significant greater number of SLN metastasized and total number of lymph-nodes metastasized.

Table 1 - Characteristics of the study sample.

	All n = 87	0 < TTL ≤ 30,000 CK19 mRNA copies/μL n = 51	TLL > 30,000 CK19 mRNA copies/μL n = 36	p
Age, median (range)	55.0 (32-78)	57.0 (37-76)	53.5 (32-78)	0.907*
Follow-up in months, median (range)	21 (7-36)	23 (12-36)	20 (7-32)	0.022
Affected breast, n (%)				0.666**
Left	39 (44.8%)	24 (47.1%)	15 (41.7%)	
Right	48 (55.2%)	27 (52.9%)	21 (58.3%)	
Previous contralateral Breast Carcinoma, n (%)				0.168**
Yes	2 (2.3%)	0 (0.0%)	2 (5.6%)	
No	85 (97.7%)	51 (100.0%)	34 (94.4%)	
BI-RADS, n (%)				0.218**
4	30 (34.5%)	20 (39.2%)	10 (27.8%)	
5	39 (44.8%)	18 (35.3%)	21 (58.3%)	
6[#]	18 (20.7%)	13 (25.5%)	5 (13.9%)	
Tumor size in mm by imaging, median (range)	17.0 (4-51)	16.0 (6-51)	20.5 (4-50)	0.060*
Tumor size in mm by pathology workup[1], median (range)	21.0 (7-90)	18.0 (7-90)	25.0 (11-65)	0.017*
cT, n (%)				0.118**
T1	52 (59.8%)	34 (66.7%)	18 (50.0%)	
T2	34 (39.1%)	16 (31.4%)	18 (50.0%)	
T3	1 (1.1%)	1 (2.0%)	0 (0.0%)	
Definitive breast surgery, n (%)				0.604**
Breast-conserving surgery	68 (78.2%)	41 (80.4%)	27 (75.0%)	
Total mastectomy	19 (21.8%)	10 (19.6%)	9 (25.0%)	
Histological type, n (%)				0.686**
Ductal	63 (72.4%)	35 (68.6%)	28 (77.8%)	
Lobular	10 (11.5%)	7 (13.7%)	3 (8.3%)	
Other	14 (16.1%)	9 (17.6%)	5 (13.9%)	
Tumor grade, n (%)				0.004**
1	14 (16.1%)	11 (21.6%)	3 (8.3%)	
2	44 (50.6%)	30 (58.8%)	14 (38.9%)	
3	29 (33.3%)	10 (19.6%)	19 (52.8%)	
Multifocality, n (%)				0.390**
Yes	15 (17.2%)	7 (13.7%)	8 (22.2%)	
No	72 (82.8%)	44 (86.3%)	28 (77.8%	
Lymphovascular invasion, n (%)				0.009**
Yes	25 (28.7%)	9 (17.6%)	16 (44.4%)	
No	62 (71.3%)	42 (82.4%)	20 (55.6%)	
Perineural invasion, n (%)				1.000**
Yes	14 (16.1%)	8 (15.7%)	6 (16.7%)	
No	73 (83.9%)	43 (84.3%)	30 (83.3%)	
Biological subtype, n (%)				1.000**
Luminal HER2-	77 (88.5%)	45 (88.2%)	32 (88.9%)	
Luminal HER2+	6 (6.9%)	4 (7.8%)	2 (5.6%)	
HER2-enriched	2 (2.3%)	1 (2.0%)	1 (2.8%)	
Triple negative/Basal-like	2 (2.3%)	1 (2.0%)	1 (2.8%)	
Adjuvant chemotherapy, n (%)				
Yes	37 (42.5%)	17 (33.3%)	20 (55.6%)	
No	50 (57.5%)	34 (66.7%)	16 (44.4%)	

Adjuvant radiotherapy, n (%) Yes No	 84 (96.6%) 3 (3.4%)	 48 (94.1%) 3 (5.9%)	 36 (100.0%) 0 (0.0%)	
Adjuvant hormone therapy, n (%) Yes No	 83 (95.4%) 4 (4.6%)	 49 (96.1%) 2 (3.9%)	 34 (94.4%) 2 (5.6%)	
Adjuvant anti-HER2 therapy, n (%) Yes No	 4 (4.6%) 83 (95.4%)	 3 (5.9%) 48 (94.1%)	 1 (2.8%) 35 (97.2%)	

[1]Includes Ductal Carcinoma in Situ. * Mann-Whitney U. ** Pearson's chi-squared test / Fisher's exact test. [#]Patients diagnosed outside breast center.

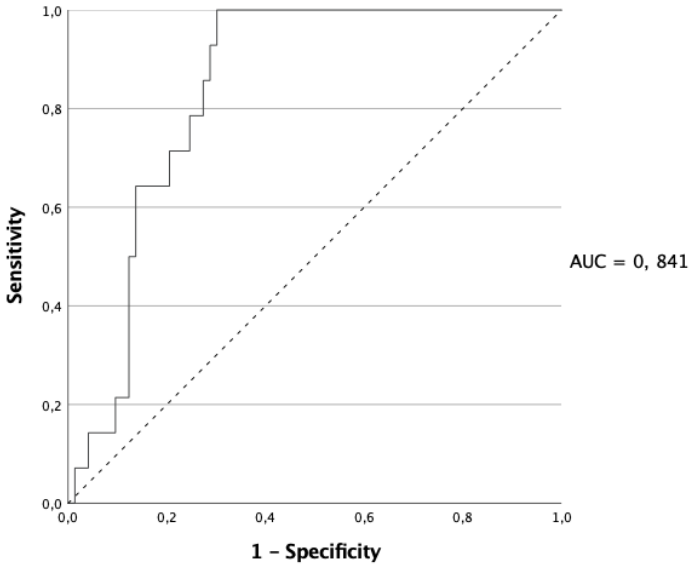

Figure 2 - TTL as a tool for distinguishing between NSLN status. AUC - Area under the ROC curve.

Table 2 - SLN and NSLN-related variables.

	All patients, n = 87				ALND patients, n = 36			
	All n = 87	TTL <= 30,000 CK19 mRNA copies/μL n = 51	TTL > 30,000 CK19 mRNA copies/μL n = 36	p	All n = 36	NSLN+ n = 14	NSLN- n = 22	p
Number of SLN removed, median (range)	2 (1-6)	2 (1-6)	2 (1-5)	0,041*	2 (1-5)	2 (1-5)	2 (1-4)	0.810*
Number of SLN metastasized, median (range)	1 (0-4)	1 (0-3)	1 (1-4)	<0,001*	1 (1-4)	2 (1-2)	1 (1-4)	0.141*
ALND, n (%) Yes No	 36 (41.4%) 51 (58.6%)	 0 (0.0%) 51 (100.0%)	 36 (100.0%) 0 (0.0%)					

Number of NSLN removed, median (range)	0 (0-23)		12 (1-23)		12 (1-23)	13 (1-23)	10 (6-20)	0.597*
Number of NSLN metastasized, median (range)	0 (0-8)		0 (0-8)		0 (0-8)	2 (1-8)	0 (0-0)	
Total number of LN metastasized, median (range)	1 (1-9)	1 (1-3)	2 (1-9)	<0,001	2 (1-9)	4 (2-9)	1 (1-4)	
TTL, median (range) 25th percentile 75th percentile	10,000 (210-4,040,000) 920 150,000	1,180 (210-26,000) 410 8,070	265,500 (31,000-4,040,000) 100,000 1,075,000		265,000 (31,000-4,040,000) 100,000 1,075,000	295,000 (31,000-3,740,000) 59,700 580,750	206,700 (45,000-4,040,000) 116,000 1,562,500	0.785*

*Mann-Whitney U test

Abbreviations: LN -Lymph-nodes; SLN-Sentinel Lymph Nodes; NSLN-Non-Sentinel Lymph Nodes; ALND-Axillary Lymph Node Dissection; TTL-Total Tumor Load

Table 3 - Tools to assist in the decision of performing an ALND.

	30,000 CK19 mRNA copies/μL	140,000 CK19 mRNA copies/μL	150,000 CK19 mRNA copies/μL	200,000 CK19 mRNA copies/μL	260,000 CK19 mRNA copies/μL
Patients spared an ALND, n (%)	51 (58.6%)	63 (72.4%)	66 (75.9%)	67 (77.0%)	68 (78.2%)
NSN+ patients not submitted to ALND, n (%)	0 (0.0%)	5 (35.7%)	5 (35.7%)	5 (35.7%)	5 (35.7%)
AUC	0.849	0.719	0.739	0.746	0.753
Sensitivity	100.0%	64.3%	64.3%	64.3%	64.3%
Specificity	69.9%	79.4%	83.6%	84.9%	86.3%
FP	30.1%	20.5%	16.4%	15.1%	13.7%
FN	0.0%	35.7%	35.7%	35.7%	35.7%
PPV	38.9%	37.5%	42.9%	45.0%	47.4%
NPV	100.0%	92.1%	92.4%	92.5%	92.6%
Accuracy	74.7%	77.0%	80.5%	81.6%	82.8%

Abbreviations: FP-False Positives; FN-False Negatives; PPV-Positive Predictive Value; NPV-Negative Predictive Value; ALND-Axillary Lymph Node Dissection; +NSN-Positive Non-Sentinel Lymph Nodes;

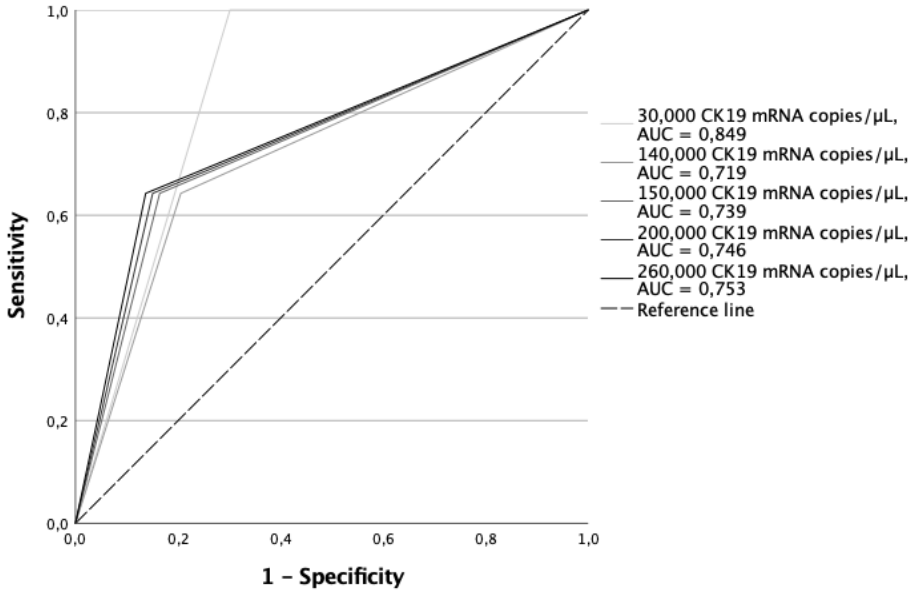

Figure 3 - AUC of the 30,000 CK19 mRNA copies/µL criterion and of the additional TTL cut-offs obtained.

4. Discussion

This study aimed to evaluate performance of a criterion derived from a national cohort [11] after two consecutive years of its application to a unicentric breast cancer population.

The TTL as a tool for discriminating between NSLN status yielded an AUC of 0.841 in our study sample, which may be considered as having an excellent degree of accuracy [22]. Besides, a statistically significant difference was found between the TTL of the 14 NSLN positive patients and all other patients. Both these findings are in line with results from previous studies that indicate the TTL is able to predict NSLN status [16,17]. Tumor size was also significantly greater in the 14 NSLN positive patients when compared to the 73 NSLN negative patients. This result is coherent with studies that show an association between tumor size and NSLN status [10].

Statistically significant differences were found between the TTL below or equal to 30,000 CK19 mRNA copies/µL group and the TTL over 30,000 CK19 mRNA copies/µL group regarding tumor size, tumor grade and presence of lymphovascular invasion. These differences are observed in variables commonly

used to predict NSLN status [10,23]. The group with less than or equal to 30.000 CK19 mRNA copies/μL had significantly more follow-up time when compared to the more than 30.000 CK19 mRNA copies/μL group. This is of no concern, as the group with greater follow up was the one that did not perform ALND, so increasing the chances of identifying clinically evident metastasis in axillary NSLN.

The 30,000 CK19 mRNA copies/μL criterion for deciding on performing an ALND yielded an AUC of 0.849, which is in another tier of discrimination in relation to the value of 0.636 obtained by *Fougo et al* in the 30,000 CK19 mRNA copies/μL criterion derivation study [11]. The 30,000 CK19 mRNA copies/μL criterion represented a positive evolution from the previous Breast Center CDR [10], having spared a greater number of patients an ALND while leaving no patients with metastasized NSLN. However, this came at a cost of an unacceptably high FP rate. Approximately one third of the study sample performed an ALND with no additional benefit in locoregional control, while inheriting the risks of an ALND procedure [8,24]

Although the follow-up time is short in our study, this mostly affects the 51 patients not submitted to an ALND. Hypothetically, if one of these patients had developed a clinically evident axillary disease after the study's follow-up time, the patient would be considered a FN of the criterion. This means that the short follow-up time only affects sensitivity and that the 30,000 CK19 mRNA copies/μL criterion would yield the same FP rate, regardless of the follow-up time.

The PPV value of the criterion was also lower than the value obtained by *Fougo et al* in the derivation study (54.8%) [11].

Application of the Z0011 criteria would allow more patients than the 30.000 CK19 mRNA copies/μL criterion to be spared an ALND, although approximately forty percent of the NSLN positive patients would remain with a surgically untreated metastasized axilla.

In an effort to find a better balance between locoregional control and morbidity, an interim ROC analysis was performed, with emphasis on specificity. We found an interval of TTL values with the same sensitivity but growing specificity. Their rounded-up values were 140,000, 150,000, 200,000 and 260,000 CK19 mRNA

copies/μL. These values were chosen because they provided the best balance between loss in sensitivity and gain in specificity.

The TTL cut-off of 260,000 CK19 mRNA copies/μL was the one that maximized specificity. Its PPV was 47.4%, which is more comparable to the 30,000 CK19 mRNA copies/μL criterion PPV obtained in the PORTTLE study [11] (54.8%) than the PPV of the 30,000 CK19 mRNA copies/μL criterion obtained in our study sample. In addition, the 260,000 CK19 mRNA copies/μL cut-off spared a greater number of ALND when compared to the 30,000 CK19 mRNA copies/μL cut-off, the previous CDR and the Z0011 criteria. This new criterion would leave approximately one third of the NSLN positive patients with a surgically untreated axilla, which is less than the value yielded by the Z0011 criteria.

In fact, with the 30,000 CK19 mRNA copies/μL criterion, approximately one third of the patients performed an unnecessary ALND (FP), whereas with the 260,000 CK19 mRNA copies/μL criterion, approximately one third would be left with a surgically untreated metastasized axilla (FN).

In this study sample of 87 patients, raising the cut-off for deciding on an ALND from 30,000 CK19 mRNA copies/μL to 260,000 CK19 mRNA copies/μL, would spare 17 additional patients an ALND of which 5 patients had positive NSLN. This translates into 17 additional patients foregoing the morbidity of an unnecessary ALND [8,24], at the cost of 5 of them maintaining a surgically untreated metastasized axilla, that would be addressed by adjuvant therapies [25].

A total of 4 of these 5 patients fulfilled the Z0011 criteria for not performing an ALND and all 5 patients had estrogen receptor positive tumors, remarkable for their usually long recurrence times [26,27]. The ALND procedure in these 5 patients upgraded the pN status of 3 patients. These 3 patients were staged as pN2 after the procedure, which could lead to further regional treatment with radiotherapy and/or systemic therapy with chemotherapy [25]. This means that the omission of ALND in these 3 patients, could potentially lead to undertreatment. Nonetheless, 2 of these 3 fulfilled the Z0011 criteria because they performed WBI and hormonetherapy. Therefore, with the Z0011 results in mind, the problem lies with the patient that performed total mastectomy and also had a total of 6 lymph nodes involved. This patient could potentially be

undertreated, with impact on overall survival and/or disease-free survival, if the ALND procedure was omitted with use of the 260,000 CK19 mRNA copies/μL criterion.

Considering the ALND patients, no statistically significant differences were found regarding the TTL of the NSLN positive versus the NSLN negative patients. However, a closer analysis of the percentiles shows that less than 50% of the NSLN negative patients would proceed to an ALND and more than 50% of the NSLN positive patients would perform an ALND if decision to raise the TTL cut-off for deciding on ALND to 260,000 CK19 mRNA copies/μL was made.

The PORTTLE study showed that the 30,000 CK19 mRNA copies/μL criterion was an independent predictor of NSLN status [11]. The analysis of the participants' characteristics was repeated for the 260,000 CK19 mRNA copies/μL criterion and it yielded statistically significant results for the tumor size and presence of lymphovascular invasion. These findings suggest that the 260,000 CK19 mRNA copies/μL criterion stands as an independent predictor of NSLN status in our study sample.

We assessed the metastatic load by the sum of number of SLN metastasized with number of NSLN metastasized. There were significantly more total lymph nodes metastasized in the more than 30,000 CK19 mRNA copies/μL and in the more than 260,000 CK19 mRNA copies/μL groups. These results are in line with studies that show an increase in number of lymph nodes metastasized, when the TTL increases [28,29].

In the 51 patients with a TTL less than or equal to 30,000 CK19 mRNA copies/μL, the median of total lymph nodes involved was 1 (range 1-3). 45 patients had 1 SLN involved. Although none of these patients performed ALND (and therefore pN cannot be determined), this suggests that SLN with a TTL up to 30,000 CK19 mRNA copies/μL correlates with small metastatic load. 17 patients had a TTL between more than 30,000 CK19 mRNA copies/μL and less than or equal to 260,000 CK19 mRNA copies/μL and therefore performed ALND (pN can be determined). In these patients, the median of total lymph nodes involved was 2 (range 1 - 6). Only 3 of them had 4 or more total lymph nodes involved (82.4%

pN1 and 17.6% pN2 patients). This suggests that up to a TTL of 260,000 CK19 mRNA copies/μL continues to represent low metastatic load with the vast majority being pN1 patients.

Ohi et al reported a frequency of 7.0% pN2 patients when the SLN contained micrometastasis and of 27.4% pN2 patients when the SLN contained macrometastasis [29]. We cannot assume the pN of the 68 patients with less than or equal to 260,000 CK19 mRNA copies/μL, because 51 of them did not perform ALND. However, if we focus on the 17 patients mentioned above, the TTL cut-off of 260,000 CK19 mRNA copies/μL is halfway between a micrometastic SLN and a macrometastic SLN in terms of proportion of pN2 patients. In a clinical sense, SLN with up to 260,000 CK19 mRNA copies/μL would be comparable to SLN with micrometastasis.

A study conducted in a cohort of 58 patients from the central region of Portugal [30] reported an AUC of 0.805 when using the TTL as tool for distinguishing between NSLN status, which is in line with our results. This study suggested the TTL cut-off of 190,000 CK19 mRNA copies/μL for deciding on ALND. Sensitivity was 73.3%, specificity was 74.4% and NPV was 88.9%. From the data provided by the authors, we estimate a PPV of just 32,0%. The 190,000 CK19 mRNA copies/μL criterion was less sensitive than the 30,000 CK19 mRNA copies/μL criterion and more sensitive than the 260,000 CK19 mRNA copies/μL criterion. Specificity was higher than the 30,000 CK19 mRNA copies/μL criterion but less than the 260,000 CK19 mRNA copies/μL criterion. Both the PPV and the NPV were inferior to the 30,000 CK19 mRNA copies/μL criterion and the 260,000 CK19 mRNA copies/μL criterion. In our study sample, 67 (77.0%) patients would be spared an ALND with the 190,000 CK19 mRNA copies/μL and 5 patients would remain with a surgically untreated metastasized axilla. These results are very similar to those reported regarding the 260,000 CK19 mRNA copies/μL criterion.

The majority (95.4%) of our study sample were estrogen receptor positive tumors. *Espinosa-Bravo et al* [16] reported that a TTL cut-off of 500,000 CK19 mRNA copies/μL in HR positive patients yielded a specificity of 87%, a NPV of 84% and a PPV of 56%. These results compare favorably to the results obtained when we used the 260,000 CK19 mRNA copies/μL criterion in all of our study sample. If

we had used the TTL cut-off of 500,000 CK19 mRNA copies/μL for deciding on ALND, only 5 patients in 87 would have performed ALND. However, 10 in 14 patients with metastasized NSLN would not have performed ALND, when compared to the 5 patients obtained with the 260,000 CK19 mRNA copies/μL criterion.

Most patients had estrogen receptor positive tumors, remarkable for their usually long natural history and dormant metastasis [26,27]. A total of 96.6% of our patients received adjuvant whole breast irradiation, 95.4% received adjuvant hormone therapy and 42.5% received adjuvant chemotherapy. Whole breast irradiation is able to deliver a small amount of radiation to axillary lymph node levels 1 and 2. The dose delivered is also dependent of prone or supine position of the procedure. However, authors consistently claim the dose delivered to the nodes in this procedure is inadequate for prophylactic coverage of the axilla [31,32]. In our study, prone or supine position of radiotherapy was not registered in the data base nor was the delivery of additional dose of radiotherapy to axillary nodes. Nonetheless, adjuvant systemic therapies target both lymph node and distant foci of disease [25,26].

Although this study was retrospective, complete follow-up was achieved and there was no missing data [33]. One pitfall of our study is that the majority of patients did not perform ALND. Studies relating to the SLN concept, such as studies that propose TTL cut-offs to decide on ALND, usually derive these cut-offs from a sample where every patient performed ALND. Nonetheless, our study sample was obtained from a center with a TTL cut-off already implemented and therefore it reflects the intention to evaluate performance of a clinical practice tool. The study design yields real world evidence, valuable for everyday clinical practice and complies to the principles of Evidence-Based Medicine [34]. Another possible critic to our study is that it is unicentric. As we stated before, the main objective of the study was to evaluate performance of a criterion derived from a national cohort, when applied to a localized population of breast cancer patients. In fact, this population proved to have a greater than average proportion of ER positive tumor patients [11,30], denoting the importance of personalized treatment.

This study emphasizes the need to find a new balance between locoregional control and morbidity, bearing in mind that, in the current status of breast cancer adjuvant treatments, focus should be on preserving the axilla.

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Supplementary material

Table 1 - Patient, tumor and node characteristics of the 5 patients not performing an ALND with the application of the 260,000 CK19 mRNA copies/μL criterion.

	Patient				
	1	2	3	4	5
Age at diagnosis	54	57	64	69	77
cT	2	1	2	1	2
Number of metastasized SLN	1	2	2	1	2
Total Tumor Load	31,000	133,000	45,800	63,000	49,800
Definite breast surgery	Breast-conserving surgery	Breast-conserving surgery	Total Mastectomy	Breast-conserving surgery	Breast-conserving surgery
Adjuvant chemotherapy	No	Yes	Yes	No	No
Adjuvant radiotherapy	Yes	Yes	Yes	Yes	Yes
Adjuvant hormone therapy	Yes	Yes	Yes	Yes	Yes
Adjuvant anti-HER2 therapy	No	No	No	No	Yes
Fulfills the Z0011 criteria for avoiding ALND	Yes	Yes	No	Yes	Yes
Biological subtype	Luminal HER2-	Luminal HER2-	Luminal HER2-	Luminal HER2-	Luminal HER2+
Number of metastasized NSLN	1	2	4	1	2
pN	1	2	2	1	2

Table 2 - Characteristics of the study sample using the TTL of 260,000 CK19 mRNA copies/μL as cut-off point.

	All n = 87	0 < TTL ≤ 260,000 CK19 mRNA copies/μL n = 68	TLL > 260,000 CK19 mRNA copies/μL n = 19	p
Age, median (range)	55.0 (32-78)	57.0 (32-77)	52.0 (35-78)	0.817*
Follow-up in months, median (range)	21 (7-36)	22.5 (12-36)	18 (7-32)	0,091*
Affected breast, n (%)				0.787**
Left	39 (44.8%)	31 (45.6%)	8 (42.1%)	
Right	48 (55.2%)	37 (54.4%)	11 (57.9%)	
Previous contralateral Breast Carcinoma, n (%)				0.046**
Yes	2 (2.3%)	0 (0.0%)	2 (10.5%)	
No	85 (97.7%)	68 (100.0%)	17 (89.5%)	
BI-RADS, n (%)				0.693**
4	30 (34.5%)	24 (35.3%)	6 (31.6%)	
5	39 (44.8%)	29 (42.6%)	10 (52.6%)	
6[#]	18 (20.7%)	15 (22.1%)	3 (15.8%)	
Tumor size in mm by imaging, median (range)	17.0, (4-51)	16.5 (4-51)	23.0 (10-46)	0.123*
Tumor size in mm by pathology workup[1], median (range)	21.0, (7-90)	21.0 (7-90)	26.0 (13-65)	0.036*
cT, n (%)				0.373**
T1	52 (59.8%)	43 (63.2%)	9 (47.4%)	
T2	34 (39.1%)	24 (35.1%)	10 (52.6%)	
T3	1 (1.1%)	1 (1.5%)	0 (0.0%)	
Definitive breast surgery, n (%)				0.345**
Breast-conserving surgery	68 (78.2%)	55 (80.9%)	13 (68.4%)	
Total mastectomy	19 (21.8%)	13 (19.1%)	6 (31.6%)	
Histological type, n (%)				0.212**
Ductal	63 (72.4%)	47 (69.1%)	16 (84.2%)	
Lobular	10 (11.5%)	10 (14.7%)	0 (0.0%)	
Other	14 (16.1%)	11 (16.2%)	3 (15.8%)	
Tumor grade, n (%)				0.068**
1	14 (16.1%)	14 (20.6%)	0 (0.0%)	
2	44 (50.6%)	34 (50.0%)	10 (52.6%)	
3	29 (33.3%)	20 (29.4%)	9 (47.4%)	
Multifocality, n (%)				1.000**
Yes	15 (17.2%)	12 (17.6%)	3 (15.8%)	
No	72 (82.8%)	56 (82.4%)	16 (84.2%	

Lymphovascular invasion, n (%)				0.009**
Yes	25 (28.7%)	15 (22.1%)	10 (52.6%)	
No	62 (71.3%)	53 (77.9%)	9 (47.4%)	
Perineural invasion, n (%)				1.000**
Yes	14 (16.1%)	11 (16.2%)	3 (15.8%)	
No	73 (83.9%)	57 (83.8%)	16 (84.2%)	
Biological subtype, n (%)				0.396**
Luminal HER2-	77 (88.5%)	61 (89.7%)	16 (84.2%)	
Luminal HER2+	6 (6.9%)	5 (7.4%)	1 (5.3%)	
HER2-enriched	2 (2.3%)	1 (1.5%)	1 (5.3%)	
Triple negative/Basal-like	2 (2.3%)	1 (1.5%)	1 (5.3%)	
Adjuvant chemotherapy, n (%)				
Yes	37 (42.5%)	26 (38.2%)	11 (57.9%)	
No	50 (57.5%)	42 (61.8%)	8 (42.1%)	
Adjuvant radiotherapy, n (%)				
Yes	84 (96.6%)	65 (95.6%)	19 (100.0%)	
No	3 (3.4%)	3 (4.4%)	0 (0.0%)	
Adjuvant hormone therapy, n (%)				
Yes	83 (95.4%)	66 (97.1%)	17 (89.5%)	
No	4 (4.6%)	2 (2.9%)	2 (10.5%)	
Adjuvant anti-HER2 therapy, n (%)				
Yes	4 (4.6%)	4 (5.9%)	0 (0.0%)	
No	83 (95.4%)	64 (94.1%)	19 (100.0%)	

[1]Includes Ductal Carcinoma in Situ. * Mann-Whitney U. ** Pearson's chi-squared test / Fisher's exact test. [#]Patients diagnosed outside breast center.

Table 3 - SLN and NSLN-related variables sample using the TTL of 260,000 CK19 mRNA copies/μL as cut-off point.

	All patients, n = 87			
	All n = 87	TTL <= 260,000 CK19 mRNA copies/μL n = 68	TTL > 260,000 CK19 mRNA copies/μL n = 19	p
Number of SLN removed, median (range)	2 (1-6)	2 (1-6)	2 (1-5)	0,191
Number of SLN metastasized, median (range)	1 (0-4)	1 (0-3)	1 (1-4)	0,011
ALND, n (%)				
Yes	36 (41.4%)	17 (25.0%)	19 (100.0%)	
No	51 (58.6%)	51 (75.0%)	0 (0.0%)	
Number of NSLN removed, median (range)	0 (0-23)	0 (0-20)	12 (1-23)	<0,001
Number of NSLN metastasized, median (range)	0 (0-8)	0 (0-4)	0 (0-8)	0,262
Number of total LN metastasized, median (range)	1 (1-9)	1 (1-6)	2 (1-9)	<0,001
TTL, median (range) 25th percentile 75th percentile	10,000 (210-4,040,000) 920 150,000	2,650 (210-253,400) 560 29,750	703,000 (261,000-4,040,000) 337,700 2,300,000	<0,001

*Mann-Whitney U test

Abbreviations: LN -Lymph nodes; SLN-Sentinel Lymph Nodes; NSLN-Non-Sentinel Lymph Nodes; ALND-Axillary Lymph Node Dissection; TTL-Total Tumor Load

Reporting guidelines

STROBE Statement—Checklist of items that should be included in reports of ***cohort studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract The present study was a retrospective cohort study. Page 7, paragraph 7: "This retrospective, unicentric, study (...)" (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found Page 7, paragraphs 6-9: "The Total Tumor Load (TTL) concept has been demonstrated to accurately predict the status of the non-sentinel lymph nodes (NSLN) in breast cancer patients. In 2019, our center implemented the TTL cut-off of 30,000 CK19 mRNA copies/μL as sole criterion for deciding on performing ALND. This retrospective, unicentric, study analyzed 87 cT1-3N0 breast cancer patients treated consecutively in a period of two years and aimed to evaluate the performance of this criterion. Secondary objectives included the comparison of the criterion versus our previous Clinical Decision Rule (CDR) versus ACOSOG Z0011 criteria for avoiding an ALND in proportion of patients spared an ALND and in proportion of patients left with a surgically untreated metastasized axilla. An interim analysis revealed new TTL cut-offs for deciding on performing an ALND. The 30,000 CK19 mRNA copies/μL criterion yielded an area under the ROC Curve (AUC) of 0.849, a false positive (FP) rate of 30.1% and a positive predictive value (PPV) of 38.9%. The 30,000 CK19 mRNA copies/μL criterion spared 58.6% of the patients an ALND versus 41.4% with CDR versus 73.6% with Z0011 and left 0.0% patients with a surgically untreated metastasized axilla versus 21.4% with CDR versus 42.9% with Z0011. The new TTL cut-off of 260,000 CK19 mRNA copies/μL for deciding on an ALND yielded an AUC of 0.753, a FP rate of 13.7% and a PPV of 47.4%. This new criterion spared 78.2% of the study sample an ALND and left 35.7% of metastasized axillae surgically untreated. This study emphasizes the need to find a new balance between locoregional control and the morbidity associated with Berg levels I+II axillary lymph node dissection."	7
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	8

		Page 8, paragraph 3: "Sentinel lymph node (SLN) biopsy has gradually replaced axillary lymph node dissection (ALND) as the preferred method for nodal staging in the clinically node-negative early stage breast cancer patient [1,2]. In current state of the art, a patient with a negative SLN can forego an ALND [3,4]. Also, the presence of isolated tumor cells in the SLN does not warrant an ALND [5]." Page 8, paragraph 5: " The IBCSG 23-01 trial showed that no-ALND was non-inferior to performing ALND in terms of disease-free survival and overall survival in patients with SLN micrometastasis [7]." Page 8, paragraph 6: " If accurate prediction of NSLN status can be achieved, many patients can be spared an ALND (...)" Page 8, paragraph 8: " So far, consensus is still not reached on how to proceed after detection of a SLN macrometastasis. Current focus is on developing a reliable and simple tool for prediction of NSLN status, which could be widely applied to most of the breast cancer population."	
Objectives	3	State specific objectives, including any prespecified hypotheses Page 9, paragraph 1: "The main objective of this study was to evaluate the performance of the 30,000 CK19 mRNA copies/μL criterion for deciding on an ALND when applied to a cohort of consecutive patients for a period of 2 years." Page 9, paragraph 2: " Secondary objectives included comparison of the 30,000 CK19 mRNA copies/μL criterion with the previous Breast Center Clinical Decision Rule (CDR) [10] and Z0011 criteria for avoiding ALND [12] in the proportion of patients spared an ALND and the proportion of NSLN positive patients not submitted to an ALND." Page 9, paragraph 3: " In an interim analysis, additional TTL cut-offs for predicting NSLN involvement were searched with emphasis on specificity."	9
Methods			
Study design	4	Present key elements of study design early in the paper Page 10, paragraph 3: " The present study is observational, retrospective and unicentric."	10
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	10, 11

		Page 10, paragraph 3: " We studied patients treated between July 1st 2019 to June 30th 2021, at the Breast Center of CHUSJ, Porto, Portugal. Data from patients with a cT1-3 cN0 tumor whose SLN was processed via OSNA assay and the TTL calculated were collected." Page 11, paragraph 6: " All patients were assessed at the Breast Center out-patient clinic every 6 months."	
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up Elegibility criteria: Page 10, paragraph 3: "Data from patients with a cT1-3 cN0 tumor whose SLN was processed via OSNA assay and the TTL calculated were collected." Exclusion criteria: Page 10, paragraph 3: "Patients submitted to neoadjuvant systemic treatment, patients who had previous ipsilateral breast carcinoma, and patients with a TTL equal to 0 CK19 mRNA copies/μL were excluded." Sources and methods for selection of participants included consultation of the clinical records platform SClínicoV2 and application of the eligibility and exclusion criteria to patients treated between July 1st 2019 to June 30th 2021. Methods of follow-up: Page 11, paragraph 6: "All patients were assessed at the Breast Center out-patient clinic every 6 months. Patients were submitted to clinical examination, breast and axillary ultrasound (US), mammography, serum tumor markers, trunk CT scan and bone scan, according to institutional follow-up protocol. Regional node recurrence was mainly assessed by clinical examination and axillary US. Suspicious nodes were submitted to US guided needle biopsy (cytology or histology were both accepted)." (*b*) For matched studies, give matching criteria and number of exposed and unexposed. Not applicable.	10,11
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable Exposure was performance (or not) of an Axillary Lymph Node Dissection (ALND). Outcomes included Non-Sentinel Lymph Nodes (NSLN) status, locoregional recurrence, distant metastases and vital status.	10, 11, 12

		Page 10, paragraph 5: "Variables collected were age at diagnosis, follow-up time, tumor size, grade and histological type, multifocality, lymphovascular and perineural invasion, estrogen and progesterone receptor status, HER2 status, surgical treatment, number of biopsied SLN, number of positive SLN, TTL, number of excised NSLN during ALND, number of positive NSLN, adjuvant treatments, locoregional recurrence, distant metastases and vital status." Exposure: Page 11, paragraph 2: "Patients with a TTL of 30,000 CK19 mRNA copies/μL or below did not undergo ALND. Patients with a TTL of more than 30,000 CK19 mRNA copies/μL underwent ALND (Berg levels I+II) [21]. ALND product was sent to pathology for histological analysis." NSLN status: Page 12, paragraph 4: "In patients with a TTL above 30,000 CK19 mRNA copies/μL, NSLN status was given by histological analysis of the ALND product and by follow-up, because the surgeon did not perform a complete three-level ALND. In patients with a TTL of 30,000 CK19 mRNA copies/μL or below, NSLN status was given by follow-up and by histological analysis of an ALND product, in case clinical axillary recurrence occurred and the patient underwent an ALND." Locoregional recurrence: Page 11, paragraph 6: "Regional node recurrence was mainly assessed by clinical examination and axillary US. Suspicious nodes were submitted to US guided needle biopsy (cytology or histology were both accepted)." Distant metastases and vital status: Page 11, paragraph 6: "All patients were assessed at the Breast Center out-patient clinic every 6 months. Patients were submitted to clinical examination, breast and axillary ultrasound (US), mammography, serum tumor markers, trunk CT scan and bone scan, according to institutional follow-up protocol."	
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group Age at diagnosis - Consultation of clinical records (SClinico). Follow-up time - Consultation of clinical records (SClinico). Tumor size - Consultation of clinical records (SClinico and VC Integrator). Tumor grade - Consultation of clinical records (SClinico and VC Integrator). Histological type - Consultation of clinical records (SClinico and VC Integrator).	11

		Multifocality - Consultation of clinical records (SClinico and VC Integrator). Lymphovascular invasion - Consultation of clinical records (SClinico and VC Integrator). Perineural invasion - Consultation of clinical records (SClinico and VC Integrator). Estrogen and progesterone receptor status Consultation of clinical records (SClinico and VC Integrator). HER2 status - Consultation of clinical records (SClinico and VC Integrator). Surgical treatment - Consultation of clinical records (SClinico and VC Integrator). Number of biopsied SLN - Consultation of clinical records (SClinico and VC Integrator). Number of positive NSLN - Consultation of clinical records (SClinico and VC Integrator). Adjuvant treatments - Consultation of clinical records (SClinico). Locoregional recurrence - Consultation of clinical records (SClinico and VC Integrator). Distant metastases - Consultation of clinical records (SClinico and VC Integrator). Vital status - Consultation of clinical records (SClinico). Page 11, paragraph 4: "Adjuvant treatments included endocrine therapy, chemotherapy and radiotherapy. A patient was considered to have received endocrine therapy if drug prescription and successive renewals were registered in the clinical records. A patient was considered to have received chemotherapy (including anti-HER2 therapy) if completion of all chemotherapy cycles as defined by a medical oncologist was documented in the clinical records. A patient was considered to have received adjuvant radiotherapy if completion of a whole breast irradiation course was documented in the clinical records."	
Bias	9	Describe any efforts to address potential sources of bias SClinico and VCIntegrator were thoroughly searched to secure completion of our data base variables, therefore avoiding missing data.	
Study size	10	Explain how the study size was arrived at The study size was arrived at by consultation of the clinical records of patients treated in Breast Center between July 1st 2019 and June 31th 2021 and by application of the inclusion and exclusion criteria. Page 10, paragraph 3: " We studied patients treated between July 1st 2019 to June 30th 2021, at the Breast Center of CHUSJ, Porto, Portugal. Data from patients with a cT1-3 cN0 tumor whose SLN was processed via OSNA	10

		assay and the TTL calculated were collected. Patients submitted to neoadjuvant systemic treatment, patients who had previous ipsilateral breast carcinoma, and patients with a TTL equal to 0 CK19 mRNA copies/μL were excluded. Total study sample consisted of 87 patients."	
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why Quantitative variables were handled using medians: Page 12, paragraph 6: "Frequencies were used for qualitative variables and medians for quantitative variables."	12
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding Page 12, paragraph 6: "A database containing patient, tumor and node characteristics was created and all statistical analyses were performed using IBM SPSS Statistics v27.0, Chicago, USA. Frequencies were used for qualitative variables and medians for quantitative variables. A receiver operating characteristic curve (ROC) analysis was performed and the area under the curve (AUC) obtained for evaluation of the TTL discriminative power of NSLN status in our study sample. The AUC was also obtained for the 30,000 CK19 mRNA copies/μL criterion discriminative power of NSLN status. In an interim analysis, ROC analysis was used to search for additional TTL cut-offs in our study sample, and their AUC obtained. Finally, sensitivity, specificity, positive and negative predictive values and accuracy were calculated for the 30,000 CK19 mRNA copies/μL criterion and for the additional TTL cut-offs obtained." (*b*) Describe any methods used to examine subgroups and interactions Not Applicable. (*c*) Explain how missing data were addressed There was no missing data. (*d*) If applicable, explain how loss to follow-up was addressed There was no loss of follow-up. (*e*) Describe any sensitivity analyses There were no sensitivity analyses.	12
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	13

		391 potentially eligible patients were examined for eligibility; 87 patients were confirmed eligible and were included in the study: page 13, Figure 1 and page 13, paragraph 2: " Total study sample included 87 patients (Figure 1)." (b) Give reasons for non-participation at each stage 52 patients were excluded because their sentinel-lymph node was processed by conventional hematoxylin and eosin technique; 58 patients were excluded because they underwent primary systemic treatment and/or had previous ipsilateral breast carcinoma; 194 patients were excluded because they had a total tumor load of 0: page 13, Figure 1. (c) Consider use of a flow diagram Page 13: Figure 1.	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders Page 13, paragraph 2: "The characteristics of the study sample are synthetized in Table 1. The majority of the study sample (95.4%) are estrogen receptor-positive tumors. Median follow-up time was 21 months (range: 7 – 36 months). All but one patient had a follow-up equal to or more than 12 months. The patient died at 7 months of follow-up." Page 13, paragraph 3: "Only one patient had isolated tumor cells (TTL of 210 CK19 mRNA copies/μL) in the SLN. 36 patients had micrometastasis in the SLN and 50 patients had macrometastasis in the SLN." Page 13, paragraph 4: "Of the 87 patients included, 36 had a TTL above 30,000 CK19 mRNA copies/μL and were submitted to ALND. A total of 51 patients had a TTL of 30,000 CK19 mRNA copies/μL or below and did not perform ALND (Tables 1 and 2)." Pages 17 & 18: Table 2. (b) Indicate number of participants with missing data for each variable of interest There was no missing data. (c) Summarise follow-up time (eg, average and total amount) Page 13, paragraph 2: "Median follow-up time was 21 months (range: 7 – 36 months)."	13, 17, 18

Outcome data	15*	Report numbers of outcome events or summary measures over time Page 14, paragraph 1: " Of the 36 patients submitted to ALND, 14 had metastasized NSLN, whereas 22 performed ALND and all their NSLN were negative." Page 14, paragraph 1: "None of the 87 patients developed locoregional recurrence or distant metastases during follow-up."	14

Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included Page 14, paragraph 4: "The 30,000 CK19 mRNA copies/μL criterion yielded a sensitivity (SN) of 100.0%, a false negative rate (FN) of 0.0%, a specificity (SP) of 69.9%, a false positive rate (FP) of 30.1%, a positive predictive value (PPV) of 38.9% and a negative predictive value (NPV) of 100.0%. Accuracy was 74.7% and the AUC was 0.849 (Table 3)." Page 15, paragraph 1: "Of the 87 patients in our study sample, the 30,000 CK19 mRNA copies/μL criterion spared 51 patients (58.6%) an ALND. The previous CDR [15] and the Z0011 eligibility criteria [19], had they been applied, would spare 36 (41.4%) and 64 (73.6%) patients an ALND, respectively. The new TTL cut-offs spared a growing number of ALND until a maximum of 68 (78.2%) patients, obtained with the 260,000 CK19 mRNA copies/μL cut-off (Table 3). Of the 14 patients with a metastasized axilla, the 30,000 CK19 mRNA copies/μL criterion left 0 patients with a surgically untreated axilla versus 3 (21.4%) and 6 (42.9%) patients, had the previous CDR and Z0011 criteria been applied, respectively. All of the new TTL cut-offs left the same 5 (35.7%) patients with a surgically untreated metastasized axilla (Table 3)." Page 15, paragraph 2: "Of these 5 patients, two were cT1 and three were cT2. While two had 1 positive SLN, the other three had 2 positive SLN. A total of 4 were treated with lumpectomy and 1 with total mastectomy. All were treated with whole breast irradiation and hormone therapy. This means that of these 5 patients, four fulfilled the Z0011 eligibility criteria for avoiding ALND [19]. The only patient not fulfilling the eligibility criteria was due to total mastectomy performed. Also, four had Luminal HER2- tumors and one had a Luminal HER2+ tumor. While four had 2 or less positive NSLN, one had 4 positive NSLN, leading to two pN1 patients and three pN2. A total of 2 patients received adjuvant chemotherapy, one of whom was the patient with 4 metastasized NSLN (see supplementary material)." (*b*) Report category boundaries when continuous variables were categorized Not applicable. (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period Not applicable.	14, 15
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses Not applicable.	

Discussion			
Key results	18	Summarise key results with reference to study objectives Page 20, paragraph 1: "The 30,000 CK19 mRNA copies/μL criterion for deciding on performing an ALND yielded an AUC of 0.849, which is in another tier of discrimination in relation to the value of 0.636 obtained by *Fougo et al* in the 30,000 CK19 mRNA copies/μL criterion derivation study [11]. The 30,000 CK19 mRNA copies/μL criterion represented a positive evolution from the previous Breast Center CDR [10], having spared a greater number of patients an ALND while leaving no patients with metastasized NSLN. However, this came at a cost of an unacceptably high FP rate. Approximately one third of the study sample performed an ALND with no additional benefit in locoregional control, while inheriting the risks of an ALND procedure [8,24]." Page 21, paragraph 1: "The TTL cut-off of 260,000 CK19 mRNA copies/μL was the one that maximized specificity. Its PPV was 47.4%, which is more comparable to the 30,000 CK19 mRNA copies/μL criterion PPV obtained in the PORTTLE study [11] (54.8%) than the PPV of the 30,000 CK19 mRNA copies/μL criterion obtained in our study sample. In addition, the 260,000 CK19 mRNA copies/μL cut-off spared a greater number of ALND when compared to the 30,000 CK19 mRNA copies/μL cut-off, the previous CDR and the Z0011 criteria. This new criterion would leave approximately one third of the NSLN positive patients with a surgically untreated axilla, which is less than the value yielded by the Z0011 criteria." Page 21, paragraph 2: "In fact, with the 30,000 CK19 mRNA copies/μL criterion, approximately one third of the patients performed an unnecessary ALND (FP), whereas with the 260,000 CK19 mRNA copies/μL criterion, approximately one third would be left with a surgically untreated metastasized axilla (FN)." Page 21, paragraph 4: "A total of 4 of these 5 patients fulfilled the Z0011 criteria for not performing an ALND and all 5 patients had estrogen receptor positive tumors, remarkable for their usually long recurrence times [26,27]. The ALND procedure in these 5 patients upgraded the pN status of 3 patients. These 3 patients were staged as pN2 after the procedure, which could lead to further regional treatment with radiotherapy and/or systemic therapy with chemotherapy [25]. This means that the omission of ALND in these 3 patients, could potentially lead to undertreatment. Nonetheless, 2 of these 3 fulfilled the Z0011 criteria because they performed WBI and hormonetherapy. Therefore, with the Z0011 results in mind, the problem lies with the patient that performed total mastectomy and also had a total of 6 lymph nodes involved. This patient could potentially be undertreated, with impact on overall survival and/or disease-free survival, if the ALND procedure was omitted with use of the 260,000 CK19 mRNA copies/μL criterion."	20, 21, 22

Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias Page 24, paragraph 2: "Although this study was retrospective, complete follow-up was achieved and there was no missing data [33]. One pitfall of our study is that the majority of patients did not perform ALND. Studies relating to the SLN concept, such as studies that propose TTL cut-offs to decide on ALND, usually derive these cut-offs from a sample where every patient performed ALND. Nonetheless, our study sample was obtained from a center with a TTL cut-off already implemented and therefore it reflects the intention to evaluate performance of a clinical practice tool. The study design yields real world evidence, valuable for everyday clinical practice and complies to the principles of Evidence-Based Medicine [34]. Another possible critic to our study is that it is unicentric. As we stated before, the main objective of the study was to evaluate performance of a criterion derived from a national cohort, when applied to a localized population of breast cancer patients. In fact, this population proved to have a greater than average proportion of ER positive tumor patients [11,30], denoting the importance of personalized treatment."	24
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence Page 25, paragraph 1: "This study emphasizes the need to find a new balance between locoregional control and morbidity, bearing in mind that, in the current status of breast cancer adjuvant treatments, focus should be on preserving the axilla."	25
Generalisability	21	Discuss the generalisability (external validity) of the study results The study results are applicable to cT1-3N0 breast cancer patients whose sentinel-lymph node(s) are processed via One-Step Nucleic Acid Amplification Assay and the Total Tumor Load obtained. Our results were obtained in a population where 95.4% were estrogen-receptor positive tumors.	
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based Page 25, paragraph 3: "This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors."	25

*Give information separately for exposed and unexposed groups.

Regras de Formatação da revista *Surgical Oncology*

Preparation

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Please ensure the figures and the tables included in the single file are placed next to the relevant text in the manuscript, rather than at the bottom or the top of the file. The corresponding caption should be placed directly below the figure or table.

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REVISED SUBMISSIONS

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Regardless of the file format of the original submission, at revision you must provide us with an editable file of the entire article. Keep the layout of the text as simple as possible. Most formatting codes will be removed and replaced on processing the article. The electronic text should be prepared in a way very similar to that of conventional manuscripts (see also the Guide to Publishing with Elsevier). See also the section on Electronic artwork.
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Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Essential title page information

• ***Title.*** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
• ***Author names and affiliations.*** Please clearly indicate the given name(s) and family name(s) of each author and check that all names are accurately spelled. You can add your name between parentheses in your own script behind the English transliteration. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
• ***Corresponding author.*** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. This responsibility includes answering any future queries about Methodology and Materials. **Ensure that the e-mail address is given and that contact details are kept up to date by the corresponding author.**
• ***Present/permanent address.*** If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Highlights

Highlights are optional yet highly encouraged for this journal, as they increase the discoverability of your article via search engines. They consist of a short collection of bullet points that capture the novel results of your research as well as new methods that were used during the study (if any). Please have a look at the examples here: example Highlights.

Highlights should be submitted in a separate editable file in the online submission system. Please use 'Highlights' in the file name and include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point).

Abstract

A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using American spelling and avoiding general and plural terms and multiple concepts

(avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention there, as well as in the footnote. Ensure consistency of abbreviations throughout the article.

Acknowledgements
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources
List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].

It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, it is recommended to include the following sentence:

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Units
Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

Footnotes
Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article.

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.
• Preferred fonts: Arial (or Helvetica), Times New Roman (or Times), Symbol, Courier.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Indicate per figure if it is a single, 1.5 or 2-column fitting image.
• For Word submissions only, you may still provide figures and their captions, and tables within a single file at the revision stage.
• Please note that individual figure files larger than 10 MB must be provided in separate source files.

A detailed guide on electronic artwork is available.
You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

Regardless of the application used, when your electronic artwork is finalized, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS (or PDF): Vector drawings. Embed the font or save the text as 'graphics'.
TIFF (or JPG): Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
TIFF (or JPG): Bitmapped line drawings: use a minimum of 1000 dpi.
TIFF (or JPG): Combinations bitmapped line/half-tone (color or grayscale): a minimum of 500 dpi is required.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); the resolution is too low.
• Supply files that are too low in resolution.
• Submit graphics that are disproportionately large for the content.

Color artwork

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graphs. Image 'polishing' is also available, where our illustrators take your image(s) and improve them to a professional standard. Please visit the website to find out more.

Figure captions
Ensure that each illustration has a caption. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text
Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Data references
This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

Preprint references
Where a preprint has subsequently become available as a peer-reviewed publication, the formal publication should be used as the reference. If there are preprints that are central to your work or that cover crucial developments in the topic, but are not yet formally published, these may be referenced. Preprints should be clearly marked as such, for example by including the word preprint, or the name of the preprint server, as part of the reference. The preprint DOI should also be provided.

References in a special issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

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Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley. Using citation plug-ins from these products, authors only need to select the appropriate journal template when preparing their article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes from different reference management software.

Reference formatting
There are no strict requirements on reference formatting at submission. References can be in any style or format as long as the style is consistent. Where applicable, author(s) name(s), journal title/book title, chapter title/article title, year of publication, volume number/book chapter and the article number or pagination must be present. Use of DOI is highly encouraged. The reference style used by the journal will be applied to the accepted article by Elsevier at the proof stage. Note that missing data will be highlighted at proof stage for the author to correct. If you do wish to format the references yourself they should be arranged according to the following examples:

Reference style
Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.
Example: '..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ....'
List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.
Examples:
Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
[2] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, 2018. The art of writing a scientific article. Heliyon. 19, e00205.
https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
[3] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[4] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your

article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
Reference to a dataset:
[dataset] [6] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/xwj98nb39r.1.
Reference to software:
[7] E. Coon, M. Berndt, A. Jan, D. Svyatsky, A. Atchley, E. Kikinzon, D. Harp, G. Manzini, E. Shelef, K. Lipnikov, R. Garimella, C. Xu, D. Moulton, S. Karra, S. Painter, E. Jafarov, S. Molins, Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88), Zenodo, March 25, 2020. https://doi.org/10.5281/zenodo.3727209.

Data visualization

Include interactive data visualizations in your publication and let your readers interact and engage more closely with your research. Follow the instructions here to find out about available data visualization options and how to include them with your article.

Supplementary material

Supplementary material such as applications, images and sound clips, can be published with your article to enhance it. Submitted supplementary items are published exactly as they are received (Excel or PowerPoint files will appear as such online). Please submit your material together with the article and supply a concise, descriptive caption for each supplementary file. If you wish to make changes to supplementary material during any stage of the process, please make sure to provide an updated file. Do not annotate any corrections on a previous version. Please switch off the 'Track Changes' option in Microsoft Office files as these will appear in the published version.

Parecer da Comissão de Ética do

Centro Hospitalar Universitário de São João / Faculdade de Medicina da Universidade do Porto

Título do Projeto: Total Tumour Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients

Nome da Investigadora Principal: Nuno Miguel Gomes Barbosa de Sousa

Onde decorre o Estudo: No Centro de Mama do CHUSJ. Apresentou declaração do Prof. Doutor José Luís Fougo, que será o profissional de ligação.

Objetivos do Estudo:

Avaliar a sensibilidade, especificidade, valor preditivo positivo, valor preditivo negativo, likelihood ratios positivos e negativos de esta regra na decisão clínica.

Estudo realizado no âmbito do Mestrado Integrado em Medicina da FMUP, sob orientação do Prof. Doutor José Luís Fougo.

Conceção e Pertinência do estudo:

Este estudo pretende aferir, retrospetivamente, a validade externa desta regra de decisão clínica quando aplicada aos utentes do Centro de Mama, uma vez que esta foi derivada de uma coorte de doentes, nacional.

Serão incluídos casos entre 1 de julho de 2019 e 30 de junho de 2021. Não estão claramente definidas as variáveis a recolher para o estudo.

Benefício/risco: Não aplicável

Confidencialidade dos dados:

Os nomes dos participantes do estudo não serão registados na base de dados.

Apresentou um pedido de reutilização de registos clínicos para Investigação e Desenvolvimento ao RAI.

Respeito pela liberdade e autonomia do sujeito de ensaio: Não aplicável

***Curriculum* da investigadora**: Adequado à investigação.

Data previsível da conclusão do estudo: janeiro de 2023

Conclusão: Proponho um parecer favorável à realização do estudo, desde que identificadas as variáveis a recolher para o estudo e que as mesmas sejam não desproporcionadas com os objectivos do mesmo.

Porto, 28 de maio de 2021

O Relator da CE, Prof. Doutor Filipe Almeida

Parecer aprovado por votação electrónica dos membros da CE em 28 maio 2021

O Presidente da CE

Prof. Doutor Filipe Almeida

O investigador respondeu satisfatoriamente à questão enunciada no parecer, descrevendo as variáveis a recolher que são adequadas ao objectivo da investigação, pelo que o parecer da CE se torna favorável sem condicionalismos.

Porto, 17 junho 2021

O Presidente da CE

Prof. Doutor Filipe Almeida

Unidade de Investigação

Tomei conhecimento. Nada a opor. À DC.

28 de Setembro de 2021

A Coordenadora da Unidade de Investigação

(Prof.ª Doutora Ana Azevedo)

DIRECÇÃO CLÍNICA
2021/9/28 n.º 187/21

SÃO JOÃO

PEDIDO DE AUTORIZAÇÃO

Realização de Investigação

Exmo. Senhor Presidente do Conselho de Administração do Centro Hospitalar de São João

CONSELHO DE ADMINISTRAÇÃO - REUNIÃO DE
Presidente do Conselho de Administração
(Prof. Doutor Fernando Araújo)
Diretora Clínica | Enfermeira Diretora | Vogal Executivo | Vogal Executivo
(Profª Doutora Maria João Baptista) (Enfª Filomena Cardoso) (Dr. Luís Pinto Gomes) (Dra. Sofia Leal)

Nome do Investigador Principal:
Nuno Miguel Gomes Barbosa de Sousa

Título da Investigação:
Total Tumor Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients.

Pretendendo realizar no(s) Serviço(s) de:
Centro de Mama

a investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador/Promotor, autorização para a sua efetivação.

Para o efeito, anexo toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de São João/ Faculdade de Medicina da Universidade do Porto respeitante à investigação, à qual enderecei pedido de apreciação e parecer.

Com os melhores cumprimentos. *O Investigador/ Promotor*

Porto, 13 *de* Abril *de* 2021 . Nuno Sousa
assinatura

SÃO JOÃO | **Encarregado de Proteção de Dados** *Data Protection Officer*
Entrada 28/06/2021

Centro Hospitalar São João
Centro de Epidemiologia Hospitalar
22/9/2021

CES-IM005-0

SÃO JOÃO

U.PORTO
FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

Questionário para submissão de Investigação

Exmo. Sr. Presidente da Comissão de Ética do Centro Hospitalar de São João/ Faculdade de Medicina da Universidade do Porto,

Pretendendo realizar a investigação infracitada, solicito a V. Exa., na qualidade de Investigador, a sua apreciação e a elaboração do respetivo parecer. Para o efeito, anexo toda a documentação requerida.

IDENTIFICAÇÃO DO ESTUDO

Título da investigação: Total Tumor Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients

Nome do investigador: Nuno Miguel Gomes Barbosa Sousa

Endereço eletrónico: nunomgbsousa@gmail.com **Contacto telefónico:** 935 298 801

Caracterização da investigação:

☒ Estudo retrospetivo ☐ Estudo observacional ☐ Estudo prospetivo

☐ Inquérito ☐ Outro. Qual? _____

Tipo de investigação:

☐ Com intervenção ☒ Sem intervenção

Formação do investigador em boas práticas clínicas (GCP): ☐ Sim ☒ Não

Promotor *(se aplicável)*: _____

Nome do orientador de dissertação/tese *(se aplicável)*: José Luís Fougo

Endereço eletrónico: joseluisfougo@gmail.com

Local/locais onde se realiza a investigação: Centro de Mama

Data prevista para início: 30 / 06 / 2021 Data prevista para o término: 01 / 01 / 2023

PROTOCOLO DO ESTUDO

Síntese dos objetivos: O Portfle Study, desenvolvido por Fougo et al, estabeleceu uma regra de decisão clínica baseada num cut-off de 30.000 cópias de mRNA de CK19 para auxiliar na decisão de esvaziamento ganglionar axilar no doente com carcinoma da mama e gânglio sentinela positivo.
O objectivo deste estudo é avaliar a sensibilidade, especificidade, valor preditivo positivo, valor preditivo negativo, likelihood ratios positivos e negativos desta regra de decisão clínica.
Assim, pretende-se aferir a validade externa desta regra de decisão clínica quando aplicada aos utentes do centro de mama.

Fundamentação ética (ganhos em conhecimento/inovação; ponderação benefícios/riscos):
A regra de decisão clínica em estudo foi derivada de uma coorte de doentes nacional. É fundamental avaliar retrospectivamente a validade externa desta regra de decisão clínica quando aplicada a uma coorte de doentes do centro de mama. Uma vez que se trata da regra de decisão clínica atualmente em uso no Centro de Mama, é necessário avaliar se os resultados que está a produzir são aceitáveis à luz da mais recente evidência científica.
Trata-se de um estudo observacional e retrospectivo, em que os parâmetros de identificação pessoal dos doentes não serão registados pelos investigadores, pelo que não possui riscos.

CONFIDENCIALIDADE

De que forma é garantida a anonimização dos dados recolhidos de toda a informação?

Os nomes dos participantes do estudo não serão registados na base de dados

O investigador necessita ter acesso a dados do processo clínico? ☒ Sim ☐ Não

Está previsto o registo de imagem ou som dos participantes? ☐ Sim ☒ Não

Se sim, está prevista a destruição deste registo após o sua utilização? ☐ Sim ☐ Não

CONSENTIMENTO

O estudo implica recrutamento de:

Doentes: ☐ Sim ☒ Não Voluntários saudáveis: ☐ Sim ☒ Não

Menores de 18 anos: ☐ Sim ☒ Não

Outras pessoas sem capacidade do exercício de autonomia: ☐ Sim ☒ Não

A investigação prevê a obtenção de Consentimento Informado: ☐ Sim ☒ Não

Se não, referir qual o fundamento para a isenção:

Trata-se de estudo observacional, retrospectivo, efectuado pela consulta do processo clínico de doentes seleccionados e análise estatística dos dados colhidos

Existe informação escrita aos participantes: ☐ Sim ☒ Não

PROPRIEDADE DOS DADOS

A investigação e os seus resultados são propriedade intelectual de:

☐ Investigador ☐ Promotor ☐ Ambos ☐ Serviço onde é realizado

☒ Não aplicável Outro: _____

BENEFÍCIOS, RISCOS E CONTRAPARTIDAS PARA OS PARTICIPANTES

Benefícios previsíveis:

Nenhum.

Riscos/incómodos previsíveis:

Nenhum.

São dadas contrapartidas aos participantes:

- *pela participação* ☐ Sim ☐ Não ☒ Não aplicável
- *pelas deslocações* ☐ Sim ☐ Não ☒ Não aplicável
- *pelas faltas ao emprego* ☐ Sim ☐ Não ☒ Não aplicável
- *por outras perdas e danos* ☐ Sim ☐ Não ☒ Não aplicável

CUSTOS / PLANO FINANCEIRO

Os custos da investigação são suportados por:

☐ Investigador ☐ Promotor ☐ Serviço onde é realizado

☒ Não aplicável Outro: _____

Existe protocolo financeiro? ☐ Sim ☒ Não

LISTA DE DOCUMENTOS ANEXOS

- [x] Pedido de autorização ao Presidente do Conselho de Administração do Centro Hospitalar de São João *(se aplicável)*
- [ ] Pedido de autorização à Diretora da Faculdade de Medicina da Universidade do Porto *(se aplicável)*
- [x] Protocolo do estudo
- [x] Declaração do Diretor de Serviço onde decorre o estudo
  (sendo um estudo na área de enfermagem deve anexar também a concordância da chefia de enfermagem)
- [x] Profissional de ligação
- [ ] Informação dos orientadores
- [ ] Informação ao participante
- [ ] Modelo de consentimento
- [ ] Instrumentos a utilizar *(inquéritos, questionários, escalas, p.ex.)*: _____
- [x] Curriculum Vitae abreviado *(máx. 3 páginas)*
- [ ] Protocolo financeiro
- [ ] Outros:

COMPROMISSO DE HONRA E DECLARAÇÃO DE INTERESSES

Declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (1960 e respetivas emendas), e da Organização Mundial da Saúde, Convenção de Oviedo e das "Boas Práticas Clínicas" (GCP/ICH) no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo, nos últimos três meses. Comprometo-me a entregar à CES o relatório final da investigação, assim que concluído.

Porto, 13 *de* Abril *de* 2021

Nome legível: Nuno Miguel Gomes Barbosa de Sousa Nuno Sousa
assinatura

Parecer da Comissão de Ética do Centro Hospitalar de São João/ FMUP Emitido na reunião plenária da CE de 28 / 05 / 2021

Aguarda esclarecimentos.

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

Centro Hospitalar **São João**.

CONSIDERADOS QUE FORAM COMO SATISFATÓRIOS OS ESCLARECIMENTOS PRESTADOS PELO(A) INVESTIGADOR(A), A CES APROVA POR UNANIMIDADE O PARECER DO RELATOR, PELO QUE NADA TEM A OPOR À REALIZAÇÃO DESTE PROJETO DE INVESTIGAÇÃO.

17/06/2021

Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

187 - 21

RESPONSÁVEL PELO ACESSO À INFORMAÇÃO

SÃO JOÃO

Pedido de Reutilização de Registos Clínicos para Investigação e Desenvolvimento (I&D)

Número do Pedido
|2|1|0|0|6|1|2|8|
(A preencher pelo Gabinete de Apoio ao RAI)

Exmo. Senhor
Responsável pelo Acesso à Informação
(Artigo 9.º da Lei n.º 26/2016, de 22 de agosto)
Dr. Rui de Vasconcellos Guimarães

SÃO JOÃO
AUTORIZADO
Responsável pelo Acesso à Informação do Centro Hospitalar de São João (Art. 9º, Lei 26/2016, de 22/8)
28/06/2021

1. Identificação do(s) Investigador(es) *Preenchimento Obrigatório*

1.1. Investigador Principal *No caso de haver mais do que um investigador mencionar nas observações*
Nome Nuno Miguel Gomes Barbosa de Sousa
Contacto telefónico |+|3|5|1|9|3|5|2|9|8|8|0|1|
Endereço eletrónico nunomgbsousa @ gmail.com

1.2. Investigador(es) Associado(s)
Número Total: |3|
Nome Nuno Miguel Gomes Barbosa de Sousa
Contacto telefónico |+|3|5|1|9|3|5|2|9|8|8|0|1|
Endereço eletrónico nunomgbsousa @ gmail.com

Nome José Luís Rosas Fouga
Contacto telefónico |+|3|5|1|9|1|9|8|9|0|4|4|0|
Endereço eletrónico joseluisfouga @ gmail.com

Nome Bárbara Neves Peleteiro
Contacto telefónico |+|3|5|1|9|6|5|8|8|6|6|8|2|
Endereço eletrónico barbara.peleteiro @ chsj.min-saude.pt

1.3. Afiliação Institucional do Investigador Principal
1.3.1. Grupo Profissional
☐ Médico(a) ☐ Enfermeiro(a) ☐ Docente ☒ Estudante
☐ Outro. Qual?_____

1.3.2. Documento de identificação pessoal ou profissional
☒ **Cartão de Cidadão** ☐ Bilhete de Identidade ☐ **Cédula Profissional**
☐ Cartão de Docente ☐ Cartão de Estudante ☐ Outro. Qual?_____
Número de Documento |1|4|0|7|9|2|2|1|0|Z|X|9|

2. Enquadramento e Identificação do Trabalho de Investigação e Desenvolvimento *Preenchimento Obrigatório*

2.1. Enquadramento da investigação
☒ Projeto ou trabalho académico de investigação e desenvolvimento:
☐ Não conferidor de grau
☒ Conferidor de grau: ☐ Licenciatura ☒ Mestrado ☐ Doutoramento

SÃO JOÃO Gabinete de Apoio ao RAI 18 06 21 Assistente Hospitalar

RAI-IM002-0

2.2. Entidade(s) que tutela(m) a investigação

☒ Centro Hospitalar de São João

Serviço: Centro de Mama

☐ Universidade do Porto

Faculdade / Instituto:

☐ Outra Instituição. *Qual?*

Há alguma parceria entre instituições?

☐ Não ☒ Sim. Qual(is)? CHUSJ - Faculdade de Medicina da Universidade do Porto

2.3. Orientador *Se Aplicável*

Contacto telefónico

Endereço eletrónico jose luis fraga @ gmail.com

2.4. Título provisório

Total Tumor Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients

Deverá posteriormente indicar o título definitivo para emissão do Certificado de Reutilização pelo RAI – DAta REuse Certificate for Research – DARE através dos contactos disponíveis no fim deste formulário.

2.5. Acesso requerido

☐ Ficheiro

Descrição do património informacional a que pretende ter acesso, identificando a informação a obter, i.e. nome, morada, diagnóstico, idade, códigos dos distritos, entre outros.

☐ Consulta de processos clínicos em ambiente papel:

☐ Bloco ☐ Consulta Externa ☐ Hospital de Dia ☐ Internamento ☐ MCDT ☐ Urgência

Deverá anexar ficheiro(s) contendo a identificação do pretendido, i.e. números de processos, episódios, números de utente, entre outros.

Anexar ficheiro no ato de envio

☒ Consulta de registos clínicos eletrónicos

Especificar os Sistemas de Informação:

SClínico ; J-one

Data previsível de fim de utilização das credenciais de acesso 2023 - 01 - 01

☐ Outro Acesso. Qual?

2.3. Pareceres e Autorizações

☐ Autorização da Hierarquia

☐ Protocolo Científico Aprovado [1]

☒ Parecer da Comissão de Ética para a Saúde (CES) [1]

☐ Parecer do Centro de Epidemiologia Hospitalar [1]

Deverá anexar ficheiro(s) contendo cópia dos documentos referentes às opções selecionadas.

Anexar ficheiro no ato de envio

[1] *Obrigatório quando aplicável.*

3. Observações *Preenchimento Facultativo*

4. Aceitação dos Termos e Condições da Reutilização

Cumulativamente com as obrigações decorrentes da lei já citada *(n.º 2 e 3 do art.º 21 e o n.º 1 e 2 do art.º 12, ambos da Lei n.º 26/2016, de 22 de Agosto)* ao submeter o presente pedido concordo e fico ainda vinculado, juridicamente, aos seguintes termos e condições:

- Comprometo-me a manter confidencial toda a informação à qual vou ter acesso;
- Após explicação do RAI do CHUSJ, embora a Lei 26/2016, de 22 de agosto, imponha como requisito a anonimização sem possibilidades de reversão, tal desiderato, é não só uma impossibilidade matemática já comprovada, como ainda resulta num prejuízo para a investigação, face à quantidade e qualidade da informação a retirar à fonte, razão pela qual, concordando com o RAI, assumimos como compromisso a pseudo anonimização, o que impõe uma avaliação e gestão do risco, num quadro ético-jurídico que aceitamos e nos comprometemos a colaborar;
- Não vou elaborar registos, suscetíveis de identificar ou tornar identificável a identidade das pessoas a quem os mesmos dizem respeito;
- Comprometo-me a consultar os processos clínicos nos termos e locais que me forem indicados para o efeito;
- Além do presente pedido para reutilizar registos clínicos, dirigido ao RAI, comprometo-me a obter os necessários pareceres quer do Encarregado da Proteção de Dados, quer da Comissão de Ética do Hospital, quer ainda do Centro de Epidemiologia Hospitalar;
- Comprometo-me a citar as fontes, sempre que publicitar o trabalho de investigação, independentemente de requerer a Certidão de Reutilização (***DA**ta**RE**use Certificate for Research* – **DARE**).
- Tomei conhecimento, que a violação de qualquer dos compromissos aqui assumidos, resultará no apuramento de responsabilidades disciplinares, civis e penais e ainda, à impossibilidade futura de aceder a informação de saúde para fins de investigação.

5. Decisão do investigador sobre requerer a DAta REuse Certificate for Research – DARE *Preenchimento Obrigatório*

☒ **Pretendo** desde já requerer a Certidão de Reutilização **(DARE)** cujo sentido, valor e significado consultei em *http://portal-chsj.min-saude.pt/pages/710*.

☐ **Não pretendo** requerer a Certidão de Reutilização **(DARE)** cujo sentido, valor e significado consultei em *http://portal-chsj.min-saude.pt/pages/710*.

Na circunstância do requerente não indicar nenhuma opção, presumimos que pretende requerer o DARE.

6. Assinatura

Nota 1: Se o presente pedido for submetido eletronicamente ou faz assinatura digital qualificada; ou posteriormente vem ao Centro Hospitalar de São João exibir o seu documento de identificação pessoal; ou no âmbito do seu espaço de liberdade e como manifestação expressa do seu consentimento envia cópia do referido documento, neste caso, concluído o processo ser-lhe-á devolvida ou eliminada a cópia do documento de identificação pessoal, conforme as indicações que dê.
Nota 2: Se o presente pedido for entregue presencialmente, assina e exibe o documento de identificação a quem recebe o pedido.

Data 2021 - 09 - 13

Nuno Soares

Investigador Principal

Em caso de dúvida no preenchimento contacte através dos endereços eletrónicos
rai.reutilizacao.id@chsj.min-saude.pt **ou** ruiguimaraes@chsj.min-saude.pt
ou pelos números de telemóvel 962 204 194 **ou** 918 880 299

SUBMETER

SÃO JOÃO

ENCARREGADO DE PROTEÇÃO DE DADOS (EPD)
CENTRO HOSPITALAR UNIVERSITÁRIO DE S. JOÃO, EPE
Paulo Alexandre Mota da Silva
Encarregado de Proteção de Dados do CHUSJ
epd@chsj.min-saude.pt

Ref.ª CES CHUSJ: **187 / 2021**

Título do Projeto	Total Tumor Load to assist in the decision for additional axillary surgery in the positive sentinel node breast cancer patients
Responsável pelo tratamento	Nuno Miguel Gomes Barbosa de Sousa
Instituição	Centro Hospitalar e Universitário de São João (CHUSJ) Faculdade de Medicina da Universidade do Porto (FMUP)
Investigador	Interno **Externo**
Contacto telefónico	935298801 — Endereço Electrónico: nunomgbsousa@gmail.com
Profissional de Ligação	Prof. Doutor José Luís Fougo (Coordenador do Centro da Mama)
Amostra	250

Análise de Risco: Tolerável | **Baixo** | Elevado | Muito Elevado

Parecer do EPD: Data: 21/09/2021

Finalidade: O Centro de Mama do CHUSJ utiliza uma regra de decisão clínica para decidir quais os pacientes que vão ser submetidos a esvaziamento ganglionar axilar. Uma vez que esta regra de decisão foi derivada a partir de uma coorte de doentes a nível nacional, o objetivo deste estudo é aferir a validade desta regra quando aplicada a uma coorte de doentes do Centro de Mama.

Licitude: fundamento previsto no artigo **9(2)(j)**, com as garantias do **89(1)** do **RGPD**, e artigo **31(1)** da **LERGPD**.

Categorias de dados pessoais: variáveis identificadas com detalhe na AIPD, datada de 21/09/2021, **ponto 12**, tendo presente o **princípio da minimização dos dados.**

Conservação: os dados serão alvo de **pseudonimização**, armazenados em **local seguro**, em área restrita ao Investigador Principal, com acesso a ficheiros **protegido por palavra-passe**, efetuando-se a conservação durante o prazo máximo de **dois (2) anos**. Os dados recolhidos **serão destruídos** após a finalização do estudo.

Comunicação de Dados: **não há partilha** de dados pessoais.

Face ao exposto, e observadas as recomendações, entende-se que a presente AIPD apresenta os elementos necessários para assegurar que o **tratamento é realizado em conformidade com o RGPD.**

Recomendações:

1. Garantir medidas de segurança adicionais no transporte dos dados com recurso a dispositivos electrónicos de armazenamento (Laptop), nomeadamente através de medidas de cifragem e autenticação;
2. Em caso de necessidade de extensão de prazo e/ou de qualquer alteração dos pressupostos atinentes ao presente parecer o Investigador Principal deverá solicitar a reapreciação do projeto de investigação junto do EPD.

Revisão AIPD:

☐ Data da próxima revisão: ___ /___ / _____ ☒ Não carece de revisão.

Anexos:

1. Processo CES n.º 187/2021
2. Parecer CES (17/06/2021)
3. AIPD (21/09/2021)

Assinado por: **PAULO ALEXANDRE MOTA DA SILVA**
Num. de Identificação: 12061004
Data: 2021.09.21 11:20:33+01'00'

CARTÃO DE CIDADÃO