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**Evaluation of the histological size of the sentinel lymph node metastases using RT-PCR assay:  
a rapid tool to estimate the risk of non-sentinel lymph node invasion in breast cancer patients.**

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## **Abstract**

**Purpose:** A RT-PCR assay (GeneSearch™, Veridex, LLC), FDA approved and CE marked to detect metastases >0.2-mm in sentinel lymph nodes (SLNs) is used intra-operatively for breast cancer patient management. The assay provides qualitative results by applying cut-off values to cycle times (Ct) for mammaglobin (MG) and cytokeratin-19 (CK19) genes. Aims of this study were to evaluate the performance of the quantitative Ct values to estimate the size of nodal metastases and the risk of additional disease in non-SLNs.

**Methods:** SLNs from 367 patients were clinically processed using both BLN assay and post-operative histology. Complementary axillary lymph node dissection (ALND) was performed concurrently in case of BLN assay positivity or tumour size >2-cm.

**Results:** BLN positivity was reported in 19.6% of the patients for a sensitivity of 89%. BLN specificity (94.5%) and negative predictive value (97.5%) clearly demonstrated its reliability to guide ALND decision. All, except one, residual axillary metastases were found in BLN-positive patients. Considering the 78 patients with SLN positivity or discordant status according to both criteria, the metastases histological size was significantly correlated to the expression level of MG ( $p=0.62$ ) and CK19 ( $p=0.64$ ) genes ( $p<10E-6$ ). Moreover, ALND status positivity was significantly associated to Ct value of MG ( $z=2.4$ ;  $p=0.018$ ) and CK19 ( $z=3.2$ ;  $p=0.001$ ).

**Conclusions:** The high intra-operative quality performance of the BLN assay minimizes the need for second surgeries for ALND. Results from this investigational study suggest that markers Ct value may provide, intra-operatively, valuable metastases size data and a risk prediction of additional disease in non-SLNs.

## Introduction

A commercially available RT-PCR assay for MG and CK-19 (the GeneSearch™ Breast Lymph Node [BLN] Assay, Veridex LLC, Warren, NJ) has been adopted in our institution as an intra-operative test for the detection of sentinel lymph node (SLN) metastases and management of breast cancer patients [1,2]. As previously reported, molecular assay performance was compared with standard postoperative histology for 253 consecutive patients with clinically node-negative T1 early breast cancer (<2cm; which is our institutional criteria for SLN evaluation). The molecular assay correctly identified 26 of 27 macro metastases and 11 of 15 micro metastases. Overall concordance with histopathology was 93%, with 87% sensitivity. The BLN assay specificity (94%) and particularly the negative predictive value (97%) clearly demonstrated the reliability of the BLN assay to guide a decision on performing an axillary lymph node dissection (ALND) [2].

Approximately 50% of patients with a positive SLN are found to have no other nodal metastases [3-5]. Consequently many patients are undergoing an unnecessary ALND with no additional therapeutic benefit or further staging information provided. Some clinicians have thus begun advising their patients to not undergo ALND even though the patients' SLN contained disease. Previous investigations found that the size of the SLN metastasis is significantly predictive of the likelihood of additional, non-SLN metastases [6]. The AJCC Cancer Staging Manual, 6<sup>th</sup> edition includes size of the metastasis as a determining factor of stage and therefore of prognosis [7]. However, assigning a lesion size to many cases can be difficult because of differences in the distribution pattern of malignant cells within the node.

Actually, the FDA approved the BLN Assay for a qualitative positive or negative result only. However, interestingly we previously reported a correlation between the level of BLN assay gene expression and the size of the nodal metastases [1]. This result, if confirmed, would allow the quantification of the disease volume for adjuvant treatment recommendation in cases of BLN assay positivity but undetected disease by histology. Estimation of the metastases size could also be useful for making intra-operative decision of whether or not to do a complementary axillary dissection.

To corroborate our preliminary data, we retrospectively assessed the performance of the BLN assay in estimating the size of nodal metastases and the risk of invasion in non-SLNs.

## **Methods**

### Patients

From November 2006 to September 2008, 367 consecutive patients with clinically node-negative early breast cancer, ie. tumor size less than 2 cm (T1), were treated with breast-conserving surgery and SLN biopsy at the Jules Bordet Institute. BLN assay was considered for patient management and an ALND was thus performed during the same surgery as the SLN biopsy when the assay results were positive. However, as the current practice of the Jules Bordet Institute is to perform ALND for all tumours >2cm in size, same-surgery ALNDs were performed (N=58) despite SLN negativity when the primary tumor size was intra-operatively measured greater than 2 cm by the pathologist.

The study was approved by the ethics committee of our Institute.

### Sampling Method

SLNs were identified using 99m technetium-labeled, nanosized, Human Serum Albumin colloids and excised surgically before the lumpectomy to avoid contamination of the lymph nodes during tumour manipulation [8]. Fresh SLN sections of 1.5 to 2.0 mm in thickness were made along the short axis of the node and alternately processed by standard postoperative histology and by intraoperative GeneSearch™ Breast Lymph Node (BLN) assay (Veridex LLC, Warren, NJ).

### BLN Assay

The assay has been extensively described previously [1,2]. Briefly, for each SLN, all node sections were homogenized and RNA purified. Real-time RT-PCR assays were performed in a one-step reaction in which the expression of three markers genes - mammaglobin (MG), CK-19 and an internal control (IC; porphobilinogen deaminase, PBGD) - was evaluated. Cycle threshold (Ct) cut-off values were predetermined and validated for each marker by the manufacturer on the basis of 2 prospective multisite U.S. clinical trials [9,10]. Cut-off Ct values were  $\leq 31$  for MG and  $\leq 30$  for CK-19. RT-PCR result interpretation is done automatically by the Cepheid SmartCycler® instrument. The BLN assay result is “positive” if 1 or both genetic markers are positive (below the Ct cut-off).

### Histological Evaluation

As previously described [2], at least one 5µm thick H&E permanent section was obtained from all paraffin-embedded 2mm node sections not tested in the BLN assay. In case of negative histology, additional sections were taken at 100µm intervals until all material was exhausted and alternatively H&E- or IHC-stained with cytokeratin (cam 5.2). SLN positivity was considered on the basis of metastasis >0.2mm as defined by the TNM classification 6th edition [7]. Pathologists were blinded to results from the BLN assay. In cases of histology-positive SLN, a second surgery for ALND was performed when the BLN assay intra-operative result was negative.

### Statistical analyses

Association between parameters was assessed using the Spearman rank correlation test (for continuous variables) and the Mann-Whitney test (for binary versus continuous variables). P-values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS15.0 (Inc., Chicago, IL, USA).

## Results

SLN status was analyzed using the BLN assay for 367 patients and the results were compared with those of the post operative evaluation. The average number of SLNs analyzed per patient was 1.25 and BLN positivity found in 19.6% (72/367) of the patients versus 17% (62/367) assessed as positive by histological evaluation.

Complementary ALND has been performed during the same surgery as the SLN biopsy in cases of BLN assay positivity or a finding of tumor size greater than 2 cm as measured intra-operatively by the pathologist (see material and methods). A total of 128 ALND (35%) have been completed for the 367 patients and 19 (14.8%) of them contained residual metastases. Importantly, 18 out of these 19 ALND positive cases were from patients with positive BLN assay whereas only 16 were from patients with nodal metastasis detected by histology.

Among the SLN-positive patients evaluated by the BLN assay, residual axillary metastases were found in 25.7% (18/70) of the cases (in 2 cases ALND data were missing). In conformity with Jules Bordet institutional criteria, 58 ALND were performed despite BLN assay negativity for the patients with tumor size greater than 2 cm. Out of these 58 cases, 53 (91%) were histologically negative and only one (1.7%) ALND invasion was reported in a patient presenting a BLN-negative/histology-negative SLN.

### Robustness of the GeneSearch™ Breast Lymph Node (BLN) Assay to detect metastases in sentinel lymph nodes

An update of our previously published data on 253 patients [2] confirmed the reliability of the BLN assay to identify SLN status with a sensitivity of 89% when compared to postoperative histological evaluation (see table 1). Moreover, the BLN assay specificity (94.5%) and particularly the negative predictive value (NPV) (97.5%) clearly demonstrated that the BLN assay is reliable enough to guide an intra-operative decision on further axillary node dissection. These results are comparable to those reported by Blumencranz et al and Julian et al [9,10].

### Investigation of the reliability of the Ct value of CK19 and of MG to estimate the sizes of the nodal metastases and thereby estimate the risk of invasion in non-SLNs

To simplify the comprehension and to help the reader to judge the usefulness of the data reported below, our results have been summarized in a REMARK diagram (figure 1)[11].

### Estimation of the metastases size using the BLN assay

For 78 cases, the levels of MG and CK19 expression (Ct value) were compared with the size of the metastases evaluated by histology. Most of these cases, 54/78 (69%), were BLN positive and histology positive, the remaining 24/78 (31%) cases presented discordant status. A significant correlation was observed between the metastases size assessed by histology and the expression level of MG ( $p = 0.62$ ) and CK-19 ( $p = 0.64$ ) genes evaluated with the BLN assay ( $p$  values  $<10E-6$ ) (figure 2). Lower Ct values clearly indicate an increased likelihood of macro metastases detected by histology as described in table 2. Consequently, the BLN assay would allow the quantification of the disease volume for adjuvant recommendation, particularly in the 17 BLN positive cases with undetectable metastasis by histology.

Considering the 7 cases that were BLN negative but histology positive, 2/5 cases had a borderline CK19 Ct of 30.9 (cutoff  $\leq 30$ ) and 6 /7 were micro-metastases. The 17 cases that were BLN positive but histology negative also presented Ct values close to borderline with a median Ct of 28.6 for CK19 and 30.7 for MG (MG cutoff  $\leq 31$ ). Thus almost all discrepant cases presented micro-metastases or with borderline expression of both assay gene markers suggesting the presence of micro metastases that may be confined to the part of the sample subjected to the BLN assay only or to the histological assay only (figure 3).

### Mammaglobin biomarker needed for the detection of >10% of the nodal metastases

Contrary to other molecular assays, which only detect CK19 gene expression, the BLN assay detects both CK19 and MG genes expression.

Considering the 71 BLN positive assay (1 case with missing Ct data), 48 were considered positive for both MG and CK19, 15 were considered positive for CK19 only and 8 (11%) were considered positive for MG only. Importantly, two out of the 8 MG positive cases present detectable metastases by histology.

### Estimation of the risk of metastases in non-SLN using the BLN assay

Considering the 78 patients with BLN Assay and histological SLN positivity or discordant status with SLN positivity, a significant association was observed between ALND status positivity and Ct expression value of MG ( $z=2.4$ ;  $p=0.018$ ) and CK19 ( $z=3.2$ ;  $p=0.001$ ) (see figure 4) as well as between ALND positivity and metastases histological size ( $z=2.45$ ;  $p=0.014$ ). As shown in table 2 there is a clear association between lower Ct value and ALND positivity.

Regarding the impact of the metastases size on ALND positivity, the 69 patients with BLN-positive SLN for whom ALND has been performed were considered (3 missing patients, 1 with no Ct values and 2 with no ALND data). ALND positivity was reported in 60% of the patients presenting a



SLN with a CK19 Ct value < 20 (9/15) versus 22.5% of those presenting a SLN with a CK19 Ct value 20><26 and less than 9% (7/31) of those presenting a SLN with a CK19 Ct value >26 (2/ 23)(figure 5).

Regarding the impact of the number of BLN-positive SLNs on ALND positivity, the 128 patients for whom ALND has been performed were considered. In the majority of the patients (288/367, 78.5%), BLN assay has been performed in only 1 SLN, however in respectively 67/367 (18.3%) and 12/367 (3%) of the patients, 2 SLNs or more than 2 SLNs have been analyzed. Less than 2% of ALND positivity was reported for BLN-negative patient versus 20% for patients with only one positive SLN and 33% for patients with >1 positive SLNs.

## Discussion

No model, including the nomogram of Van Zee, is ideal for providing an estimate of the likelihood of finding additional disease in non-SLNs [6]. However, the size of the SLN is significantly predictive of additional non-SLN metastases and size measurement is therefore essential for staging and management of breast cancer patients [7].

We investigated the reliability of the Ct value of CK19 and of MG to estimate the sizes of the nodal metastases and thereby estimate the risk of invasion in non-SLNs. A statistically significant association was observed between the size of the metastases evaluated by histology and the expression levels of both markers. Thus, the BLN assay could be useful in estimating the volume of disease in cases with metastases undetected by histology, which represent 24% of the BLN positive patients. Moreover, even in cases of histologically detected metastases, it could often be difficult to assign a size because of the difference in the distribution pattern of malignant cells within the node. The GeneSearch™ BLN assay technology provides an objective and more thorough SLN assessment with a standardized evaluation of 50% of the node versus less than 5% of the node typically viewed microscopically with post-operative histological procedures.

The small number of cases with discordance between the BLN assay and postoperative histology status were associated with borderline Ct value of the BLN markers that most likely correspond to micro metastases in either the sample analyzed by the BLN assay or by histology only. The discrepancy is thus attributable to the sample processing (sample error) as 2mm pieces of the SLN were alternatively processed by BLN or histology. This hypothesis was supported by the fact that about one-third of the histology SLN node-negative patients of our Institute with BLN assay positive results presented nodal involvement either in other SLNs or in non-sentinel ALNs [2]. The calibration of the molecular assay is appropriate thus preventing possible over-treatment of these patients.

A significant association was observed between a high level of BLN marker expression and a higher risk of metastases in non-SLNs. Considering the CK19 biomarker, we were able to identify a high risk group with 60% of ALND positivity, an intermediate risk group with ~20% of ALND positivity and a low risk group with less than 10% of ALND positivity. The BLN assay marker expression can thus provide the surgeon with an intraoperative estimate of the risk for additional disease.

Additionally, the number of positive SLNs may also provide the surgeon with an estimate of the risk for additional disease. ALND invasion was reported in 20% of the patients with only one positive SLN versus in more than 30% of the patients with two or more positive SLNs. We thus recommend analysing 2 SLNs with the BLN assay for an optimal SLN status evaluation. First of all because we observed that the removal of more than one SLN allowed the reduction of histologic false negative cases and increased histological agreement with BLN assay positivity [2] and secondly because it provides an intraoperative risk prediction of additional disease in non-SLNs.

Validation that a cutoff value for CK19 of 26 assumes less than 10% of invasion in non-SLNs, will provide the surgeons with a valid predictor of non-SLN involvement. For some groups of patients, a cutoff value for CK19 less than 26 should even be considered to avoid the morbidity of completion ALND. Patient age has no predictive value for additional disease; nevertheless several studies have shown that most patients with SLN positivity but no ALND positivity were older individuals [12]. This suggests that a patient's age is associated with the amount of risk clinicians are willing to assume. Moreover, it would appear that ALND, and any therapies which are offered as a consequence of receiving ALND, confers no survival benefit over simple lumpectomy/mastectomy in women over 70 years with clinical stage I or II breast cancer. It is therefore reasonable to omit ALND for a subset of SLN positive elderly patients by using a cut off value for CK19 lower than 26 [13].

Contrary to other molecular based metastasis detection systems, the BLN assay detects MG in addition to CK19. In our study, although the majority of the BLN positive cases were CK19 and MG positive or CK19 positive, we also observed around 10% of cases only positive for MG. Therefore even if the OSNA assay offers a high sensitivity (95%) and specificity (97%) [14], this assay may miss some positive cases.

**Conclusion.** Results from this investigational study clearly suggest that the marker Ct value may provide, intra-operatively, valuable metastasis size data and thus allow an estimation of risk for additional disease in non-SLNs. The FDA approved the BLN assay for qualitative positive or negative results; these data are thus investigational and not currently available for intra operative use.

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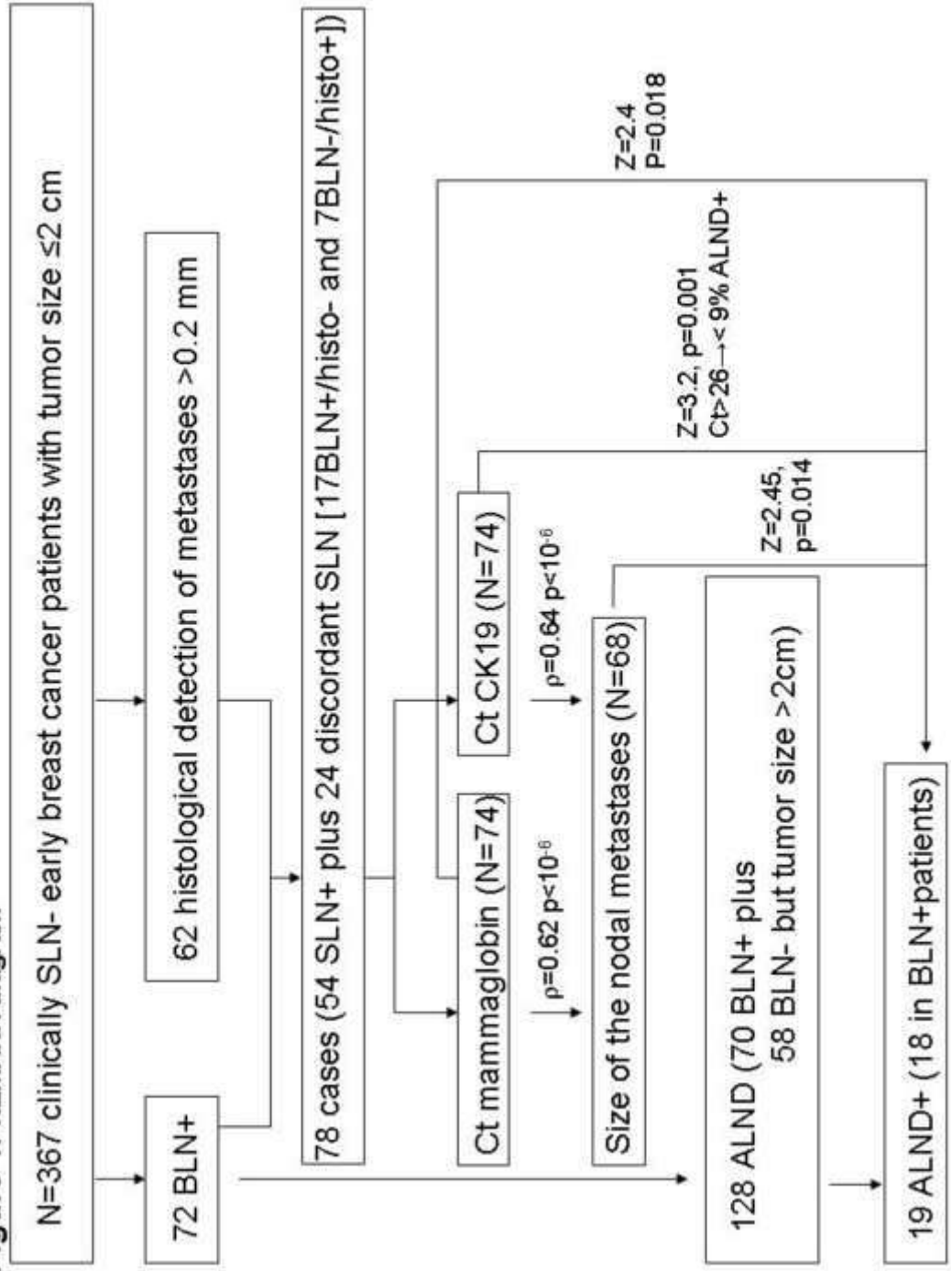
## Figure legend

### Figure 1: Remark diagram.

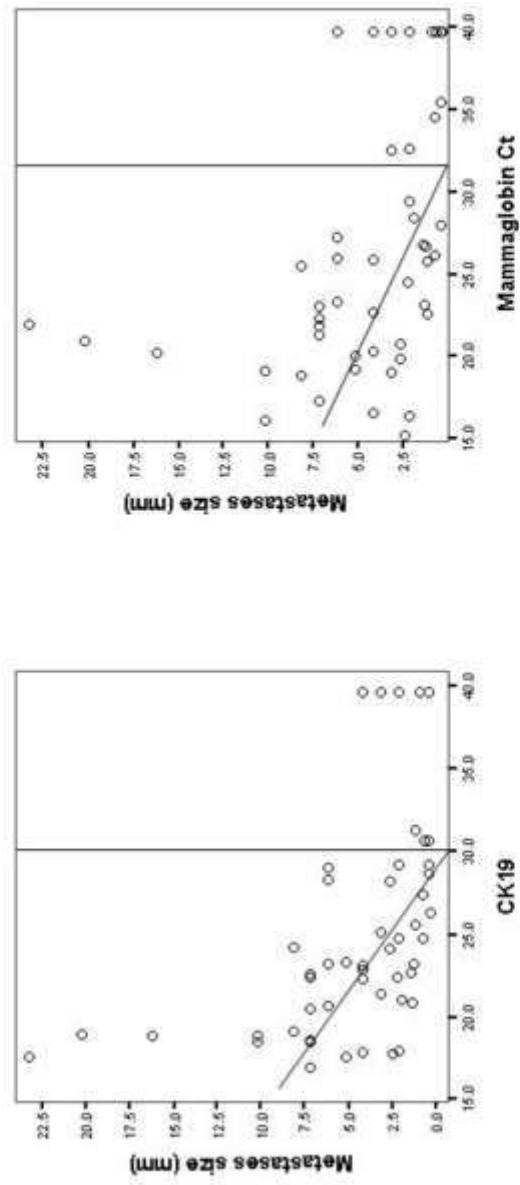
Sentinel lymph nodes (SLN) status has been evaluated on a population of 367 patients with early breast cancer. Positivity has been reported in 72 patients according to the BLN assay versus 62 patients according to histological evaluation. Complementary axillary lymph node dissection (ALND) has been performed in 128 patients with positive BLN assay or breast tumors >2cm in size.

To investigate of the reliability of the Ct value of CK19 and of MG to estimate the sizes of the nodal metastases and thereby the risk of invasion in non-SLNs, a subpopulation of 78 patients was considered. This subgroup was composed of patients with positive SLN according to both the BLN assay and the histological evaluation (N= 54) and of patients with SLN of discordant status according to both criteria (N=24).

**Figure 1. REMARK diagram**

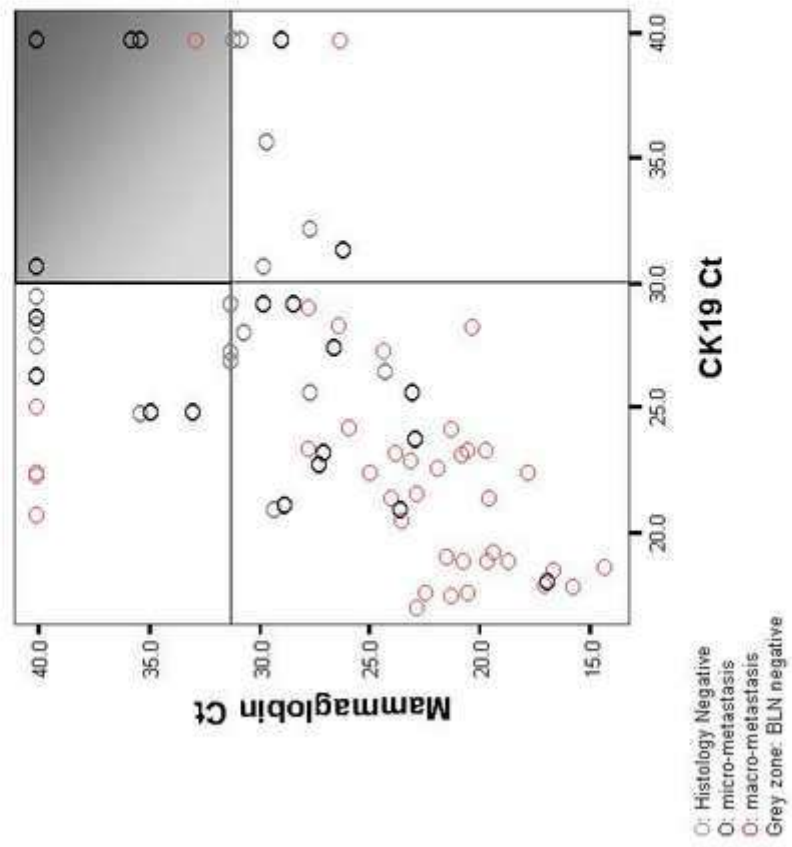


**Figure 2. Positive correlation between metastases size and CK and MG genes expression**

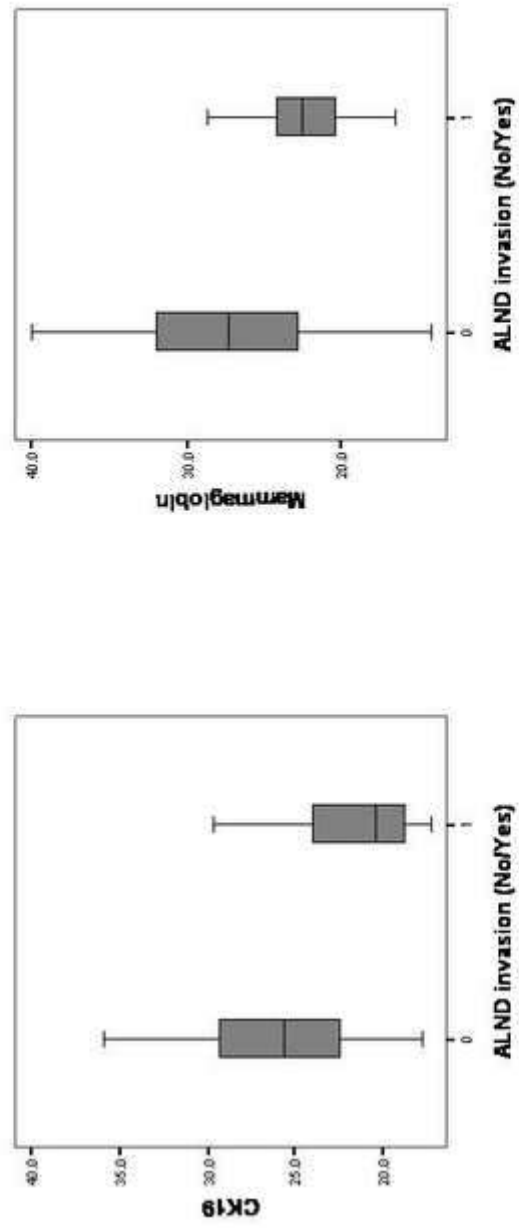




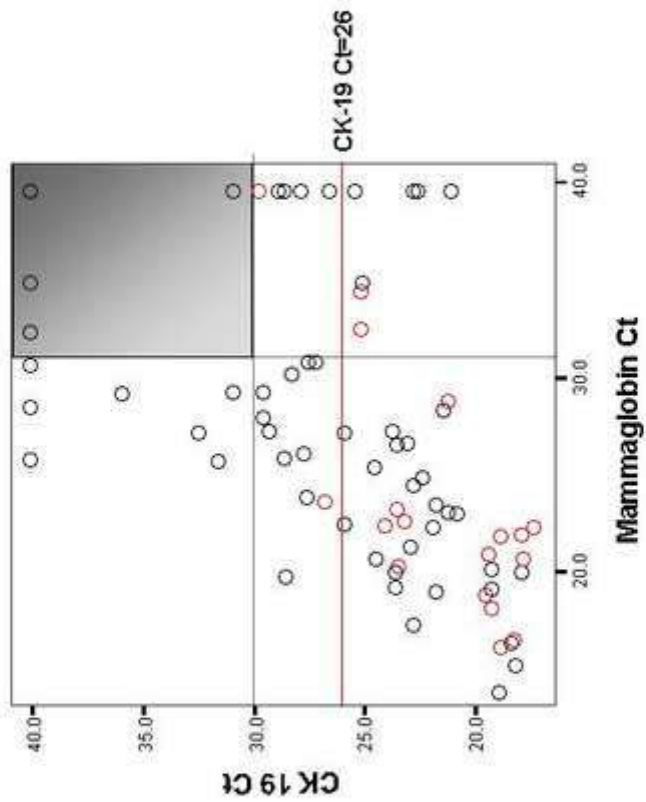
**Figure 3. Size of the metastases according to CK and MG genes expression**



**Figure 4. Association between non-SLNs invasion and CK and MG genes expression**



**Figure 5. Estimation of the risk of metastases in non-SLNs using the BLN assay**



**In red: ALND positive patients (N=18; 1 missing Ct values)**

**Table 1. BLN assay performance**

	Histological assay negative	Histological assay positive	Total
BLN assay negative	288	7	295
BLN assay positive	17	55	72
Total	305	62	367

NPV= 97.5% (288/295); PPV= 76.5% (55/72).  
 Specificity= 94.5 (288/305); Sensitivity= 89% (55/62)

**Table 2. Association between a higher metastases size and a higher expression of CK and MG genes (lower ct)**

MG	CK19	N	% H&E Positive	% MACRO-metastases*	% MICRO-metastases*	% ALND Positive
≤ 31.0	-	56	(46/56) 82	57	21	(15/55) 27
-	≤ 30.0	63	(52/63) 82	55	24	(18/62) 29
≤ 31.0	≤ 30.0	48	(44/48) 92	67	23	(15/48) 31
<hr/>						
< 25.0	-	34	(33/34) 97	85	11	(14/34) 41
-	≤ 23.5	34	(33/34) 97	78	18	(13/34) 38
< 25.0	≤ 23.5	26	100	91	9	(12/26) 46
<hr/>						
>31.0	>30.0	295	(7/295) 2.4	0.3	1.7	(1/58) 1.7

\* exclude H&E positives of unknown specific size