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Predictive factors of non-sentinel axillary lymph nodes in breast cancer patients. Molecular markers of primary tumor and variability of patient's microenvironment.

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ABSTRACT

Background: The management of breast cancer has changed dramatically over the past two decades, from radical mastectomy to breast conservative surgery. Axillary lymph node dissection (ALND) has moved from a complete dissection to a less invasive procedure, the sentinel lymph node biopsy (SNLB), which has less morbidity compared to ALND and is used to evaluate lymph node status. Patients with positive sentinel lymph node (SNL) undergo a complete ALND of axillary level I and II lymph nodes. This operation is burdened by relevant postoperative morbidity, such as upper limb lymphedema, wound complications, nerve injury, limited mobility, neuropathic pain, numbness and sensory loss. Axillary lymph node metastasis in early T stage breast cancer are often be confined within the SNL(s), which offers no information about the presence of additional non-SNL (NSNL) metastasis, which may not occur in 40% to 70% of cases. The identification of these low risk patients would prevent them the morbidity associated with a full ALND. In order to avoid unnecessary, highly morbid ALND, it has become imperative for breast surgeons to find diagnostic tool that can identify SNL positive patients at low risk of NSNL metastasis and also who can safely avoid an ALND.

Material and Methods: We analysed whether a panel of molecular genes, expressed in primary breast cancer patients who underwent SNLB for breast cancer at our Institution, could predict NSNL metastasis and the influence of polymorphisms of genes related to inflammatory and angiogenetic pathways (IL8 *rs4073,* VEGF-2 *rs11133360*) on axillary lymph node status and to predict NSNL metastasis.

Molecular Markers:

- Pilot study: We calculated the expression of a panel of genes in 24 frozen tissue samples from selected patients with positive SNL received ALND, 12 with negative NSNL and 12 with positive NSNL. We customized our PCR arrays that include the genes of RT2 Profiler 'human modified breast cancer' PCR arrays (SABiosciences) and 4 genes (NDUFA7, MERTK, FN1, PSMB6) obtained from a previous microarray study performed in our department (unpublished). These 88 genes are involved in tumor classification, signal transduction and pathways such as angiogenesis, adhesion, proteolysis, cell cycle, and apoptosis.
- Validation study: We evaluated the expression of five significant genes (THBS1, IGF1, ERBB2, GRB7, MGMT), obtained from previous pilot experiment, in 171 frozen tissue samples from primary breast cancer of patient, who underwent breast surgery and SNLB for invasive breast cancer, between 2000 to 2013 in 1st Surgical Clinic, with RT-PCR.

Polymorphisms (IL8 rs4073 VEGFr-2 rs11133360):

Genomic DNA was isolated from peripheral whole blood/buffy coat of 234 patients. 10 to 20 ng of DNA of each patient were used for TaqMan SNP genotyping assays (Applied Biosystems). Genotyping was performed by Real-Time PCR using allelic discrimination in the 7300 RT-PCR System (Applied Biosystems). Post run data were by 7300 SDS software (Applied Biosystems) and Automatic calls were assigned with approximately 99.8% quality analysed.

Results:

Molecular Markers: THBS1 (p:0.0012) and IGF1 (p:0,05) were statistically differently expressed between patients with positive SNLs and negative NSNLs and patients with positive SNLs and positive NSNLs. ERBB2, GRB7, MGMT were differently expressed between the two groups, not statistically significant (p:0,07; p:0,07; p:0,08), but we included in the validation study. The RT-PCR for THBS1, IGF1, ERBB2, GRB7, MGMT was performed in 171 patients. Only GRB7 level was differently expressed in the interest group of our research. The median expression level of GRB7 in patients with positive SNLB and negative NSNL was 3,14 ng/ul, in the group of patients with positive SNL and positive ALND the median level was 6,76 ng/ul (p:0,014). The other genes (IGF1, MGMT, ERBB2, THBS1) had a similar expression in these groups of patients

Vascular invasion (IV) and frozen section analysis were predictive factors of NSNLs status on univariate logistic regression analysis ($p<0,05$). Multivariate logistic regression analysis confirmed that GRB7 was an independent predictive factor of NSNLs in patients with positive SNLB (p:0,017). Moreover, frozen section analysis proved to be statistically different in the two groups on multivariate analysis (p:0,047).

The discrimination of the multivariable model was assessed with the area under the receiver ROC curve and AUC was 0,77.

Polymorphisms (IL8 rs4073 VEGFr-2 rs11133360): We considered two models for both polymorphism: dominant and recessive. When we analysed the correlation between the genotype and NSNLs status in patients with positive SNLs, no statistically significant results were obtained for both models. The two polymorphisms investigated were no predictive of axillary status in patients with positive SNL, but only IL8 rs4073, in recessive model, was significantly different between patients with axillary metastasis (N+) and patient with negative axillary status (N0)

Conclusion: Our data suggest that the expression level of GRB7 could be a predictive tool of axilla status in patients with positive SNLB and could help the surgeon to decide the better axillary surgery in this group of patients. The polymorphisms investigated are not predictive of NSNL status, but a homozygous woman for the allele A of the polymorphism IL-8 rs4073 has a probability 2.28 times greater than the rest of the sick population of developing axillary metastasis from breast cancer.

BACKGROUND

BREAST CANCER SURGERY

The dominant model for breast cancer in the 19th century was a mechanistic model, describing the disease as a local phenomenon within the breast spreading centrifugally along lymphatics to first and then second echelon nodes, with progressive systemic spread. Radical local surgery, the Halstead radical mastectomy, was a therapeutic consequence of this model. In the late 1960s, this model was replaced by a biological model described by Fisher[1] , in which disease outcome is pre-determined by the extent of micrometastatic dissemination, early on in the natural history of the disease. Depending on the assessment of recognised prognostic markers, at diagnosis, prognosis is now believed to be either 'favourable' or 'unfavourable' rather than disease being regarded as 'early' or 'late'. The National Surgical Adjuvant Breast Project B04 trial[2] reported that axillary lymph node dissection was important for achieving local control and obtaining prognostic information, but prophylactic removal of the lymph nodes did not improve survival. This supports the biological model rather than the mechanical model of stepwise spread. The therapeutic consequence of this model has been the development of adjuvant systemic therapy using cytotoxic drugs or endocrine manipulation. In keeping with this model, surgical treatment of breast cancer has evolved substantially in the last three decades away from extensive surgical resection (radical mastectomy) towards a more tailored and conservative approach to the breast (lumpectomy followed by radiation therapy, that is the so called "breast conservation surgery"). Two randomised controlled trials[3] [4], with over 20 years of follow-up, demonstrated that breast conserving surgery followed by radiotherapy is as effective as mastectomy with no detrimental effect on patient survival.

A comparable trend has been seen with axillary surgery, where the surgical option has evolved from complete ALND to SNLB, which has significantly less morbidity compared to ALND and has become widely used to evaluate lymph node status in patients with breast carcinoma.

By definition, the sentinel lymph node is frequently the first node in the lymphatic basin that receives drainage from an anatomic region and is immunologically responsible for that region. Its relatively low false negative rate of 5 to 10% (patients with negative sentinel lymph node who developed lymph nodes metastasis during their follow-up) and high sensitivity rate of 90 to 95% in the detection of cancer to the lymph node basin has made this minimally invasive operation a standard.

Patients with a positive SNLs undergo a complete ALND of level I and II lymph nodes. ALND is burdened by meaningful postoperative morbidity, such as upper limb lymphedema, wound complications, nerve injury, limited mobility, neuropathic pain, numbness, and sensory loss in a significant number of patients.

Through aggressive screening programs and educational campaigns for patient self-exam, breast cancer is now detected at an earlier stage, resulting in fewer axillary metastasis, which are detected in 4-37% of American Joint Committee on Cancer (AJCC) TNM stage I and II.

Although SNLB is far less morbid than ALND, it is not without risks or morbidity. It is still an invasive procedure and complications include pain, paraesthesia and arm swelling up to 24 months

after SNLB surgery[5]. It offers no information about the presence of additional NSNL metastasis, which may not occur in 40% to 70% of cases.

PREDICTIVE FACTORS OF AXILLARY STATUS

Recent studies have shown that axillary metastasis in early T stage breast cancer, if present, may be confined only to the SNLs. The identification of these low risk patients would avoid them the morbidity associated with a full ALND.
In order to avoid the over-treatment suffering brought about by ALND, it has become imperative for breast cancer surgeons to find diagnostic tool that can identify SNL positive patients at low risk of NSNL metastasis and also who can safely avoid an ALND.
It is important to make a distinction between prognostic and predictive factors[6 7]. Prognostic factors are clinical, pathologic or biological features of cancer patients that predict clinical outcome such as disease-free or overall survival. Predictive factors are clinical, pathologic or biological features that estimate the likelihood of axillary lymph node metastasis. Developments in scientific methodology have generated an ever-increasing number of markers for primary and metastatic disease but none of these are in widespread clinical use.
Using biological parameters of the primary tumor to estimate the risk of axillary lymph node involvement could provide a rationale for proceeding to SNLB or axillary sampling.
Predictors may be of particular clinical utility in cases where the SNLB is equivocal (contains micro-metastasis) or negative, offering a strategy to reduce the false negative rate. They may also offer an alternative to invasive techniques in prognostication and planning of adjuvant treatment.
The benefit of accurate predictors of lymph node status is likely to be greatest in those patients who are axillary lymph node negative at the time of diagnosis, these individuals could be spared the risk, cost and distress of a negative axillary procedure. Predictors may also find utility in situations where complete lymph node sampling is not possible, for example, in the context of neo-adjuvant chemotherapy, which affects the ability to perform accurate surgical staging and situations where co-morbidity, logistical, or financial considerations prevent ALND. Ultimately, the introduction of these tools in clinical practice will lead to more accurate and less invasive axillary staging and improve breast cancer patient management and quality of life.
Several studies have investigated these predictive factors, some groups have focused on finding predictive factors of axillary metastasis, while others, more recently, have studied the predictive factors of NSNLs in patients with positive SNLs.

PREDICTIVE FACTORS OF AXILLARY METASTASIS

Clinical characteristics

Clinical features including young age at diagnosis, palpability of the primary tumor and axillary lymph nodes have been correlated with axillary lymph node status[8 9].
Clinical examination of the axilla is notoriously unreliable in detecting axillary lymph node involvement. Clinical characteristics are often subjective and lack the accuracy and consistency to guide surgical intervention, however, they may provide justification for further diagnostic tests, including imaging or image-guided biopsy.

Radiological characteristic

Very few studies evaluated imaging of the primary tumor as a means of predicting axillary lymph node involvement. Santamaria et al.[10] used power Doppler ultrasound to grade the vascularity of 97 primary tumors. Number of arteries and tumor size were found to be significant independent predictors of lymph node status. Mussurakis et al.[11], using dynamic contrast-enhanced magnetic resonance imaging (MRI), found that maximum enhancement ratio in regions of interest within the primary tumor was a strong predictor of nodal status.

Modern imaging modalities including ultrasound (US), MRI and positron emission tomography (PET) have been used to image the axilla.

With diagnostic imaging there is always a trade-off between sensitivity and specificity, depending on the radiological criteria used, cut-off points, inter-observer variation and patient population. Results to date are inconsistent with higher accuracies achieved when two imaging modalities are combined. With US, the reported sensitivities and specificities range from 50% to 84% and 80% to 92%, respectively, with higher sensitivity achieved when US is combined with fine needle aspiration cytology (FNA)[12 13 14]. Two prospective studies showed moderate accuracy with FDG-PET for the detection of axillary lymph node involvement, with a sensitivity of 61% to 85%, specificity of pathological characteristic 80% to 99% and positive predictive value of 62% to 98%[15 16]. FDG-PET cannot therefore be routinely recommended for axillary staging. More recently, the combination of US and 99mTc-sestamibi scintimammography was demonstrated to be superior to US alone with sensitivity, specificity and accuracy for the combination of 92%, 93% and 92%, respectively.

Contrast-enhanced MRI for local staging is well established, particularly as an adjunct to determine suitability for breast conserving surgery and nipple preservation. There is, however, little published evidence on its accuracy with respect to imaging the axilla, perhaps as a result of poor contrast enhancement of lymph nodes with gadolinium based contrast agents used for imaging the breast. This may change with the use of ultra-small super paramagnetic iron oxide (USPIO) contrast agents, which are specifically phagocytosed by lymphocytes[17].

Pathological characteristics

Tumor size

The maximum diameter of the invasive component of the primary tumor correlates both qualitatively and quantitatively with axillary lymph node involvement [18], for example, in tumors of size <10 mm and < 20mm, 15% and 25% of cases, respectively, were found to be axillary lymph node positive. The seemingly simple relationship between tumor size and lymph node status is, however, clinically unreliable. In two studies of patients with invasive breast carcinoma, tumor size and peritumoral vascular invasion have emerged as the two most powerful independent predictors of SNL metastasis, these variables were not, however, found to be predictive of the extent of axillary lymph node involvement [19 20].

Tumor grade and morphology

The correlation between tumor grade and axillary lymph node involvement is well recognised[21]. The modified system of Bloom and Richardson includes a measure of cellular proliferation, pleomorphism and tissue differentiation. Of these three important parameters, the extent of tumor cell mitosis or Ki67 index (which correlates with S-phase fraction and mitotic index) is an independent prognostic indicator and is correlated with lymph node metastasis[22 23].

Furthermore, the proliferative index of intratumoral fibroblasts has also been shown to be associated with the presence of axillary lymph node involvement[24].
The histological tumor type has also been correlated with axillary lymph node involvement with favourable tumor types including tubular, mucinous, medullary and cribriform carcinomas[20].

Primary tumor extent
The multifocality or multicentricity of the primary tumor has also been shown to be an independent predictor of axillary lymph node metastasis[20 25]. Multifocal tumors arise at different points along a single duct system in the same segment of the breast, whilst multicentric tumors arise from different duct systems in different segments of the breast. Positive resection margins may correlate with an aggressive phenotype, rather than inadequate surgery, and have been found to be associated with axillary lymph node involvement[26].

Tumor vascularisation
Neo-angiogenesis is a prerequisite to tumor growth and is intimately involved with metastasis[27]. Intra-tumoral microvessel density (IMVD), evaluated with the vascular marker CD-31, has been identified as a surrogate marker for tumor angiogenesis and is correlated with lymph node status[28]. IMVD has been identified as an independent prognostic factor particularly for node negative disease[29].

Lymphangiogenesis
Lymphovascular invasion of primary breast cancer has long been associated with axillary lymph node involvement[20 30] and is defined as the presence of tumor within an endothelial celllined space. Since the discovery of markers of lymphangiogenesis (including D2-40 and LYVE-1) there has been a growing body of evidence that correlates lymphangiogenesis with breast cancer metastasis[25 31 32].

Hormone receptor status
Progesterone receptor status as measured by nuclear immunoreactivity correlated with axillary lymph node involvement[20 33], but in other studies the immunohistochemical determination of oestrogen, progesterone or HER-2 receptor status has not been found to be consistently related to lymph node status[25 34 35 36]

Molecular characteristics
- Protein markers

Proteins with diverse cellular functions are altered in breast cancer, some of which also correlate with the likelihood of lymph node metastasis. Chelouche-Lev et al.[37] reported the small heat-shock protein alpha B-crystallin as a marker of axillary lymph node involvement in breast cancer. CD44 (standard variant) is a cell surface adhesion molecule and CD44 negativity has been shown to be significantly associated with axillary node involvement[38]. Immunohistochemical staining of human breast cancer tissues with antibodies against the mucinous carcinoma associated antigen (MCA) has also been correlated with axillary lymph node involvement and metastasis, but analysis of the data did not show any association with disease-free survival[39]. RhoCGTPase is a novel tissue biomarker involved in cell polarity and motility and increased levels of this protein are associated with biologically aggressive breast cancers, in particular those with axillary lymph node metastasis[40].

Expression of the enzyme Cyclo-oxygenase-2 has also been found to be associated with lymph node metastasis[41].

Reduced expression of both Bax and Bcl-2, which are proteins involved in the regulation of apoptosis, has been found to correlate with axillary metastasis[42]. Lectins (carbohydrate binding proteins) have been used to study the glycosylation of proteins and lipids on primary breast cancers. Staining with the lectin Helix pomatia agglutinin (HPA) has been shown to be a powerful predictor of axillary lymph node involvement and mortality in breast cancer[43] and this relationship has been shown to be stronger in the context of DNA ploidy[44].

Sauer et al.[45] compared the protein expression in preoperative core needle biopsy of the primary lesion with the protein expression in lymph nodes with sandwich immunoassays and demonstrated that FGF-2, Fas and TIMP-1 were predictive of nodal status ($p<0.05$ logistic regression).

- Genetic markers

Many changes in oncogenes and tumor suppressor genes have been reported in breast cancer, some of which correlate with lymph node metastasis and are prognostic and predictive markers[46]. Hirata et al.[47] used fluorescence in situ hybridisation and found that polysomy of chromosome 7 was positively correlated with the number of metastatic lymph nodes and overall survival. In keeping with these findings, it has been reported that microsatellite instability is correlated with lymph node metastasis[48]. Kataoka et al.[49] studied the transcription product of a novel human gene designated HRad17 in human breast cancer and found statistically significant relationships with both the proliferative index and lymphatic permeation of the primary tumor and lymph node metastasis. Grey et al.[50] performed artificial neural network-based analyses of the oncogene S100A4, the tumor suppressor gene nm23 and steroid receptor expression (oestrogen and progesterone) and found this to be an effective predictor of nodal status.

Analysis of gene expression profiles using high-throughput technologies such as microarrays has been used in conjunction with both non-supervised and supervised cluster analyses. The data from these studies show that many genes are upregulated in breast cancer and some of these correlate with overall survival[51 52] as well as lymph node metastasis[48]. These analyses have yielded many putative predictive markers of outcome and are useful for the classification of, for example, tumor sensitivity to chemotherapy or radiotherapy[53 54 55 56].

Other group[57] identified differential protein peaks (metal binding polypeptides at 4,871 Da and 8,596) in primary breast cancer that predict ALN metastasis using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS).

Other proteomic study using a combination of 2D-SDS-PAGE and Lc-MC/MS showed 19 proteins up-regulated and 3 proteins down-regulated in node-positive breast carcinoma compared to node-negative breast carcinomas[58].

Hao et al.[59] tried to elucidate molecular event associated with metastatic process and with microarray data, observed statistically significant overexpression of IGFBP-5, down-regulation of cyclin D1, and unchanged MDM-2 in metastatic tumor cells. Nonetheless, although fibronectin and *MMP2* mRNA expression levels were decreased in many metastasis specimens, expression levels of the corresponding proteins in the extracellular matrix were elevated in most metastasis.

Other group did not get the same results, but observed similarities of the overall genomic and proteomic profiles between primary tumors and matched ALN metastasis, suggest that key biological characteristics of the primary breast tumor are maintained in the corresponding lymph node metastasis[60].

PREDICTORS OF NON SENTINEL LYMPH NODES IN PATIENTS WITH POSITIVE SENTINEL LYMPH NODES

Clinicopathologic characteristic of primary tumor and sentinel lymph nodes

Multiple published studies have identified pathologic characteristics of the primary tumor and SNL that are associated with an increased likelihood of additional positive NSNLs in patients with positive SNLB. As shown in this table from a meta-analysis[61] tumor size and metastasis size are most frequently find to be predictors of NSNLs (Fig.1).

TABLE 2
Association between Clinicopathologic Features and NSN Metastasis by Study

Study	Tumor size	Metastasis size	Extranodal extension	LVI	Histologic grade	Age	ER	PR	Histologic type	No. of positive SNs	Total no. of SNs	HER-2-*neu*	Tumor palpable	Tumor location
Abdessalam et al.[5]	○	●[a]	●	●	○	○	○	○	○	○		○	○	
Weiser et al.[7]	●	●		●	○	○			○					○
Rahusen et al.[8b]	○	●		○	○	○	○			●			○	
Wong et al.[9]	●				○	○				●	○			○
Turner et al.[10b]	●	●[a]	●	●	●	○	○	○		●		○	○	
Hwang et al.[11]	●	●	○	●	○	○	○	○	○	○	●	○		
Reynolds et al.[12b]	●	●		○	○	○	●	○	○					
Cserni[13]	●	●	●		○									
Kamath et al.[14]	●	●[a]												
Mignotte et al.[15]	○[c]	○[a,c]												
Canavese et al.[16]	○[c]	○[c]												

NSN: nonsentinel lymph nodes; LVI: lymphovascular invasion; ER: estrogen receptor; PR: progesterone receptor; SNs: sentinel lymph nodes; Open circles; feature evaluated but not found to be statistically significant by univariate analysis; Solid circles: feature found to be Statistically significant by univariate analysis.

[a] Included stratification for lymph nodes positive by immunohistochemistry only.

[b] Statistical significance calculation based on data that included nonsentinel lymph nodes determined to be positive with routine use of immunohistochemistry.

[c] Statistical analysis not reported.

Fig 1 from *Clinicopathologic features of metastasis in nonsentinel lymph nodes of breast carcinoma patients.* Degnim AC et al. Cancer. 2003[61]

In addition to this, two most recent studies have confirmed the correlation between the size of the tumor and the presence of metastasis in NSNLs in positive SNLs patients[62 63] .
In the previous meta-analysis there was no correlation between the age of the patient and the probability of metastasis of NSNLs, whereas two recent studies have shown the opposite[63 64].
Histological grade does not seem to affect the probability of NSNLs metastasis, only two authors have obtained a significant correlation[62-64].
Vascular invasion confirms to be a strong predictor of not only axillary status, but also of the presence of additional metastasis in NSNLs, as seen from meta-analysis, but also from more current articles[64 65 66].
The histological type did not prove to be a good predictive factor as showed by meta-analysis[61] and other recent studies[64-66], only Nogi et al. described a statistically significant correlation between histotype and presence of metastasis in NSNLs. This study is retrospective with a small number of patients and the analysis conducted is only univariate[67].
Discordant are the results on the role of hormone receptors[64], although most authors have not obtained correlation between expression of hormone receptors and additional metastasis in patients with positive SNLs[63 67 68 69]. Only one study showed that HER2/neu over-expression predicted the presence of NSNL involvement[69].

The metastatic sentinel lymph node features are strongly related to the presence of other metastasis in the axilla. The main characteristics studied are: size of metastasis, presence of extranodal extension and number of positive SNLs[61 62 66 67 70]. Chae et al[69] considered also the number of SNL removed, ratio of SNL+/SNL removed, ratio metastatic diameter/SNL diameter, area of metastasis, ratio metastatic area/SNL area and total metastatic area. All these parameters were predictors of additional metastatic disease in NSNLs ($p<0,05$ multivariate analysis).

However, none of these characteristics by themselves can identify a subset of patients for whom ALND is unnecessary.

Several mathematical models have been developed to predict NSNLs status in patients with breast cancer with SNL metastasis[70] (Fig.2). These models use an array of primary tumor characteristic (tumor size, histologic grade, lymphovascular involvement and hormonal receptors) and nodal factors (number of positive SNLs, detection method and size of metastasis) to calculate the risk of NSNL metastasis. These include four nomograms: the Memorial Sloan-Kettering Cancer Center (MSKCC) nomogram, the nomogram developed by Degnim et al (Mayo nomogram), the nomogram developed by Pal et al (Cambridge nomogram), and the nomogram developed by Kohrt et al (Stanford nomogram); three scoring systems: the Tenon score, the score from The University of Texas M. D. Anderson Cancer Center (MDA score), and the score developed by Saidi et al (Saidi score), and two recursive partitioning (RP) tools developed by Kohrt. Only two models are available as online calculators (MSKCC and Stanford) (Fig.3).

Table 2. Description of Models to Predict Nonsentinel Node Involvement in Breast Cancer Patients With Sentinel Node Metastasis

Characteristic	MSKCC Nomogram[12]	Mayo Nomogram[11]	Cambridge Nomogram[15]	Stanford Nomogram[14]	MDA Score[6]	Tenon Score[10]	Saidi Score[16]	RP-ROC[14]	CART[14]
Type	Nomogram	Nomogram	Nomogram	Nomogram	Score (−2 to 4)	Score (0 to 7)	Score (0 to 4)	RP	RP
Threshold	≤ 10%	≤ 10%	≤ 10%	≤ 10%	≤ 0	≤ 3.5	≤ 2	≤ 10%	≤ 10%
Age of patient according to estrogen receptor status		X							
Histologic tumor size		X		X*	0 pt when ≤ 10 mm, 1 pt when > 10 mm	0 pt when ≤ 10 mm, 1.5 pt when 0 to 20 mm, 3 pts when > 20 mm	2 pts when > 10 mm, 1 pt if not	X	X
Palpable mass or not							1 pt, 0 if not		
Tumor type	X								
Histologic grade			X						
Lymphovascular invasion	X			X†	1 pt, 0 if not		1 pt, 0 if not	X	X
Multifocality	X								
Estrogen receptor status	X								
No. of SNs removed					−2 pt when ≥ 3				
No. of negative SNs	X	X							
No. of positive SNs	X	X							
Extracapsular extension		X‡					1 pt, 0 if not		
The size of SN metastasis		X	X§	X†, X*	2 pts when macrometastasis, 0 if not	2 pts when macrometastasis, 0 if not		X	X
Method of detection of SN metastases	X								
Ratio of positive SNs to all removed SNs			X			0 pt when ≤ 0.5, 1 pt when 0.5-1, 2 pts when = 1			

Abbreviations: MSKCC, Memorial Sloan-Kettering Cancer Center; MDA, The University of Texas M. D. Anderson Cancer Center; RP-ROC, recursive partitioning with receiver operating characteristic; CART, boosted Classification and Regression Trees; RP, recursive partitioning; pt, point; SN, sentinel lymph node.
*Composite variable: tumor size × (size of SN metastasis)^2.
†Composite variable: lymphovascular invasion × size of SN metastasis.
‡No. of positive SNs according to the presence or absence of extracapsular extension.
§Overall size of SN metastases.

Fig.2 from *Comparison of models to predict nonsentinel lymph node status in breast cancer patients with metastatic sentinel lymph nodes: a prospective multicenter study.* Coutant C et al. J Clin Oncol. 2009 [70].

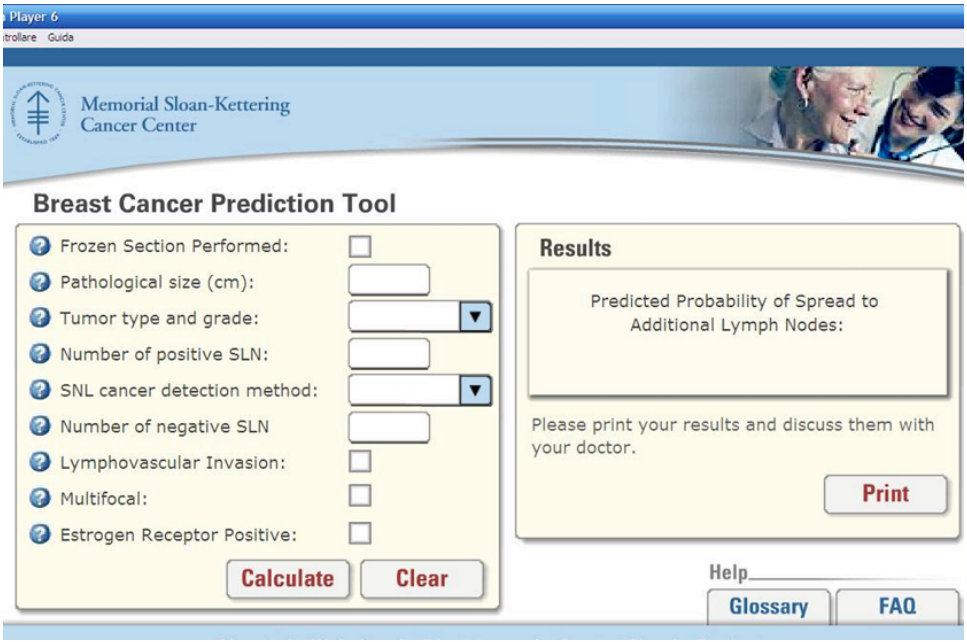


Fig.3 MSKCC nomogram online

Several group validated these models in retrospective study and compared the different score systems[71 72 73 74 75 76 77 78 79].

Coutant et al.[71] evaluated and compared the tools in an independent, multicenter cohort of patients with breast cancer and positive SNLs. The group studied the performance of the nine models in terms of discrimination (AUC), calibration, FN rate, and clinical utility (number of patients at low risk for positive NSNLs). Only two of nine models had an AUC greater than 0.75. Three models were well calibrated. Two models yielded an FN rate less than 5%. Three models were able to assign more than a third of patients in the low-risk group. Overall, the Memorial Sloan-Kettering Cancer Center nomogram and Tenon score outperform other methods for all patients, including the subgroup of patients with only SNL micrometastasis or ITC .

All of these studies suggest that all models do not perform equally, the routine clinical practice and patient characteristics varied among different hospitals, thereby, greatly influencing the accuracy, consistency, and reproducibility of these models and hampering their extensive application.

Molecular markers

Few authors have investigated the role of molecular markers in predicting the status of NSNLs. Several studies focused on the role of molecular subtypes classification (luminal A, luminal B, HER2 over-expression and triple negative**)** rather than on the search for real molecular markers[62 64 66]. Luminal A and Luminal B relative to triple negative subgroups showed a higher risk of NSNLs metastasis in patients with positive SNL.

Known et al. tried to find the biological markers of the primary tumor by immunohistochemical analysis: E-cadherin, CD44, cyclin D1, p21, p53, CK7, CK18, CK19, CK5, p63 did not appear to be helpful predictors[65].

Some authors investigated molecular markers not in primary tumor but in SNL metastasis, using one-step nucleic acid amplification (OSNA), to quantify total tumoral load (TTL) in the positive SNL[68 80]. This assay has been reported as a molecular detection procedure that analyses lymph node metastasis by detection and amplification of cytokeratin 19 mRNA. TTL was a predictor of NSNL

additional metastasis[68].

Other authors[67] investigated the role of breast cancer stem cells (CD44+CD24-) in NSNLs involvement. These cancer stem cells have the capacity to self renew and to differentiate, recent data have shown a subpopulation of this cells in the bone marrow in early breast cancer patients and this study demonstrated that these cells have an impact of NSNLs metastasis. Positive NSNL metastasis were significantly associated with CD24- expression (p:0,04), CD44+ expression (p:0,01) and CD44+CD24- expression (p:0,02).

The molecular markers most studied in this field are the proteins of human matrix metalloproteases (MMPs). An elevated expression of MMPs and their specific inhibitors (TIMPs) by mononuclear inflammatory cells from the stroma (MICs) of primary tumors seems to be significantly associated with the evidence of distant metastasis in breast cancer[81 82]. These results suggest to study the expression of this family proteins in primary breast tumor with positive SNL and the role of MMPs and TIMPs as predictors of NSNLs status. MMP1 and TIMP-2 expression by MICs was significantly associated with metastatic spread to NSNLs. One study used to calculated the expression of the proteins a multiplex sandwich immunoassays and the other group an immunohistochemical analysis[63 83]. Some author[84] tried to find biomarkers that can predict NSNL status using next-generation RNA sequencing and the table below showed the results (Fig 4).

Table 3 Top ten of differentially expressed genes in the NSLN positive group

Gene	NSLN negative	NSLN positive	Log2(FC)	FDR	Description
Up-regulated genes					
KLK11	0.93	185.48	7.64	0.00015	Kallikrein-related peptidase 11
SCGB3A1	8.06	1231.39	7.26	4.89E-05	Secretoglobin, family 3A, member 1
CLEC3A	0.98	143.59	7.19	2.96E-06	C-type lectin domain family 3, member A
CYP2A6	0.45	63.31	7.12	0.00029	Cytochrome P450, family 2, subfamily A, polypeptide 6
KLK10	0.17	19.11	6.80	0.0034	Kallikrein-related peptidase 10
KLK12	0.89	94.90	6.73	0.0011	Kallikrein-related peptidase 12
KLK13	0.57	49.98	6.45	7.42E-05	Kallikrein-related peptidase 13
CYP2A7	0.33	26.37	6.32	0.0052	Cytochrome P450, family 2, subfamily A, polypeptide 7
OBP2B	0.43	33.39	6.29	0.0391	Odorant binding protein 2B
KCNC2	0.32	21.60	6.09	0.0040	Potassium voltage-gated channel, Shaw-related subfamily, member 2
Down-regulated genes					
KRT20	17.19	0.07	−7.95	0.00019	Keratin 20
KRT4	6.42	0.05	−7.14	0.048	Keratin 4
VPREB1	25.93	0.19	−7.10	0.012	Pre-B lymphocyte 1
RBP2	31.24	0.58	−5.75	0.0014	Retinol binding protein 2, cellular
ALDOB	4.64	0.09	−5.66	0.011	Aldolase B, fructose-bisphosphate
BIRC7	7.80	0.16	−5.57	0.017	Baculoviral IAP repeat containing 7
FIBCD1	7.75	0.19	−5.38	0.00091	Fibrinogen C domain containing 1
MUC13	8.85	0.23	−5.24	0.0022	Mucin 13, cell surface associated
FCAMR	50.50	1.38	−5.19	4.97E-12	Fc receptor, IgA, IgM, high affinity
DHRS2	19.04	0.55	−5.10	0.0085	Dehydrogenase/reductase (SDR family) member 2

FC fold change of (NLSN positive/negative)

Fig. 4 from *Molecular biomarkers screened by next-generation RNA sequencing for non-sentinel lymph node status prediction in breast cancer patients with metastatic sentinel lymph nodes.* Liang F et al. World J Surg Oncol. 2015[84]

Molecular tests may hold significant promise, because they are more objective, more standardized, and easier to popularize. Unfortunately, currently available biomarkers remain limited and their practical value still needs additional verification.

Polymorphisms

We know that the metastatic process does not depend only on intrinsic tumor factors, but is a result of interaction between the tumor and the microenvironment of the host. Particularly the pathways of inflammation play an important role. Numerous studies have shown how inflammatory and angiogenetic pathways are related to the risk of developing the tumor, but also affect its progression and prognosis. The balance between inflammatory and anti-inflammatory actions is essential for proper control of the immune response and protection against underlying tissue damage. However, factors such as bacterial or viral infections, diet and lifestyle exposure, as well as individual genetic makeup may disrupt this balance and lead to a heightened state of inflammation resulting in tissue damage. Interleukins (ILs) are a group of cytokines that control cell growth and differentiation, cell migration and inflammatory and anti-inflammatory responses of the immune system. Cytokines are potentially central to the carcinogenic process, since they are key regulators of immune response.
Pro-inflammatory ILs generally include IL-1, IL-2, IL-6, IL-8, IL-15 and IL-17, whereas anti-inflammatory ILs include IL-4 and IL-10. ILs have been linked to tumor progression. In particular, serum IL-8 has been shown to promote malignant progression of breast tumors and has been associated with inflammatory pathways and angiogenesis. IL-8 can bind to two different forms of the IL-8 receptor: IL-8 receptor alpha also called CXCR1 and IL-8 receptor beta also called CXCR2. Numerous studies investigated genetic polymorphisms in inflammatory response genes and their associations with breast cancer risk. NFKB1, NFKB1a, IL-8, IL-10, IL-6, IL-1a, IL1b and TNF polymorphisms could serve as useful predictive biomarkers for breast cancer risk[85 86 87 88 89].
Several studies showed that the polymorphism of some cytokines (IL6, IL8, IL1) are also associated with aggressive phenotype of breast carcinoma, as defined by the high histological grade, axillary lymph node metastasis and large tumor size[87 88 89 90].
Snoussi et al.[90] demonstrated a significantly increased risk of breast carcinoma with heterozygous IL-8 (-251) TA and homozygous IL-8 (-251) AA. A significant association between IL-8 (-251) AA homozygous genotype and the aggressive phenotype of breast carcinoma (axillary's lymph node metastasis) was found[90 91].
Also the polymorphism of the IL-8 receptors (CXCR2) was associated with increased breast cancer risk and decreased overall survival and disease-free survival[91].
Current evidence indicates that angiogenesis plays an important role in the pathogenesis of several malignancies, including breast cancer. Angiogenesis is critical for the growth and metastasis of invasive tumors and constitutes an important component in the suppression of cancer formation. The process of transporting excess nutrients, producing some risk factors, and forming tumor blood vessels and a route for tumor cell egress induces tumor aggression, growth, and dissemination. Vascular endothelial growth factor (VEGF) acts as an angiogenic inducer that is an endothelial cell-specific mitogen, and as a mediator of vascular permeability, playing a central role in the regulation of this process. The vascular endothelial growth factor (VEGF) pathway has been investigated extensively, due to its important role in angiogenesis. The major mediator of tumor angiogenesis is VEGF-A, frequently referred to as VEGF, which activates the VEGF receptor-2. The VEGF gene is located on chromosome 6 and constitutes a highly polymorphic gene. Numerous polymorphism (SNPs) in the promoter, 5'- and 3'-untranslated regions (UTR) of VEGF gene have been recognized. This genetic variability possibly influences the production and function of VEGF. Subsequently, the VEGF SNPs may have an impact on breast cancer risk and disease outcome[92 93].
Patients with the −634CC genotype had significantly higher VEGF mRNA in breast cancer tissue

than those with −634GG or −634GC genotypes (P<0.001). High VEGF mRNA was shown to be associated with a tumor size >2 cm (OR:2.476; p:0.039), the presence of lymphovascular invasion (OR:2.406; p:0.021), and the presence of axillary nodal metastasis (OR:2.288; p:0.025)[93].
Other authors have not shown a statistically significant association between VEGF polymorphisms and the risk of breast cancer[94 95]. The most frequent polymorphism studied was +936 C/T, but current data on the association between VEGF +936 C/T and breast cancer susceptibility have shown a great discrepancy. Recent results from meta-analysis and review suggests that this polymorphism may not be associated with breast cancer risk[96 97].

AIMS OF THE STUDY

Characteristics of primary tumor, such as size, grade, and hormone receptor status have been previously associated with NSNL involvement. Several studies identified clinical parameters and histo-pathological characteristics of primary tumor and its SNL metastasis that are associated with the risk of additional metastasis in the NSNLs. Predictive models based on retrospective analysis of patients' and tumors' characteristics (e.g., age, histological type, tumor size, lymphovascular invasion, and hormone receptor status), such as the Nomogram of Memorial Sloan-Kettering Cancer Center and the scoring systems of MD Anderson, Tenon, Cambridge, and Stanford, are the most frequently considered. However, the routine clinical practice and patient characteristics varied among different hospitals, thereby, greatly influencing the accuracy, consistency, and reproducibility of these models and hampering their extensive application.
Molecular markers of primary tumor to estimate the risk of NSNL has not been studied so much. Breast cancer with specific gene expression or fusion have more invasive behavior and is at higher risk of lymph node metastasis. On the other and, neoplastic transformation, growth, invasion, and metastasis depend upon the establishment of a pro-angiogenic environment, which determine local angiogenesis together with the over-expression of pro-angiogenic and inhibitors factors.
The main aims of this project are two:

- investigate a panel of molecular genes expressed in primary breast cancer patients who underwent SNLB to predict NSNL metastasis.
- investigate the influence of polymorphisms of genes related to inflammatory and angiogenetic pathways (IL8 *rs4073,* VEGF-2 *rs11133360*) on axillary lymph node status and to predict NSNL metastasis.

MATERIALS AND METHODS

This research was conducted using the resources of Tissue Bank of 1st Surgical Clinic: 189 frozen section tissue from breast primary tumor and 234 blood/buffy coat from patients who underwent breast surgery for invasive breast cancer in our department between 2000 to 2016.

Molecular Markers:

- Pilot study: We calculated the expression of a panel of genes in 24 frozen tissue samples from selected patients with positive SNL received ALND, 12 with negative NSNL and 12 with positive NSNL. We customized our PCR arrays that include the genes of RT2 Profiler 'human modified breast cancer' PCR arrays (SABiosciences) and 4 genes (NDUFA7, MERTK, FN1, PSMB6) obtained from a previous microarray study performed in our department (unpublished). These 88 genes are involved in tumor classification, signal transduction and pathways such as angiogenesis, adhesion, proteolysis, cell cycle, and apoptosis.
- Validation study: We evaluated the expression of five significant genes (THBS1, IGF1, ERBB2, GRB7, MGMT), obtained from previous pilot experiment, in 171 frozen tissue samples from primary breast cancer of patient who underwent breast surgery e SNL biopsy for invasive breast cancer (sample of population) between 2000 to 2013 in 1st Surgical Clinic.

Laboratory techniques:

A. RNA extraction and reverse transcription

Each surgical specimen was snap-frozen in liquid nitrogen within 15 minutes of collection and stored in liquid nitrogen. Total RNA was extracted using the TRIzol reagent lysis buffer (Thermofisher Scientific, Waltham, MA USA) according to the manufacturer's protocol. In brief:

1. HOMOGENIZATION

50-100 mg of tissue were homogenized in 1 mL of TRIZOL Reagent using an homogenizer and 5mm stainless steel beads (TissueLyser, QIAGEN, Hilden, Germany) for 2-5 min.

2. PHASE SEPARATION

The homogenized samples were incubated for 5 minutes at 15 - 30°C to permit the complete dissociation of nucleoprotein complexes. 0.2 mL of chloroform were added and samples were shaken vigorously for 15 seconds and incubated at 15 - 30°C for 2 to 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 2 - 8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. RNA PRECIPITATION

RNA was precipitated from the aqueous phase by mixing it with 0.5 mL isopropyl alcohol. Samples were incubated at 15 - 30°C for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 2 - 8°C.

4. RNA WASH

The RNA pellet was washed once with 75% ethanol, adding at least 1mL of 75% ethanol. The samples were mixed by vortexing and centrifuged at 7,500 x g for 5 minutes at 2 - 8°C.

5. REDISSOLVING THE RNA
At the end of the procedure, the RNA pellet was briefly air-dried. The RNA was dissolved in 30 μL RNase-free water, passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 - 60°C.
Quantification and quality assessment of total RNA were performed with Nanodrop 1000 spectrophotometer (Thermofisher Scientific, Waltham, MA USA).
A further purification step with RNeasy Mini Kit (QIAGEN, Hilden, Germany) was applied to RNA samples employed in the pilot study, requested for the application of the RT2 Profiler PCR arrays protocol (SABiosciences - QIAGEN, Hilden, Germany).
1μg of total RNA was reverse transcribed employing MultiScribe™ Reverse Transcriptase kit (Thermofisher Scientific, Waltham, MA USA) following the manufacturer's instructions. After an incubation of 10 minutes at 25°C, the reaction was carried out for 2 hours at 37°C and then stopped incubating 5 minutes at 85°C. MultiScribe™ Reverse Transcriptase is a recombinant moloney murine leukemia virus (rMoMuLV) reverse transcriptase that has been optimized for TaqMan-based assays. RNA samples employed in the pilot study were reverse transcribed with RT2 First Strand Kit (SABiosciences - QIAGEN, Hilden, Germany). In this case the reaction was carried out for 15 minutes at 42°C and then stopped incubating 5 minutes at 95°C.

B. qRT-PCR

A real-time quantitative PCR (qRT-PCR) was performed in an LightCycler 480II (Roche Molecular Diagnostics Pleasanton, CA, USA) using the relative quantification method ($2^{-\Delta\Delta Ct}$ method) as previously described[98]. The genes were analyzed using the following TaqMan assays (Thermofisher Scientific, Waltham, MA USA):

Hs00962908_m1 THBS1
Hs01037698_m1 MGMT
Hs01547656_m1 IGF1
Hs00917999_g1 GRB7
Hs01001580_m1 ERBB2
Hs00187842_m1 B2M

Data were analyzed with the *LightCycler*® 480 Instrument *Software* Version 1.5, adopting an automatically-set baseline and a fluorescence threshold adjusted to measure quantification cycle (Cp) values. Using the $2^{-\Delta\Delta Ct}$ method the data were presented as the fold-change in gene expression normalized by a reference gene and relative to a calibrator sample. As the reference gene in this study we used Hs00187842_m1 B2M, one of the most commonly used housekeeping genes. The cDNA derived from the patient #191 was used as the calibrator source in our study.

Polymorphisms (IL8 rs4073 VEGFr-2 rs11133360)
We investigated these two polymorphisms in 234 patients who underwent breast surgery for breast cancer in our department between 2002 to 2015 and the respective blood sample, collected in our tissue biobank. We included in statistical analysis 188 patients, because the other patients had non invasive cancer (DCIS), or received neo-adjuvant treatment or we missed some data.

Laboratory techniques:

A. DNA extraction

Genomic DNA was isolated from peripheral whole blood employing the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.
In brief, 20 µl of QIAGEN Protease (or Proteinase K) were added to 200 µl of buffy coat. 200 µl Buffer AL were added to the sample and were mixed by pulse-vortexing for 15 s. The reaction was incubated at 56°C for 10 min. 200 µl ethanol (96–100%) were added to the sample, and were mixed again by pulse-vortexing for 15 s.
The reaction mixture was applied to the QIAamp Spin Column and centrifuged at 6000 x ***g*** for 1 min. 500 µl Buffer AW1 were added to the QIAamp Spin Column and centrifuged at 6000 x ***g*** for 1 min. 500 µl Buffer AW2 were added to the QIAamp Spin Column and centrifuged at full speed (20,000 x ***g***) for 3 min. DNA was eluted in 200 µl Buffer AE.
Quantification and quality assessment were performed with Nanodrop 1000 spectrophotometer (Thermofisher Scientific, Waltham, MA USA).

B. Patient genotyping

Ten to 20 ng of DNA of each patient were used for TaqManSNP genotyping assays according to the manufacturer's instructions (Thermofisher Scientific, Waltham, MA USA).
The genes of interest were analyzed using the following TaqMan assays:

IL8 rs4073 C__11748116_10
VEGF-2 rs11133360 C__26111278_10

Genotyping was performed by real-time PCR using allelic discrimination in the 7300 RT-PCR System (Thermofisher Scientific, Waltham, MA USA), using the primers provided by the manufacturer. PCR parameters involved an initial denaturation at 95˚C for 10 min followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Two blank (water) controls in each 96-well plate were used for the assay quality control. Post run data were analysed by 7300 SDS software (Thermofisher Scientific, Waltham, MA USA) and Automatic calls were assigned with approximately 99.8% quality.
A call rate >95% was considered the cutoff to consider genotyping successful.

Statistical method:
Descriptive statistic was used to assess the frequency distribution among the study population. The associations between gene expression value, NSNL status, and other considered clinicopathologic characteristics were analyzed with chi-square test (categorical variables) and Student's T-test (continuous variables). Also univariate logistic regression analysis was performed. Multivariable logistic regression analysis was used to analyze the association of each covariate which was significantly associated with NSNL metastasis at univariate analysis.
Correlation between polymorphism expression with both a recessive and a dominant model and the presence of metastasis in NSNLs was calculated using chi-square test.

The discrimination of the multivariable model was assessed with the area under the receiver operating characteristic curve (ROC curve, AUC). For all results two-tailed p-values <0,05 were considered statistically significant.

RESULTS

Molecular Markers

Of the 88 genes that we tested, with the pilot study, in 24 patients with positive SNLB who underwent lymphadenectomy, 5 genes were differently expressed in the two groups (Table 1). THBS1 (p:0.0012) and IGF1 (p:0,05) are statistically differently expressed between patients with positive SNLs and negative NSNLs and patients with positive SNL and positive NSNLs. ERBB2, GRB7, MGMT are not so far to be significant different expressed between the two groups (p:0,07; p:0,07; p:0,08) and so we included in the validation study.

Table 1. Pilot study

	THBS1	IGF1	EGFR	ERBB2	GRB7	MGMT
pz1	-2,17	-10,85	-5,58	1,27	4,38	1,53
pz2	-1,22	1,16	1,04	1,80	1,83	1,01
pz3	1,30	4,39	1,99	-1,10	1,46	-1,05
pz4	-1,26	-2,79	-3,31	2,93	3,25	1,26
pz6	-2,41	2,06	2,16	1,10	2,12	-1,02
pz11	1,85	-1,57	-1,40	11,55	12,47	-1,48
pz12	2,11	-1,40	-1,27	50,56	56,49	1,13
pz17	2,06	1,45	1,78	-3,61	1,56	1,13
pz16	1,04	-1,84	1,88	5,28	5,70	1,77
pz19	1,56	-1,89	-2,28	4,60	4,83	2,06
pz22	3,48	-4,99	1,11	60,55	60,13	1,69
pz 10	1,00	1,00	1,00	1,00	1,00	1,00
pz5	-2,16	-2,93	-2,91	-1,08	1,71	-1,07
pz7	-1,55	-1,72	-1,35	-1,39	-1,23	1,01
pz8	1,37	6,88	3,01	-1,16	1,48	1,10
pz9	-2,07	1,68	2,17	-1,22	1,89	-1,01
pz13	-2,48	4,57	2,64	-2,64	1,08	-1,23
pz14	-1,01	1,55	-1,25	1,52	1,40	-1,39
pz15	-1,23	1,01	1,43	1,08	-1,05	1,02
pz18	-1,58	-1,06	2,06	2,84	4,04	-2,05
pz20	-1,82	-1,12	-1,13	-1,19	-1,40	-1,48
pz21	-3,70	1,25	3,82	1,82	4,48	-1,01
pz23	-2,96	3,95	13,20	3,19	7,22	2,07
pz24	-2,72	6,76	9,16	-4,48	-3,77	1,50
P	**0,0012**	**0,052**	**0,07**	**0,07**	**0,076**	**0,088**

In the validation study we considered frozen tissue sample from 171 patients who received breast surgery in 1st Surgical Clinic between 2000-2013, as a sample of population, as shown in Table 2. 43 patients had no axillary metastasis in SNLB or in lymphadenectomy (N0), 49 had positive ALND (N3), but didn't receive SNLB, because the status of the axilla was already known (clinical positive metastatic axilla, positive fine needle aspiration from suspicious nodes) and 79 patients had metastasis in SNL and underwent ALND. Of these patients 53 (67%) had no metastasis in NSNLs (N1) and 26 (33%) had positive ALND (N2).

Table 2. Clinico-pathological characteristic of 171 patients

		PZ TOT	PZ N0	PZ SNL+ NSNL - (N1)	PZ SNL+ NSNL+ (N2)	PZ N+ (N3)
Number (%)		171	43 (25,1)	53 (31)	26 (15,2)	49 (28,7)
age		62	63,9	61,1	56,1	64,9
size		22,4	21,1	20,8	20,9	25,9
histotype	*dutt inf*	138 (80,7)	35 (81,4)	40 (75,5)	21 (80,8)	43 (87,7)
	lob inf	25 (14,6)	6 (13,9)	11 (20,7)	5 (19,2)	2 (4,1)
	altro	8 (4,7)	2 (4,7)	2 (3,8)	0	4 (8,2)
G	*1*	12 (7)	6 (14)	4 (7,5)	0	2 (4,1)
	2	85 (49,7)	26 (60,4)	28 (52,8)	14 (53,8)	17 (34,7)
	3	74 (43,3)	11 (25,6)	21 (39,7)	12 (46,2)	30 (61,2)
IV	*yes*	54 (31,6)	10 (23,2)	11 (20,7)	11 (42,3)	22 (44,9)
	no	117 (68,4)	33 (76,8)	42 (79,3)	15 (57,7)	27 (55,1)
MIB1-ki67		23,7	23,1	22,9	25,5	24,2
ER	neg	29 (16,9)	9 (20,9)	8 (15,1)	3 (11,5)	9 (18,4)
	pos	142 (83,1)	34 (79,1)	45 (84,9)	23 (88,5)	40 (81,6)
HER2 (90 pz)	*3+*	12 (13,3)	1 (6,3)	3 (10,7)	0	8 (26,6)
	0/1+/2+	78 (86,7)	15 (93,7)	25 (89,3)	16 (100)	22 (73,3)
LNF tot +		3,19	0	1,11	6,27	6,61
LNF tot		18,65	17,48	17,87	18,92	20,38

We compared each group with each other in relation to the characteristics and there was same difference statistically demonstrated as shown in Table 3 (chi-square for categorical variables and Student's T-test for continuous variables).

The median age was 62, N2 group had the youngest age (p:0,024 N0vsN2). The median size of the primary tumor was 22,4mm and in case of multifocal tumors we considered the size of the largest tumor. As is logical the tumors with metastatic axilla (N3) had larger tumors, comparing this group with N1 and N0 this difference is statistically significant, there was no difference between N3 and N2 and between N1 and N2 (p:0,004 N0vsN3; p:0,03 NvsN3; p:0,08 N2vsN3; p:0,97 N1vsN2).

80,7% of patients had invasive ductal carcinoma, 14,6% had invasive lobular carcinoma, the remain patients had other histotype (tubular, medullary, mixed). In situ carcinoma (DCIS) was excluded from the study. Patients with positive SNLs and negative NSNLs or positive NSNLs (N1 and N2) had more invasive lobular carcinoma comparing with N3 group (p:0,04 OR:0,2 chi-q:4,054 N2vs N3; p:0,0082 OR:0,15 chi-q: 6,99 N1vsN3).

85 patients had moderately differentiated invasive cancer (G2), obviously in group N3 and N2 (metastatic axilla) there were more poorly differentiated carcinomas (G3) (p:0,015 N0vsN2; p:0,0005 N0vsN3).

Most of patients had no vascular invasion (IV) in the tumor (68,4%), but the presence of IV increased when the lymph nodes were metastatic. The patients of group N3 had more IV comparing with N0 and N1 group (p:0,03 OR:0,37 chi-q:4,73 N0vsN3; p:0,045 OR:0,36 chi-q:4,033 N1vsN2; p:0,009 OR:0,32 chi-q:6,78 N1vsN3). The vascular invasion increased as the axillary involvement increased.

The median Ki-67 of all tumor was 23,7 and most of them expressed oestrogen receptor, there were no difference between the four groups for both of this parameter.
The data about Her2 expression in the tumor was incomplete, we recovered the Her2 status only in 90 of 171 patients. The Human epidermal growth factor receptor 2 (HER2) testing was introduced for early breast cancer in the guidelines after 2005, before was performed only in metastatic setting, this dataset also includes patients received surgery before 2005. The patients with HER2 over-expressed (3+) were 13,3% of the 90 patients, patients with positive metastatic axilla (N3) were Her2 3+ more frequently comparing with patients of N2 group (p:0,02 N2vsN3).
The median number of lymph nodes removed during axillary dissection was 18,6 (range 1-40) in all four group. In N3 group we removed more lymph nodes comparing with N1 group (p:0.05 N1vsN3). Also in group N0 (patients with negative axillary status) no different numbers of lymph nodes (LNF) removed, because only 15 patients of group N0 received SNL biopsy without lymphadenectomy, the other 28 patients underwent surgery before that sentinel lymph nodes procedure was introduced in our surgical department (2002).

Table 3. univariate analysis between all groups

Parameter		**chi-square test and Student's T-test**
age		p:0,024 N0vsN2
size		p:0,004 N0vsN3; p:0,03 N1vs N3; p:0,08 N2vs N3; p:0,97 N1vs N2
histotype	*dutt inf*	p:0,04 OR:0,2 chi-q:4,054 N2vs N3 p:0,0082 OR:0,15 chi-q:6,99 N1vs N3
	lob inf	
	altro	
G	*1*	p:0,015 N0vsN2; p:0,0005 N0vsN3
	2	G1+G2vsG3: p:0,08 OR:2,49 chi-q:3,086 N0vsN2 p:0,0006 OR:4,59 chi-q:11,77 N0vsN3
	3	
IV	*yes*	p:0,03 OR:0,37 chi-q:4,73 N0vsN3 p:0,045 OR:0,36 chi-q:4,033 N1vsN2 p:0,009 OR:0,32 chi-q:6,78 N1vsN3
	no	
HER2	*3+*	p:0,02 N2 vs N3
	0/1+/2+	
LNF tot		p:0.05 N1vsN3

The results of validation study for gene expression analysis are summarized in Table 4.

Table 4 Gene expression in all patients, Student's T-test

Gene expression level	**PZ TOT**	**PZ N0**	**PZ SNL+ NSNL - (N1)**	**PZ SNL+ NSNL+ (N2)**	**PZ N+ (N3)**	**p**
GRB7 (ng/ul)	8,26	6,7	**3,14**	**6,76**	15,71	**p:0,014 N1vsN2**
MGMT (ng/ul)	20,6	17,14	18,52	18,38	27,1	NS
THBS1 (ng/ul)	8,16	9,48	6,61	7,89	8,11	NS
IGF1 (ng/ul)	-8,2	-10,8	-9,14	-7,97	-5,03	**0,02 N0vsN3**
ERBB2 (ng/ul)	11,3	10,1	9,36	11,48	14,26	NS

The RT-PCR for all selected genes was performed in 171 patients and the median level was 8,26ng/ul for GRB7, 20,6ng/ul for MGMT, 8,16 ng/ul for THBS1, -8,2ng/ul for IGF1 and 11,3ng/ul for ERBB2. Gene's expression value of each group (N0-N1-N2-N3) was compared and only two genes were different express in the groups. The median expression level of GRB7 in patients with positive SNL biopsy and negative NSNL was 3,14 ng/ul, in the group of patients with positive SNL and positive ALND the median level was 6,76 ng/ul. This different expression was statistically significant with p:0,014. IGF1 level was lower in patients with negative axillary involvement (N0) comparing with patients with positive ALND (N3) (p:0,02).
In the other group (N0 and N3), there was a different expression of the genes, but these difference was not statistically significant.
Only GRB7 level was differently expressed in the interest group of our research (N1 and N2), the other genes (IGF1, MGMT, ERBB2, THBS1) had a similar expression in these groups of patients.

Whereas the aim of our project is to find predictive factors of NSNLs in the patients with positive SNL, we focused our correlation analysis on the two groups that reflect our population of interest (N1-N2). The characteristics of these two group are summarized in Table 5.
The median age was 60, the median size of tumor was 20,7mm. We performed breast conservative surgery in 51 patients and mastectomy in 28 patients. During the operation all patients underwent SNLB. The SNLs were identified by technetium-99m sulfur colloid as radioactive tracer, lymphoscintigraphy was performed a day before surgery and SNLs were localized with a navigator of the Gamma Guidance System in operating room. In 62 patients one SNL was identified, in 10 patients 2 SNLs were found and 7 patients had 3 or more SNLs in axilla. Once localized, the SNLs were removed and sent to the pathology department. For frozen-section examination, nodes with diameters of ≤0.5 cm were bisected, while nodes measuring >0.5 cm were sectioned each 2 to 3 mm. For each sample, two frozen sections made at 40 μm intervals were examined. The frozen tissue was then thawed, fixed and embedded to obtain permanent sections. For definitive histological examination, two consecutive 5μm thick tissue sections were cut from a paraffin block at two levels, 40 μm apart from each other. The sections were then stained with haematoxylin-eosin and immunostained with monoclonal antibody to cytokeratin. In 65 cases the surgeon asked the frozen section analysis, in 14 patients we sent the SNLs to the pathologist only for permanent section. In this situation, NSNLs were more likely to be negative (p:0,002 OR:8,13 chi-2:5,12).
In most patients the median number of positive SNL found at pathology examination was one, in two patients the SNL was negative, but the pathologist found metastasis in NSNLs, these two cases were false negative SNL (FN).
Most of the tumor was invasive moderately differentiated ductal carcinomas and no difference was reported between the patients with positive NSNLs and the patients with negative NSNLs.
The median Ki-67 of all tumor was 23 and most of them expressed oestrogen receptor, there were no difference between the two groups for both of this parameter.
We recovered the Her2 status only in 44 of 79 patients. The patients with HER2 over-expressed (3+) were 6,8% of the 44 patients and the HER2 expression was no statistically different between the two groups.

Table 5 Clinical-pathological characteristic and chi-square test and Student's T-test when we consider the interest group (SNLB positive patient N1 vs N2).

Parameter		**PZ TOT**	**PZ SNL+ NSNL - (N1)**	**PZ SNL+ NSNL+ (N2)**	**p**
Number (%)		79	53 (67)	26 (33)	
age		60	61,1	56,1	NS
size		20,7	20,8	20,9	NS
surgery	bcs	51 (64,5)	35 (66)	16 (61,5)	NS
	mastectomy	28 (45,5)	18 (44)	10 (48,5)	
histotype	*dutt inf*	61 (77,2)	40 (75,5)	21 (80,8)	NS
	lob inf	16 (20,2)	11 (20,7)	5 (19,2)	
	altro	2 (2,6)	2 (3,8)		
G	*1*	4 (5,1)	4 (7,5)	0	NS
	2	42 (53,2)	28 (52,9)	14 (53,8)	
	3	33 (41,7)	21 (39,6)	12 (46,2)	
IV	*yes*	22 (27,9)	11 (20,7)	11 (42,3)	**chi-q:4,033 p:0,045 OR:2,80**
	no	57 (72,1)	42 (79,3)	15 (57,7)	
MIB1-ki67		23	22,9	25,5	NS
ER	neg	11 (13,9)	8 (15,1)	3 (11,5)	NS
	pos	68 (86,1)	45 (84,9)	23 (88,5)	
HER2 (44 pz)	*3+*	3 (6,8)	3 (10,7)	0	NS
	0/1+/2+	41 (93,2)	25 (89,3)	16 (100)	
GRB7 (ng/ul)		4,24	**3,14**	**6,76**	**p:0,014**
MGMT (ng/ul)		18,42	18,52	18,38	NS
THBS1 (ng/ul)		7,47	6,61	7,89	NS
IGF1 (ng/ul)		8,89	-9,14	-7,97	NS
ERBB (ng/ul)		9,32	9,36	11,48	NS
frozen section	*yes*	65 (82,3)	40 (75,5)	25 (96,1)	**chi-Q:5,12 p:0,002 OR:8,13**
	no	14 (17,7)	13 (24,5)	1 (3,9)	
n° SNL	*1*	62 (78,5)	40 (75,5)	22 (84,6)	
	2	10 (12,6)	7 (13,2)	3 (11,5)	NS
	>3	7 (8,9)	6 (11,3)	1 (3,9)	
n° SNL -	*0*	58	38	20	NS
	1	15	9	6	
	>2	7	6	1	
n° SNL +	*0 (FN)*	2 (2,5)	2 (3,8)	0	NS
	1	68 (86,1)	46 (86,8)	22 (84,7)	
	2	5 (6,3)	4 (7,5)	1 (3,8)	
	3	4 (5,1)	1 (1,9)	3 (11,5)	
Size SNL meta	*micro*	8 (10,1)	8 (15,1)	0	**chi-Q:3,94 p:0,047 OR:44,27**
	macro	71 (89,9)	45 (84,9)	26 (100)	
LNF tot		18,06	17,87	18,92	NS

Patients with positive SNLs and presence of IV had higher probability to have positive NSNL (p:0,045 OR:2,80 chi-q:4,033), this result was already demonstrated by numerous studies[64 65 66].
All patients with positive NSNLs had macrometastatic involvement of SNLs, the size of metastasis in SNL was a good predictive factors on NSNL status (p:0,047), as a lot of studies shown [61 62 66 67 70.]
In presence of SNLs' micrometastasis there was lower probability to have other axilla metastasis, the actual guidelines (AIOM, NCCN) suggest to avoid ALND in these case, based on results from important trial[99]. The patients considered in these studies underwent surgery when these guidelines were not yet updated, and we performed ALND, also when SNLs were micrometastatic.
We removed the same number of SNLs in the two groups and there were no difference in positive or negative number of SNLs between the two groups (p>0,05).
We performed also univariate logistic regression analysis for all variables in patients with positive SNLB. The results of these statistical analysis confirmed the predictive value of GRB7, frozen section analysis and vascular invasion, as shown in Table 6. Diameter of metastasis was not confirmed to be statistically significant different between the two group at univariate logistic regression, probably for the small number of events (only 16 patients with micrometastasis).

Table 6. Univariate logistic regression analysis

axilla	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
grb7	1.118091	.0510541	2.44	**0.015**	**1.022374**	**1.222769**
IV	2.733333	1.427824	1.92	**0.054**	**.9818534**	**7.609192**
frozen section	8.333333	8.907565	1.98	**0.047**	**1.025564**	**67.71343**

Multivariate logistic regression analysis confirmed that GRB7 level was an independent predictive factor of NSNLs in patients with positive SNLB. Also frozen section analysis was statistically different in the two groups at multivariate analysis (Table 7).

Table 7. Multivariate logistic regression

	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf. Interval]	
grb7	1.116578	.0516715	2.38	**0.017**	1.01976	1.222587
frozen section	8.664052	9.41185	1.99	**0.047**	1.03051	72.84338
IV	2.560662	1.467849	1.64	0.101	.8325644	7.875657

We calculated the Receiver operating characteristic (ROC) curves for our predictive factors at univariate logistic regression analysis (GRB7 level, IV and frozen section analysis) as shown in Fig.5.
Discrimination of multivariable model was quantified with the area under the receiver operating characteristic (ROC) curve (AUC), and the AUC was 0,77.

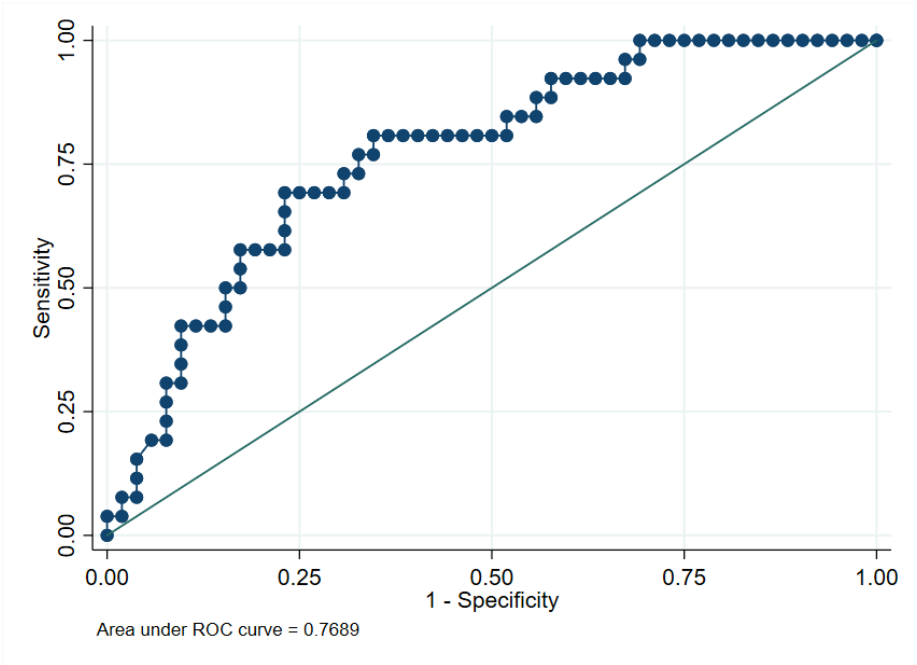


Fig.5 ROC curves for our predictive factors (GRB7 level, IV, frozen section) of NSNL status in patients with positive SNLB

RESULTS

Polymorphisms

We analysed the results of genotyping for IL8 rs4073 and VEGF-2 rs11133360 of 188 patients from 234 blood samples of patients who underwent breast surgery in our Surgical Unit between 2002-2015. The characteristics of the patients are shown in Table 8.

46 patients were eliminated from the analysis because the data were incomplete or patients received neo-adjuvant chemotherapy (22). All of 188 underwent surgical axillary staging. 96 patients had no metastasis invasion in SNLs, 27 patients received ALND that was positive, without SNLB, because the status of axilla was already known before surgery (clinical positive metastatic axilla, positive fine needle aspiration from suspicious nodes). 65 patients had positive SNLs, 42 (64,5%) had negative NSNLs after lymphadenectomy, 23 (35,5%) had positive ALND.

Table 8. Clinico-pathological characteristic patients genotyping for polymorphisms

		PZ TOT	**PZ N0**	**PZ SNL+ NSNL - (N1)**	**PZ SNL+ NSNL+ (N2)**	**PZ N+**
		188	96	42	23	27
age		61,2	60,9	63	56,8	63,2
size(mm)		20,5	20,4	20,8	20,6	28,3
histotype	*dutt inf*	147	76	30	17	23
	lob inf	22	6	8	6	2
	ca i.d.	7	7			
	altro	10	5	3	0	2
G	*1*	20	12	5	0	3
	2	100	61	22	11	6
	3	68	23	15	12	18
IV	*yes*	149	82	36	15	16
	no	39	14	6	8	11
ki67		20,6	18,6	20,7	22,6	26,1
ER	*neg*	44	23	8	5	8
	pos	144	73	34	18	19
LNF tot +		2,14	0	1,12	6,7	7,4
LNF tot		13,4	7,9	17,3	17,8	23,3

We considered two models for both polymorphism: dominant and recessive. The stratification of patients, according to genotypes of IL8 rs4073 and VEGF-2 rs11133360 gene polymorphism are summarized in figure 6.

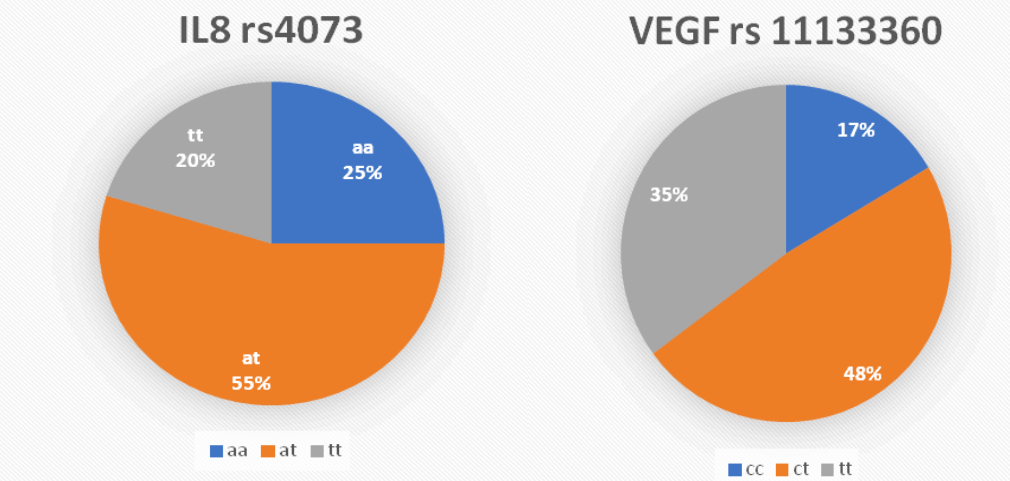
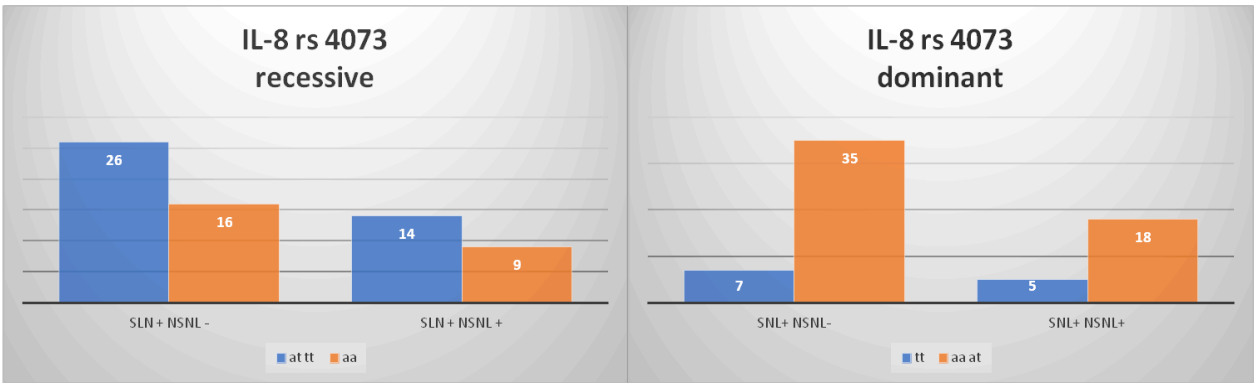


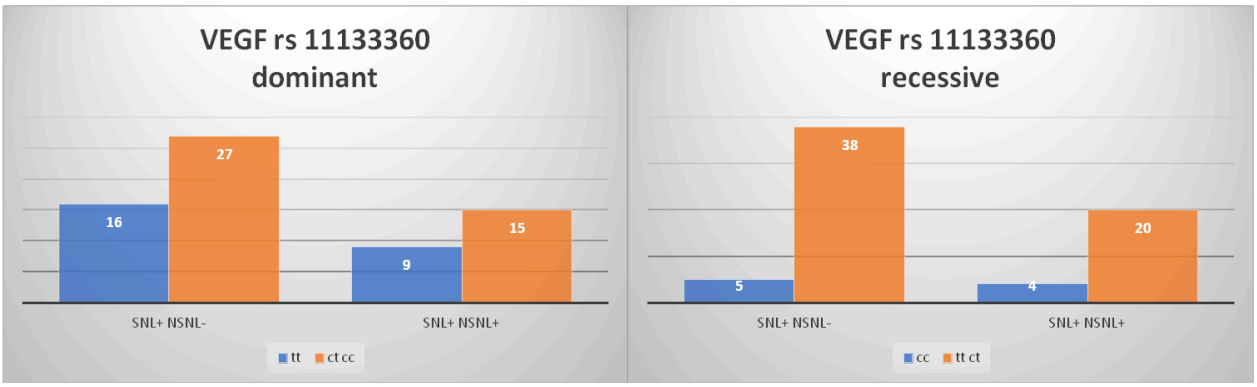
Fig.6 stratification of patients according to genotypes

When we analysed the correlation between the genotype and NSNLs status in patients with positive SNLs, no statistically significant results were obtained for both models (Fig. 7-8).
The two polymorphisms investigated were no predictive of axillary status in patients with positive SNL (Fig.7-8), but only IL8 polymorphism in recessive model was significantly different between patients with axillary metastasis (N+) and patient with negative axillary status (N0) (Fig.9).



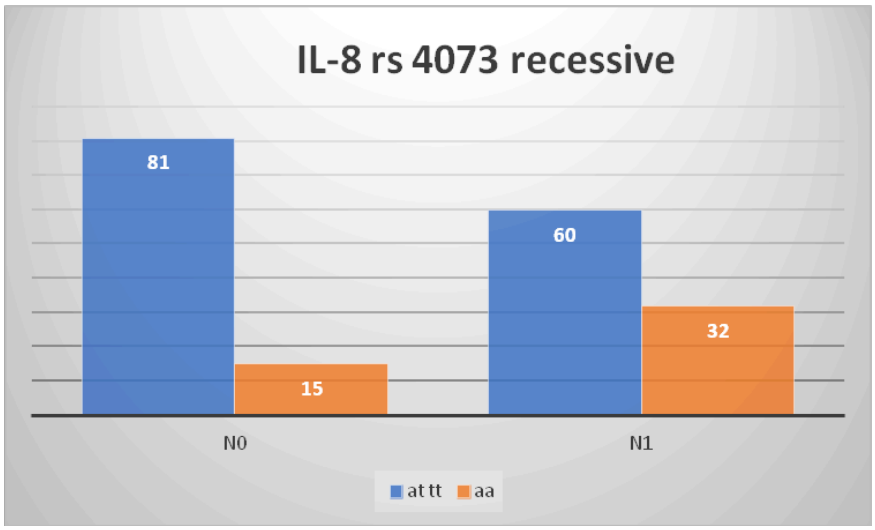
p:0.9 **p:0.67**

Fig 7 Correlation between recessive and dominant polymorphism IL-8 rs 4073 and NSNLs status



p: 0.67 **p:0.98**

Fig.8 Correlation between recessive and dominant polymorphism VEGF rs 11133360 and NSNLs status



p:0.0024 OR:2,28 chi-q:9,19

Fig. 9 Correlation between recessive polymorphism IL8 rs 4073 and axillary status

In other words, a homozygous woman for the allele A of the polymorphism IL-8 rs4073 has a probability of 2.28 times greater than the rest of the sick population of developing axillary metastasis from breast cancer. These results were already demonstrated by other groups[90 91].

DISCUSSION

Our data suggest that GRB7 could be an independent predictive marker of NSNLs status in patients with positive SNLB. No other genes demonstrated the same predictive role.
This result was very important, because the level of GRB7 was demonstrated statistically significant different with all statistical tests used (p:0,014 t-Student; p:0,015 univariate logistic regression; p:0,017 multivariate logistic regression), confirming the predictive role of this test.
This result could be explained by the biological role of GRB7. GRB7 is an adaptor molecule mediating signal transduction from multiple cell surface receptors to diverse downstream pathways[100]. GRB7, along with Grb10 and Grb14, make up the Grb7 protein family. This protein family has been shown to be over-expressed in certain cancers and in cancer cell lines[101 102]. Grb7 over-expression has been linked to enhanced cell migration and metastasis, has been implicated as a downstream mediator of integrin- FAK signal pathways in the regulation of cell migration, although the molecular mechanisms are still not well understood[103]. Other authors showed that high Grb7 expression was strongly associated with decreased survival in all patients and in the node positive subset (p:0.0034 and p:0.0019). In this study on multivariable analysis, it remained an independent prognostic marker (p:0.01)[104].
The important role of GRB7 as prognostic marker is demonstrated by the fact that this gene is one of 21 genes included in Oncotype DX[105].
Higher GRB7 level probably increase the attitude of cancer cell to migration and explain the higher invasion of NSNLs in patient with positive ALND.
In our series the level of GRB7 was calculated in all patients (171) and was compared between all the 4 groups, that represented all possible scenario in the axilla, as a sample of population. The Group N0 that are patients without axillary metastasis are not representative of population, are only 43 patients, 25% of our series. The actual population underwent axillary dissection has a higher probability of negative SNLB, thanks to screening programs. Our series probably has this low number of non-metastatic patients because most patients underwent surgery between 2000 to 2006, when early diagnosis was less common and also we started to introduced SNLs biopsy. Our bank has predominantly palpable tumors as we can see from the medium size of the tumors (22mm), because there was agreement with the pathologist to pick up the tissue for bio bank only in medium size tumors, during the surgical operation. We were not allowed to take a sample of small tumor in order not affect the histological diagnosis. In the future we will try to solve this problem by taking the sample of no palpable lesions, the smaller ones, for biobank, during the diagnostic biopsies, often performed by radiologists.
The groups N1 and N2 are more representative of the current population, 67% of patients with positive SNLs had negative NSNLs and 33% had positive NSNLs, as reported in numerous studies[61-80].
In our study some clinical-pathologic characteristics seem to be a good predictive factors of NSNLs status: IV and frozen section analysis, confirmed also on univariate logistic regression analysis.
A lot of studies had already demonstrated that vascular invasion is a predictive factor of axillary staging[20-30] and also increased the risk of NSNL metastasis in patient with positive SNLs[64-66]. The vascular invasion is one of the pathologic characteristic of tumor included in Nomogram of Memorial Sloan-Kettering Cancer Center and in scoring systems of MD Anderson[70-79].

The patients received frozen section analysis of positive SNLs had 8.8 times greater then patients received routine SNLs histologic examination of having metastasis in NSNLs (chi-Q:5,12 p:0,002 OR:8,13; p:0,046 multivariate logistic regression). This finding could be explained by the fact that the surgeon asks for the frozen section analysis, when he thinks there is a good chance that lymph node will be positive based on the characteristics of primitive tumor, or because during the biopsy procedure SNLs has suspected macroscopic characteristics. The surgeon requests an intra-operative assessment to pathologist, when probably a ALND is necessary to avoid a second surgical procedure to the patients.

The size of metastasis in SNLs has an important role in predicting the NSNLs status: in our series no micrometastasis (size of metastasis 0,2mm ≤2mm) were found in SNLs of patients with positive NSNLs, confirmed the predictive power (p:0,047 Student's T-test). This result was not confirmed on logistic regression analysis and multivariate logistic regression analysis, probably for small size of our series. Regardless of this result, the current guidelines[99] do not indicate the need for ALND in patients with micrometastasis in SNLs.

The discrimination of the multivariable model was assessed with the area under the receiver operating characteristic curve ROC (Fig.5) and illustrated the high diagnostic ability of our predictive test. The current nomograms[71] have lower AUC comparing with AUC of 0,77, as reported in our series.

In this study the correlation between molecular subtype (luminal A, luminal B, HER2 over-expression and triple negative) was not done because the lack of information about HER2 test.

The second part of this study investigated the role of IL-8 and VEGF polymorphisms to predict the NSNLs status in patients with positive SNLs. We knew from the literature[89-91] that IL8 rs 4073 polymorphism increased the risk of developing breast cancer and influenced prognosis, promoting greater axillary involvement. That is why we wanted to understand whether this polymorphism could also increase the risk of positivity of NSNLs in positive SNLs patients. As shown before this polymorphism not influenced the NSNLs status (Fig.7-8). Our results confirmed only the literature data that patients homozygous for the allele A of the polymorphism IL-8 rs4073 has metastasis in axillary lymph nodes greater than the rest of the sick population. VEGF polymorphism was demonstrated not to correlate with breast cancer aggressiveness.

CONCLUSION

This study suggests that the surgeon could use the expression level of GRB7 as a predictive tool of axilla status in patients with positive SNLB and this test could help the surgeon to define better axillary surgery approach in this group of patients.

On the other hand, some limitations still exist in the present study: all data result from retrospective analysis and the population with positive SNL is small in number. We need to validate these results in a larger population and in a prospective study. The other limit is that the level of GRB7 was tested in frozen tissue, that was accurate, but only few hospitals have a tissue bank, so this test was not accessible and hampering extensive application. Further investigations are required to determine whether this result will can be successfully translated to paraffin-embedded tissue.

Our outcomes suggest that molecular markers could be a good predictive factors of NSNLs in positive SNLs patients and is necessary to extend the research to more genes, in order to create a genomic test (such as Oncotype DX) predictive of NSNLs status.

In the era of AMAROS trial[106] and ACOSOG Z0011[107], finding predictive marker of NSNLs with high accuracy, sensibility and sensitivity will be very important, to improve the current surgical practice in staging the axilla, aiming to precision medicine for less invasive treatment for patients.

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