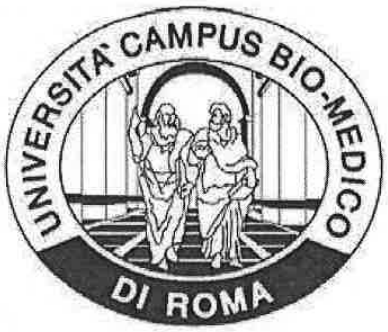

Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in Scienze Biomediche Integrate e Bioetica
XXXIII ciclo a.a. 2017-2018

Prognostic value of Lymphovascular invasion (LVI) in early breast cancer: pathological definition and comparison with PROSIGNA gene test results

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16 Giugno 2021

Chapter 1- Breast Cancer: Overview

1.1 Breast cancer epidemiology

Breast cancer is the most common malignancy in women accounting for about 25% of all cancers worldwide. In Italy, 54.976 new breast cancers were diagnosed in 2020 with the highest rate in women less than 50 years old (41% of cases)[1]. From 2008 to 2016, breast cancer showed a progressive incidence increase in all age subclasses with the highest rate in women below 50 years old (+1,6%/year). This increase can be partially explained by advancement in screening programs with bilateral mammography together with more sensitive diagnostic tools, resulting in earlier detection of the disease. Improvements in localized disease management also resulted in a significant decrease in the mortality rate with a 6% reduction in 2015-2020[1]. After lung cancer, breast cancer is the second leading cause of cancer death in women and more than 12000 deaths for breast cancer occurred in 2017 in Italy. About 6-7% of patients receive diagnosis of metastatic breast cancer. Thanks to treatment improvements and a multidisciplinary approach, the overall survival has dramatically increased over the time, resulting in 87% of patients alive after 5 years of diagnosis[1]. Many of the established risk factors are linked to oestrogens (see *Fig. 1*). Risk is increased by early menarche, late menopause, and obesity in postmenopausal women, and prospective studies have shown that high concentrations of endogenous oestradiol are associated with an increase in risk. Childbearing reduces risk, with greater protection for early first birth and a larger number of births; breastfeeding probably has a protective effect. Both oral contraceptives and hormonal therapy for menopause cause a small increase in breast-cancer risk, which appears to diminish once use stops. Alcohol increases risk, whereas physical activity has been shown to be protective. Mutations in certain genes (i.e., BRCA 1/2, PALB2, CEK) greatly increase breast-cancer risk, but these account for a minority of cases (around 10%). Breast cancer is a heterogeneous disease comprising multiple entities with different histological and molecular features characterized by distinctive clinical behaviours and response to treatment. Thus, a central component of the treatment of breast cancer is the knowledge of its extent and biological properties. National and international guidelines provide precise criteria for pathological classification and staging, giving an essential tool for treatment management in a such complex disease.

Risk factor	*Relative risk*	*High risk group*
Age	>10	Elderly
Reproductive risk factors		
Age at menarche	3	Menarche before age 11
Age at menopause	2	Menopause after age 54
Age at first pregnancy	3	Nulliparous or first child in early 40s
Lifestyle factors		
Diet	1.5	High intake of saturated fat
Body weight (postmenopausal)	2	Body mass index > 35
Alcohol	1.3	Excessive intake
Hormonal status		
Oral contraceptives	1.24	Current use
Hormone replacement therapy	1.35	Use for > 10 years
Radiation	3	Abnormal exposure after age 10
Family history	≥2	Breast cancer in first degree relative when young

Fig. 1 Risk factors for breast cancer [2]

1.2 Immunohistochemical classification

Currently, four immunohistochemical biomarkers are used in routine pathological reports of an invasive breast cancer: oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and KI67.

- **Hormone receptors:** Overall, more than 75% of breast carcinomas express the hormone receptors ER and/or PR, measured by immunohistochemistry (IHC). The percentage of cancer cells stained for those biomarkers has valuable prognostic and predictive information (see below). ER is an intracellular protein mostly expressed in breast, endometrium, ovarian stroma, and hypothalamus. PR is also an intracellular protein and its gene is transcriptionally activated by ER by binding to ER binding sites, so-called ERE, present upstream to PR gene. The expression of PR, thus, correlates to that of ER, and, for this reason, the existence of ER-negative/PR-positive breast cancers is highly controversial. Starting from 2010, the ASCO-CAP guidelines set the new cut-off of ≥1% to define hormone-positive cases at the clinically significant level, even if hormone responsiveness with ER between 1% to 9% is debated[3].
- **Human epidermal growth factor receptor 2 (HER2):** HER2, also known as HER2/neu or ErbB-2, is a trans-membrane receptor member of the Epidermal Growth Factor (EGF) Receptor Tyrosine Kinase (RTK) family. It is encoded by the ERBB2 gene located on the long arm of the chromosome 17 (17q21-q22). HER2, which normally regulates cell growth, differentiation, and survival, is overexpressed in 15-20% of invasive breast cancers and correlates with more aggressive cancer features[4]. HER2 receptor, which has no high-affinity ligand, is activated for homodimerization or heretodimerization with other HER receptors and, possibly, for auto-cleavage of the extra-cellular domain. The binding to HER3 receptor generates a dimer of high-

signalling potency. The diagnosis of HER2-positive breast cancer is made via immunohistochemistry (IHC), which identifies overexpression of the HER2 gene product and fluorescence in situ hybridization (FISH) analysis, which identifies amplification of the HER2 gene. According to the College of American Pathology (CAP) guidelines, tumours that have indeterminate results by IHC (2+) should have reflex testing by FISH. The "HER2 Testing in Breast Cancer - 2018 Focused Update[5] provides current criteria for HER2 testing and analysis.

- **Ki67**: KI-67 is a non-histonic nuclear protein expressed at crescent levels during all the active phases of the cell cycle and is the most used marker of cell proliferation in solid tumours. Biologically, Ki-67 enables the motility of chromosomes and their interaction with the mitotic spindle, during cell division. Tumour proliferation rate is generally assessed as the number of cell nuclei positively stained for Ki67 antibody, among the whole number of scored malignant cells. Even if most of the studies consistently appointed Ki67 as an independent prognostic factor of disease-free survival in early breast cancer, IHC for Ki-67 analysis lacks reproducibility across laboratories and, therefore, cannot be consistently interpreted when performed in a broad range of laboratories[6]. For this reason, ASCO recommendations on appropriate use of breast tumour biomarker assays stated IHC for Ki-67 is not recommended for broad clinical use to determine whether a patient should receive adjuvant chemotherapy or not[7].

Based upon the abovementioned biomarkers, breast cancer has traditionally been classified into four IHC subtypes (Fig.2), which traditionally guided clinicians for treatment tailoring: *Luminal A and B, HER2-positive and triple negative (TNBC)* subtypes. Based on work by Prat et al., who determined that patients with IHC-based luminal A tumours had better disease-free survival (DFS) if PR was >20%, the 2013 St. Gallen update defined luminal A as ER positive (ER+), PR ≥20%, HER2 negative, Ki67 <14%[8]. Luminal B-like (HER2-negative) tumours are ER+, HER2 negative, and at least one of the following: Ki67 ≥20%, PR negative or <20%. Luminal B-like (HER2-positive [HER2+]) tumours are ER+, HER2+, any Ki67 level, and any PR level. HER2+ (non-luminal) tumours are defined as HER2+ and ER and PR negative. Triple-negative (ductal) tumours are defined as ER, PR, and HER2 negative. These definitions are frequently used in clinical practice today. However, these IHC-based markers are only a surrogate and cannot establish the intrinsic subtype of any given cancer, with discordance rates between IHC-based markers and gene-based assays as high as 30%[9]. Since breast tumours with similar histopathological appearances can exhibit divergent clinical presentations, disease aggressiveness and treatment responsiveness, systematic investigations of gene expression

patterns and their correlation with specific features of phenotypic diversity are changing the way of classifying, at the molecular level, the phenotypes of breast cancers.

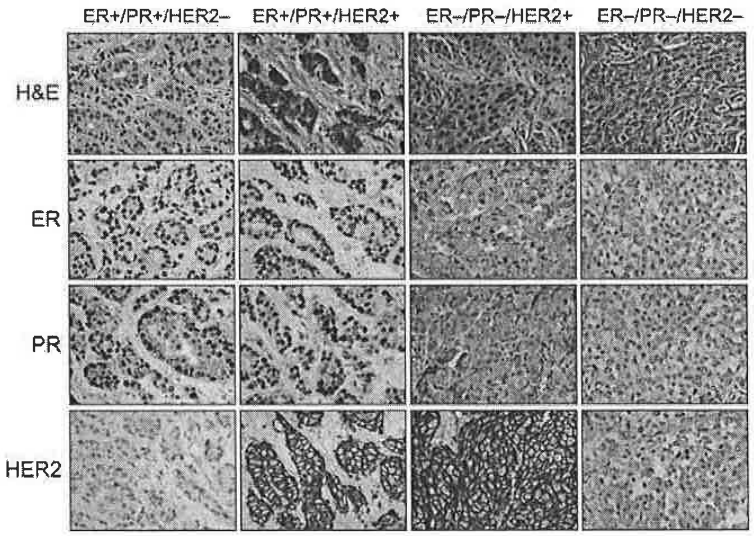


Fig. 2 Clinical classification of invasive breast cancer based on expression of ER, PR, and HER2. Cancer histology is depicted using H&E staining; expression of ER, PR, and HER2 is visualized using immunohistochemistry. Breast cancers are generally classified as positive or negative for hormone receptors ER and PR and for HER2, resulting in four major clinical groupings: ER+/PR+/HER2−, ER+/PR+/HER2+, ER−/PR−/HER2+, and the triple-negative ER−/PR−/HER2−. Original magnification, ×40.

1.3 Gene profiling and molecular intrinsic subtyping of breast cancer

During the last decades breast cancer research experienced a real revolution thanks to the emergence of novel technologies based on high throughput gene expression analysis. Gene-expression profiling has had a considerable impact on our understanding of breast cancer biology and clinical researchers moved from "semantic" classification of breast cancer subclasses by pathology-based biomarkers (e.i., oestrogen receptor, progesterone receptor and HER2) into new genomic classifiers. Apart from prognostic value, molecular profiling's field of application will be prediction of treatment efficacy and forecasting of outcomes for individual patients with breast cancer. Thanks to gene-expression profiling, breast cancer can be categorized at least in five different biologic subtypes, namely, luminal A, luminal B, HER2-enriched, basal-like, and normal breast–like (Fig.3)[10]. It is also true that the exact number of molecular subclasses of breast cancer is currently unknown: up to 30% of cases do not fit

into any of the recognized four molecular categories, and, as genomic studies evolve, new molecular classes are being defined, such clauding-law in basal-like disease[11]. Each intrinsic subclass is well plotted to an IHC-defined subtype except for the normal-like tumours (7.8% of all breast tumours), which share a similar IHC status with the luminal A subtype and are characterized by a normal breast tissue profiling. Main features are here highlighted for each subtype:

- **LUMINAL subtypes**: Following Sorlie et al.[12] and Van't Veer et al.[13] studies, Sotiriou et al.[14] created a combined data set identifying two main luminal-like subclasses corresponding to luminal-A and luminal-B. A higher level of ER and lower levels of proliferation related genes characterize Luminal A tumours (50%-60% of all breast cancers) with subsequent sensitivity to endocrine manipulation and relative resistance to standard cytotoxic agents. Patients with luminal-A breast cancer have a good prognosis and relapse rate is significantly lower than the other subtypes but it can occur even decades after surgery[15]. Luminal B breast tumours (15%-20% of cases) have much lower expression of ER-related genes, a variable expression of an HER2 cluster of genes, and a relatively higher expression of proliferation-related genes (i.e., MKI67 and AURKA), conferring a more aggressive phenotype and a worse prognosis[16].
- **HER2-enriched subtype:** HER2 enriched breast cancer (15-20% of cases) are defined by high expression of HER2/neu proliferation genes and low expression of luminal epithelial cytokeratins (CK). Morphologically, these tumours are highly proliferative, 75% have a high histological and nuclear grade and more than 40% have p53 mutations[17]. Clinically, 70% of the tumours classified as HER2-enriched by gene expression profiles are also HER2-positive, as well as many HER2-amplified/ER-positive cancer are rather classified as Luminal B.
- **Basal-like subtype:** The basal-like intrinsic breast cancer subtype represents 10%–25% of all tumors. The gene expression pattern, shared with basal epithelial cells, includes keratin 5,6, and 17, integrin-β4, laminin, and fatty-acid binding protein. These tumours are frequently ER-negative, PR-negative, HER2-negative by IHC. They also show high frequency of BRCA1 (breast cancer type 1 susceptibility gene) mutations, increased genomic instability, high expression of the proliferation cluster of genes, and a high histologic grade[18]. Basal-like is not synonymous of triple negative disease and the immunohistochemistry-defined triple-negative subtype is currently being subdivided into several molecularly distinct subtypes with potential future clinical and therapeutic implications. Indeed, this subgroup presents the highest intrinsic diversity depending on the complex genomic landscape.
- **Normal-like subtype:** this is a category showing gene expression features usually expressed by the adipose tissue and clustering with fibroadenoma and normal breast tissue. However, the

clinical relevance of this subtype is still unclear, and many consider it as a mere artifact, likely attributable to a specimen contamination by normal tissue.

Even though IHC and gene expression based intrinsic subtypes moderately correlate to each other, they are not synonymous. Intrinsic subtypes are, in fact, represented in each IHC based subgroup and their identification has demonstrated clinical value. Indeed, HER2-enriched subgroup includes approximately 35% of HER2-negative cancers as defined by IHC, and only 52% of the tumours are ER-negative /HER2-positive. A moderate inconsistence has also been demonstrated between TNBC IHC-surrogate and basal-like subtype. TNBC is a highly diverse group composed of many cancer subtypes among whom basal-like tumours predominate (~70% of the cases, when claudin-low are ignored). Within basal-like category, approximately 85% of the cases are classified as TNBC, whereas ER-positive as well as HER2-positive subtype is also significantly represented[19]. Of interest, TNBCs and non-TNBCs within basal-like tumours show a nearly complete overlap in the pattern of expressed genes, which strengthen the notion of their unique biology.

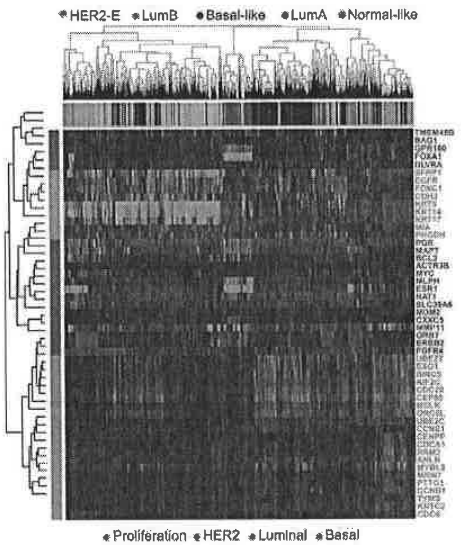


Fig. 3 PAM50 unsupervised gene expression heatmap of 1,834 breast cancer samples. The subtype calls of each sample are shown below the array tree. Each square represents the relative transcript abundance[9].

1.4 Prognostic and Predictive factors in early breast cancer

Prognostic and predictive factors are universally utilized in the management of breast cancer and can be used to stratify patients into two groups: (1) those who are expected to derive the most benefit from adjuvant systemic therapy, and (2) those for which the risks and costs of adjuvant therapy outweigh the expected benefit[20]. By definition, a prognostic factor is a clinical or biologic characteristic that is objectively measurable and provides information on clinical outcome at diagnosis, independently of the treatment. In cancer, prognostic markers are usually indicators of growth, invasion, and metastatic potential. A predictive factor is a clinical or biologic characteristic capable to provide information on the likelihood of response to a given therapy and may serve to identify subpopulations of patients with a higher probability to benefit from a certain treatment. Some factors in breast cancer function both as prognostic and predictive markers (e.i. HER2). A **biomarker**, defined as a general biologic or molecular condition that distinguishes one patient group from another, should strictly fulfil three criteria: analytic validity, clinical validity, and clinical utility[6].

1) *Analytic validity*: it refers to the accuracy, reliability, and reproducibility of the assay as demonstrated by preanalytical, technical, and scoring or interpretation methods.

2) *Clinical validity*: it refers to the ability of a tumour biomarker test to divide one population into two or more groups that differ either biologically or clinically. For example, a tumour biomarker test has clinical validity if a group of patients with early-stage breast cancer is found to have a worse disease-free survival (DFS) or overall survival (OS) if their tumour is positive for the marker compared with those that are negative. Clinical validity often is illustrated in Kaplan-Meier curves and expressed as relative or proportional differences in outcomes in accordance with marker status, with associated measures of uncertainty or likelihood that the differences are due to chance alone (eg, confidence limits, P values).

3) *Clinical utility*: a clinically useful biomarker is a marker that impacts clinical decision making and patient outcomes when compared with a clinical situation in which it is not used. Proven analytic and clinical validity do not imply clinical utility. This is the case, for instance, of a biomarker that does not show to be independent from predictors already in use in clinical practice, despite an outstanding clinical validity. High-quality data are required to prove the clinical utility of a biomarker.

- Predictive factors

Predictive factors enable treatment tailoring by providing tools for the identification of subjects with higher or lower likelihood to respond to a certain treatment, sparing from unnecessary therapies non-responsive patients. Up to date, only ER positive status and HER2 overexpression have been shown to be predictive of hormone therapy and HER2-directed therapy benefit, respectively. The results from EBCTCG metanalysis of over 100.000 patients included in 123 trials revealed that benefit from adjuvant chemotherapy is independent of age, ER status, grade, tumour size, nodal involvement, and adjuvant tamoxifen[21]. The potential predictive value of genomic profiles is currently under investigation and will be discussed in the next session.

- Prognostic factors

Prognostic factors in early breast cancer can be grouped as follows:

Clinical factors:

- AGE: Both younger and older age is associated with poorer prognosis. Patients < 35 years at diagnosis have a worse absolute 5-year survival even after adjustment for tumour stage, histopathologic characteristics and given treatments, indicating an intrinsic aggressive biology. Women >65 years diagnosed with breast cancer have an increased mortality mainly due to later stage at diagnosis, comorbidities, and less aggressive therapies.

Pathologic factors:

- PRIMARY TUMOUR SIZE: Primary tumour size is defined as the largest diameter of the primary tumour. It is directly related to an increasing probability of regional metastasis, an increasing average number of involved axillary lymph nodes (especially for Luminal cancers)[22] and an increasing probability of recurrence and death. In many analyses it is second only to axillary node status as an independent prognostic factor. The 5-year survival decreases from 91% for cancer <2 cm to 63% for those >5 cm.
- AXILLARY LYMPH NODES INVOLVEMENT. Together with the total number of positive nodes, the presence or absence of metastasis to axillary lymph nodes is the single most influential predictor of post-treatment recurrence and death. The 5-year survival rate for tumours localized to the breast vs. tumours that spread to the regional lymph nodes is 99% and 85%, respectively, independently of tumour size[23]. In addition, the presence of micro-

metastasis (<2mm) in the examined axillary nodes is associated with worse prognosis in comparison with no metastasis whereas no difference in survival emerged between node negative patients and those with isolated tumour cells.

- TNM STAGE: Staging based on clinical or pathologic information is limited to providing a static picture of the disease and does not take into account the complex biology behind each cancer. Within each stage are cases with differing biologic potential and speed of progression and a broad spectrum of prognoses. The most important components of anatomic staging are the size of the primary tumour and the extent to which regional lymph nodes are involved. These two variables are independent, but they are closely related.
- TUMOUR MORPHOLOGY: Lobular carcinoma is associated to a lower risk of recurrence compared to ductal carcinoma in the first 6 years after diagnosis but confers a significantly higher risk after six years. 99 Tubular, papillary, mucinous, medullary, and adenoid cystic carcinomas have a better prognosis while micro-papillary and metaplastic are associated with shorter survival.
- HISTOLOGIC GRADE: Histologic grade is a prognostic marker that allows risk stratification within a given tumour stage. Nottingham histological grading, currently used in pathology reports, assesses the degree of tumour differentiation (tubule formation and nuclear pleomorphism) and proliferative activity (mitotic index) by giving a score to each of these features. Grades from 1 to 3 indicate progression from well differentiated (low or good grade) to poorly differentiated (high or poor grade)[24]. Histologic and nuclear grade are subordinate to node status and tumour size as prognostic features, but both are significant predictors of overall mortality for node-positive and node-negative patients[25]
- PERITUMORAL LYMPH-VASCULAR INVASION: discussed in Chapter 3.
- HORMONE RECEPTORS: Patients with ER-positive tumours have prolonged disease-free survival after primary treatment, superior overall survival, and longer survival after recurrence compared with patients with ER-negative tumours, and this advantage is independent of axillary node status. However, the value of ER status as an independent prognostic variable is diminished by its association with other established indicators of favourable prognosis and by its relationship to successful hormone therapy. ER-positive cancers generally have low-grade histology, favourable nuclear grade, a low S-phase fraction, a normal complement of DNA, a low proliferative index, and a low thymidine labelling index[26]. PR is a well-known prognostic factor of time to recurrence and overall survival and adds prognostic value to the IHC definition of breast cancer subtypes refining the identification of good outcome.

- HER2 OVEREXPRESSION: As a single variable, overexpression of ERBB2 is associated with poor prognosis, but the prognostic discrimination is almost entirely confined to node-positive patients[27]. In node-negative patients, the influence on prognosis has been inconsistent and not clearly independent of other prognostic factors[28].
- KI67: Using 20 percent labelled cells as the cut-off to define high and low proliferation indices, Veronesi et al[29] reported that Ki-67 predicted four-year survival independently of node and ER status. Among node-positive patients, Railo et al[30] found a significant difference in disease-free survival favouring Ki-67-positive/ER-negative patients over Ki-67-negative/ER-positive patients.

Genomic profiles:

PAM50; 21-gene Recurrence Score (RS); 70-gene signature; EndoPredict; Genomic Grade Index (GGI); Breast Cancer Index (BCI). Those tests will be discussed in the 1.5 paragraph.

Emerging biomarkers:

- TUMOUR INFILTRATING LYMPHOCYTES (TILs): Tumour-infiltrating lymphocytes (TILs), a surrogate marker of adaptive immune response, have shown their association with improved prognosis in early-stage BC[31]. Many studies suggested the biological association between TILs and primary breast tumours may differ between ER-positive and ER-negative breast cancer, with high TIL expression acting as a poor prognostic marker in ER-positive patients and a marker of good prognosis in ER-negative patients (HER2 +/ER- included)[32]. In TNBC patients, a higher quantity of TILs has been identified as a biomarker of increased pathological response after neoadjuvant chemotherapy and prognostic factor in patients treated with adjuvant chemotherapy[33]. Latest pooled analysis of patients treated with chemotherapy showed that node-negative TNBC patients with at least 30% stromal TILs (sTILs) had excellent survival, raising the possibility of using sTILs to identify a subgroup of TNBC patients with good prognosis who may need less or no systemic chemotherapy. However, data regarding the prognostic effect of TILs in the absence of chemotherapy has been limited and are currently under investigation.

1.5 Gene-expression profiling tests: overview and focus on PROSIGNA® assay

Tumour gene signatures were initially developed to help clinicians address the two main questions related to the management of early breast cancer patients: "Should adjuvant treatment be prescribed?" and "Which type of adjuvant treatment should be prescribed?". The aim of gene-expression profiling technology is to provide a better prediction of clinical outcome than the traditional clinical and pathological parameters. Guidelines from professional societies, have recommended that the decision to use systemic adjuvant therapy requires considering balancing risk of disease recurrence with local therapy alone, the magnitude of benefit from applying adjuvant therapy, toxicity of the therapy and comorbidity. Thus, based on genomic tests (GEPs) trial-derived clinical utility, several guidelines have included the use of GEP tests to prognosticate distant recurrence in early-stage invasive breast cancer for adjuvant chemotherapy treatment decision-making, including the American Society of Clinical Oncology[34], the National Comprehensive Cancer Network (United States)[35], the St. Gallen International Expert Consensus[36], the European Society of Medical Oncology[37], and the National Institute for Health and Care Excellence (NICE)[38]. Of interest, some of these tests also provide intrinsic subtyping of breast cancer (i.e. Prosigna) but their clinical application is confined to luminal breast cancers, with exclusion of both HER2 positive and Triple-negative subtypes. First-generation assays developed for use in ER+/HER2- breast cancer included MammaPrint[39] and the Oncotype DX Breast Recurrence Score[40], both of which are prognostic in early-stage breast cancer but can only be performed in central laboratories and do not include clinicopathological factors. Further technological and scientific advances led to the development of second-generation prognostic assays, including EndoPredict[41] and Prosigna[42], which have now been validated and can be performed in local laboratories, potentially reducing the overall cost of the assay and lead times for results. These tests include clinicopathological factors and have been demonstrated to improve risk prediction[43]. For most of these assays, clinical utility has been demonstrated. Main features from the most relevant and currently commercially available GEP tests are presented in TABLE 1.

First generation:

- ONCOTYPE DX RECURRENCE SCORE (RS): The 21-gene (16 tumour-associated and 5 controls genes) RS is among the earliest and best-validated prognostic assays in early breast cancer. Oncotype DX is based on RNA isolation from FFPE breast cancer tissue followed by RT-PCR, providing a stratification of the 5-year or 10-year risk of distant relapse into risk groups: low risk where the clinical benefit of chemotherapy is expected to be small [recurrence score (RS < 18)], intermediate risk where it is uncertain whether the beneficial effect of

chemotherapy outbalance the risks and complications mediated by its toxic lateral effects (RS 18–31), and high risk where there is a high probability of cancer of recurrence, and the benefits of chemotherapy are should surpass the risks of side effects (RS >31). The higher expression of genes in the ER-pathway, GSTM1, BAG1 is associated with favourable prognosis and results in low RS, whereas expression of proliferation related genes, such as Ki67 and cyclin B1, genes within the HER2 and invasion pathway produce higher RS score. RS has been validated as prognostic tool to identify very low-risk patients among those with ER-positive, HER2-negative, node negative tumours, which could be safely spared from chemotherapy[37]. Prediction of chemotherapy benefit was prospectively investigated in two randomized controlled trials, namely TAILORx and RxPONDER trials, in node-negative and node-positive (1 to 3 positive) luminal breast cancers, respectively. The TAILORx study was designed to prospectively validate the 21-gene RS in a population of patients with ER positive, HER2-negative, node negative tumours for whom adjuvant chemotherapy was indicated based on clinicopathologic features (tumour size >1.1 cm or 0.6-1.0 cm but intermediate-high histologic grade). The first results of the TAILORx trial indicated that patients with RS ≤10, appointing a very low-risk of relapse, may forgo adjuvant chemotherapy and receive endocrine therapy alone. In fact, in this group risk of distant relapse was less than 1%, of any relapse was in the range of 2-5% and overall survival rate 98% at 5-year follow-up[37]. In 2018, Sparano and colleagues reported the definitive results from TAILORx, clarifying the effect of chemotherapy for women considered to be at intermediate risk for recurrence. Patients in this group were randomized to receive endocrine therapy with or without chemotherapy. The authors established that chemotherapy may be spared in all women older than 50 with RS results of 11 to 25 and all women age 50 or younger with RS results of 11–15[44]. At 2020 SABCS, results from RxPONDER trial (ClinicalTrials.gov identifier: NCT01272037) were presented for patients affected by ER+/HER2- breast cancer that has spread to one to three lymph nodes. RxPONDER found that postmenopausal women with HR-positive, HER2-negative breast cancer with one to three positive nodes and a 21-gene recurrence score (RS) of ≤ 25 (Oncotype DX) derived no further benefit from chemotherapy added to endocrine therapy and can safely avoid adjuvant treatment with it. On the other hand, premenopausal women with the same characteristics experienced a 45% relative risk reduction in invasive disease–free survival events with the addition of chemotherapy.

- MAMMAPRINT: MammaPrint is a 70-gene signature as a prognostic test cleared by the FDA to stratify patients with ER-positive or ER-negative breast carcinomas into a high vs. low risk

for relapse[45]. Prospective indication of the predictive ability of MammaPrint in early-stage luminal breast cancer for adjuvant chemotherapy became available in the MINDACT trial (level1A evidence), which included 6,693 women with early-stage breast cancer (lymph node negative or 1-3 lymph node positive). This study showed that chemotherapy could be spared in women who had a low genomic risk for recurrence according to MammaPrint and who were at high clinical risk for relapse defined using clinicopathological parameters (on the basis of Adjuvant!Online). Women with discordant risk prediction (low genomic risk/high clinical risk) were randomly assigned to adjuvant chemotherapy or endocrine therapy. Approximately 80% of the enrolled patients had negative axillary lymph nodes. In the discordant group, women with high clinical but low genomic risk who received chemotherapy had 95.9% rate of metastasis-free survival at 5 years vs. 94.7% for those treated with endocrine treatment alone. However, the study was not powered to exclude a benefit from chemotherapy and did not demonstrate a clinical usefulness in demonstrating efficacy of chemotherapy in the small subset of women diagnosed with clinical low-risk/genomic high-risk tumours[39].

Second-generation:

- PAM50/PROSIGNA: Prosigna is an FDA-cleared and CE-marked GEP assay developed to guide adjuvant chemotherapy decisions in patients with early-stage ER+/HER2- breast cancer, and it can predict recurrence-free survival at 10 years after initiation of treatment[46]. The assay assumes 5 years of endocrine therapy and measures the mRNA expression of 50 genes (PAM50) in formalin-fixed paraffine embedded tumour tissues. The 50 genes (PAM50) are compared with prototypical gene expression profiles of the intrinsic breast cancer subtypes to identify the intrinsic molecular subtype of the tumour (LUMINAL A, LUMINAL B, HER2-ENRICHED, BASAL-LIKE). When combined to tumour size and nodal status (negative or one to three positive nodes), PAM50 provides a continuous ROR-score (ROR-S), which ranges from 0 to 100 and stratify patients with ER-positive disease in low, intermediate, and high-risk subgroups on the basis of the 10-year risk of recurrence. Additional studies are needed to support recommendations about adjuvant chemotherapy in patients with an intermediate Prosigna/PAM50 ROR score. The prognostic value of the Prosigna PAM50 ROR score, Oncotype Dx recurrence score, Breast Cancer Index, EndoPredict, CTS, and IHC4 were evaluated in a pre-planned, retrospective biomarker analysis of the ATAC trial in 774 postmenopausal women with early ER+/HER2- breast cancer who had received endocrine therapy for 5 years[47]. In patients with node negative disease, Prosigna provided the most

prognostic information during Years 0–10 (hazard ratio [HR]: 2.56; 95% confidence interval [CI]: 1.96–3.35), followed by Breast Cancer Index (HR: 2.46; 95% CI: 1.88–3.23) and EndoPredict (HR: 2.14; 95% CI: 1.71–2.68), and Prosigna also provided the most prognostic information during Years 5–10 (HR: 2.77; 95% CI: 1.93–3.96). In patients with node positive disease, EndoPredict provided the most prognostic value for late distant recurrence (HR: 1.87; 95% CI: 1.27–2.76), followed by Prosigna (HR: 1.65; 95% CI: 1.08–2.51). Although the prognostic value of Prosigna/PAM50 has been clarified, there is a lack of prospective clinical studies that show the predictive value of this signature. The ongoing OPTIMA trial of Prosigna, a randomised controlled trial with a projected recruitment of 4,500 patients in the UK, will provide evidence of long-term patient outcomes following Prosigna scoring[48]. The study aims to validate use of the assay to help guide clinical decisions for adjuvant chemotherapy for patients with hormone-sensitive, HER2- and LN+ (up to nine nodes) early-stage breast cancer and will provide valuable information for future updates to the international guidelines evaluation of GEP tests in early breast cancer, which currently does not recommend any GEP tests for LN+ disease. In the real-life setting, PROSIGNA test application resulted in a 44% decrease in the indication for chemotherapy in the intermediate risk group supporting its promising aid for clinicians choice on adjuvant treatments[49]

- ENDOPREDICT: This test combines the expression of three proliferative and five ER-signalling-associated genes together with four normalisation and control genes and can be measured in formalin-fixed, paraffin-embedded tissue sections by quantitative real-time polymerase chain reaction (qRT-PCR) in decentralised laboratories[50]. EPclin incorporates information on nodal status and tumour size and is used as the diagnostic algorithm in the clinical setting. EPclin has been validated as a prognostic test in pre- and postmenopausal women with ER-positive, HER2-negative breast cancer[51]. In the GEICAM 9906 trial, EndoPredict was able to independently predict the risk of relapse in low and high-risk categories according to EPclin but did not demonstrate ability to predict the benefit deriving from adding paclitaxel to antracyclines[52]. Furthermore, EndoPredict and EPclin were highly prognostic of recurrence after endocrine therapy.

| Clinical trials implicated in the development of multigene prognostic signatures.

	Number of genes/proteins	Candidate patients for adjuvant chemotherapy assessment (recommended by guidelines)	ASCO / NCCN recommendations	AJCC staging	Molecular subtyping	Clinical trial	Combination with clinical parameters in score assessment
OncotypeDX	21 genes (16 genes + 5 reference genes)	ER/PgR+, HER2, node - ER/PgR+, HER2, node +	Yes (strong)	Yes	No	NSABP B14 (retrospective) NSABP B20 (retrospective) SWOG 8814 (retrospective) TransATAC (retrospective) TAILORx (prospective) RxPonder (prospective)	No
MammaPrint	70 genes	ER/PgR+, HER2, node - ER/PgR+, HER2, node +	Yes (strong)	Yes	Yes (BluePrint)	TRANSBIG (retrospective) RASTER (prospective) MINDACT (prospective)	Yes (Adjuvant! Online)
Prosigna/PAM50	50 genes (+ 5 reference genes)	ER/PgR+, HER2-, node -	Yes - (middle size)	Yes	Yes	ABCSG8 (retrospective) ATAC (retrospective)	Yes (Proliferation score, tumor size)
EndoPredict	12 genes (8 genes + 3 RNA reference genes + 1 DNA reference gene)	ER/PgR+, HER2-, node -	Yes (moderate)	Yes	No	GEICAM 9906 ABCSG6 (retrospective) ABCSG8 (retrospective)	Yes (tumor size and nodal status (EPclin))

Table 1. Most relevant GEPs tests available in early breast cancer.

Chapter 3 - Lymphovascular invasion as prognostic factor in breast cancer

In breast cancer lymphovascular invasion (LVI) including both lymphatic vessel invasion (LVI) and blood vessel invasion (BVI), has been defined as presence of tumour cells within an endothelial-lined space in the area surrounding the invasive carcinoma[53] (Fig.4). The first study on the prognostic significance of peritumoral lymphovascular invasion in breast cancer was reported in 1964[54], and, since then, several independent studies have investigated the prognostic significance of LVI in breast cancer in both lymph node negative and positive tumours. Accumulating evidence has showed that LVI has an unfavourable prognostic effect on breast cancer survival and recurrence across all molecular subtypes[55] and LVI is regarded as one of the crucial steps in breast cancer metastasis[56]. At the molecular level, there are several biochemical and biophysical interactions that cancer cells utilise to facilitate their metastatic progression through vascular and lymphatic channels: vascular and lymphatic invasion mechanisms are regulated by the expression of different vascular endothelial growth factors and their ligands, presented by malignant cells. Initially, the extracellular matrix (ECM) is the field of the vibrant interactions between malignant cells and stromal non-tumoral cells in the event of LVI, and when malignant cells induce significant molecular modifications in the ECM,

they will secure their progressive pathways of invasion and, hence, metastasis[57]. Those remodelling actions are the synthesis, alignment, crosslinking and proteolysis of the ECM, and malignant cells will select a combination of remodelling actions to set up the ECM micro-environment to their optimum conditions to invade the targeted vessels and disseminate to distal sites of the body. The strong interaction between malignant cells, lymphatic and blood vessels, and microenvironment is responsible for local recurrence and distant metastasis[58]. Certain tumour morphological features are associated with LVI, including tumour type, grade, and size. One of the characteristic features of inflammatory BC (IBC) is extensive LVI, particularly the involvement of dermal lymphatics. There is a direct correlation between LVI and tumour grade and primary tumour size. For instance, LVI is rarely seen in grade 1 tumours while up to one half of grade 3 tumours are associated with LVI. In fact, PVI is considered as a mirror of tumour cell dissemination to axillary lymph nodes and spread to distant sites, and it directly correlates with pathological nodes involvement. There is also a correlation between LVI and BC molecular subtype. Triple-negative or basal-like molecular classes showed the least association with LVI despite their poor prognosis and higher tendency to local recurrence[59]. Contrasting this, luminal and HER2-positive classes showed the highest incidence of LVI. One explanation attributed this difference to the lower expression of claudin tight junction protein and the higher expression of proteins that are crucial to transform the malignant epithelial cell to the mesenchymal form in triple-negative and basal-like tumours compared with luminal and HER2 BC classes.

Beyond molecular mechanisms, several studies have shown that LVI is an independent poor prognostic factor in patients with invasive breast cancer[60-61]. However, not all commentators agreed on its clinical importance[62], especially in node-positive patients. Most of them have underscored the role of LVI as predictor of poor prognosis, in terms of overall survival (OS) and disease-free survival (DFS), and whether the presence of lymphovascular invasion should be considered sufficient to reclassify breast cancer patients who are at a low risk of recurrence into a high-risk category remains unclear[63]. In 2005, during the 9th St. Gallen consensus conference, the panel included lymphovascular invasion among the adverse prognostic factors to define early breast cancer patients's risk category. According to the panel, LVI status should be considered relevant only for patients with node-negative disease: in this subgroup the presence of LVI moved the risk class from low to intermediate risk, even if the decision for adjuvant chemotherapy should not be taken based on LVI presence alone[64]. LVI role in lymph node positive disease is still unclear and matter of controversy. *Hwang et al.* compared lymphatic invasion (LI) and vascular invasion (VI) effects on overall survival (OS) and disease-free survival (DFS) in the different intrinsic molecular subtypes of breast cancer[65]. They observed that positive LVI increased risk of local and systemic disease recurrence in luminal A and triple negative breast cancer, both in

terms of OS and DFS. LVI role has been also investigated in neoadjuvant chemotherapy (NAC)-treated patients. It is known that the principal aim in patients treated with NAC is the achievement of pathological complete response, a surrogate marker of survival. Many studies have analysed the role of LVI in predicting pCR achievement after NAC and its prognostic role in terms of disease-free survival and overall survival. Hamy et al.[66]tried to evaluate LVI degree as independent prognostic factor in neoadiuvant setting for treatment of BC. These studies demonstrate as LVI presence and its degree in histological examination after surgery, in patients who have received NAC, correlates with an increased risk of local-relapse or distant metastasis and a worse DFS and OS, especially in TNBC[67]. Also, LVI degree can be considered as one of the important factors to predict the NAC efficacy in patients with invasive breast cancer[68]. They found that LVI, large tumour size and hormonal receptor-positivity define a subgroup of patients who had a decreased response to chemotherapy and a lower pCR rate. In the molecular era of breast cancer genomic profiling, after the underlined role of Mutai et al. tried to find an answer to the difficult question about which are the characteristics that support adjuvant chemotherapy in luminal breast cancer[69]. They decided to correlate lymphovascular invasion status and recurrence score (RS) on Oncotype DX assay. They found that LVI was not significantly associated with ODX RS, but LVI presence determines worse 5 years-overall survival in intermediate risk patients. The same effect has not shown in low or high-risk patients.
According to Mutai et al., patients with intermediate RS and LVI could benefit from adjuvant chemotherapy.

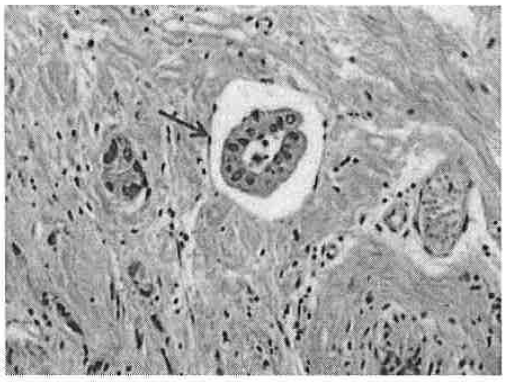


Fig 4. H&E-stained section of invasive ductal breast carcinoma showing tumoral emboli inside a lymphatic vessel (red arrow)

Chapter 4 - Prognostic value of Lymphovascular invasion (LVI) in early breast cancer: pathological definition and comparison with PROSIGNA test results

Background

Breast cancer is a heterogeneous disease characterized by variant pathological features, disparate response to therapeutics, and substantial differences in long-term patient survival[70]. Beyond the immunohistochemical classification, gene-expression profiling identified four main classes, the so-called intrinsic molecular subtypes (Luminal A, Luminal B, HER2-enriched, and Basal-like), each one associated with different clinical outcomes. Clinical classification has a major influence on treatment decisions for individual patients. In the adjuvant setting, patients with luminal breast cancers are typically treated with anti-estrogenic drugs (e.g., tamoxifen, aromatase inhibitors) with or without chemotherapeutic drugs, while HER2+ and TN breast cancer patients undergo chemotherapy (plus anti-HER2 drugs, as trastuzumab, in case of HER2+). After performing surgery, the use of adjuvant chemotherapy in hormone-receptor–positive, HER2-negative breast cancer remain questionable, with effects that are proportionally greater in younger women but that are little affected by nodal status, grade, or the use of adjuvant endocrine therapy[71]. Those traditional clinicopathological markers continue to be a standard for guiding the use of chemotherapy but the clinician may be confronted with equivocal results that require additional testing. Recently, gene expression-based assays (Oncotype DX, MammaPrint, PAM50/Prosigna, EndoPredict, and many others) have been developed to refine physician decision-making process helping the estimate of the absolute benefits expected from systemic adjuvant chemotherapy or extension of adjuvant endocrine therapy. The ability of Oncotype DX to predict chemotherapy benefit in luminal patients has been validated in both N0 and N+ (from one to three positive nodes) population in two large randomized prospective trials (Tailor X and RxPonder), while the other molecular tests are still waiting for prospective validation even if have been already endorsed in the AJCC 8th staging edition, as well as in professional societies' guidelines. The prognostic potential of lymphovascular invasion (LVI), defined as the presence of malignant cells in lymphatic and/or vascular vessels close to the invasive carcinoma, has been explored repeatedly, but whether its presence provides additional information to the most relevant traditional clinic-pathological factors (such tumour dimension or nodal status) or acts as an independent high-risk criterion beyond genomic tests remains controversial. In 2005, during the ninth St. Gallen consensus conference, the Panel of experts included lymphovascular invasion among the negative prognostic factors defining early BC patient's risk category[60]. LVI status was considered relevant only for patients

with node-negative luminal breast cancer where its presence moves patient's risk class from "low" to "intermediate". In node-positive disease LVI prognostic value is this still unclear and matter of controversy. For these reasons, LVI has not been incorporated into internationally recognized staging systems, prognostic indices, algorithms, and guidelines such as AJCC TNM, Nottingham Prognostic Index, and Adjuvant!Online. Moreover, difficulties in identifying LVI in histopathological specimens convincingly, let this information not being uniformly found and described in breast cancer pathology reports. The aim of the present work is to review the pathological definition and biological role of LVI in early luminal BC and to determine whether there is an association between LVI status and ROR score/10year risk of recurrence according to PAM50/PROSIGNA gene test.

4.2 Study Objectives

Primary objectives

The main objectives of this study are to determine LVI occurrence in luminal breast cancer and to explore the correlation between LVI status and genomic risk of relapse based on PAM50 ROR score in a LUMINAL breast cancer population.

Secondary objectives

The secondary objectives of this study are:

- To evaluate the association among LVI status and traditional clinicopathological features (tumour dimension, node status, grading, KI67 index, Luminal A or B subtypes)
- To explore the correlation between LVI status and adjuvant chemotherapy benefit calculated by the online tool PREDICT

4.3 Patients and Methods

We retrospectively analysed data about 82 patients who underwent surgery and subsequent PAM50 PROSIGNA® genomic test at Campus Bio-Medico of Rome. Clinical data were collected between 01 Jan 2014 and 31 Dec 2015, to get a minimum clinical follow up of 5 years. Formalin-fixed paraffin-embedded (FFPE) tissue recollection was performed starting April 2018 and ended in June 2020. Data analyses were completed on December 16th, 2020.

Patient's Selection

Inclusion criteria

- Postmenopausal women with node-negative (Stage I or II) or 1-3 node-positive (Stage II) who underwent breast surgery and hormonal therapy (plus chemotherapy if indicated)

- Histologically proven diagnosis of invasive hormone receptor-positive (HR+) breast cancer (ductal, lobular, mixed or NOS)

- Formalin-fixed paraffin-embedded (FFPE) tumour tissue samples with > 10% tumour (4 mm^2 minimum tumour area)

- Available data for the following clinical and pathological features:

- Age
- Diagnosis (screening, symptoms, unknown)
- Size of primary tumour in mm (T stage)
- Oestrogen, progesterone and HER2 receptor status
- Proliferation index expressed by KI-67
- Grading
- LVI status
- Lymph nodes involvement (negative *versus* 1-3 positive, N stage)
- PAM50 PROSIGNA® genomic test ROR score and 10-year recurrence rate

Exclusion criteria

- ER negative OR HER2 positive/amplified OR HER2-enriched/basal-like subtype by PAM50
- Pre-surgical chemotherapy, endocrine therapy, or radiotherapy for breast cancer
- Metastatic at time of diagnosis
- Clinical follow up less than 5 years or lost.
- Unavailable FFPE tissue samples

Methods

Pathological definition of lymph nodes involvement

Lymph nodes involvement was classified according 2020 ASCO CAP criteria (v. 4.4.0.0) as following:

- **Number of nodes examined:** The total number of nodes includes sentinel nodes, non-sentinel nodes, nodes from axillary dissections, and intramammary nodes.

- **Size of metastases:** Metastases are classified into 3 groups:

- Isolated tumor cell clusters (ITCs) are defined as small clusters of cells not larger than 0.2 mm, or single cells, or fewer than 200 cells in a single cross-section. *Nodes containing only ITCs are not included in the total number of positive nodes for N classification.*

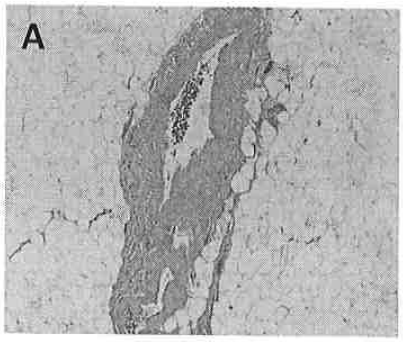
- Micrometastases measure more than 0.2 mm, but not more than 2 mm, and/or comprise more than 200 cells in a single cross-section. *If only micrometastases are present, the N classification is pN1mi. If at least 1 macrometastasis is present, nodes with micrometastases are included in the total node count for N classification.*

- Macrometastases measure more than 2 mm.

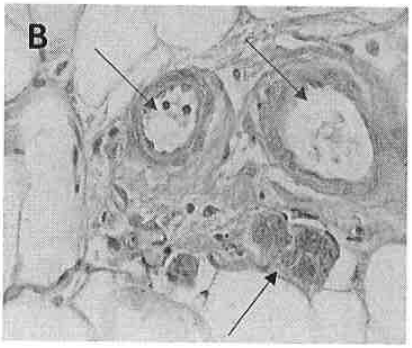
Pathological definition of LVI status

In collaboration with the Department of Pathology of our Institution a literature review was conducted to summarize the appropriate definition of LVI positivity in breast cancer. The timeline for the literature review was of four weeks (April 2018) and a total of four studies[72,73,74,75] were identified as resuming the criteria of LVI individuation on invasive breast cancer tissues. Of them, the study from *Rosen et al.*[63] provided the backbone criteria for the pathological definition of LVI with a very precise description of the diagnostic standards (see below). Since LVI assessment reported by the ASCO-CAP 2020 guidelines (v. 4.4.0.0)[76] is according to *Rosen et al.*, the following rules were selected for the pathological revision of our case series (Table 2):

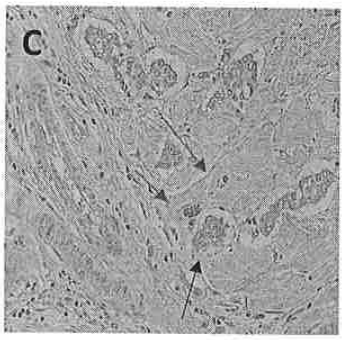
- LVI must be diagnosed outside the border of the invasive carcinoma. The most common area to find LVI is within 1 mm of the edge of the carcinoma (Fig. A)



- Distinguishing lymphatic channels from blood vessels is unnecessary. Lymphatics are defined by exclusion as an endothelial-lined channel devoid of red block cells and without a smooth muscle wall (Fig. B)



- Lymphovascular invasion may be seen in stroma between uninvolved lobules and can sometimes be mistaken for DCIS if the cells completely fill the lymphatic space. Intralymphatic tumor emboli generally have the shape similar but not identical with that of the space in which they lie (Fig. C).



Guidelines issued by the 2011 St. Gallen International Expert Consensus Conference included recommendations based on the presence of "extensive" LVI but do not define the term "extensive"[77]. Since there are conflicting results on the significance of the number of foci of LVI, we decided to report the number of foci or the number of blocks with LVI as a measure of extent, according to *Colleoni et al*[65]: *Focal* (one focus) versus *Extensive* (two or more foci).

Criteria for Lymphovascular Invasion (LVI)

1.	LVI must be diagnosed outside the border of the invasive carcinoma. The most common area to find LVI is within 1 mm of the edge of the carcinoma.
2.	The tumor emboli usually do not conform exactly to the contours of the space in which they are found. In contrast, invasive carcinoma with retraction artifact mimicking LVI will have exactly the same shape.
3.	Endothelial cell nuclei should be seen in the cells lining the space.
4.	Lymphatics are often found adjacent to blood vessels and often partially encircle a blood vessel.

Data derived from Rosen

Table 2. LVI criteria by the ASCO-CAP 2020 guidelines (v. 4.4.0.0)[69].

Since accurate detection of LVI is crucial to its prognostic validity and depends on high-quality tissue preservation techniques during handling, fixation, and preparation, we identified hematoxylin and eosin (H&E) histological specimens as the ones favouring true LVI presence[78]. H&E staining highlights features are a definite endothelial lining around the tumour embolus, invasion into a vessel lumen with nearby cancer glands that have minimal or no retraction, and an embolus different in shape from the surrounding clear LV space.

The pathological revision of hematoxylin and eosin-stained slides was performed by three Anatomo-pathologists in a two-step revision from June 2018 to May 2020. LVI was reported as:

+ **Not identified**

+ **Present**

If present, LVI was defined as:

+ **Focal** (one detected focus)

+ **Extensive** (two or more detected foci)

Statistical analysis

LVI was dichotomized in negative (score 0) and positive (score 1 for *focal* positivity and score 2 for *extensive* positivity) *or* non-extensive (score 0+1) versus extensive (score 2). Categorical variables were compared using two-sided Fisher'sexact test or the $\chi 2$ test. Numerical variables were compared using the Mann-Whitney U test. Multiple linear regression was used to model the relationship between PROSSIGNA 10-year recurrence score and clinicopathological variables. A P value < 0.05 was considered statistically significant. All analyses were conducted using IBM SPSS statistics version 26.0 (Armonk, NY, USA) and Graphpad Prism version 8.0 (San Diego, California USA).

4.4 Results

Descriptive analysis

A total of 82 luminal breast cancer patients were enrolled at time of analysis. Of them, 2 patients were excluded from the final analysis because of non-luminal subtyping by PAM50: one patient resulted affected by a HER2-enriched and one patient from basal-like breast cancer when molecular intrinsic subtype was determined by PROSIGNA test. The patients' clinical histories and tumour characteristics were retrieved from the database. This was a well-characterized series of patients treated uniformly in a single institution. Features of the overall population (N=80) are reported in Table 3:

Characteristics of the Overall Population (N=80)		
Age		63 (44-84)
T size (mm)		18 (7-45)
N status	N0	59,8% (49)
	N+	40,2% (31)
	N1mic	25,8% (8)
	N1a	74,2% (23)
Grading	G1	9,8% (8)
	G2	70% (56)
	G3	20,2% (16)
Ki67 (%)	< 20%	42,5% (34)
	> 20%	57,5% (46)
PAM50 LUM sub	LUM A	56,3% (45)
	LUM B	43,7 % (35)
IHC LUM sub	LUM A	40% (32)
	LUM B	60% (48)
Risk class according to PROSIGNA (N0)	Low	34% (17)
	Inter	46% (23)
	High	20% (10)
Risk class according to PROSIGNA (N+)	Low	8,4% (2)
	Inter	22,6% (7)
	High	68% (21)

Table 3. Overall population clinicopathological features (n=80).

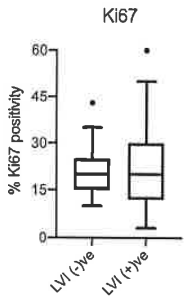
Their median age was 63 years (range, 44–84). The vast majority (72%) had negative or micrometastatic nodal disease. LVI status was reported as positive in 25 patients (20%). Data on LVI were missing for 32 patients (25%). As clearly reported in the table, population's characteristics recapitulate the so-called "grey zone" for clinicians at the time of prognosis definition and adjuvant chemotherapy benefit prediction. Most of patients present a Luminal B breast cancer (which absolute frequency was increased by using gene expression analysis by PAM50, resulting 60% *versus* 43,7% by IHC), with a median tumour size of 18 mm (pT1c stage), N0 (60%), G2 (70%) and a median KI67 of 21,4 %. Most of patients with node-negative disease were classified as intermediate risk of recurrence by PROSIGNA test (46%) while node-positive disease resulted in a high genomic risk in 68% of the cases.

4.4.1 LVI presence increased after ASCO-CAP criteria systematic application in the pathological revision.

Lymphovascular invasion (LVI) in not uniformly found or reported in breast cancer tumour examination and, very often is a lacking information on pathology reports. In our case series, 25% of the pathology reports missed LVI status definition, while positive cases were about 20% with no distinction between *focal* or *extensive* LVI presence. After performing the 2-step revision, about 54% of the tumour samples resulted as positive cases for LVI. Of them, 58% of patients presented an *extensive* LVI positivity *versus* the remaining 42% that were classified as *focal*. Given its prognostic value, we can conclude that LVI should be carefully evaluated and reported in final pathological examinations.

4.4.2 LVI presence is independent from breast cancer luminal subtyping.

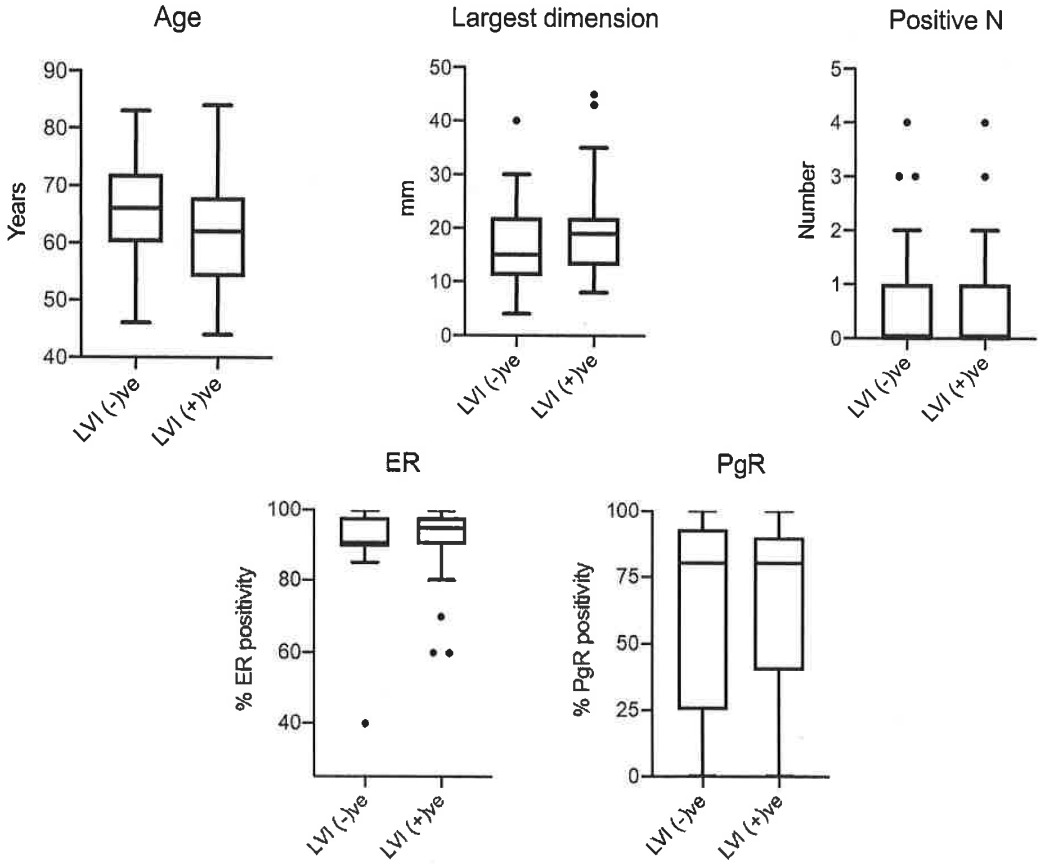
As previously reported in literature, the presence of LVI did not differ between luminal subtypes (A *versus* B) or according to KI-67 levels (p=0.477) in our case series (Table 4). Lum A and Lum B subclasses were defined by both IHC (*with LUM A defined as ER and PgR positive with KI67 < 20% and LUM B as PgR negative or KI67 > 20%*) or genomic subtyping by PAM50. The analysis was repeated in the overall population (N0/N+) and N0/N+ cases separately.



Characteristics (N0/N+)	LVI negative (n =38)	LVI positive (n =44)		p value
		Focal (n=20)	*Extensive (n=24)*	
Luminal call according to IHC				**0,575**
LUM A	16	8	8	
LUM B	22	12	16	
Luminal call according to PAM50				**0,246**
LUM A (PAM50)	24	9	8	
LUM B (PAM50)	14	11	16	

Table 4. LVI distribution according to Luminal subclasses (according to St. Gallen, Luminal A-like cancers were ER/PR ≥ 1% and Ki67 <20% and Luminal B-like (HER2-negative) were ER≥ 1%, PR ≥ 1% and KI67 ≥ 20% ore PR negative and any KI67).

4.4.3 Extensive LVI positivity correlate with largest tumour dimension independently from nodal status. Other traditional clinicopathological features are not associated to LVI status in both N0 and N+ positive population.



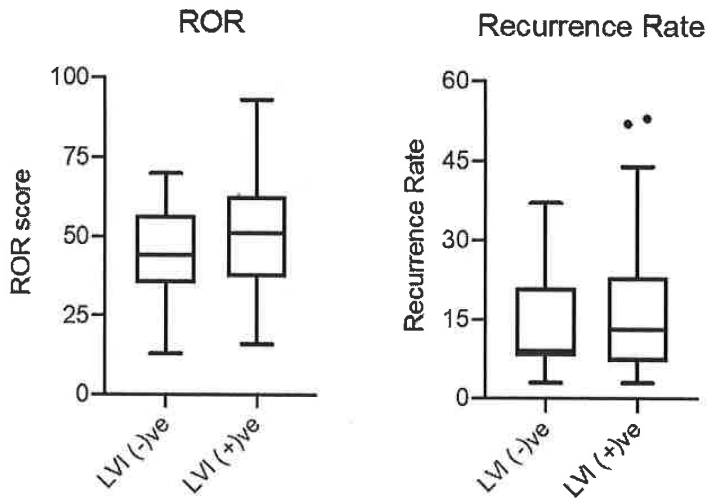
Characteristics (N0/N+)		LVI negative (n =38)	LVI positive (n =44)		p value
			Focal (n=20)	*Extensive (n=24)*	
AGE					0,435
Tumour dimension (mean, mm)		16,4	16	21	0,04
ER (mean, %)		89	89	90	0,404
PgR (mean, %)		61,8	59,2	64,1	0,477
Grading					0,149
	G1	6	1	2	
	G2	30	10	8	
	G3	2	9	14	

Table 5. LVI distribution according to traditional clinicopathological factors

As reported in table 5, extensive LVI positivity was associated with largest tumour dimension (p= 0.04) in the overall population (N0/N+). The same association was present when N0 subpopulation

was analysed separately (p= 0.04) but was lost in N+ patients (p=0.645). All other traditional clinicopathological features (ER and PR % staining, age, histology, nodal status) did not show significant correlations with LVI status.

4.4.4 Extensive LVI positivity do not correlate with Risk of Recurrence (ROR) score calculated by PAM50/PROSIGNA test but it correlates with 10-year probability of Distant Recurrence



Characteristics (N0)	LVI negative (n =22)	LVI positive (n =29)		p value
		Focal (n=12)	*Extensive (n=15)*	
ROR score				0,356
low	7	5	5	
intermediate	12	4	6	
high	3	5	4	
10y DFRS (mean, %)	9,90%	15,60%	18,30%	0,004

Characteristics (N+)	LVI negative (n =15)	LVI positive (n =15)		p value
		Focal (n=6)	*Extensive (n=9)*	
ROR score				0,317
low	1	0	0	
intermediate	3	2	2	
high	11	4	7	
10y DFRS (mean, %)	15,80%	15,20%	23,50%	0,501

Table 6. LVI distribution according to ROR score risk classes (low, intermediate, high risk) and 10y Distant-Recurrence rate (as mean), assessed by PAM50/PROSIGNA test in N0 (upper table) and N+ populations (lower table).

In node positive (N+) population, the rate of extensive LVI positivity was higher in the PROSIGNA ROR high-risk group (78%) than in the intermediate-risk (22%) low-risk group (0%) but the difference

was not significant (p = 0.501). In node-negative cases, ROR distribution was similar in both positive and negative LVI patients (p=0.356). Interestingly, only in N0 population, extensive LVI was statistically significant associated with higher 10-year recurrence rate (p=0,004) provided by the PROSIGNA test.

However, in multivariate linear regression analysis, neither the presence of LVI (score 0 versus score 1-2) nor extensive LVI status (score 0-1 versus score 2, Table 7) had independent predictive value for PROSIGNA 10-year recurrence score besides node status, grading, KI67 and tumour dimensions.

Model		95,0% Confidence Interval for B	
		Lower Bound	Upper Bound
1	(Constant)	-5,036	,825
	Age	-,026	,024
	DimMax	,106	,175
	N_bin	1,518	2,523
	G	1,223	2,284
	ER	-,038	,009
	PR	-,005	,008
	KI67	,017	,068
	IHC	-1,196	,138
	SubPRO	-1,335	,168
	RORscore	-,012	,038
	Histo	-1,014	,448
	LVI_01v2	-,737	,274

a. Dependent Variable: OS10yScore

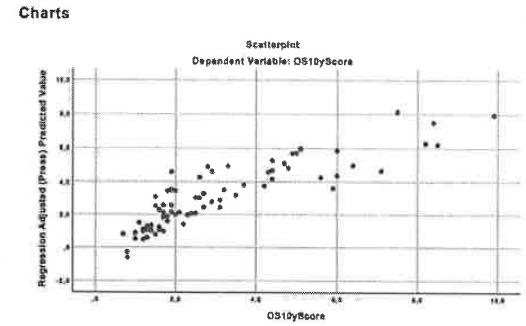
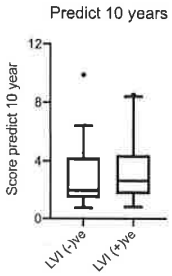


Table 7. Multivariate linear regression analysis (dependent variable 10y Recurrence Score)

4.4.4 Extensive LVI positivity is associated with 10year chemotherapy benefit calculated by PREDICT tool in node-negative luminal breast cancer patients.



Characteristics (N0)	LVI negative (n =21)	LVI positive (n =29)		p value
		Focal (n=12)	*Extensive (n=15)*	
10y CHEMO BENEFIT by PREDICT				0,022
< 3%	19	4	3	
≥ 3%	2	8	12	

The PREDICT breast cancer prognostication and treatment benefit prediction model (v1) was developed in 2010[79] using data from the East Anglia Cancer Registration and Information Centre (ECRIC) for model fitting and data from the West Midlands Cancer Intelligence Unit for model validation[70]. The algorithm uses tumour size, node status (including micrometastatic disease), tumour grade, oestrogen receptor (ER) status and mode of detection (clinical/screening) to estimate breast cancer-specific mortality at 5 and 10 years, as well as age to estimate non-breast cancer mortality at 5 and 10 years. Moreover, this tool provides the predicted benefit of adjuvant chemotherapy classified as first-, second- or third generation and adjuvant hormone therapy. PREDICT was implemented as a web-based tool for clinicians in January 2011 (www.predict.nhs.uk), and since then the use of the tool has increased steadily and is the only breast cancer prognostic model currently available online that has been endorsed by the American Joint Committee on Cancer[80].

In our case series, extensive LVI was associated with 10-year benefit from adjuvant third-generation chemotherapy (anthracyclines and taxanes-based combination) only in N0 population (p=0.022) but not in N+ patients (p=1.0) or the overall population (p=0.124). However, univariate linear regression analysis (followed by leave one out cross-validation) showed that LVI status had not independent predictive value for ROR definition (as continuous variable) beside 10-year PREDICT survival (R2 0.209 vs 0.204).

4.5 Discussion

Outcomes for early-stage breast cancer (BC) patients have improved over recent decades as a result of better diagnostic accuracy, comprehension of disease complex biology and more effective drug therapies. Unfortunately, 3-15% of ER+ (or *luminal*) patients will experience long-term metastatic distant recurrences, with individual cumulative risk depending on tumour characteristics. Over the years, several histopathological features have been recognized as strong independent prognostic factors in early breast cancer, including tumour size, lymph node status and histological grade. Today, breast cancer management is already changing considering the new molecular analysis that is becoming more accessible in daily clinical practice thanks to commercially available genetic prognostic tests, such as PAM50/PROSIGNA®. The integration of clinical features of the patient, such as tumour size and nodal status, with genomic profiling of individual breast cancer can help clinicians better estimate disease outcome and safely tailor adjuvant treatments.

An early event in the development of metastasis is lymphovascular invasion (LVI), which provides not only a route for cancer cell dissemination but also an indication of the biological ability of the tumour

to invade lymphovascular spaces and survive within the lumens of vascular channels. Although the prognostic value of LVI in breast cancer has been repeatedly investigated, conflicting results resulted into the omission of this marker from TNM staging and prognostic indices/algorithms that guide clinician to select the adjuvant strategy. The present study examined the prognostic role of LVI in early breast cancer in the current era of molecular profiling starting from accurate definition of LVI status on pathological reports. First, to standardize the use of LVI in patient management, it was clear that the method of detection of LVI was the primary issue to be addressed. To do that, a 2-step revision of H&E stained-formalin-fixed paraffin-embedded (FFPE) tumour tissues have been performed after conducting a literature review of LVI status criteria. In the present work, the proportion of patients with LVI (54%) was slightly higher than reported in studies using a similar approach (21–42%)[81]. LVI higher rate can be explained by both increased diagnostic accuracy (which reduced false LVI cases detected by the same team of three pathologists) and clinical characteristics of our population (40% of patients were node-positive, with enhanced probability of LVI presence due to nodal metastases). Since there is no information on the clinical implication of the extent of LVI (focal or extensive) and whether such an evaluation might accurately identify patients at higher risk, we investigated them separately: in our cohort, 58% of patients presented an extensive LVI positivity versus the remaining 42% that were classified as focal. In the present study, we showed different prognostic implications for extensive LVI (defined as the presence of multiple foci of LVI in more than 1 tumour block) compared with a less extensive vascular invasion, suggesting that not only qualitative but also quantitative assessment of LVI might be relevant, as previously proposed by Colleoni et al[65]. Extensive LVI was significantly associated with larger tumour size (p=0.04) in both node-negative and node-positive patients, while no statistically significant association were found with all the other traditional clinicopathological parameters (age, grading, KI67, ER and PR expression). When compared to prognostic results from PROSIGNA, we found that the rate of LVI positivity was higher among node-positive patients with a higher Risk of Recurrence (ROR), but the association did not reach statistical significance. Nevertheless, an association with 10-year recurrence rate calculated by PROSIGNA was found in node-negative population (p=0.004) against node-positive cases (p=0.501). These findings are consistent with most previous studies which reported LVI prognostic value confined to node-negative disease. However, when multivariate linear regression analysis was performed, neither the presence of LVI (score 0 versus 1-2) nor extensive LVI status itself provided independent prognostic information for PROSIGNA 10-year recurrence score besides node status, grading, KI67 and tumour dimensions. Taken together, these results suggested that the analysis of LVI might provide independent prognostic information beyond advanced molecular testing, but it remains controversial. The hypothesis to be tested are numerous. First, LVI quantitative pathological detection in H&E-stained

tissues may not recapitulate the biological process of lymphovascular invasion. There are several lines of evidence to indicate the occurrence of LVI in breast tumours lacking morphological evidence of LVI, such as the demonstration of circulating tumour cells, bone marrow micrometastases, and lymph node positivity. This may indirectly influence our ability to understand the biology driving LVI in breast cancer. Second, the molecular mechanisms of LVI and associated genes that may represent therapeutic targets or biomarkers remain to be identified. Several gene signatures predictive of LVI have been reported[82] and interestingly none of the upregulated genes (most of them belonging to Epithelial–mesenchymal transition (EMT)-implicated genes) is present in PAM50 signature. This observation may explain why LVI presence, even if associated with worst recurrence rate, did not correlate to PAM50-derived ROR score (p=0.351) nor LUMINAL intrinsic subtyping (p=0.246) in our case series. In favour of these hypothesis, two studies sought to evaluate the association of LVI status with the recurrence score (RS) on the multigene Oncotype DX (ODX). Both studies showed that LVI was not significantly associated with a higher ODX RS, but it may infer a worse outcome, especially in ODX intermediate risk patients[63-83]. When chemotherapy benefit calculated by PREDICT online was investigated, extensive LVI correlated with higher percentage of 10-year survival rate gain from third-generation therapy in node-negative population (p=0.022). Some studies have shown that LVI is associated with "chemoresistant" cancers[84] and that its absence on core biopsies is associated with a complete pathological response (pCR) and improved survival[85]. Up to date, LVI predictive value for chemotherapy benefit have not been tested in luminal breast cancer and should not be used to guide choice of adjuvant chemotherapy. According to this, univariate regression analysis done in the present work, showed that LVI status did not provide additional prognostic information besides 10-year PREDICT overall survival. Our study presents several limitations. First, it is limited by the single-centre, retrospective design, which may have unknown inherent biases. The histopathological parameters evaluated were based on the routine pathology reports and were not re-evaluated for the study by a central laboratory. Second, our cohort was relatively small and even if increased diagnostic accuracy resulted in higher number of cases of LVI (48 patients), the study was unpowered for subgroup analyses. Additionally, given the good prognosis of the included population (early-stage, hormone receptor-positive, HER2-negative disease, 60% node-negative), there were few DFS events after a five-year follow-up and the impact of LVI on survival outcomes could not be analysed.

4.6 Conclusion

In conclusion, the presence of extensive lymphovascular invasion is associated with larger tumour dimension and worse recurrence rate in node-negative luminal breast cancer. As a marker that has the strength to upgrade apparently low-risk cases to a higher category, more convincing data from large series studies and real-world experiences are needed, including molecular analysis. Understanding the biology driving LVI in breast cancer will be essential to define its prognostic role besides the most relevant clinicopathological parameters and molecular testing.

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