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BIOLOGICAL AND CLINICAL CHARACTERISTICS OF INVASIVE DUCTAL CARCINOMA WITH LOBULAR PATTERN

Final Degree Project

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I would like to thank the pathology unit of Dr. Josep Trueta Hospital for their dedication to decency, despite their busy and important schedules and their welcoming of all students with their best intentions.

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ABSTRACT

Introduction: Invasive Breast Cancer (IBC) has a great epidemiological impact in our society; it is the first most frequent cancer among women and the first cause of death from cancer in women worldwide. Invasive ductal carcinoma (IDC) represents the largest group of malignant tumours of the breast, comprising up to 75% of the cases. It is a heterogeneous entity with no clear definition and its diagnosis is made by the exclusion of other special types of Breast Cancer. Women presenting with Invasive Ductal Carcinoma have a wide range of prognostic outcomes, thus, there is a need to reassess tumours classified as Invasive Ductal Carcinomas in an effort to elaborate more accurate subclassifications of this heterogeneous entity. It is in this context that, in the pathology unit of Josep Trueta Hospital of Girona, a possible morphological variant of Invasive Ductal Carcinoma has been recognized. It could be described as an Invasive Ductal Carcinoma exhibiting a growth pattern in linear cords, typically seen in Invasive Lobular Carcinoma, yet not fulfilling the criteria to be classified as mixed tumours. To our knowledge, these tumours have not been assessed in past studies.

Objective: To assess the clinicopathological characteristics of Invasive Ductal Carcinoma with Lobular Pattern (IDCLp) regarding, age of presentation, nationality, menopausal status, family history, primary tumour size, histological tumour type, histological grade, lymph node status, bilateralism, oestrogen and progesterone receptors, HER2 overexpression, staging and treatment procedures, disease-free survival and survival time, and to determine whether these characteristics significantly differ from the classic types of Invasive Ductal Carcinoma and Invasive Lobular Carcinoma (ILC).

Methods: This is a retrospective, observational and analytical study. Information will be reviewed and collected from the Cancer Registry of Girona database which contains epidemiologic information of all Breast Cancer cases of the province of Girona since 1994. Three cohorts will be analysed retrospectively, between 1994 and 2016, regarding their clinicopathological characteristics: Invasive Ductal Carcinoma with Lobular Pattern cases, Invasive Ductal Carcinoma, and Invasive Lobular Carcinoma.

Key words: Invasive Lobular Carcinoma, Invasive Ductal Carcinoma, Morphological variant, Lobular pattern, Invasive Breast Cancer, Breast Cancer, Invasive Carcinoma of No Special Type

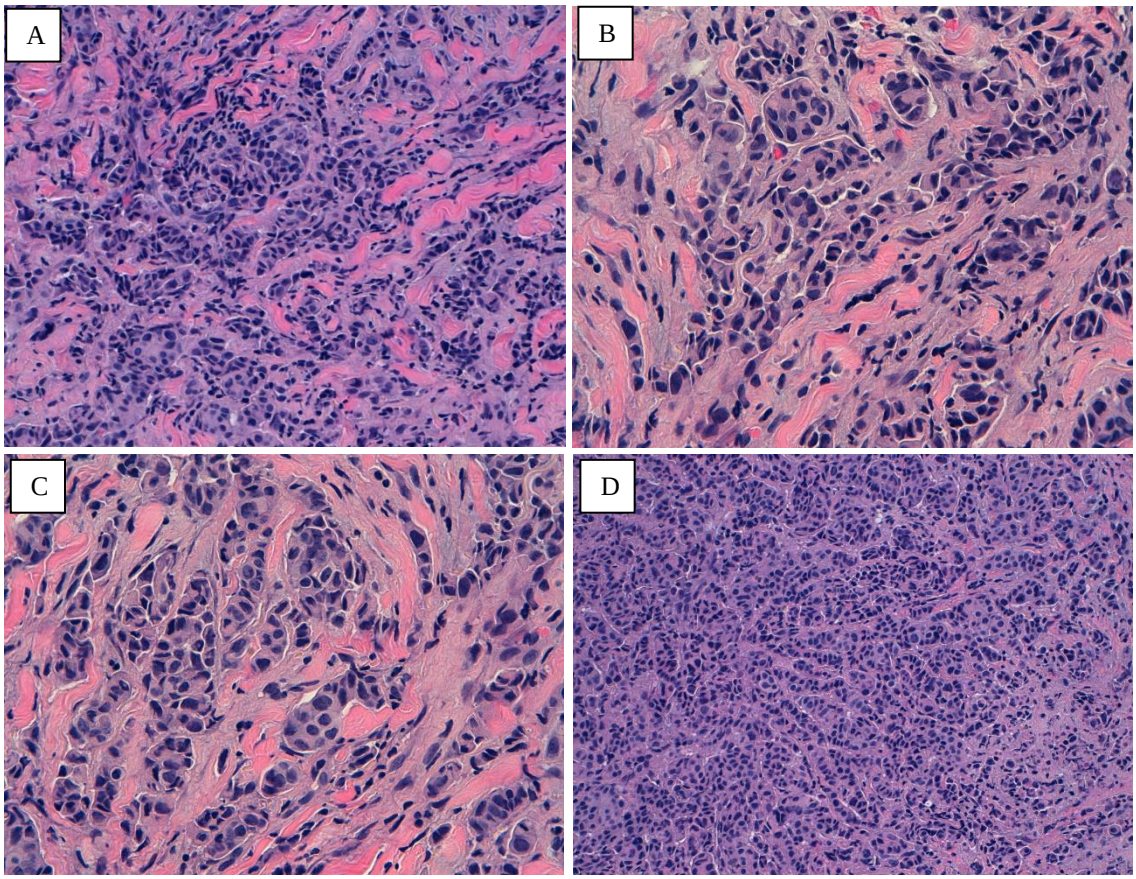
ABREVIATIONS

ALNS	Axillary Lymph Node Status
ASCO	American Society of Clinical Oncology
BC	Breast Cancer
CAP	College of American Pathologist
DCIS	Ductal Carcinoma in Situ
DFS	Disease Free Survival
ER	Oestrogen Receptors
IBC	Invasive Breast Cancer
IBCNST	Invasive Breast Carcinoma of No Special Type
IDC	Invasive Ductal Carcinoma
IDCLp	Invasive Ductal Carcinoma with Lobular Pattern
IHC	Immunohistochemistry
ILC	Invasive Lobular Carcinoma
ISH	In Situ Hybridization
ITC	Isolated Tumour Cells
LN	Lobular Neoplasia
LVI	Lymph Vascular Invasion
MRI	Magnetic Resonance Imaging
NPI	Nottingham Prognostic Index
NST	No Special Type
OS	Overall Survival
PLC	Pleomorphic Lobular Carcinoma
PR	Progesterone Receptors
RCG	Cancer Registry of Girona
SD	Standard Deviation
WHO	World Health Organization

1. INTRODUCTION

1.1. Plausible findings of a morphologic variant of Invasive Ductal Carcinoma

In the pathology unit of Dr. Josep Trueta Hospital of Girona, a possible morphologic variant of Invasive Ductal Carcinoma (IDC) has been recognized. A consistent number of cases exhibiting the classic characteristics of invasive ductal carcinoma (see section 1.3.4) has been found to express a growth pattern in linear cords around a classic lesion of invasive ductal carcinoma. This pattern of growth is characteristic of the Invasive Lobular Carcinoma (ILC, see section 1.4.5), yet it lacks the necessary characteristics for a diagnosis to be made, or even to be categorized as an Invasive Breast Cancer (IBC) with mixed features (see section 1.3.3), mainly because it lacks the discohesive property of the lobular proliferations, expresses positive E-cadherin by immunohistochemistry (IHC) and the cellular appearance is concordant with that of IDC tumours. Thus, tumours expressing this differentiated growth pattern could potentially behave in a very different way than classic IDC, as histological morphology frequently correlates with clinical and biological behaviour.



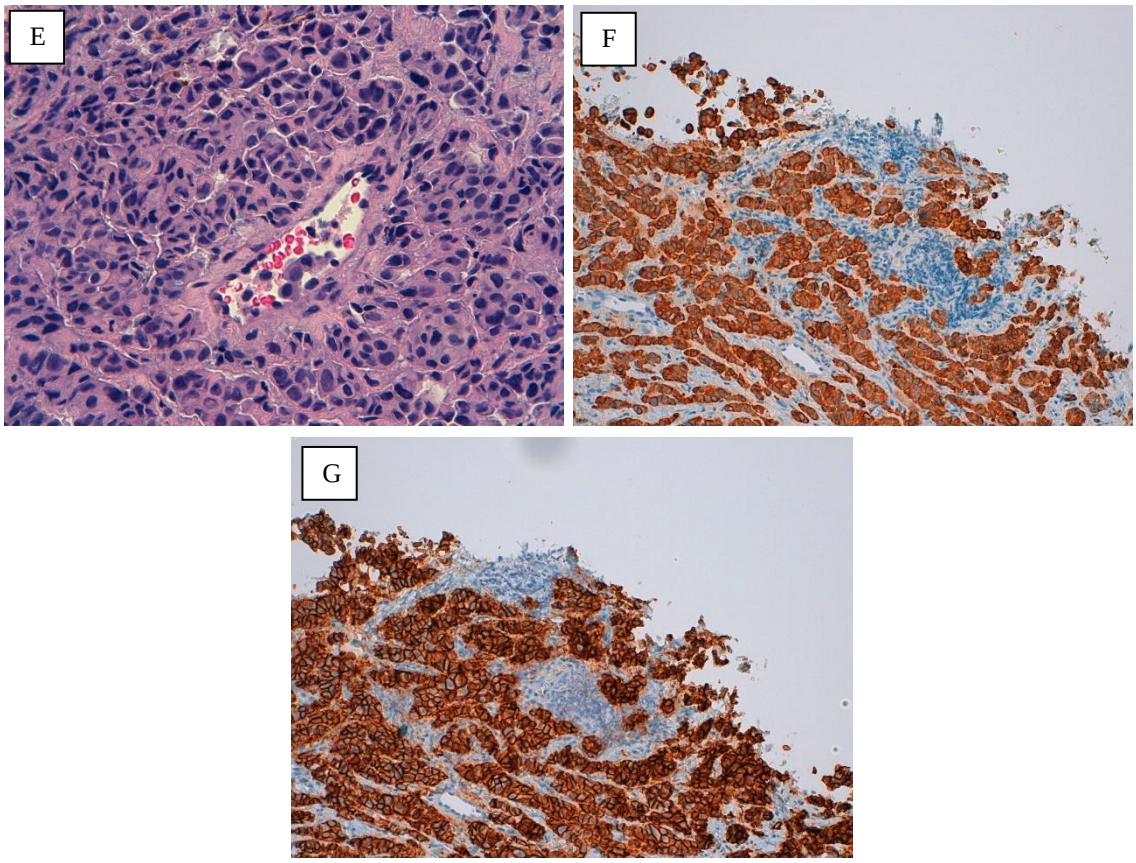


Figure 1: Invasive Ductal Carcinoma with Lobular Pattern (IDCLp). **A, B C** and **D** invasive cells appear to have a growth pattern forming linear cords similar to the characteristic growth pattern of Invasive Lobular Carcinoma (ILC). **E** Same tumour expressing solid nest and Lymph-vascular invasion (LVI), more characteristic of Invasive Ductal Carcinoma (IDC). **F** positivity for E-cadherin characteristic of IDC. **G** positivity for cytokeratin CK19, characteristic of all Breast Cancer (BC) neoplastic epithelial cells. Images obtained from the pathology laboratory of Josep Trueta Hospital.

For the purpose of this study, we are going to refer to these tumours as Invasive Ductal Carcinoma with Lobular Pattern (IDCLp). The aim of this study is to assess the clinicopathological characteristic of such tumours.

1.2. General characteristics of invasive breast carcinoma

1.2.1. Epidemiology and impact of breast cancer

Breast cancer (BC) is by far the most frequent type of cancer in women with an estimated 1.67 million new cancer cases diagnosed in 2012, representing 25% of all cancer in women worldwide and 10.9% of all cancer worldwide in both men and women. It is the most common cancer type in women both in the developed and developing world with incidence rates varying from 27 per 100,000 women in Eastern Africa to 92 per 100,000 women in North America, maintaining a characteristic high incidence in developed countries and lower incidence in developing countries.

Nevertheless, the mortality rate of BC is much less than expected by its incidence. BC ranks fifth in overall cancer mortality both in men and women, despite being the most frequent cause of death from cancer for women in less developed regions, and the second-most frequent cause of death from cancer in developed regions after lung cancer (1,2). This is due to the more favourable survival of BC in developed countries with a high incidence, owing to the combined effect of population screening and adjuvant hormonal treatment and chemotherapy (3). However, it is still the most frequent cause of cancer death in women both in developing and developed regions.

In Spain, the BC incidence rate is around 63 per 100,000 (4). Among all cancers, BC is the fifth-most frequent cancer found among men and women, and the fourth cause of death from cancer accounting for a 4.9% of deaths from all cancers. However, among women only, BC is the first most frequent cancer, and the first cause of female mortality from cancer accounting for a 15.5% of deaths from cancer, followed by colorectal cancer (1).

In Girona, data obtained from the Cancer Registry of Girona (RCG) (5) reveals that among men and women BC is the third-most incident cancer type, with an average of 409 cases per year, which represents a 27.8% relative incidence among the ten most frequent cancers. Among women, BC was the second-most frequent incidence cancer after skin cancer with an average of 405 cases per year. Regarding mortality among men and women, BC is the third cause of death from cancer, but among women alone, it is the first cause of death from cancer, accounting for an average of 96 deaths per year followed by colorectal cancer.

1.2.2. Clinical Features

A palpable mass is the most common clinical sign of invasive breast carcinoma (IBC), although skin retraction, nipple inversion, nipple discharge and, less commonly, change in skin colour or texture or enlargement of the axillary lymph nodes may also be seen (3,6). Benign breast disease can cause similar symptoms; therefore, the differential diagnosis becomes essential for early detection of cancer and administration of the most adequate treatment.

The diagnosis of IBC can be very challenging, as both benign and malignant lesions in the breast share similar clinical features. The most common tools that physicians use to make a differential diagnosis are mammography, ultrasound, Magnetic Resonance Imaging (MRI) and histopathological study of diverse samples of mammary tissue.

Mammography is the baseline method for the detection of breast cancer in women aged older than 40. This technique allows physicians to determine the presence of calcifications, speculated masses, architectural distortion, or focal asymmetric density.

Ultrasound alone is the method of choice for BC detection in women aged younger than 40, but it is a complementary technique to mammography to assess the size, extent, and presence of multifocality of suspected lesions. It can also be very useful to complement mammography in women with very mammographically dense breasts, to assess the axillary lymph nodes and to guide biopsies of suspicious lesions.

MRI is the most sensitive method for detecting IBC, but it is also a costly one, most of the time being unaffordable for whole population screening of cancer. It can be used to improved pre-treatment staging, especially in badly defined lesions that cannot be assessed by mammography.

Once suspicious lesions are recognized, histological confirmation is required for diagnosis. The method of choice is ultrasound-guided core biopsy (3,6).

1.2.3. Prognostic factors

The adequate management of patients with IBC requires robust prognostic and predictive factors to support decision-making by patients and physicians, in relation to choosing the best therapeutic procedures from several potentially suitable treatment options to give the best possible quality of life for the patients.

The three strongest prognostic determinants in operable breast cancer used in routine clinical practice internationally are (7,8) :

- Tumour histological grade
- Lymph node stage
- Primary tumour size

The Nottingham Prognostic Index (NPI) includes these 3 variables with the objective of discriminating subgroups of patients with different clinical outcomes (9,10). It mainly divides patients into three prognostic groups: Good, moderate, and poor.

NPI is calculated based on the formula:

$$NPI = (0.17 \times Size) + (0.76 \times lymph - node\ stage) + (0.82 \times Tumour\ grade)$$

In clinical practice, a more simplified formula is used, given the fact that it gives very similar results to the more complex formula previously mentioned:

$$NPI = (0.2 \times size) + stage + grade$$

1.2.4. Grading

Several studies have been reported to justify the determination of tumour grade because of its prognostic value (7). The most widely used histological grading system of breast cancer is the Nottingham combined histological grade, based on the assessment of three criteria (7,11):

- Tubule and gland formation
- Nuclear pleomorphism
- Mitotic count

Each factor is assessed independently, receiving a score between 1 and 3 (see annex 2). Glandular formation is assessed over the whole tumour carefully avoiding possible confounding artefacts that simulate glandular lumen, like clefs induced by shrinkage. Nuclear Pleomorphism is determined by tumour area with the worst degree of pleomorphism and the mitotic count is determined by the tumour area with the highest count of mitosis, preferably in the peripheral area of the tumour (see Annex 3).

The overall result classifies tumours into 3 categories, each with different associated survival and disease-free intervals (11):

- Grade 1: well differentiated
- Grade 2: moderately differentiated
- Grade 3: poorly differentiated

1.2.5. Staging

Staging gives important information about two out of the three main prognostic factors in BC (12,13). It is assessed by the TNM system published by the American Joint Committee on Cancer (14). This system synthetises information about the primary tumour site (T), the invasion of regional lymph nodes (N), and the spread to other distant metastatic sites (M). see Annex 1: *TNM Classification of tumour of the breast*.

All the information obtained from T, N, and M is combined and categorized into 5 stages (see Annex 1). This categorization is essential to give the patient the most adequate treatment in terms of control of the local disease and the utility of systemic therapy, but it also is valuable to research, for organizing groups of patients with similar characteristics for comparison in clinical trials.

1.2.6. Lymph-vascular invasion

Lymph-Vascular Invasion (LVI) is defined as the presence of neoplastic emboli within unequivocal vascular lymphatic or capillary Lumina in areas adjacent to, but outside, the margins

of the carcinoma. LVI has been reported as an independent prognostic factor for overall survival (8,15,16).

LVI is associated with lymph nodal status. Although women with both positive LVI and lymph nodal status have a worse prognosis than either one alone, it is more important as a prognostic factor in axillary node negative women (3,8).

The association between lymph nodal status and LVI is not true for all histological types of breast cancer. LVI is not common in lobular carcinomas, however they are often positive for infiltrated lymph nodes; this is possibly due to lack of cellular adhesion, which is characteristic of this tumour, impeding the adhesion of the neoplastic cells to the walls of the vessels.

Vascular space involvement can serve as reservoir for neoplastic cells. These cells cannot be removed with conventional surgery, and it seems like this mechanism also confers certain resistance to chemotherapy agents (3).

1.2.7. Lymph node status

For most BC, Axillary Lymph Node Status (ALNS) is the single most important prognostic factor in BC (1). ALNS has been reported to correlate to tumour size, both behaving as independent but additive prognostic factors. A study published by Carter CL et al. (12) which analysed the relation between size and ALNS found a linear correlation between tumour diameter and the percentage of cases with positive lymph node involvement.

Disease-free survival and overall survival diminish with each additional positive node as reported by Michaelson et al. (13) for women with tumours of equivalent sizes. They reported an increase of 6% chance of death for every extra positive node. Regarding size, for women with equal lymph node status every millimetre of tumour diameter increases the chance of death by 1% (13).

Despite ALNS being a good prognostic factor, axillary lymph node dissection does not appear to have major consequences in the overall survival, as stated by Giuliano et al. (17)

Axillary lymph node metastasis can be classified as (see lymph node staging, annex 1):

- macrometastases, defined as been >0.2 cm in size
- micrometastases: defined as being between 0.02 cm to 0.2 cm in size
- Isolated tumour cluster cells (ITC): size <0.02 cm or <200 cells in a single nodal cross-section

Macrometastases have been proven to have major prognostic significance as reported by Pugliese M. et al. (18), and it can be reliably detected by thinly sectioning breast tissue samples into paraffin blocks. On the other hand, micrometastases and isolated tumour cluster cells require

additional levels of thinly sectioned slices of breast tissue or inmmunohistochemical analysis. Women with occult micrometastases and isolated tumour clusters cells, which have been reported as lymph node negative in an initial evaluation, do have a statistical significantly worse outcome than women with negative nodes. However, the difference in outcome in 5 years' time is small (1%), thus not justifying the need for additional evaluation to detect micrometastases and ITC, as reported by Weaber D. L. et al. (19).

In present clinical practice, the use of Sentinel Node Biopsies (SNB) is widely used to separate ALNS positive women from ALNS negative women. SNB is at least just as effective to detect metastasis nodal disease as Axillary Lymph Nodal Dissection (ALND), as reported by Kell MR. et al. (20). But incidence of residual morbidity is much higher in the patients that undergo ALND in terms of risk of infection, seroma, swelling and numbness, thus SNB is recommended as the optimum technique in evaluating ALNS in clinically node negative women.

Although ALNS negative women have a very favourable prognostic factor compared to their positive counterparts, 10 to 30% of these women will develop distant metastases. Presumably in these patients, the tumour has spread by other routes to distant sites (12).

1.2.8. Molecular testing for estrogen receptor, progesterone receptor and HER2

These three molecular biomarkers are used in clinical practice in women with IBC, because they are all targets or indicators of effective therapies, thus making their assessment essential and even mandatory.

1.2.8.1. Oestrogen receptors

Oestrogen Receptors (ER) like progesterone receptors are nuclear ligand activated transcriptor factors that regulate gene expression in the presence of steroids. When the receptor is activated, it stimulates the growth of normal breast epithelial cells. Neoplastic cells that express this receptor will eventually result in stimulation of proliferation of these cells and progression of the tumoral lesion (21).

In today's clinical practice, ER is assessed mainly by IHC of samples of breast tissue, a method that has been proven to be inexpensive, sensible and specific (22).

Presence of ER has been reported to be a strong predictive factor for response to hormonal therapy, according to the Early Breast Cancer Trialist Collaborative Group (EBCTCG) in a study that focussed on different systemic adjuvant therapies over a period of 15 years (23). Their conclusion was that endocrine therapies such as tamoxifen that block ER and, in turn, blocking

estrogen stimulated growth, reduce the annual BC death rate by 31% in ER positive women of any age.

The response to hormonal therapy correlates to the grade of expression of ER. About 74% of breast cancer tumour express ER, with a IHC grade ranging from 2 to 8 (22). (The inmmunohistochemical grade named Allred Score is calculated by the sum of the proportion of positive ER cells in mammary tissue samples where **0**, none; **1**, < 0.01; **2**, 0.01-0.1; **3**, 0.1-0.33; **4**, 0.33-0.66; **5**, >0.66; and the intensity score where **0**, none; **1**, weak; **2**, intermediate; **3**, strong, thus producing a score from 1 to 8). However even tumours expressing very low (≥2) inmmunohistochemical grade for ER have a significant benefit from endocrine therapy compared to those patients with no positivity to ER (grade < 2) (22), thus defining ER positivity as any breast tumour expressing any IHC grade for ER from 2 to 8 (corresponding to >1% positive cells).

The guidelines published by the American Society of Clinical Oncology (ASCO) and College of American Pathologist (CAP) for ER and PR testing (24) can be seen in Annex 4.

1.2.8.2. Progesterone Receptors

Progesterone receptors (PR) are also nuclear receptor (see section 1.2.8.1), routinely assessed in clinical practice for patients with BC.

ER regulate the expression of PR, thus the expression of PR reflects a functional ER pathway (21). PR activates by the binding of progesterone and its receptor, producing stimulation of growth in tumour cells with such receptors.

PR is expressed in about 60-70% of IBC, with an IHC grade (which applies the same IHC grading used for ER, named Allred Score, see section 1.2.8.1) ranging from 0 to 8. Like ER, even very low expression of PR (grade >2) correlates to a significantly better outcome in terms of disease-free interval and overall survival than negative PR patients, as reported by Mohsin S. K. et al. (25).

ER and PR expression is correlated, according to Bardou V. J. et al. (26), and the combined expression of these two biomarkers results in different outcomes than the expression of ER and PR alone. This gives rise to four phenotypes of BC patients with different outcomes:

- ER-positive / PR-positive: the most frequent phenotype, with the best rate of response to endocrine therapy.
- ER-negative / PR-negative: the next category in frequency, with almost no response to endocrine therapy

- ER-positive / PR-negative: these tumours tend to have worse disease-free and overall survival compared to ER-positive / PR-positive tumours, but without significant differences, according to Bardou V. J. et al (26).
- ER-negative / PR-positive: there is controversy about whether this tumour really exists, since ER is known to be a prerequisite to PR presence. The small amount of cases with these characteristics makes it difficult to draw conclusions about their behaviour.

1.2.8.3. HER2 oncogene and oncoprotein

HER2, also referred as HER2/*neu* and *erbB2*, is a proto-oncogene located in the chromosome 17q, and encodes a growth factor receptor (transmembrane tyrosine kinase receptor) on the surface of normal epithelial cells of the breast. Amplification of HER2 gene is an acquired molecular alteration that promotes the maintenance and growth of carcinoma.

Gene amplification for HER2 has been reported to be present in about 15% of IBC, and this amplification is correlated to the overexpression of the protein (27,28).

The relation between HER2 status and clinical outcome is complex and varies with the setting. This relation seems to depend on the type of therapy. There are many studies addressing this issue; some have reported that HER2-positive IBCs are resistant to certain types of cytotoxic chemotherapies such as cytoxanmethotrexate-5-fluoracil combination, but sensitive to others such as anthracyclines and taxanes. Other authors have suggested that HER2-positive status might be related to resistance to hormonal therapies. The most relevant findings are that a HER2-positive status responds well to new antibody based targeted therapies. The main reason for assessing HER2 status is to identify possible candidates who would benefit from this targeted therapy (28).

In clinical practice, HER2 status can be evaluated by measuring protein expression by IHC or measuring gene amplification by FISH. There is controversy regarding which method is better to evaluate HER2 status, but recent studies show that when properly performed, both methods are equivalent (28,29).

The guidelines published by the American Society of Clinical Oncology (ASCO) and College of American Pathologist (CAP) for HER2 testing (29) can be seen in Annex 5 and 6.

1.3. Invasive ductal carcinoma

1.3.1. Definition

Invasive ductal carcinoma represents the largest group of malignant tumours of the breast, comprising between a 40% and a 75% of cases (3,6,30). It represents a heterogeneous group of tumours that do not express sufficient characteristics to be classified as special type of breast

carcinoma such as ILC, or tubular carcinoma; thus, it is not a defined entity but categories of BC that include all non-specialized BC tumours.

There have been many names to describe this type of breast carcinoma: Invasive carcinoma not otherwise specified, Invasive carcinoma of no special type and invasive ductal carcinoma. The term that has been most used in the past is "invasive ductal carcinoma", but it is based on the misconception that this type of BC arises from within the ductal epithelium, unlike lobular carcinoma which were thought to arise only from the lobules. To resolve this issue, the latest edition of the World Health Organization (WHO) classification of tumours (3) proposed the name "invasive breast carcinoma of no special type" to recognize the non-specific nature of the term "Invasive ductal carcinoma". However, the WHO classification in the same edition retains the terms "atypical ductal hyperplasia" and "ductal carcinoma in situ". In this study, this type of tumour will be referred to as "Invasive ductal carcinoma" (IDC), which we consider more appropriate.

1.3.2. Epidemiology

As previously mentioned, IDC comprises between 40 to 75% of cases of BC. This big difference in reported cases can be attributed to the lack of strict criteria for inclusion in the special type of BC categories, and the fact that tumours with combined characteristic of IDC and any other special type of BC are not universally considered in a "mixed category" and may be classified as IDC instead (3,6).

Regarding the specific epidemiology of this tumour, it is very similar to BC in general (see section 1.2.1).

1.3.3. Invasive ductal carcinoma with mixed histological features

IDC represents a heterogeneous group of tumours that might exhibit one or more histological features of other special types of BC, but do not have sufficient criteria to be categorized as one. Approximately one third of the lesions characterized as IDC express one or more combined features, according to Edwin R. et al (31). Such mixed patterns can be exhibited in a truly "mixed" pattern, meaning that the different histological types are well-defined inside the breast lesion, or in a "mixed hybrid" morphology, meaning that there are features of different BC histological types that converge together in the breast lesion (6).

According to the latest edition of the WHO classification of breast cancer (3), to categorize a tumour as an invasive carcinoma of no special type or IDC it must have a non-specialized pattern in >50% of its mass, judged by a thorough examination of its representative sections. This definition refers to the quantification of well-defined patterns.

If the non-specialized pattern comprises between 10% and 49% of the tumour mass, the rest being of a recognized special type pattern, then it will be classified into two groups:

- Mixed invasive no special type and a Special type
- Mixed invasive no special type and lobular carcinoma

Combinations of IDC with invasive lobular carcinoma (ILC) comprise approximately 6% of BC tumours, according to Edwin R. et al (31) and Li C.I. et al (32). A study carried out by Rakha et al (33) analysed the biological and clinical characteristics of mixed ductal and lobular morphology, concluding that such mixed ductal and lobular tumours are a distinct entity that intermediate features between ILC and IDC. However, the population of the study was selected from a database of the Nottingham Tenovus Primary Breast Carcinoma from a series of cases of operable breast carcinomas with a diagnosis of mixed IDC and ILC, which was based on the recognition of well-defined histological components (ILC and IDC) in the paraffin samples. This implies that the tumours that were selected for the study are tumours that express different proportions of well-defined patterns of IDC and ILC in the same breast lesions.

The findings of our group in Dr. Josep Trueta Hospital do not fulfil the criteria to be classified as mixed tumours because they do not express well-differentiated patterns of a no special type BC and another special type. In addition, they exhibit E-cadherin positivity, the histological characteristics of the cells that compose them are typical of IDC, but the growth pattern is typical of lobular proliferations. To our knowledge, these tumours have not been recognized in the literature, and might have been misclassified as IDC.

In a different section of the WHO classification of BC (3), rare morphological variants of IDC are mentioned, which include: Pleomorphic carcinoma, Carcinoma with osteoclast-like stromal giant cells, carcinoma with choriocarcinomatous features and carcinoma with melanotic features. Nevertheless, there is no mention of a morphological variant of IDC combining features of ILC, which is what corresponds to the findings of our group in the pathology unit of Josep Trueta Hospital.

1.3.4. Histopathology

The designation of IDC is basically a process of exclusion of recognized special types of BC (3,30). Invasive carcinomas vary in their extent of differentiation and growth pattern, but although their atypical morphology does not usually represent a challenge to recognize their malignant nature, it can be challenging to differentiate the type of BC. The diagnosis of invasive carcinoma always requires the presence of both cytological atypia and conventional histological evidence of destructive tissue invasion (34).

1.3.4.1. Shape of well-differentiated lesions of IDC

IDC appears as expanding occupying masses made up of malignant cells, which causes a reactive response of the stromal tissue. The overall effect is the creation of new tissue which can be irregular or stellate because of invagination of "tongues" made up of malignant cells that penetrate the surrounding tissue.

1.3.4.2. Structure of well-differentiated IDC

Well-differentiated IDC consist of a structureless mass of neoplastic glands and stroma mixed with variable amounts of pre-existing mammary parenchyma. The absence of an internal structure is what helps differentiate IDC from other benign common lesions of the breast.

Well-differentiated IDC usually forms tubules and glands, but neoplastic cells can also grow in small clusters or as individuals. The glands vary in shape and size, which might express angulated ends or open lumens (see figure 2). The difference in shape and size of the glands is a very informative characteristic but does not constitute a fundamental criterion for the diagnosis of invasive cancer.

Regarding their cytological features, carcinomatous cells that compose the glands appear slightly bigger compared to their benign counterparts. This larger cell size is due to an increased nucleus and cytoplasm. The nucleus contains homogenous chromatin and inconspicuous nucleoli.

Another histological feature of IDC is that the epithelial neoplastic aggregates lack myoepithelial cells, but this feature has no diagnostic value if it is found isolated.

The stroma surrounding the neoplastic lesions can also present diverse changes. A desmoplastic appearance is often observed due to the proliferation of reactive fibroblasts within a myxoid ground among collagen bundles. This characteristic is not present in other benign lesions, which adds diagnostic value.

When neoplastic glands invade adipose tissue, the presence of focal fat necrosis, inflammation and fibrosis can be observed. Since malignant lesion do not always infiltrate fat tissue, the diagnosis of invasion cannot be made only with this characteristic.

Regarding peripheral epithelial elements, the presence of proliferative glands in the periphery of the neoplastic lesion and their destructive invasion of the surrounding tissue is the most consistent evidence of invasive carcinoma (34). It is present in all invasive BC and no benign lesion will exhibit it. The destructive nature of invasive carcinoma can be recognized by the encirclement or penetration of pre-existing benign glandular structures and the destruction of collagen bundles.

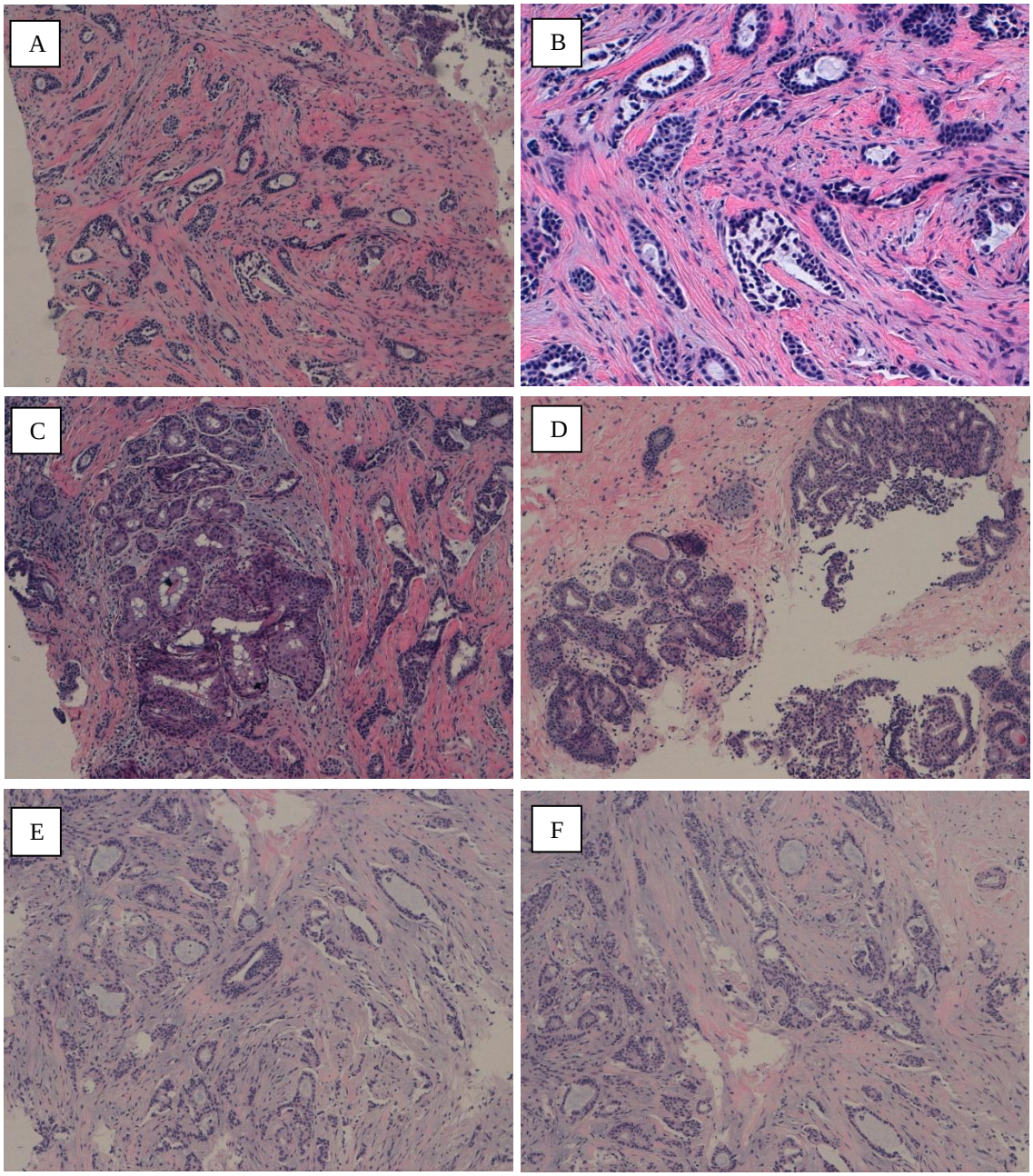


Figure 2: Well differentiated Invasive Ductal Carcinoma (IDC). **A** Glands with different shapes and sizes. **B** A Augmented 20x. **C** and **D** Nest of aberrant neoplastic glands with cells exhibiting slight augmented size and hyperchromatic nuclei. **E** and **F** Aberrant glands with desmoplastic reaction. Images obtained from the pathology laboratory of Josep Trueta Hospital

It is also worth mentioning that IDC is often accompanied by ductal carcinoma in situ (DCIS). The grade of the adjacent DCIS usually appears similar to the grade of the IDC. The presence of the latter does not confirm the malignant nature of the lesion nor does it exclude it, but it is often used by pathologists as an aid in the diagnosis of IDC (34).

1.3.4.3. Histological Characteristics of less differentiated IDC

In other less differentiated lesions of IDC, the histological characteristics can be a little different to their well-differentiated counterparts.

Regarding tumour margins, we can find all types, from highly infiltrative, altering the surroundings structures, to continuous pushing margins.

Architecturally, neoplastic cells lack the morphologic features of other special type of BC and can be arranged in cords, clusters and trabeculae, or even a predominantly solid pattern with little or no associated stroma (3,6). The presence of glands in the lesion is a marker of tumour differentiation. Areas of targetoid features can also be seen, but these areas lack the cytomorphology characteristic of ILC (3,6).

Regarding cytological features, neoplastic cells can have a very wide range of appearances. The cytoplasm can be abundant and eosinophilic. Nuclei can be regular and uniform or highly pleomorphic with prominent and multiple nucleoli. Mitotic activity may range from virtually absent to extensive.

The stromal component can also be extremely variable. A desmoplastic reaction (see section 1.3.4.2), scant connective tissue or hyalinization can be observed. Focal necrosis might also be present with secondary formation of cysts.

Lympho-vascular tumour emboli can be observed throughout the tumour, but only if it is present outside the tumour does it have prognostic value. Perineural invasion can also be present but has no prognostic value (30).

As well as the differentiated IDC counterparts, the IDC is often accompanied by DCIS.

1.3.5. Molecular characteristics

For the purpose of this study, we are only going to mention the characteristic E-cadherin expression of the IDC, as it is a widely-utilized tool used by pathologists to differentiate lobular proliferations from ductal ones in case of equivocal morphologic features.

Ductal proliferations in general, from moderate to strong, express E-cadherin positivity. Approximately 90% of IDC present some kind of positivity to E-cadherin (35,36).

1.3.6. Prognostic and predictive factors

IDC comprises the biggest group of IBC, and its prognostic characteristics are slightly worse compared to IBC as a whole in terms of overall survival, as reported in a study made by Ellis IO et al (37), The overall survival in the IDC group was 47% at 10 years compared with 50-60% respectively.

Prognosis of IDC is determined by classic prognosis factors of breast cancer: ALNS, histological grade, size of the tumour, LVI and distant metastasis.

Regarding predictive factors, the most important ones are the expression of ER, PR and HER2. Approximately 70% of IDC express hormone receptors and approximately 50% of IDC that are ER-positive are also PR-positive. Another 25% of IDC ER-positive are PR-negative and about 20% of IDCs are both ER and PR negative. IDCs with ER-negative / PR-positive phenotype are rare (6).

About approximately 15% of IDCs are HER2-positive, indicating high levels of protein expression, thus representing a group of tumours that responds well to targeted antibody therapies (3,28).

1.4. Invasive lobular carcinoma

1.4.1. Definition

ILC is an invasive carcinoma that comprises five fundamental cellular properties: cellular proliferation, discohesive cells, lack of cellular polarization, cytologic atypia and architectural atypia.

1.4.2. Epidemiology

ILC comprises between 5% to 15% of BC cases (3,6,32,38–40), being the second most common subtype of BC. The incidence of ILC has been observed to be increasing since the 1980s in comparison to the incidence of IDC, especially in women older than 50 years (38,41). The mixed ductal and lobular cases, referring to those who present different proportions of more than one well-defined histological type (see section 1.3.3), has also been reported to be increasing since the 1980s (38). This increase may be attributable to the increase of use of hormonal replacement therapy (38,40,42).

1.4.3. Clinical features

The most common way of presentation of ILC is an ill-defined mass located in any part of the breast tissue.

Regarding screening techniques, the most common findings associated to ILC observed in mammography are a speculate opacity, but it can often manifest as a poorly identified opacity, parenquimal asymmetry or architectural distortion, and can also be mammographically occult. It is not common to find calcifications associated to ILC (43,44). Mammography has a low sensitivity to detect ILC as reported by Krecke K. N. et al.(44). In a study of 184 patients diagnosed with ILC, a rate of false negative of 19% with a sensitivity of 81% was found. This low diagnostic rentability can be attributed to the histological and macroscopic characteristics of ILC and its growth pattern (see section 1.4.5 and 1.4.6) and the lack of presence of calcifications.

In the detection of ILC, ultrasounds have been reported as a very useful tool. It has also been reported to have better sensitivity than mammography, which can vary from 78-95%, in the detection of ILC (45–47). The most common findings have been reported to be a hypoechoic heterogeneous mass with irregular or indistinct margins and posterior acoustic shadowing.

Magnetic Resonance Imaging (MRI) has been reported to have even better sensitivity to diagnose ILC compared to both mammography and ultrasound. In addition, it also seems more accurate in size determination and assessment of multifocal, centric and contralateral disease (48).

1.4.4. Macroscopy

ILC often present as an irregular, poorly defined mass, which not clearly demarcated. This is due to its basic histopathology. (see section 1.4.5)

1.4.5. Histopathology of lobular neoplasia

It is worth mentioning that ILC is a malignant neoplasia that arises from a precursor lesion known as Lobular Neoplasia (LN). The origin of this LN is not known but it seems to originate from cells basally pointed in the mammary epithelium, distinct from the luminal epithelium (34). LN is divided into two categories: Atypical Lobular Hyperplasia (ALH) and Lobular Carcinoma In Situ (LCIS). Both lesions exhibit identical morphologic characteristics but differ in the amount of cells that compose them.

When LN surpasses the basal membrane acquires their characteristic invasive properties, which is then named ILC. But LN and ILC share some of the same histological characteristics and, for the purpose of this study and the general understanding of ILC, it is worth mentioning some of the most relevant characteristics of lobular neoplasia, which are also characteristics of ILC.

There are five fundamental characteristics (34) that define lobular proliferations, which are the following:

- Cellular proliferations

Cellular proliferation in the lobular neoplasm can be observed as a thickening of the epithelium that fills the lumen of the glands, later producing distention of the terminal duct-lobular units. This characteristic is often very easy to identify.

- Intercellular cohesion

The lack of intercellular cohesion is one of the characteristic features of lobular neoplasms. This characteristic distinguishes lobular neoplasms from all the other types of BC. It is recognized by the presence of a gap between cells. However, this characteristic can be easily underestimated

due a very high cellular density, or overestimated if there are artefacts in the sample that simulate intercellular spaces, for example autolysis or cauterization.

The lack of cellular cohesion is related to an acquired or inherited mutation in the proteins involved in intercellular adhesion, like E-cadherin and catenin's. It is very well documented in the literature that one of the hallmarks of lobular neoplasms is the lack of expression of E-cadherin, occurring approximately in 90% of ILCs. This property has become a robust aid for pathologist to recognize lobular carcinomas, especially from ductal neoplasms, due to the fact that the latter characteristically retains some kind of E-cadherin expression in nearly all cases. Nevertheless, there are also reported cases of aberrant expression of E-cadherin, both positive for ILCs and negative for IDC, therefore pathologists must be careful in using this tool to recognize these IBC histological types (3,6,49–51).

- Cellular polarization

The lack of cellular polarization is the second most fundamental characteristic of lobular proliferations. Compared to normal epithelial cells that compose the glands in the mammary tissue, which are mainly composed by columnar epithelial cells with eccentric nucleus, the lobular proliferation stands for its globular shapes with a nucleus sitting in the centre of the cells, unless there is a cytoplasmic structure displacing it, like vacuoles, or the cell is being deformed by external forces. This feature of lobular proliferations does not differentiate it from other types of proliferative epithelial cells, like seen in ductal proliferations.

- Cytological atypia

The grade of cytological atypia varies considerably. The classic case of a low-grade atypia can be described as epithelial cells with an enlarged nucleus that contains finely dispersed chromatin, and a small augmented cytoplasm, with nucleoli sometimes being present. However, in cases expressing high grade atypia, markedly anaplastic cells can be observed.

- Architectural atypia

Because lobular neoplastic cells lack polarization, they cannot collectively express architectural atypia other than a stratification of cells. The presence of architectural atypia such as cribriform spaces or roman bridges excludes the diagnosis of a lobular neoplasia; instead the latter grows as a dishesive sheet.

1.4.5.1. Architectural characteristics

The cells of lobular neoplasia exhibit a consistent and characteristic growth pattern: the cells proliferate as individuals in small clusters interspersed among pre-existing benign cells and those well-developed focci form structureless sheets (34). Classic lobular neoplasia does not give rise to true glandular structures; instead they fill the lumen of the glands producing distention.

Lobular neoplasia can also present unusual spreading patterns known as “pagetoid spread” (34). Most carcinomas grow as a unified mass that destroys benign cells while LN grows in clusters or as individual cells between the benign epithelial cells present in the lumen and the myoepithelial cells, showing a coexistence between benign cells and malignant. This property of lobular proliferations partly explains its clinical behaviour and the difficulties in its diagnosis.

1.4.5.2. Structural alterations of the lobules

LN tends to alter the structure of the lobules. But these changes are not in any way specific to lobular proliferations but are common to a variety of epithelial proliferations. The recognitions of these structural changes help in the confirmations of a suspected LN.

These structural alterations can be (34):

- Enlarged lobules: normal lobules already have some physiological variability in their sizes making this alteration difficult to assess, especially when the difference in size between normal and neoplastic lobules is small. But it can be assessed by comparing a series of 10 to 20 normal lobules with the lobules suspected of having LN.
- Enlarged acini and variation in size: like the above, its assessment is carried out by comparing normal acini with suspected ones.
- Increased number of acini, some with unusual shapes: the altered acini might lose their round shape, and they might exhibit irregular or teardrop shapes.
- Lack of response of stroma to presence of neoplastic cells: this is a fundamental characteristic of lobular proliferations. Little changes in the stroma translates into irregular or no distinctive macroscopic lesion, which makes it difficult to detect with clinical explorations. Furthermore radiologically, if there are no differences in density of the benign and malignant tissue it is very difficult to be detected by screening tests, contributing to the low sensitivity of mammography to detect this BC type (see section 1.4.3).

1.4.6. Histopathology of ILC

The first description of ILC was made by Foote and Stewart (52), which has been accepted as the definition of the classic pattern of ILC. It is characterized by a proliferation of cells that infiltrate the fibrous stroma arranged in single linear cords that appear loosely dispersed throughout, lacking cellular cohesion (see figure 3). These cords are mostly composed of one or two cells across. Broader cords constitutes a distinctive growth pattern known as trabecular ILC (3,6,30,34).

Another characteristic of the ILC are its very varied growth patterns. Some of them feature a growth pattern in which the neoplastic cells are arranged around the ducts and lobules in a concentric way. This gives the tumour a microscopic targetoid appearance (see figure 3). Another growth pattern has also been reported in a small number of cases in which the linear cord pattern is not evident and tends to grow mainly in several dispersed focci of neoplastic cells invading the stroma. This pattern usually presents no discrete mass macroscopically, or no evidence of malignancy (3,6,30,34).

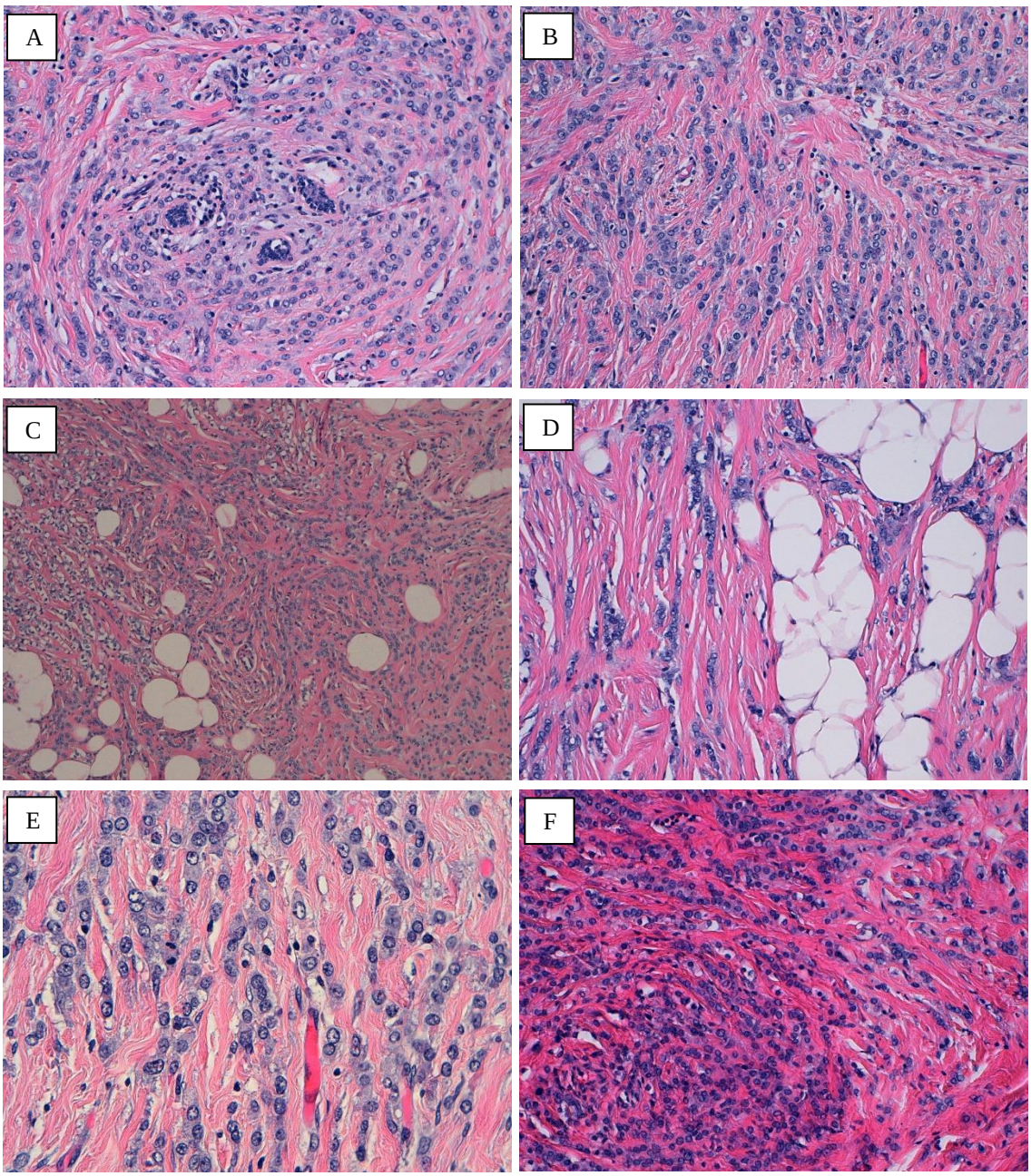


Figure 3: Invasive Lobular Carcinoma (ILC). **A** and **B** typical infiltrative pattern of ILC, exhibiting linear cords of neoplastic cells with little alteration of the surrounding stroma, A exhibits a targetoid appearance. **C** and **D** Classic ILC pattern, infiltrating fat. **E** and **F** linear cords of cells exhibiting cellular atypia. Images obtained from the pathology laboratory of Josep Trueta Hospital

The cellular characteristics of ILC are similar to their predecessor LN, where cells appear larger than normal epithelial cells. This augmented sized it is due to a larger cytoplasm and larger nuclei. Malignant cells have round or oval shapes and the nucleus tends to occupy the centre of the cell and can have a varied range of shapes. However, classic ILC is composed of malignant cells that tend to exhibit round nucleus.

The cytoplasm of malignant ILC cells is typically homogenous and stains faintly with eosin. Sometimes mucin inclusion in the cytoplasm can be observed, and such cells sometimes constitute the dominant population. When the inclusions if mucin coalesce, the lesion can express the appearance of signet ring configuration (3,6,30,34).

1.4.6.1. Variants of classic ILC

ILC exhibiting a different architecture to the classic type, described as an invasion of malignant cells arranged in single cell linear cords have been denominated variants of the classic form of ILC. The described variants are as follows (3,6,30,34):

- Solid pattern: in this pattern the cells are arranged in irregular shapes, solid nests, or in circumscribed round masses. No differences has been reported between the solid pattern and the classic ILC, according to a study carried by Fechner RE. (53), but the few number of cases with this phenotype makes it difficult to draw conclusions about their behaviour.
- Alveolar pattern: it is described as aggregates of at least 20 malignant cells arranged in globular shapes.
- Trabecular pattern: it is composed of linear cords of malignant cells, but unlike the classic type, they are composed of more than 3 cells in thickness.
- Tubulo-lobular: these tumours exhibit small tubules as well as cords of neoplastic cells in a lobular configuration, typical of ILC. A study published by E. fisher et al (54) concludes that these tumours represent a tubular variant of ILC, being better differentiated, exhibiting less necrosis and less cellular reaction, and less treatment failure compared to IDC. Compared to ILC, the tubule-lobular variant exhibits better differentiation, greater likelihood of contained intracytoplasmic mucin and a slightly lower rate of treatment failure.
- Pleomorphic lobular carcinoma (PLC): the PLC has the same growth pattern of the classic ILC, but the cells that compose it express a higher degree of cellular atypia, pleomorphism and a higher mitotic rate. These cells can show abundant eosinophilic cytoplasm, larger than the cytoplasm of classic ILC. The nucleus in some cases is hyperchromatic and eccentric, with a distinct plasmocitoid appearance, which is why they have been described

as myoid, histiocytoid and pleomorphic cells. It has often been reported associated with LCIS, which expresses the same degree of atypia and pleomorphism (6).

- Mixed groups: this category refers to ILC composed of a combination of different types of patterns. In about 5% of the cases of BC a mixture of IDC and ILC can be observed (31,32) (see section 1.3.3).

1.4.7. Prognostic and predictive factors

80 to 95% of ILCs are ER-positive (39,55), however, the rate of positivity is higher in the alveolar variant of ILC, as reported by Shousha S. et al (56). PR status is reported to be positive in 60-70% and HER2 overexpression occurs in 3-5% of classic ILC, but this percentage might vary with ILC morphological variants (57).

1.4.7.1. Histological grading in ILC

Histological grading in ILC as a prognostic factor has been a topic of controversy due to the fact that ILC exhibits no glandular formation, the neoplastic cells that compose them are uniform, and because of their low mitotic index, thus exhibiting lower grade than IDC in general.

Most classic ILC are going to be categorized as grade 2 (58) (see section 1.2.5), because of their histological properties, leaving grade 3 ILC tumours to be mostly non-classic tumour types.

However, as reported by Rakha EA et al.(58) histological grade assessed by the NPI (see section 1.2.4) provides a strong predictor of outcome in patients with ILC.

1.5. Clinicopathological differences between Invasive Ductal Carcinoma and invasive lobular carcinoma and therapeutic implications

For the purpose of this study, it is important to describe the differences found between ILC and IDC, as according to the findings of the anatomy unit in Trueta hospital seem to be an intermediate histological type between IDC and ILC. To our knowledge there is no evidence in the literature of such tumours, thus their behaviour has not been assessed. However, it is only logical to think that this morphologic variant will behave somewhere in between these two types of breast cancer. Nevertheless, if there is no clinical difference between IDC and ILC, perhaps it would reduce the clinical relevance of the study. In past years, the clinicopathological differences between ILC and IDC has been a topic of debate, with studies shown contradictory results. One of the reasons for this was the small number of cases analysed in such studies. In recent years, larger studies have shown increasing evidence of the differences between IDC and ILC as described below:

One of the hallmarks of ILC is its lack of expression of E-cadherin, with approximately 90% of ILCs failing to exhibit this adhesion protein. This property has become a robust aid for pathologists to recognize lobular carcinomas.

Patients with ILC tend to be older than patients presenting IDC. In a large study conducted by Arpino G. et al. (39), the median age of 4,140 women presenting ILC was 64.6 compared to 60.6 in 45,169 women presenting IDC.

Their findings also conclude that a higher proportion of ILC tumours tend to be larger than 2 cm, and 5 cm, compared to IDC, but this difference persists even on a stage match comparison, as reported by Wasif. N. et al. (40). ILC patients also tend to have more multifocal/multicentric tumours that are well-differentiated, according to Garcia Fernandez, A. et al. (59), with less LVI and less tumoral necrosis. ILC exhibit lower mitotic index than their IDC counterparts (39,59). The proportion of ER-positive tumours is higher for ILC, being 92.7% compared to 81.2% for IDC. PR-positivity was exhibited in 67.4% of ILC and 60.2% of IDC. HER2 was overexpressed in a higher proportion (25%) of IDC´s, compared to 11% of ILC´s as reported by Arpino G. et al (39), other authors have reported similar results (40,59,60).

The ALNS was reported to be similar in both IDC and ILC, according to Arpino G. et al. (39), despite the size of ILC being bigger than IDC. However, when doing a stage match comparison between IDC and ILC, ILC have a higher mean number of positive nodes, as reported by Wasif N. et al. (40) and Garcia Fernandez, A. et al. (59). Contralateral breast tumours are reported to be more frequent among women presenting ILC (39,59).

The metastatic patterns of both BCs also differ significantly. ILC is more likely to infiltrate the peritoneum, ovary and gastrointestinal system, and IDC is more likely to infiltrate lungs, pleura and central nervous system (39).

Regarding the difference in treatment, women presenting ILC were treated more frequently with mastectomy, compared to women presenting IDC (39,59). This could be due to the difficulties in the diagnosis and the determination of the margin in lobular carcinoma, because of its growth pattern, making it difficult for surgeons to resect the tumour with free margins, and for radiologists and pathologists to identify the margins of the lesions. Furthermore, women with IDC derive greater benefit from chemotherapy than those with presenting with ILC (57). Considering breast conserving surgery ILC has been reported to require more frequent re-excision and/or mastectomy because of positive resection margins (60).

Despite all the good predictor factors present in ILC such as lower mitotic index, lower grade, higher ER and PR expression, the overall survival for patients presenting ILC was not significantly different from those presenting with IDC, as reported by Arpino G. et al. (39).

However, when doing a staged match comparison, ILC was associated with a better 5-year disease specific survival. When we look at disease-free survival, Arpino G. et al. (39) found a difference favouring women presenting ILC in the first 5 years. But this difference disappears when a longer period of time is taken into account.

It is clear that IDC and ILC are different entities with different biological phenotypes and different clinical courses. However, the overall survival seems to be similar for these two types of BC.

2. JUSTIFICATION

Invasive Ductal Carcinoma (IDC) it is the largest group of malignant mammary tumours. It is a heterogeneous entity with no clear definition, with a wide range of prognostic outcomes; some women have the same life expectancy as women without breast cancer, other women have only a 13% probability of being alive in five years (6,61,62). There is a need to reassess tumours classified as Invasive Ductal Carcinomas in an effort to elaborate more accurate subclassifications of this heterogeneous entity.

In the pathology unit of Dr. Josep Trueta Hospital of Girona, a possible morphologic variant of Invasive Ductal Carcinoma has been recognized, which it is going to be referred as Invasive Ductal Carcinoma with Lobular Pattern (IDCLp) in this document. A consistent number of cases exhibiting the classic characteristics of Invasive Ductal Carcinoma, have been found to express a growth pattern in single linear cords around a classic lesion of invasive ductal carcinoma. This pattern of growth is characteristic of the Invasive Lobular Carcinoma (ILC), yet it lacks the necessary characteristic to make its diagnosis, or even to be categorized as an Invasive Breast Cancer (IBC) with mixed features mainly because it lacks the discohesive property of the lobular proliferations, expresses positive E-cadherin by immunohistochemistry (IHC) and the cellular appearance is concordant with that of IDC tumours.

To our knowledge there is no description of this tumours in the literature. They have not been recognized, and may have been misclassified as Invasive Ductal Carcinoma.

Invasive Ductal Carcinoma with Lobular Pattern (IDCLp) tumours expressing this differentiated growth pattern could potentially behave in a very different way than classic Invasive Ductal Carcinoma, as histological morphology frequently correlates with clinical and biological behaviour. Although there is no evidence about their behaviour it is only logical to think that these tumours will behave somewhere in between the ductal and lobular proliferations.

Such a concept of an intermediate breast cancer type between Invasive Ductal Carcinoma and Invasive Lobular Carcinoma is not new in the literature. There is growing evidence that, although Invasive Ductal Carcinoma and Invasive Lobular Carcinoma are distinct histological types of Breast Cancer (BC); they share a common pathogenic path, which at some point breaks, thus differentiating into the different types (50). But perhaps it is not unreasonable to assume that there could be the circumstances in which an intermediate form can be consolidated.

Nevertheless, if there is no clinical difference between Invasive Ductal Carcinoma and Invasive Lobular Carcinoma, perhaps it would take away clinical relevance from the topic of this study. However, Invasive Lobular Carcinomas have demonstrated to have a different metastatic pattern,

present a bigger size at diagnosis, present more multifocal/multicentric disease, require more mastectomies and reinterventions to widening surgical margins, and an overall different response to different pharmacological treatments, including, chemotherapy, endocrine treatments and HER2 specific therapies.

The biggest limitation of this study is the impossibility to select a sample number, since the number of diagnosed Invasive Ductal Carcinoma with Lobular Pattern cases is unknown. Yet it is a simple study to carry out, it is relatively cheap, and preliminary results can be obtained for further analysis.

The relevance of this study relies on the great epidemiologic impact of Invasive Ductal Carcinoma, and could determine a significant population of women presenting Invasive Ductal Carcinoma being treated in a more effective way with no additional cost to the healthcare system, with the simple recognition of Invasive Ductal Carcinoma with Lobular Pattern by trained pathologists.

3. HYPHOTESIS

The aim of this study is to assess the clinicopathological characteristics and survival of Invasive Ductal Carcinoma with Lobular Pattern (IDCLp) compared with classic Invasive Ductal Carcinoma (IDC) and classic Invasive Lobular Carcinoma (ILC), utilizing information from the Cancer Registry of Girona (RCG).

4. OBJECTIVES

4.1. Primary objective

To assess the clinicopathological characteristics of Invasive Ductal Carcinoma with Lobular Pattern (IDCLp) regarding, age of presentation, nationality, menopausal status, family history, primary tumour size, histological tumour type, histological grade, lymph node status, bilateralism, estrogenic and progesterone receptors, HER2 overexpression, staging and treatment procedures, disease free survival and survival time, and determine whether these characteristics significantly differ from the classic types of Invasive Ductal Carcinoma (IDC) and Invasive Lobular Carcinoma (ILC).

4.2. Secondary objectives

- To assess the clinicopathological differences between Invasive Ductal Carcinoma (IDC) and Invasive Lobular Carcinoma (ILC), regarding, age of presentation, nationality, menopausal status, family history, primary tumour size, histological tumour type, histological grade, lymph node status, bilateralism, estrogenic and progesterone receptors, HER2 overexpression, staging and treatment procedures, disease free survival and survival time.

5. METHODS

5.1. Study design

This is a retrospective, observational, analytic study. Information will be reviewed and collected from the Cancer Registry of Girona database which contains epidemiologic information of all Breast Cancer cases of the province of Girona since 1994. Three cohorts will be analysed retrospectively, between 1994 and 2016, regarding their clinicopathological characteristics: 1. Invasive Ductal Carcinoma with Lobular Pattern cases (IDCLp), 2. Invasive Ductal Carcinoma (IDC), 3. Invasive Lobular Carcinoma (ILC).

5.2. Study population

The population of the study are women diagnosed with operable BC, between 1994 and 2016, and registered in the Cancer Registry of Girona (RCG).

The RCG maintains a database of breast cancer patients treated in several institutions belonging to the province of Girona since 1994 until today. This database contains information about patient demographics (age, sex and nationality, family history), primary tumour characteristics (size, extent, grade, hormone receptors status, bilaterality), nodal staging (number of nodes examined, number of nodes involved), primary surgical treatment performed (lumpectomy vs mastectomy), lymph node staging (sentinel ganglion dissection vs axillary lymph node dissection), vital status, and survival. Residual biologic specimens utilized for the diagnostic process of these patients are stored and saved in the Biobank.

There is not sampling in this study as it is going to be a retrospective population analysis. From this database, we are going to select 3 study groups:

- **IDC group:** from the total number of cases diagnosed as IDC, we are going to select all those that fulfil the selection criteria:
    - Inclusion criteria
        - all patients diagnosed with the code M8500/3 corresponding to the International classification of disease for oncology, third edition, code for IDC, between 1994 and 2016 in the RCG. All patients selected are going to be classified into stages according to the AJCC 7th edition (14). (see Annex 1)
    - Exclusion criteria
        - Patients whose extent of disease or AJCC staging corresponds to distant metastases

        - Patients diagnosed with in situ cancer
        - Male patients with BC
        - Cases with reported mixed histology
        - Cases with no histological confirmation of the diagnosis
        - Cases identified from autopsy reports only
        - Patients that did not undergo surgical treatment
        - Diagnosis of IDC with lobular pattern
        - Cases with no registered information about ER status, PR status or HER2 status

- **ILC group:** from the total number of diagnosed ILC cases, we are going to select all those that fulfil the selection criteria:
    - Inclusion criteria
        - all patients diagnosed with the code M8520/3 corresponding to the International classification of disease for oncology, third edition, code for ILC, between 1994 and 2016 in the RCG database. All patients selected are going to be classified into stages according to the AJCC 7th edition (14) (see Annex 1).
    - Exclusion criteria
        - Patients whose extent of disease or AJCC staging corresponds to distant metastases
        - Patients diagnosed with in situ cancer, or lobular neoplasia
        - Male patients with BC
        - Cases with reported mixed histology
        - Diagnosis of rare ILC morphologic variants: pleomorphic, tubulolobular.
        - Cases with no histological confirmation of the diagnosis
        - Cases identified from autopsy reports only
        - Patients that did not undergo surgical treatment
        - Cases with no registered information about ER status, PR status or HER2 status
- **IDC with lobular pattern group:** a revision of all patients that fulfil the criteria for IDC group are going to be reviewed by expert pathologists, looking for histological characteristics described in section 1.1 of this document. Cases that fulfil the criteria to be diagnosed as IDCLp, are going to compose this group.

5.3. Variables

5.3.1. Independent variables

The independent variable in this study corresponds to the histological type of BC tumours. In this study, we are going to assess three histological types: ILC, IDC and IDCLp.

- IDC cases are going to be selected by previous diagnosed tumours from the RCG, coded M8500/3 corresponding to the International classification of disease for oncology, third edition.
- ILC cases are going to be selected by previous diagnosed tumours from the RCG, coded M8520/3 corresponding to the International classification of disease for oncology, third edition.
- IDCLp cases are going to be selected from a review of the IDC cases that fulfil selection criteria, and respectively re-diagnosed according to the histological criteria described in section 1.1 of this document.

  To avoid misclassification of this category, A session of diagnostic training will be carried out before the revision of the IDC cases. This session will consist of a theoretical session of histological criteria for diagnosing IDCLp, and clinical exercises in which the participants would practice their ability to recognize IDCLp.

  Later an assessment of the IDC group is going to be realized twice for each case, by two different pathologist and results will be compared. The unconcordant results will be then resolved with the aid of a third expert opinion, or excluded from the study if no agreement can be reached.

For the statistical analysis, a qualitative nominal variable is going to be considered.

5.3.2. Dependent variables

In this study the dependent variable is the clinicopathological characteristic of the different BC histological types measured by:

5.3.2.1. Age of diagnosis

The information about age of diagnosis is going to be obtained from the RCG. It is going to be considered a continues quantitate variable for the statistical analysis.

5.3.2.2. Race, ethnicity and nationality

We do not dispone of information of race and ethnicity in the RCG. However, in an effort to minimize all possible variables that could influence our results, we are going to measure nationality since this variable is available in the RCG. Therefore, nationality will be considered a qualitative nominal variable for the statistical analysis.

5.3.2.3. Family history of BC

Information about family history of BC will be obtained from the RCG. We will classify patients of this study into:

- Cases with a 1st degree relative with BC
- Cases without a 1st degree relative with BC

For statistical analysis, this variable will be considered as a nominal dichotomous qualitative.

5.3.2.4. Menopausal status

Information about the menopausal status will be obtained from RCG. According to clinical records, the cases of the study will be classified as: Premenopausal and Postmenopausal

Postmenopausal women will be considered when, at the time of diagnosis, 12 months have passed after the patient's last menstrual period.

For the statistical analysis, this will be considered a qualitative dichotomous nominal variable.

5.3.2.5. BC Staging

All the cases of this study are going to be staged according to the 7th edition of Breast Cancer Staging by the American Joint Committee on Cancer (14) (see table 1 and annex 1), which comprises five stages and nine categories in total. Information will be obtained from the clinical records of RCG.

The BC staging will be considered a qualitative ordinal variable for statistical analysis.

Table 1: Classification of Breast Cancer in to prognostic Stages. Extracted from (14)

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N1**	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Notes

* T1 includes T1mi

** T0 and T1 tumours with nodal micrometastases only are excluded from stage IIA and are classified stage IB.

- M0 includes M0(i+)
- The designation pM0 is not valid; any M0 should be clinical
- If a patient presents with M1 prior to neoadjuvant systemic therapy, the stage is considered Stage IV and remain Stage IV regardless of response to neoadjuvant therapy.
- Stage designation may be changed if postsurgical imaging studies reveal the presence of distant metastases, provided that the studies are carried out within four months of diagnosis in the absence of disease progression and provided that the patient has not received neoadjuvant therapy.
- Postneoadjuvant therapy is designated with "yc" or "yp" prefix. Of note, no stage group is assigned if there is a complete pathologic response (CR) to neoadjuvant therapy, for example, ypT0ypN0Cm0.

For more information about the meaning of each of the categories T, N and M, consult Annex 1.

5.3.2.6. Size of primary tumour (T)

Information about the size of the primary tumour at diagnosis is going to be obtained from the RCG. Length in the greatest dimension of the BC lesion at diagnosis is going to be considered. For statistical analysis, this is going to be considered a continuous quantitative variable.

5.3.2.7. Regional lymph node status

Information about the regional lymph node status is going to be obtained from the RCG. It is going to be summarized in proportion of the cases of the corresponding histological types that are classified as: pNx, pN0, pN1, pN1a, pN1b, pN1c, pN2, pN2a, pN2b, pN3, pN3a, pN3b, pN3c, according to the 7th edition of the American Joint Committee on Cancer, Breast Cancer Staging (14), see Annex 1.

For the statistical analysis, this is going to be considered a qualitative nominal variable.

5.3.2.8. Bilateral breast cancer disease

Information about bilateral BC involvement will be obtained from the RCG and is going to be classified as positive or negative. For statistical analysis, this is going to be considered as a dichotomous nominal qualitative variable.

5.3.2.9. ER expression

Information about ER is going to be obtain from the RCG. ER information is going to be recorded using the denominated Allred Score described in section 1.2.8.1 of this document. ER positivity is going to be considered any score from 2 to 8 on the Allred Score, corresponding to ≥ 1% of tumour cell nuclei being inmunoreactive.

Cases of this study will be classified according to their ER status in to ER-positive and ER-negative. For the statistical analysis, we will consider this variable a qualitative dichotomous nominal variable.

5.3.2.10. PR expression

Very like ER, PR information is going to be obtained from the RCG. The Allred Score described in section 1.2.8.1 of this document is going to be used to summarize this variable. PR positivity is going to be considered scores ≥ 2, corresponding to PR expression in ≥ 1% of the cells in tissue sample.

Cases of this study will be classified according to their PR status in to PR-positive and PR-negative. For the statistical analysis, we will consider this as a qualitative dichotomous nominal variable.

5.3.2.11. ER/PR phenotype

The phenotype of BC is going to be determined by the results of ER expression and PR expression and classified in to four categories for further analysis:

- ER-positive/PR-positive
- ER-Positive/PR-negative
- ER-negative/PR-negative
- ER-negative/PR-negative

This is going to be considered a qualitative nominal variable.

5.3.2.12. HER2 expression

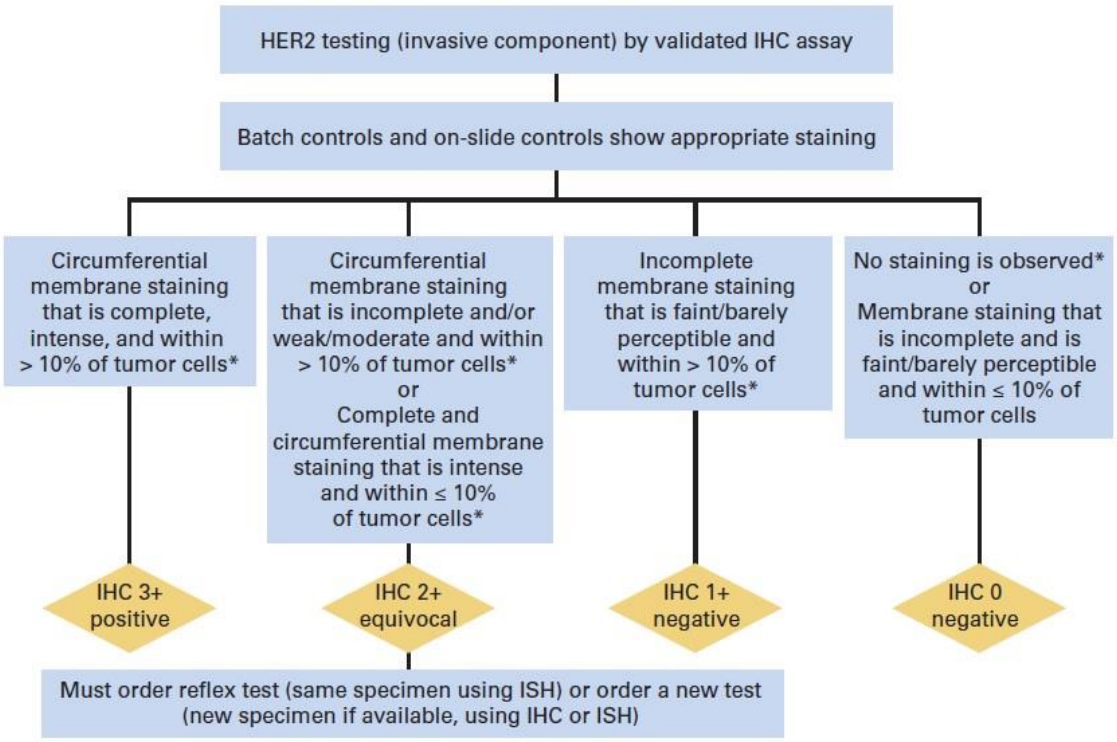


Figure 4: Algorithm for evaluation of HER2 protein expression by immunohistochemistry (IHC) in a breast cancer specimen. Extracted from (29). The final result assumes that there is no apparent histopathologic discordance observe by the pathologist.

Information about HER2 status will be obtained from RCG. HER2 positivity will be considered on the basis of one or more HER2 test results. The criteria that defines HER2 positivity, when observing within an area of tumour that amounts to > 10% of contiguous and homogenous tumour cells, there is evidence of protein overexpression (IHC) or gene amplification (HER2 copy number or HER2/CEPT17 ratio by In Situ Hybridization (ISH) based on counting at least 20 cells within the area) using a single prove ISH or dual prove ISH. The criteria is based on the ASCO and CAP recommendations for HER2 testing in BC (29) as can be summarized in the algorithms seen in figures 4, 5 and 6.

If results are equivocal, as described in figure 4, 5 and 6, another test should be performed using alternative assay IHC or ISH (single or dual prove as explained in Figure 5 and 6).

If results seem discordant with other histopathologic findings, repeated testing should be considered. For more information about the criteria to consider discordant pathologic findings consult Annex 6.

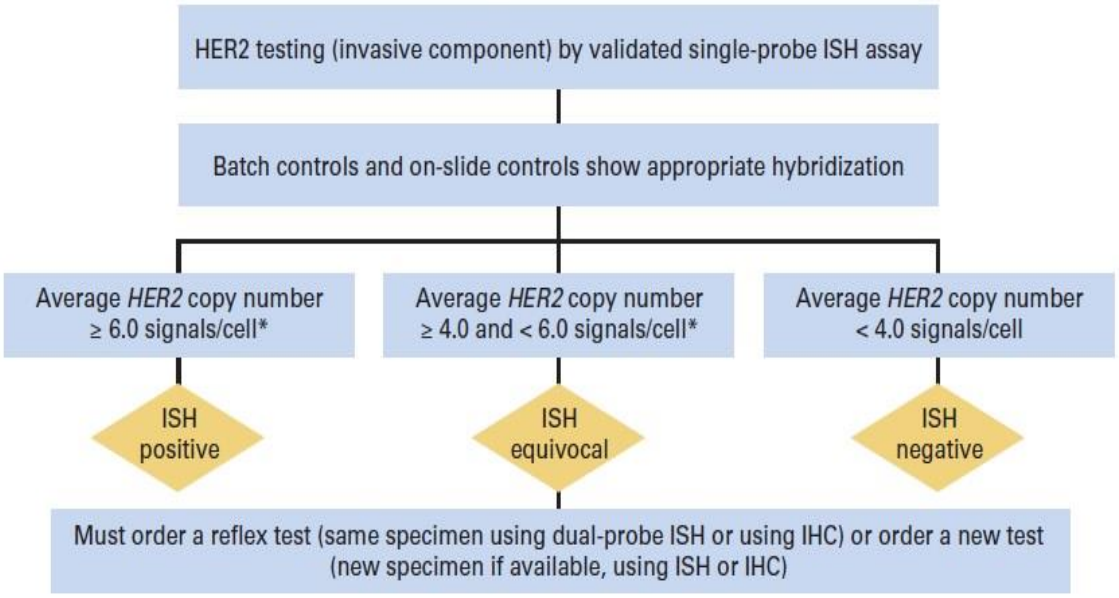


Figure 5: Algorithm for evaluation of HER2 gene amplification by in situ hybridization (ISH) assay in a breast cancer specimen using a single-signal (HER2 gene) assay. Amplification in a single -probe ISH assay is defined by examining the average HER2 copy number. The final result assumes that there is no apparent histopathologic discordance observe by the pathologist. Extracted from (29).

Negative expression of HER2 will be considered if a single test (or all) performed in a BC tumour specimen show negative IHC or/and negative ISH using a single or dual prove.

A HER2 test result will be considered indeterminate if technical issues prevent one or both test performed in a BC specimen from being reported as positive, negative, or equivocal. Another specimen should be requested for testing if available.

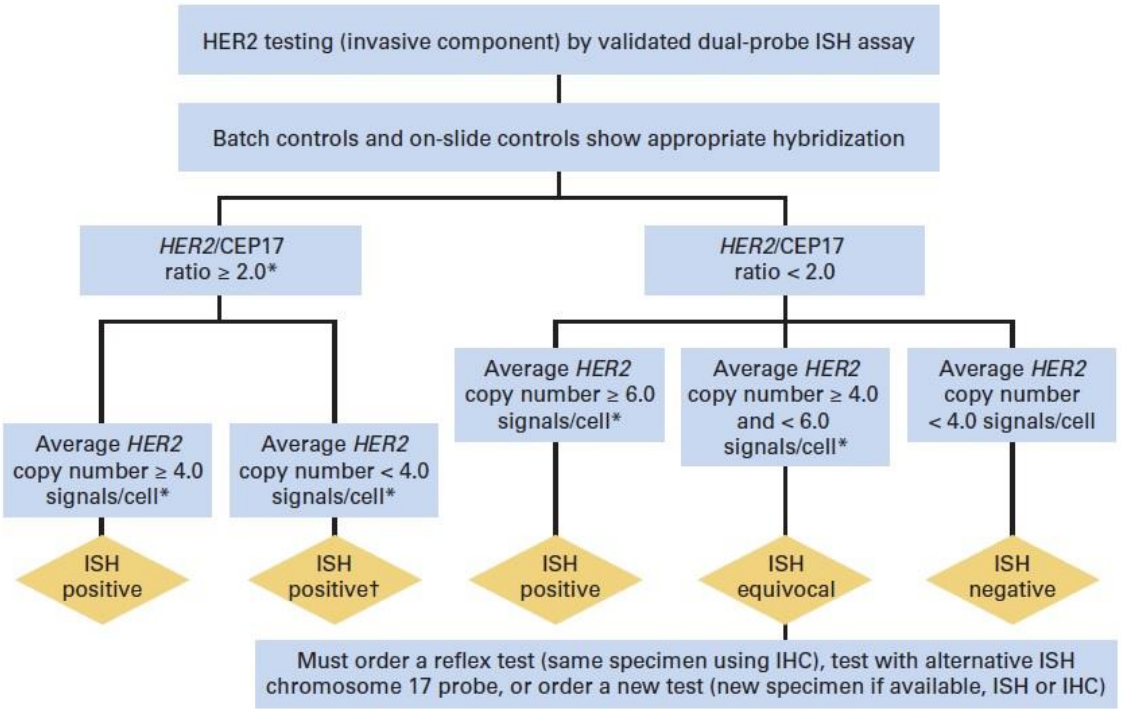


Figure 6: Algorithm for evaluation of HER2 gene amplification by in situ hybridization (ISH) assay of a breast cancer specimen. Amplification in a dual-probe ISH assay is defined by examining first the HER/CEP17 ratio followed by the average HER2 copy number. The final result assumes that there is no apparent histopathologic discordance observe by the pathologist. Extracted from (29)

For more information about the preanalytical process and analytic process the summary of 2007 and 2013 HER2 test guidelines published by the ASCO and CAP will be attached to this document in Annex 5.

For the statistical analysis, only the categories HER2-positive and HER2-negative are going to be considered and undetermined or equivocal results will be excluded from this study. Thus, we will consider this variable a qualitative dichotomous nominal variable.

5.3.2.13. Histological grade

Information about histological grade is going to be obtained from RCG. Histological grade is going to be summarized using the Nottingham combined histological grade which classifies BCs tumours in to three categories: well differentiated, moderately differentiated and poorly differentiated, assessing 3 characteristics: glandular formation, cellular atypia, mitotic count (see Annex 2-3).

For statistical analysis, this will be considered as an ordinal qualitative variable.

5.3.2.14. Surgical treatment

Information about the surgical procedure performed for treatment and/or diagnosis will be obtained from the RCG. There are two surgical procedures in BC: lumpectomy and mastectomy. Cases of this study will be classified into four categories according to the procedure performed:

- Lumpectomy
- Mastectomy
- Lumpectomy plus reoperation to widening margins
- Lumpectomy with a later mastectomy

For the statistical analysis, this will be considered as a nominal qualitative variable.

5.3.2.15. Lymph node staging surgical procedure

Information about the surgical procedure performed to treat and/or diagnose the patients of this study will be obtained from the RCG. There are two surgical procedures for lymph node staging: SGB or ALND. Cases of this study will be classified in to these two categories. For the statistical analysis, this will be considered as a dichotomous nominal qualitative variable.

5.3.2.16. Overall Specific Survival

Overall survival (OS) was defined as the interval of time between the diagnostic biopsy and death from BC specific cause. Death will be scored as an event, and patients who are still alive will be censored at the time of last follow-up.

Information about the vital state will be obtained from the *Índice Nacional de Defunción*, and patients who do not appear in this source will be considered alive. The cause of death will be obtained from the *Registre de Mortalitat de Catalunya*.

For the statistical analysis, it is going to be consider as a continues quantitative variable.

5.3.2.17. Disease-free survival

Disease-free survival (DFS) is defined as the interval of time from the date of first diagnostic biopsy, with first recurrences, local or distant, being scored as an event, with censoring of patients at the time of last follow-up or death.

Local recurrence is defined as tumour arising in the treated breast, chest wall or axilla.

Information about recurrences will be obtained from RCG, and information about Vital state of the cases will be obtained from the *Índice Nacional de Defunción*, and patients who do not appear in this source will be considered alive.

For statistical analysis, it is going to be considered a continuous quantitate variable.

5.4. Data Collection

To record all the necessary information for this study, a computer-based filling form will be created for all investigators to review the information in the RCG. A computer-based database will be created using Microsoft Access to keep all the information acquired during the process of this study.

A reliability test of the data collection process will be carried out to ensure the quality of the data collection process, regarding the content of the filling form to introduce data, the stability of the database, and the design of protocols to translate the clinical registries into measurable variables. In this test, we will use data that will not enter the study. The test will have a duration of fourteen days.

Personal and clinical information that we will have access to will be treated according to the "*Ley Orgánica 15/1999, de 13 de Diciembre, de Protección de Datos de Carácter Personal"* and the "*Royal Decret 994/1999*" the regulates security measures for automated files containing personal data.

5.5. Statistical analysis

For the statistical analysis, **the primary question** that will be used is: ¿is IDCLp a different entity regarding, age of presentation, nationality, menopausal status, family history, primary tumour size, histological tumour type, histological grade, lymph node status, bilateralism, estrogenic and progesterone receptors, HER2 overexpression, staging and treatment procedures, disease free survival and survival time, compared to classic IDC and ILC?

The **null hypothesis** for a hypothesis contrast is: there is no difference between IDCLp compared to IDC and ILC regarding, age of presentation, nationality, menopausal status, family history, primary tumour size, histological tumour type, histological grade, lymph node status, bilateralism, estrogenic and progesterone receptors, HER2 overexpression, staging and treatment procedures, disease free survival and survival time.

The level of significance it will considered a P value < 0.05. on both sides (bilateral analysis).

In the Univariate analysis, the different characteristic of the different histological types of BC cases will be determined for normal distribution with probability plots and Kolmogorov-Smirnov test statistics. Categorical variables are going to be expressed in absolute and relative frequencies. Quantitative variables are going to be expressed by mean ± Standard Deviation (SD) if normal distribution is found. If normal distribution is not found they will be expressed by median and percentiles.

In bivariate analysis, Dichotomous and non-dichotomous categorical variables will be compared in a contingency table and asses' associations with $\chi 2$ test.

Association of continuous quantitative variables normally distributed data with categorical variables will be assessed with an Analysis of variance, or Kruskal-Wallis test for not normally distributed continuous quantitative variables.

we are going to perform 3 group comparisons regarding OS and DFS variable:

- IDCLp vs IDC
- IDCLp vs ILC
- IDC vs ILC.

OS and DFS curves are going to be drawn using Kaplan–Meier estimates, and are going to be compared using log rank tests.

Multivariate analyses of DFS and OS, will be performed using Cox proportional hazard regression models. Variables that are going to be included in this test will be: age, nationality, bilateral BC disease, ER status, PR status, HER2 status, tumour grade, lymph node status, T stage, histology type of BC.

6. Limitations of the study

The biggest limitation of this study is the fact that the number of cases diagnosed with IDCLp is unknown; therefore, there is an implicit risk that, if the number of cases with such diagnosis is small, it would be very difficult to obtain reliable conclusions that could be applied to the general population.

Another limitation is a possible selection bias, since all cases re-diagnosed with IDCLp will be included in the study to maximize the sample. Thus, the sample may not represent the general population of women in Girona, and there could be over- or miss-represented populations. To control this factor, all variables that could interfere in the selection process like age, nationality and menopausal status are going to be measured for a later adjustment of results.

Another disadvantage is that the cases of BC included in this study have been diagnosed and treated in very different periods of time, and using different procedures according to what was considered the best therapeutic approach at the time. There can also be differences in surgical procedures due to the different strategies that physicians have implemented as treatment for breast cancer.

Information about the latest discovered therapeutically options may be missing, including ER expression, PR expression, and HER2 status. Such cases were decided to be excluded from the study since, although many of them have saved biological specimens in the Biobank, which could be accessed and the necessary tests could be performed, doing so would dramatically increase the budget of this study.

As this is a retrospective descriptive study, confounding variables strongly influence conclusions. To avoid this influence, we are going to determine what variables could be influencing our results, based on other prognosis studies, measure them, and then later adjust the result with a multivariate analysis.

Despite the drawbacks of the study, it is important to highlight that it is a pioneer study assessing the clinicopathological characteristics of IDC presenting with lobular pattern, it is also a simple study to make, it is relatively cheap, and preliminary results can be obtained for further analysis.

Furthermore, the impact of these findings could help to better understand the wide range of tumours that are comprised inside the category of IDC, which are the most common cause of BC and have a huge impact on the health women worldwide.

7. Clinical relevance of this study

The clinical relevance of this project relies on the great epidemiological impact of Invasive Breast Cancer; it is the first most frequent cancer among women and the first cause of death for women from cancer worldwide.

From all Breast Cancer, Invasive Ductal Carcinoma (IDC) accounts for up to 75% of cases, but it still is a heterogeneous entity with no clear definition, and its diagnosis is made by the exclusion of other special types of Breast Cancer. Women presenting with Breast Cancer also have a very wide range of outcomes, as stated by Singletary, Eva et al (62) in their revision of the American Joint Committee on Cancer Staging System for Breast Cancer, but also Rosen, Paul Peter in his book breast pathology (6) states when describing IDC that, there is a need to reassess tumours classified as Invasive Ductal Carcinomas (IDC) in an effort to elaborate more accurate subclassifications of this entity, thus providing a better understanding, and consequently a better treatment for these patients.

The simplest way to understand the behaviour of a cellular tissue is usually by analysing their morphology, as morphology and architecture correlates with the clinicopathological behaviour of neoplastic tumours. With this study, we could potentially recognize a morphological variant of IDC.

The recognition of a new morphological variant implies the need for further investigation into the ethiopatogeny of such lesions and their clinicopathological characteristics with larger studies, thus helping us to better understand these tumours and, more importantly, to plan better therapeutic strategies to fight the disease or even discover new ones to treat patients affected with breast cancer.

The ultimate consequences of the recognition of this morphological variant could impact thousands of women and even men around the world, who would receive a better treatment for their disease and a better quality of life.

8. Ethical aspects

This study was designed in accordance to the ethical principles for medical research established by the World Medical Association in the Declaration of Helsinki of Ethical Principles for Medical Research Involving Human Subjects elaborated in 1964, and last reviewed in May of 2015 (63).

The elaboration of this protocol was made in accordance with the recommendations of "*Libro Blanco de la Anatomía Patológica en España*" (64).

Both institutions that we will acquire information for our study, the Josep Trueta Hospital and the Cancer Registry of Girona, have recognized reputation on the reliability and authenticity of their data, which is acquired in complete accordance with the principles of medical research mentioned above, and respecting in all its limits the four pillar principles of Bioethics.

All confidential information that we will have access to accomplish the goals of this study will be treated in accordance with the "*Ley Orgánica 15/1999, de 13 de Diciembre, de Protección de Datos de Carácter Personal"*, the "*Royal Decret 994/1999*" the regulates security measures for automated files containing personal data.

8.1. Work plan and timeline

- **1st stage: Preparation and coordination (2 months)**
  - Objective 1: Sending proposal protocol to the Ethics Committee for its approval.
  - Objective 2: Hypothesis and objectives approach, meeting of the research team: 2 pathologists (Main investigator: Cristina Melendez, Collaborator Pathologist, Medicine student, and statistical consultant) to clarify inclusion and exclusion criteria for data collection, and characteristics of the variables.
  - Objective 3: meeting of the research team to create a data collection sheet, and designing of the database.
  - Objective 4: Meeting in RCG to introduce the research team to the working protocols of RCG.
- **2nd stage: Data collection (15 months)**
  - Objective 1: training session carried out by the main investigator for the diagnosis of IDCLp
  - Objective 2: Selection of cases of IDC and ILC that grossly fulfil the inclusion and exclusion criteria in RCG.
  - Objective 3: reliability of the collection data protocol test. (14 days)
  - Objective 4: detailed review of each IDC case twice, separately, one by the main investigator and the other by the collaborating pathologist, filling the collection data sheet, and making the diagnosis of IDCLp, if pertinent. ILC cases will be reviewed and data collection sheet will be filled by the medicine student.
  - Objective 5: selecting incongruent diagnosis of IDCLp between the two expert pathologists (main investigator and collaborator histopathologist), and resolving the issues with the aid of a third consultant pathology expert.
- **3rd stage: Statistical analysis and interpretation of results (2 months)**
  - Objective 1: results will be analysed, and statistical analysis will be performed with the help of a consultant statistician.
  - Objective 2: Discussion of results will be held, determining the conclusions
- **4th stage: publication and dissemination of results (1 month)**
  - Objective 1: preliminary writing of scientific article, presenting the results
  - Objective 2: participation in congresses on Breast Pathology and divulgation activities
  - Objective 3: publication of article in national or international journal

CRONOGRAM		2017												2018					
		Jan	Feb	Mar	Apr	May	Jun	Jul	Ago	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
1st stage	Ethical evaluation	A/C																	
	Hypothesis and objectives approach	T																	
	Creation of database and data collection sheets		A/B/C																
	Meeting in RCG to get to know working protocols		A/B/C																
2nd stage	Training session for diagnosis of IDCLp			A/B/C															
	Gross selection of IDC/ILC cases			A/B/C															
	reliability of the collection data protocol test (14 days)				A/B C														
	Detailed review of IDC/ILC cases + diagnosis of IDCLp					A/B/C													
	Selection of incongruent results of diagnosis of IDCLp and resolving with a third expert opinion															A/B/C/P			
3rd stage	Statistical analysis																A/B/C/E		
	Discussion of results + conclusions																	A/C	
4th stage	Preliminary writing of article																	A/C	
	participation in congresses of pathology and activities of diffusion																		A/B/C
	Publication on national or international Journals																		A/B/C

A Main investigator; **B** Collaborator pathologist; **C** Medical student; **E** Statistician consultant; **T** Whole team; **P** Pathology expert

9. Budget

The Josep Trueta Hospital and the Cancer Registry of Girona will be the physical places where this study is going to be carried out. The investigation team will not receive compensation for their work on this study.

Concept	Cost in Euros
1. Staff cost	**0,00**
2. Services	
• Data manager. 40€/hour * 20 hours	**800,00**
• Statistical consultant. 40€/hour * 40 hours	**1.600,00**
• Expert Pathologist. 50€/hour*40 hours	**2000,00**
• Necessary software: IBM software SPSS and Microsoft access	**200,00**
3. Diffusion activities	
• National conference attendance for three people, accommodation, travel, and inscription expenses	**1.400,00**
• Publication and divulgation expenses	**1.000,00**
Total amount claimed	**7.000,00**

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11. ANNEXES

11.1. Annex 1: Breast Cancer Staging

Primary Tumour (T)		
Tx	Primary tumour cannot be assessed	
T0	No evidence of primary tumour	
Tis	Carcinoma in situ	
	DCIS	Ductal carcinoma in situ
	LCIS	Lobular carcinoma in situ
	Paget's	Paget´s disease of the nipple not associated with invasive carcinoma or carcinoma in situ in the underlying breast parenchyma.
T1	Tumour ≤ 20mm in greatest dimension	
T1mi	Tumour ≤ 1mm in greatest dimension	
T1a	Tumour > 1mm but ≤ 5mm in greatest dimension	
T1b	Tumour > 5 mm but ≤ 10mm in greatest dimension	
T1c	Tumour > 10mm but ≤ 20mm in greatest dimension	
T2	Tumour > 20mm but ≤ 50mm in greatest dimension	
T3	Tumour >50 mm in greatest dimension	
T4	Tumour of any size with direct extension to the chest wall and/or to the skin (ulceration of skin nodules) **Note**: Invasion of the dermis alone does not qualify as T4	
T4a	Extension to the chest wall, not including only pectoralis muscle adherence/Invasion	
T4b	Ulceration and/or ipsilateral satellite nodules and/or edema (including *peau d'orange*) of the skin, which do not meet the criteria for inflammatory carcinoma	
T4c	Both T4a and T4b	
T4d	Inflammatory carcinoma	

Distant metastases (M)	
M0	No clinical or radiographic evidence of distant metastases
cM0(i+)	No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumour cells in circulating blood, bone marrow, or the nonregional nodal tissue that are no larger than 0.2 mm in a patient without symptoms or signs of metastases
M1	Distant detectable metastases as determined by classic clinical and radiographic means and/or histologically proven larger than 0.2mm

Extracted from AJCC. Cancer Staging Manual. 7th ed. Edge E, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. Springer; 2009. 648 p. (14)

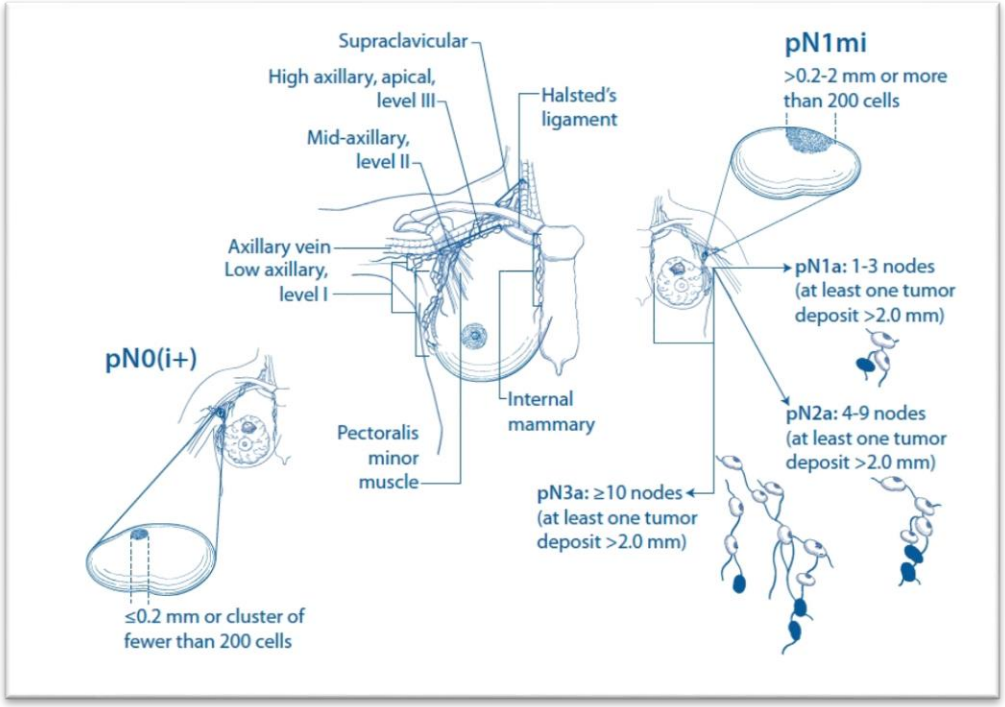
Regional Lymph nodes	
Clinical (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastases to movable ipsilateral level I, II axillary lymph node(s)
N2	Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected* ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastases
N2a	Metastases in ipsilateral level I, II axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastases only in clinically detected* ipsilateral internal mammary nodes and in the absence of clinically evident level I, II axillary lymph node metastases
N3	Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected* ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement.
N3a	Metastases in ipsilateral infraclavicular lymph node(s)
N3b	Metastases in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastases in ipsilateral supraclavicular lymph node(s)

Pathological (PN)*	
pNX	Regional Lymph nodes cannot be assessed (for example, previously removed, or not removed for pathologic study)
pN0	No regional lymph node metastases identified histologically Note: isolated tumour cells clusters (ITC) are defined as small clusters of cells not greater than 0.2mm, or single tumour cells, or a cluster of fewer than 200 cells in a single histological cross-section. ITC´s may be detected by routine histology or by immunohistochemically (IHC) methods. Nodes containing only ITC's are excluded from the total positive node count for purpose of N classification but should be included in the total number of nodes evaluated.
pN0(i-)	No regional lymph node metastases histologically, negative IHC
pN0(i+)	Malignant cells in regional lymph node(s) no greater than 0.2mm (detected by H&E or IHC including ITC)
pN0(mol-)	No regional lymph node metastases histologically, negative molecular findings (RT-PCR**)
pN0(mol+)	Positive molecular findings (RT-PCR), but no regional lymph node metastases detected by histology or IHC
pN1	Micrometastases; or metastases in 1-3 axillary lymph nodes; and/or in internal mammary nodes with metastases detected by sentinel lymph node biopsy but not clinically detected***
pN1mi	Micrometastases (greater than 0.2 mm and/or more than 200 cells, but non-greater than 2.0mm)
pN1a	Metastases in 1-3 axillary lymph nodes, at least one metastases greater than 2.0mm
pN1b	Metastases in internal mammary nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***
pN1c	Metastases in 1-3 axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected
pN2	Metastases in 4-9 axillary lymph nodes; or clinically detected**** internal mammary lymph nodes in the absence of axillary lymph node metastases
pN2a	Metastases in 4-9 axillary lymph nodes (at least one tumour deposit greater than 2.0mm)

Notes

∗ "clinically detected" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics highly suspicious for malignancy or a presumed pathologic macrometastases based on fine needle aspiration biopsy with cytologic examination.
Confirmation of clinically detected metastatic disease by fine needle aspiration without excision biopsy is designated with an (f) suffix, for example, cN3a(f). Excisional biopsy of a lymph node or biopsy of a sentinel node, in the absence of assignment of a pT, is classified as a clinical N, for example, cN1. Information regarding the confirmation of the nodal status will be designated in site-specific factors as clinical, fine needle aspiration, core biopsy, or sentinel lymph node biopsy. Pathologic classification (pN) is used for excision or sentinel lymph node biopsy. Pathologic classifications (pN) is used for excision or sentinel lymph node biopsy only in conjunction with a pathologic T assignment.

Extracted from AJCC. Cancer Staging Manual. 7th ed. Edge E, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. Springer; 2009. 648 p. (14)



Graphic explanation of different levels of axillary lymph nodes, and the pN0(i+), pN1mi, pN1a, pN2a and pN3a stages

pN2b	Metastases in clinically detected**** internal mammary lymph nodes in the absence of axillary lymph node metastases
pN3	Metastases in 10 or more axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or in clinically detected**** ipsilateral internal mammary lymph nodes in the presence of one or more positive level I, II axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***, or in ipsilateral supraclavicular lymph nodes
pN3a	Metastases in 10 or more axillary lymph nodes (at least one tumour deposit greater than 2.0mm); or metastases to the infraclavicular (level III axillary lymph) nodes
pN3b	Metastases in clinically detected**** ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with micrometastases or macrometastases detected by sentinel lymph node biopsy but not clinically detected***
pN3c	Metastases in ipsilateral supraclavicular lymph nodes

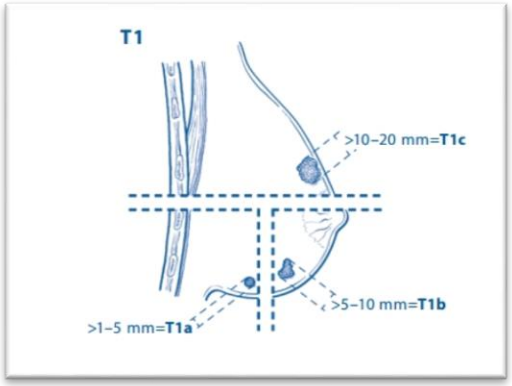
Notes

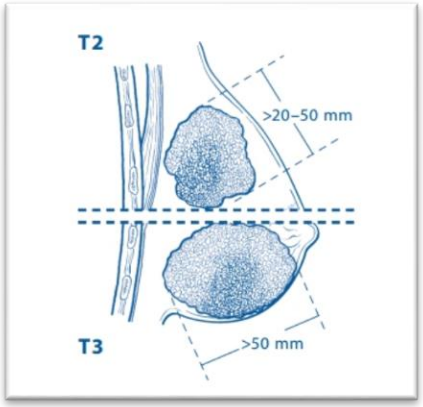
* Classification is based on axillary node dissection with or without sentinel lymph node biopsy. Classification based solely on sentinel lymph node biopsy without subsequent axillary lymph node dissection is designated (sn) for "sentinel node", for example, pN0(sn).

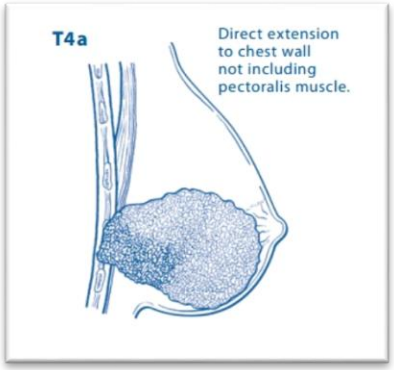
** RT-PCR: reverse transcriptase/polymerase chain reaction.

*** "not clinically detected" is defined as not detected by imaging studies (excluding lymphoscintigraphy) or not detected by clinical examination.

**** "clinically detected" is defined as detected by imaging studies (excluding lymphoscintigraphy) or by clinical examination and having characteristics that are highly suspicious for malignancy or presumed pathological macrometastases based on fine needle aspiration biopsy with cytologic examination.







Graphic representation of primary tumour (T) staging

ANATOMIC STAGE/PROGNOSTIC GROUPS			
Stage 0	Tis	N0	M0
Stage IA	T1*	N0	M0
Stage IB	T0	N1mi	M0
	T1*	N1mi	M0
Stage IIA	T0	N1**	M0
	T1*	N1**	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N1**	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Notes

* T1 includes T1mi

** T0 and T1 tumours with nodal micrometastases only are excluded from stage IIA and are classified stage IB.

- M0 includes M0(i+)
- The designation pM0 is not valid; any M0 should be clinical
- If a patient presents M1 prior to neoadjuvant systemic therapy, the stage is considered Stage IV and remain Stage IV regardless of response to neoadjuvant therapy.
- Stage designation may be changed if postsurgical imaging studies reveal the presence of distant metastases, provided that the studies are carried out within four months of diagnosis in the absence of disease progression and that the patient has not received neoadjuvant therapy.
- Postneoadjuvant therapy is designated with “yc” or “yp” prefix. Of note, no stage group is assigned if there is a complete pathologic response (CR) to neoadjuvant therapy, for example, ypT0ypN0Cm0.

Extracted from AJCC. Cancer Staging Manual. 7th ed. Edge E, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. Springer; 2009. 648 p. (14)

11.2. Annex 2: Assessment of grade in Breast Cancer

Semi-quantitative method for assessing histological grade in breast tumours. Extracted from (11) *ELSTON CW, ELLIS IO. pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long???term follow???up. Histopathology [Internet]. 1991 Nov [cited 2016 Dec 23];19(5):403–10. Available from: http://doi.wiley.com/10.1111/j.1365-2559.1991.tb00229.x*

Feature	Score
Tubule and gland formation	
• Majority of the tumour (>75%)	1
• Moderate degree (10-75%)	2
• Little or none (<10%)	3
Nuclear pleomorphism	
• Small, regular uniform cells	1
• Moderate increase in size and variability	2
• Marked variation	3
Mitotic counts	
• Dependent on microscope field area (see Annex 3)	1-3
Final grading	
Add the 3 corresponding scores:	Total Score
• Grade 1	3-5
• Grade 2	6 or 7
• Grade 3	8 or 9

11.3. Annex 3: Assessment of mitotic count

Assessment of mitotic count. Extracted from (3) *Lakhani S., Ellis IO, Schinitt SJ, Tan PH, M.J. V de V, editors. WHO clasification of tumours of the breast. Lyon: IARC; 2012.*

Field diameter (mm)	Mitotic count (score)		
	1	2	3
0,40	≤ 4	5-9	≥ 10
0,41	≤ 4	5-9	≥ 10
0,42	≤ 5	6-10	≥ 11
0,43	≤ 5	6-10	≥ 11
0,44	≤ 5	6-11	≥ 12
0,45	≤ 5	6-11	≥ 12
0,46	≤ 6	7-12	≥ 13
0,47	≤ 6	7-12	≥ 13
0,48	≤ 6	7-13	≥ 14
0,49	≤ 6	7-13	≥ 14
0,50	≤ 7	8-14	≥ 15
0,51	≤ 7	8-14	≥ 15
0,52	≤ 7	8-15	≥ 16
0,53	≤ 8	9-16	≥ 17
0,54	≤ 8	9-16	≥ 17
0,55	≤ 8	9-17	≥ 18
0,56	≤ 8	9-17	≥ 18
0,57	≤ 9	10-18	≥ 19
0,58	≤ 9	10-19	≥ 20
0,59	≤ 9	10-19	≥ 20
0,60	≤ 10	11-20	≥ 21
0,61	≤ 10	11-21	≥ 22
0,62	≤ 11	12-22	≥ 23
0,63	≤ 11	12-22	≥ 23
0,64	≤ 11	12-23	≥ 24
0,65	≤ 12	13-24	≥ 25
0,66	≤ 12	13-24	≥ 25
0,67	≤ 12	13-25	≥ 26
0,68	≤ 13	14-26	≥ 27
0,69	≤ 13	14-27	≥ 28

Evaluation of mitotic figures requires care, and relies on optimal tissue fixation and good preparation of sections. Observers must count only definite mitotic figures; hyperchromatic and pyknotic nuclei are ignored since they are more likely to represent apoptosis rather than cells in mitosis. Mitotic count requires standardization to a fixed field area. Cut-off points for scoring depend on field area. Calibrating the microscope is essential and is done by measuring the diameter of the high power field on a 40x objective.

11.4. Annex 4: Recommendations for ER/PR testing

Table 7. Summary of Guideline Recommendations for ER and PgR Testing by IHC in Breast Cancer Patients

	Recommendation	Comments
Optimal algorithm for ER/PgR testing	Positive for ER or PgR if finding of ≥ 1% of tumor cell nuclei are immunoreactive. Negative for ER or PgR if finding of < 1% of tumor cell nuclei are immunoreactive in the presence of evidence that the sample can express ER or PgR (positive intrinsic controls are seen). Uninterpretable for ER or PgR if finding that no tumor nuclei are immunoreactive and that internal epithelial elements present in the sample or separately submitted from the same sample lack any nuclear staining.	These definitions depend on laboratory documentation of the following: 1. Proof of initial validation in which positive ER or PgR categories are 90% concordant and negative ER or PgR categories are 95% concordant with a clinically validated ER or PgR assay.[3] 2. Ongoing internal QA procedures, including use of external controls of variable ER and PgR activity with each run of assay, regular assay reassessment, and competency assessment of technicians and pathologists. 3. Participation in external proficiency testing according to the proficiency testing program guidelines. 4. Biennial accreditation by valid accrediting agency.
Optimal testing conditions	Large, preferably multiple core biopsies of tumor are preferred for testing if they are representative of the tumor (grade and type) at resection.	Specimen should be rejected and testing repeated on a separate sample if any of the following conditions exist: 1. External controls are not as expected (scores recorded daily show variation). 2. Artifacts involve most of sample. Specimen may also be rejected and testing repeated on another sample if: 1. Slide has no staining of included normal epithelial elements and/or normal positive control on same slide. 2. Specimen has been decalcified using strong acids. 3. Specimen shows an ER-negative/PgR-positive phenotype (to rule out a false-negative ER assay or a false-positive PgR assay). 4. Sample has prolonged cold ischemia time or fixation duration < 6 hours or > 72 hours and is negative on testing in the absence of internal control elements.
	Interpretation follows guideline recommendation.	Positive ER or PgR requires that ≥ 1% of tumor cells are immunoreactive. Both average intensity and extent of staining are reported. Image analysis is a desirable method of quantifying percentage of tumor cells that are immunoreactive. H score, Allred score, or quick score may be provided. Negative ER or PgR requires < 1% of tumor cells with ER or PgR staining. Interpreters have method to maintain consistency and competency documented regularly.
	Accession slip and report must include guideline-detailed elements.	
Optimal tissue handling requirements	Time from tissue acquisition to fixation should be as short as possible. Samples for ER and PgR testing are fixed in 10% NBF for 6 to 72 hours. Samples should be sliced at 5-mm intervals after appropriate gross inspection and margins designation and placed in sufficient volume of NBF to allow adequate tissue penetration. If tumor comes from remote location, it should be bisected through the tumor on removal and sent to the laboratory immersed in a sufficient volume of NBF. Cold ischemia time, fixative type, and time the sample was placed in NBF must be recorded. As in the ASCO/CAP HER2 guideline, storage of slides for more than 6 weeks before analysis is not recommended. Time tissue is removed from patient, time tissue is placed in fixative, duration of fixation, and fixative type must be recorded and noted on accession slip or in report.	

Continued		
	Recommendation	**Comments**
Optimal internal validation procedure	Validation of any test must be done before test is offered. See separate article on testing validation (Fitzgibbons et al[64]). Validation must be done using a clinically validated ER or PgR test method. Revalidation should be done whenever there is a significant change to the test system, such as a change in the primary antibody clone or introduction of new antigen retrieval or detection systems.	
Optimal internal QA procedures	Initial test validation. See separate article on testing validation (Fitzgibbons et al[64]). Ongoing quality control and equipment maintenance. Initial and ongoing laboratory personnel training and competency assessment. Use of standardized operating procedures including routine use of external control materials with each batch of testing and routine evaluation of internal normal epithelial elements or the inclusion of normal breast sections on each tested slide, wherever possible. Regular, ongoing assay reassessment should be done at least semiannually (as described in Fitzgibbons et al[64]). Revalidation is needed whenever there is a significant change to the test system. Ongoing competency assessment and education of pathologists.	
Optimal external proficiency assessment	Mandatory participation in external proficiency testing program with at least two testing events (mailings) per year.	
	Satisfactory performance requires at least 90% correct responses on graded challenges for either test.	Unsatisfactory performance will require laboratory to respond according to accreditation agency program requirements.
Optimal laboratory accreditation	On-site inspection every other year with annual requirement for self-inspection.	Reviews laboratory validation, procedures, QA results and processes, and reports. Unsuccessful performance results in suspension of laboratory testing for ER or PgR.

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; IHC, immunohistochemistry; QA, quality assurance; NBF, neutral buffered formalin; ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; HER2, human epidermal growth factor receptor 2.

Extracted from (24), Hammond MEH, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, et al. American society of clinical oncology/college of American pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Arch Pathol Lab Med. 2010;134(7).

11.5. Annex 5: Recommendations for HER2 testing

Table 1. Summary of 2007 and 2013 HER2 Test Guidelines and Recommendations

Topic	2007 Recommendation	2013 Recommendation
Specimens to be tested	All primary breast cancer specimens and metastases should have at least one HER2 test performed	All newly diagnosed patients with breast cancer must have a HER2 test performed. Patients who then develop metastatic disease must have a HER2 test performed in a metastatic site, if tissue sample is available.
Optimal algorithm for HER2 testing	Positive for HER2 is either IHC HER2 3+ (defined as uniform intense membrane staining of > 30% of invasive tumor cells) or FISH amplified (ratio of *HER2* to CEP17 of > 2.2 or average *HER2* gene copy number > 6 signals/nucleus for those test systems without an internal control probe	Must report **HER2 test result as positive for HER2 if:**[a,b] • IHC 3+ based on circumferential membrane staining that is complete, intense[c,d] • ISH positive based on: Single-probe average *HER2* copy number ≥ 6.0 signals/cell[c,e] Dual-probe *HER2*/CEP17 ratio ≥ 2.0[c,e] with an average *HER2* copy number ≥ 4.0 signals per cell Dual-probe *HER2*/CEP17 ratio ≥ 2.0[c,e] with an average *HER2* copy number < 4.0 signals/cell[b] Dual-probe *HER2*/CEP17 ratio < 2.0[c,e] with an average *HER2* copy number ≥ 6.0 signals/cell
	Equivocal for HER2 is defined as: IHC 2+ or FISH *HER2*/CEP17 ratio of 1.8-2.2 or average *HER2* gene copy number 4-6 *HER2* signals/nucleus for test systems without an internal control probe	Must report **HER2 test result as equivocal** and order reflex test (same specimen using the alternative test) or new test (new specimen, if available, using same or alternative test) if:[a,b] • IHC 2+ based on circumferential membrane staining that is incomplete and/or weak/moderate[f] and within > 10% of the invasive tumor cells[d] *or* complete and circumferential membrane staining that is intense and within ≤ 10% of the invasive tumor cells[d] • ISH equivocal based on: Single-probe ISH average *HER2* copy number ≥ 4.0 and < 6.0 signals/cell[e,f] Dual-probe *HER2*/CEP17 ratio < 2.0 with an average *HER2* copy number ≥ 4.0 and < 6.0 signals/cell[e,f]
	Negative for HER2 is defined as: • IHC HER2 0: no staining • IHC HER2 1+: weak incomplete membrane staining in any proportion of tumor cells or weak, complete membrane staining in < 10% of cells • FISH *HER2*/CEP17 ratio of < 1.8 or average *HER2* gene copy number of < 4 signals/nucleus for test systems without an internal control probe	Must report **HER2 test result as negative** if a single test (or both tests) performed show:[a,b] • IHC 1+ as defined by incomplete membrane staining that is faint/barely perceptible and within > 10% of the invasive tumor cells[d] • IHC 0 as defined by no staining observed[d] or membrane staining that is incomplete and is faint/barely perceptible and within ≤ 10% of the invasive tumor cells[d] • ISH negative based on: Single-probe average *HER2* copy number < 4.0 signals/cell Dual-probe *HER2*/CEP17 ratio < 2.0 with an average *HER2* copy number < 4.0 signals/cell
	Indeterminate for HER2	Must report **HER2 test result as indeterminate** if technical issues prevent one or both tests (IHC and ISH) from being reported as positive, negative, or equivocal. Conditions may include: • Inadequate specimen handling • Artifacts (crush or edge artifacts) that make interpretation difficult • Analytic testing failure Another specimen should be requested for testing to determine HER2 status. Reason for indeterminate testing should be noted in a comment in the report.

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Table 1. Summary of 2007 and 2013 HER2 Test Guidelines and Recommendations (continued)

Topic	2007 Recommendation	2013 Recommendation
ISH rejection criteria	Test is rejected and repeated if: • Controls are not as expected • Observer cannot find and count at least two areas of invasive tumor • > 25% of signals are unscorable due to weak signals • > 10% of signals occur over cytoplasm • Nuclear resolution is poor • Autofluorescence is strong	Same and report **HER2 test result as indeterminate** as per parameters described immediately above.
ISH interpretation	Interpretation performed by counting at least 20 cells; a pathologist must confirm that counting involved invasive tumor criteria followed	The pathologist should scan the entire ISH slide prior to counting at least 20 cells or use IHC to define the areas of potential *HER2* amplification. If there is a second population of cells with increased *HER2* signals/cell, and this cell population consists of more than 10% of tumor cells on the slide (defined by image analysis or visual estimation of the ISH or IHC slide), a separate counting of at least 20 nonoverlapping cells must also be performed within this cell population and reported. For bright-field ISH, counting requires comparison between patterns in normal breast and tumor cells because artifactual patterns may be seen that are difficult to interpret. If tumor cell pattern is neither normal nor clearly amplified, test should be submitted for expert opinion.
Acceptable (IHC and ISH) tests[g]		Should preferentially use an FDA-approved IHC, bright-field ISH, or FISH assay.[g,h]
Optimal IHC testing requirements	Test is rejected and repeated or tested by FISH if: • Controls are not as expected • Artifacts involve most of sample • Sample has strong membrane staining of normal breast ducts (internal controls)	Same
IHC interpretation criteria	Positive HER2 result requires homogeneous, dark circumferential (chicken wire) pattern in > 30% of invasive tumor. Interpreters have method to maintain consistency and competency	Should interpret IHC test using a threshold of more than 10% of tumor cells that must show homogeneous, dark circumferential (chicken wire) pattern to call result 3+, HER2 positive.
Reporting requirements for all assay types	Report must include guideline-detailed elements	Same except for changes to reporting requirement and algorithms defined in this table (Data Supplements 9 and 10).
Optimal tissue handling requirements	Time from tissue acquisition to fixation should be as short as possible; samples for HER2 testing are fixed in 10% neutral buffered formalin for 6-48 hours; cytology specimens must be fixed in formalin. Samples should be sliced at 5- to 10-mm intervals after appropriate gross inspection and margins designation and placed in sufficient volume of neutral buffered formalin	Duration of fixation has been changed **from 6-48 hours to 6-72 hours.** Any exceptions to this process must be included in report.
Optimal tissue sectioning requirements	Sections should ideally not be used for HER2 testing if cut > 6 weeks earlier; this may vary with primary fixation or storage conditions	Same
Optimal internal validation procedure	Validation of test must be performed before test is offered	Same (Data Supplement 12 lists examples of various external quality assurance schemes)
Optimal initial test validation	Initial test validation requires 25-100 samples tested by alternative validated method in the same laboratory or by validated method in another laboratory	Laboratories performing these tests should be following all accreditation requirements, one of which is initial testing validation. The laboratory should ensure that initial validation conforms to the published 2010 ASCO/CAP recommendations for IHC testing of ER and PgR guideline validation requirements with 20 negative and 20 positive for FDA-approved assays and 40 negative and 40 positive for LDTs. This requirement does not apply to assays that were previously validated in conformance with the 2007 ASCO/CAP HER2 testing guideline or to those who are routinely participating in external proficiency testing for HER2 tests, such as the program offered by CAP (Data Supplement 12).
	Proof of initial testing validation in which positive and negative HER2 categories are 90% concordant with alternative validated method or same validated method for HER2	Laboratories are responsible for ensuring the reliability and accuracy of their testing results, by compliance with accreditation and proficiency testing requirements for HER2 testing assays. Specific concordance requirements are not required (Data Supplement 11).

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Table 1. Summary of 2007 and 2013 HER2 Test Guidelines and Recommendations (continued)

Topic	2007 Recommendation	2013 Recommendation
Optimal monitoring of test concordance between methods	Concordance testing must be performed prior to initiation of testing, optimally as the form of testing validation. If concordance is below 95% for any testing category, that category of test result of either FISH or IHC must be automatically flexed to alternative method before final interpretation	See text under Optimal Laboratory Accreditation.
Optimal internal QA procedures		Should review and document external and internal controls with each test and each batch of tests.
	Ongoing quality control and equipment maintenance	Same
	Initial and ongoing laboratory personnel training and competency assessment	Same
	Use of standardized operating procedures including routine use of control materials	Same
	Revalidation of procedure if changed	Same
	Ongoing competency assessment and education of pathologists	Should perform ongoing competency assessment and document the actions taken as a part of the laboratory record.
Optimal external proficiency assessment	Participation in and successful completion of external proficiency testing program with at least two testing events (mailings) a year	Same
	Satisfactory performance requires at least 90% correct responses on graded challenges for either test	Same
	• Unsatisfactory performance will require laboratory to respond according to accreditation agency program requirements	
Optimal laboratory accreditation	Onsite inspection every other year with annual requirement for self-inspection	Same (Data Supplement 11)
	• Reviews laboratory validation, procedures, QA results and processes, results, and reports	
	• Unsatisfactory performance results in suspension of laboratory testing for HER2 for that method	

NOTE. For all recommendations, evidence quality and recommendation strength are strong, except as noted. Bold font indicates changes in the updated version.

Abbreviations: ASCO, American Society of Clinical Oncology; CAP, College of American Pathologists; ER, estrogen receptor; FDA, US Food and Drug Administration; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, in situ hybridization; LDT, laboratory-developed test; PgR, progesterone receptor; QA, quality assurance.

[a]If a reflex test (same specimen/same tissue) ordered after an initial equivocal HER2 test result does not render a positive or negative HER2 test result, the pathologist should review histopathologic features, confer if possible with the oncologist regarding additional HER2 testing, and document it in the pathology report. The pathologist may pursue additional HER2 testing without conferring with the oncologist. This should be accomplished using: (1) the alternative test (IHC or ISH) on the same specimen, (2) either test on another block (same specimen), or (3) either test on another specimen (eg, core biopsy, surgical resection, lymph node, and/or metastatic site). Because the decision to recommend HER2-targeted therapy requires a HER2-positive test result, additional HER2 testing should be attempted in equivocal specimens to attempt to obtain a positive or negative HER2 test result and most accurately determine the HER2 status of the tumor specimen.

[b]See Data Supplement 2E for additional information on rare scenarios.

[c]Observed in a homogeneous and contiguous population and within > 10% of the invasive tumor cells.

[d]Readily appreciated using a low-power objective.

[e]By counting at least 20 cells within the area.

[f]Observed in a homogeneous and contiguous population.

[g]Alternatively, a laboratory accredited by CAP or another accrediting entity may choose to use an LDT, in which case its analytical performance must be documented in the same clinical laboratory that will use the assay, and documentation of analytical validity of the assay must be available.

[h]A list of HER2 assays approved by the FDA as in vitro companion diagnostic devices to aid in the assessment of patients for whom trastuzumab treatment is being considered can be found in the Medical Devices section of the US FDA Web site (http://www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm?start_search=1&search_term=HER2&approval_date_from=&approval_date_to=07/14/2013&sort=approvaldatedesc&pagenum=10; last checked July 14, 2013). The product package insert for trastuzumab and pertuzumab prepared by the FDA indicates that "HER2 testing should be performed using FDA-approved tests by laboratories with demonstrated proficiency."[77,78]

Extracted from (29), Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med [Internet]. 2007;131(1):18–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19548375

11.6. Annex 6: Criteria for possible HER2 test discordance

Table 2. Histopathologic Features Suggestive of Possible HER2 Test Discordance

Criteria to Consider*
New HER2 test should not be ordered if the following histopathologic findings occur and the initial HER2 test was negative:
Histologic grade 1 carcinoma of the following types:
Infiltrating ductal or lobular carcinoma, ER and PgR positive
Tubular (at least 90% pure)
Mucinous (at least 90% pure)
Cribriform (at least 90% pure)
Adenoid cystic carcinoma (90% pure) and often triple negative
Similarly, a new HER2 test should be ordered if the following histopathologic findings occur and the initial HER2 test was positive:
Histologic grade 1 carcinoma of the following types:
Infiltrating ductal or lobular carcinoma, ER and PgR positive
Tubular (at least 90% pure)
Mucinous (at least 90% pure)
Cribriform (at least 90% pure)
Adenoid cystic carcinoma (90% pure) and often triple negative
If the initial HER2 test result in a core needle biopsy specimen of a primary breast cancer is negative, a new HER2 test must be ordered on the excision specimen if one of the following is observed:
Tumor is grade 3
Amount of invasive tumor in the core biopsy is small
Resection specimen contains high-grade carcinoma that is morphologically distinct from that in the core
Core biopsy result is equivocal for HER2 after testing by both ISH and IHC
There is doubt about the specimen handling of the core biopsy (long ischemic time, short time in fixative, different fixative) or the test is suspected by the pathologist to be negative on the basis of testing error

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, in situ hybridization; PgR, progesterone receptor.
*Criteria to consider if there are concerns regarding discordance with apparent histopathologic findings and possible false-negative or false-positive HER2 test result.

Extracted from (29), Wolff AC, Hammond MEH, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast