From Department of Oncology and Pathology

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PROGNOSTIC FACTORS IN BREAST CANCER WITH A FOCUS ON THE ROLE OF TUMOUR PROLIFERATION

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PROGNOSTIC FACTORS IN BREAST CANCER WITH A FOCUS ON THE ROLE OF TUMOUR PROLIFERATION.

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“In purity and holiness I will preserve my life and my art”
From the Physician’s Hippocratic Oath
Hippocrates (≈ 460 BC - 370 BC)

“Con innocenza e purezza custodirò la mia vita e la mia arte”
Dal Giuramento di Ippocrate
Ippocrate (≈ 460 a.C. - 370 a.C.)

To the patients, to their fortitude

ABSTRACT

Ki67 is the most commonly used marker of proliferation in breast cancer. The general aim of the thesis was to investigate the prognostic role of Ki67 and its interplay with other prognostic factors in breast cancer cohorts.

In Paper I, the prognostic value of Ki67 as analysed in metastasis biopsies (mKi67) and the change in Ki67 from primary tumour (pKi67) to corresponding first site of relapse was studied in patients diagnosed and treated for metastatic breast cancer (MBC) at Karolinska University Hospital (Stockholm, Sweden). A significantly longer median post-relapse overall survival (OS) was demonstrated for low-mKi67 (≤20%) compared with high-mKi67 (>20%) group (25 vs.17 months, p 0.01 by log-rank test). mKi67 was associated with OS regardless of pKi67. Ki67 varied from primary tumour to metastasis in a significant number of patients (p 0.01 by McNemar's test) and the change from high to low was correlated to better OS in comparison with stable Ki67 levels.

In paper II, the prognostic value in terms of post-relapse OS of breast cancer subtypes and genomic signatures as assessed in primary tumour tissue was investigated, beyond classical clinical and pathological prognostic determinants, in patients diagnosed and treated for MBC at Karolinska University Hospital. Immunohistochemistry-(IHC) and PAM50-based intrinsic subtypes showed a significant but not independent prognostic value after distant relapse. Moreover, low and medium-risk categories according to PAM50 risk of relapse score (ROR-S) were independently associated with longer post-relapse OS in comparison with the high-risk category. In contrast, the 21-gene Recurrence Score and the 70-gene signature were not independently prognostic of post-relapse survival. The PAM50-derived proliferation score also independently correlated with survival and the additional clinical information deriving from combining ROR-P (ROR-S weighted for the proliferation score) with the other prognosticators was also highly significant ($p < 0.001$).

In paper III, the additional prognostic information deriving from the combination of genomic signatures and IHC markers, namely Ki67 alone or added to oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 receptor (HER2) to generate IHC subtypes, compared with either classifier alone was investigated in two cohorts. Cohort 1 included patients with diagnosis of primary breast cancer from the Stockholm Breast Cancer Registry (SBCR) while cohort 2 was composed of women diagnosed with primary tumour in Uppsala county (Sweden). In cohort 1, 21-gene Recurrence Score and PAM50 added relevant prognostic information beyond Ki67/IHC subtypes. All the investigated genomic signatures provided additional prognostic information when combined with Ki67/IHC subtypes in the group of ER-positive/lymph node positive tumours while no signature reached the statistical significance when ER-negative tumours were studied. IHC subtypes, but not Ki67 alone, showed additional prognostic ability when combined with all genomic signatures except PAM50, in the overall cohort 1 and ER-negative subgroup, but not in ER-positive/lymph node negative and ER-positive/lymph node positive tumours. In

cohort 2, the findings were substantially comparable but the statistical significance reduced likely due to the smaller sample size.

In Paper IV, the change in survival after local and loco-regional relapse of breast cancer over 34 years (1980-2014) was studied in a cohort of patients from the SBCR. Survival was compared between three cohorts according to years of relapse diagnosis: 1980-1989; 1990-1999; 2000-2014. In total, 1922 women were diagnosed with local and 776 with loco-regional relapse. In the group of the local recurrence, median post-relapse event-free survival (EFS) and OS significantly improved over time, regardless of age. Conversely, age-related trends in survival were demonstrated in the group of women who experienced a loco-regional relapse. Relative survival was consistent with the observed EFS and OS. In addition, a decrease in mortality over time was demonstrated only in younger patients diagnosed with a loco-regional relapse in 2000-2014 (EMR 0.48; 95% CIs 0.42-0.72), regardless of other prognostic factors. The outcome was unchanged when the analysis was restricted to the years 1980 through 2009.

LIST OF SCIENTIFIC PAPERS

I. **Falato C**, Lorent J, Tani E, Karlsson E, Wright P K, Bergh J, Foukakis T. *Ki67 measured in metastatic tissue and prognosis in patients with advanced breast cancer.* Breast Cancer Res Treat. 2014 Sep; 147(2): 407-14.

II. **Falato C**, Tobin N P, Lorent J, Lindström L S, Bergh J, Foukakis T. *Intrinsic subtypes and genomic signatures of primary breast cancer and prognosis after systemic relapse.* Mol Oncol. 2016 Apr; 10(4):517-25.

III. Lundberg A, Lindström LS, Harrell JC, **Falato C**, Carlson JW, Wright PK, Foukakis T, Perou CM, Czene K, Bergh J, Tobin NP. *Gene expression signatures and immunohistochemical subtypes add prognostic value to each other in breast cancer cohorts.* Clin Cancer Res. 2017 Sep 29.

IV. **Falato C**, Taylor S K, Szulkin R, Nordblom A, Eriksson L, Sofiadis A, Fredriksson I, Hartman J, Bergh J, Foukakis T. *Prognosis in patients diagnosed with loco-regional failure of breast cancer: 34 years longitudinal data from the Stockholm – Gotland cancer registry. (Manuscript)*

LIST OF RELATED PUBLICATIONS

I. Foukakis T, **Falato C**, Bergh J. A 21-gene expression assay in breast cancer. N Engl J Med. 2016 Apr 7; 374 (14):1386-7.

II. Kessler L, **Falato C**, Margolin S, Bergh J, Foukakis T. A retrospective safety and efficacy analysis of the first patients treated with eribulin for metastatic breast cancer in Stockholm, Sweden. Acta Oncol. 2015 Apr;54(4):522-9.

CONTENTS

LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
AR	Androgen Receptor
BCI	Breast Cancer Index
C -index	Concordance Index
EFS	Event Free Survival
EGFR	Epidermal Growth Factor Receptor
EMR	Excess Mortality Ratio
ER	Oestrogen Receptor
ESR1	Oestrogen Receptor 1
FFPE	Formalin-fixed Paraffin-embedded
FNAC	Fine-needle Aspiration Cytology
GGI	Genomic Grade Index
HER2	Human Epidermal Growth Factor 2
HR	Hazard Ratio
IHC	Immunohistochemistry
LR-χ^2	Likelihood Ratio
LRF	Loco-Regional Failure
MBC	Metastatic Breast Cancer
mKi67	Ki67 from first distant metastasis
OS	Overall survival
pKi67	Ki67 from primary tumour
PR	Progesterone Receptor
RFI	Recurrence Free Interval
ROR	Risk Of Relapse score
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RS	Recurrence Score
SBCR	Stockholm Breast Cancer Registry
TK	Tyrosine-kinase
TILs	Tumour Infiltrating Lymphocytes
TMA	Tissue micro-array
TNBC	Triple Negative Breast Cancer

BACKGROUND

1.1 EPIDEMIOLOGY OF BREAST CANCER

Breast cancer is the most common malignancy in women accounting for about 25% of all cancers worldwide. Overall, 1.670.000 new breast cancers were diagnosed in 2012 with slightly more cases identified in less developed (883.000) than in more developed (794.000) countries (of these, 362.000 in European Union). In the years 2008-2012, there were approximately 6.232.000 incident cases of breast cancers across the world, of whom 1.444.000 in European Union. Incidence rates vary nearly four-fold with the lowest estimates of 27 per 100.000 women in Middle Africa and Eastern Asia to 90 per 100.000 women in Western Europe. [1] Figure 1 presents the age-standardized rates of breast cancer per 100.000 in the female population. Recently, a systematic analysis for the Global Burden of Disease Study reported a further increase in breast cancer incidence with a total of 2.400.000 new cases in 2015. [2]

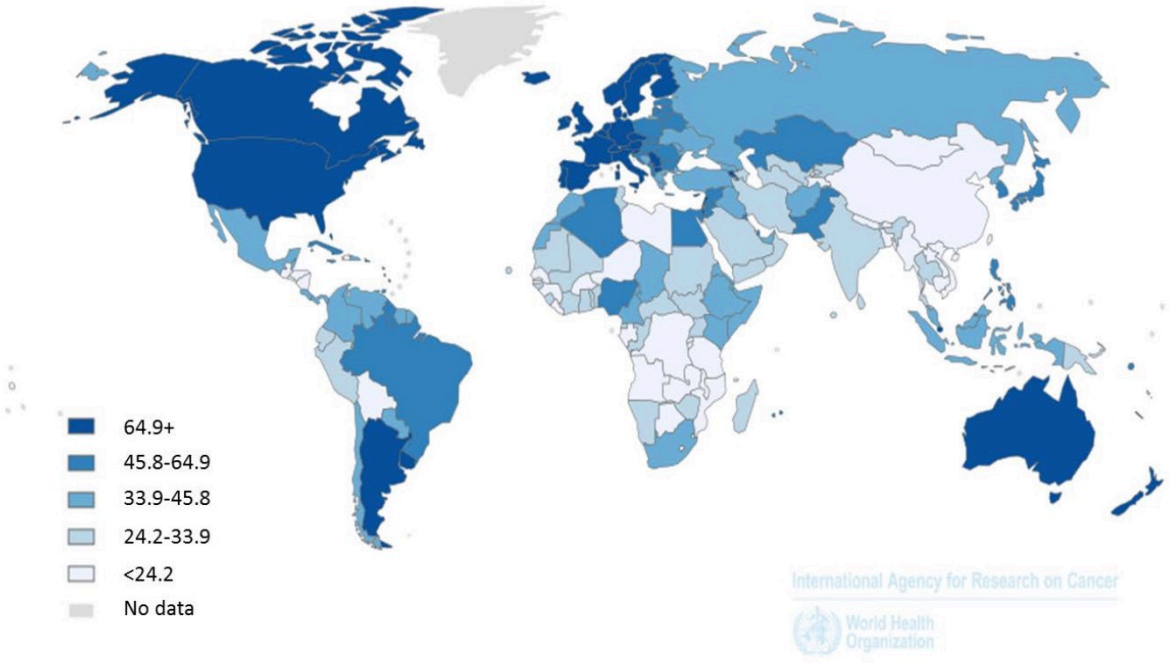


Figure 1. *Estimated age-standardized incidence rates of breast cancer per 100.000 women (GLOBOCAN 2012, IARC).*

In 2012, breast cancer was estimated as the fifth cause of death from malignancy in the world leading to 522.000 deaths. After lung cancer, it represented the second most frequent cause of death due to malignancy in industrialized countries accounting for about 15.8% of all cancer deaths, while it ranks as the main cause of death (14.3%) among all cancers in less developed regions. In 2012, the mortality rates varied from 6 per 100.000 in Eastern Asia to 20 in Western Africa. The range in mortality between world regions is less pronounced than the range in incidence thanks to the favourable prognosis of breast cancer, especially in high-incidence developed countries (Figure 2). [1] Recent estimates from the Global Burden of Disease Collaboration appointed breast tumour as the leading cause of cancer death in women in 2015. [2]

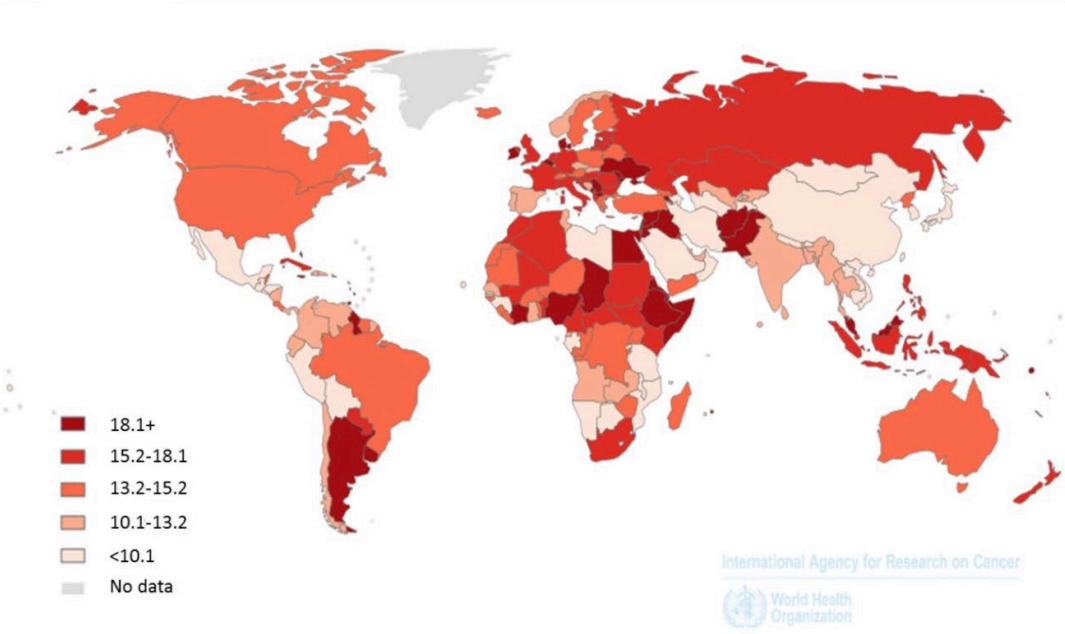


Figure 2. *Estimated age-standardized mortality rates of breast cancer per 100.000 women (GLOBOCAN 2012, IARC).*

In Sweden, breast cancer is the most frequent form of cancer within the female population accounting for 30% of all cancers. [3] As Figure 3 illustrates, the age-standardized incidence has doubled since 1960, while mortality has declined by nearly 7% over time in the overall Swedish population. [4]

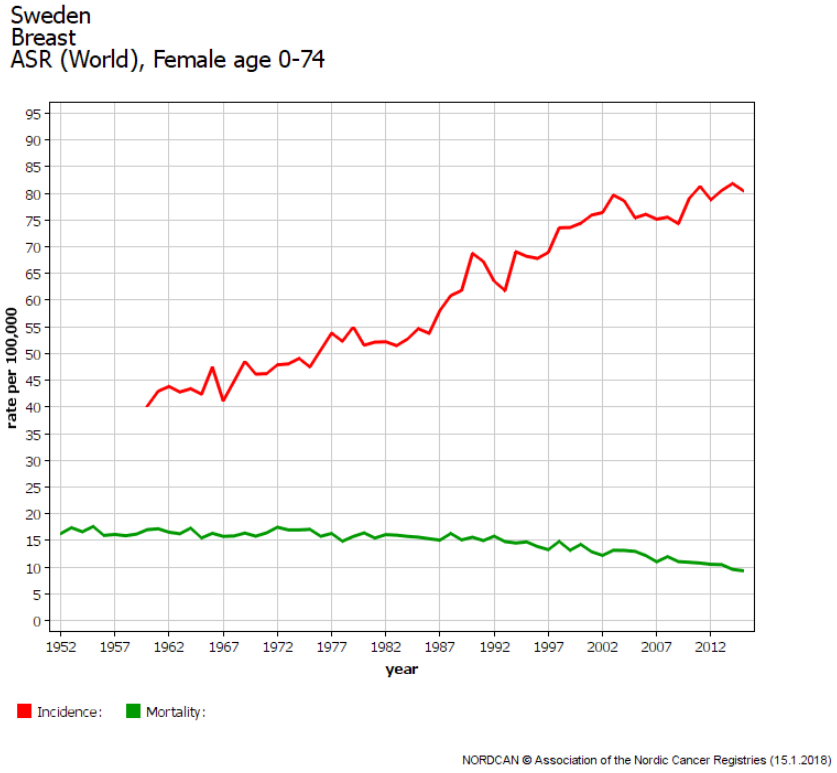


Figure 3. *Age-standardized incidence and mortality rates of breast cancer per 100.000 women (age 0-74 years) in Sweden (NORDCAN 2014)*

Albeit more women died from lung and colon-rectal cancer in 2012 in the general population, breast cancer was the most common cause of cancer death among women aged 65 or younger. Overall, 10-year breast cancer survival has generally increased from 50% to approximately 80% since the beginning of 1960, with a parallel improvement registered in all age groups. The 5-year survival, which is strictly dependent on the disease stage ranging from nearly 100% for stage 0-1 to 20% for stage 4-breast cancer, has also improved from 60% to 90% since 1960. [3]

The combination of mammography screening and improved adjuvant therapies has led to a reduction in death rates for breast cancer in the United States by approximately 30% since 1970s, although the net screening contribution remains controversial. [5] Besides stage, oestrogen receptor (ER) is the longest established prognostic and predictive factor in breast cancer. [6] Based upon this evidence, trends in mortality variations by screening and early

disease systemic treatment have been reinterpreted in a molecular context indicating greater absolute death rate declines in ER-positive (median 17 per 100.000 women, range=13-21) than ER-negative cancers (median 5 per 100.000 women, range=3-6) in the years 1975 trough 2000, largely owing to the use of tamoxifen. In contrast, similar decrease in death rates was observed for ER-positive and ER-negative cases (median 16.7% vs. 14.0%, respectively), where no adjuvant treatment was assumed. Interestingly, among only screening-detected invasive tumours (thus excluding over-diagnoses) the overall 5-year survival probability was higher in ER-negative tumours (35.6% vs. 30.7%), mainly as an effect of the absolute higher survival gain obtained by diagnosis at an earlier stage in this tumour subgroup (25.6% vs. 20.2%). [7] These data provide further support to the molecular and clinical heterogeneity of breast cancer [8,9] and motivate novel screening approaches that might more efficiently detect fast-growing tumours in high-risk population, ex. BRCA1-mutation carriers. [7]

Despite advances in systemic therapies, metastatic breast cancer is still treated with a palliative intention. In contrast to reports from population-based studies suggesting that survival for patients diagnosed with systemic disease between 1970 and 2000 has modestly improved over time in the United States [10-12], no general improvement was observed in the same years in a large cohort of women who received adjuvant systemic treatment within 11 randomized trials. [13] An analysis from the Breast Cancer Registry in Stockholm (Sweden) revealed that age-related trends in survival exist and that prognosis is more favourable for women 60 years or younger diagnosed with a distant relapse after 2000 in comparison with previous time periods, presumably thanks to the availability of newer and more efficacious drugs. [14]

1.2 CLASSIFICATION OF BREAST CANCER

Breast cancer is a heterogeneous disease comprising multiple entities with different histological and molecular features characterized by distinctive clinical behaviours and response to treatment. Thus, a central component of the treatment of breast cancer is the knowledge of its extent and biological properties. In clinical practice, categorization of tumours is a tool to guide or standardize treatment, planning for follow-up, selecting clinical trials and strengthening translational research. Great advances in refining breast cancer molecular classification and prognostication have characterized the last two decades. Here, a

traditional histo-pathologic classification of breast neoplasm is presented, along with more integrative biological and molecular characterization.

1.2.1 MORPHOLOGICAL CLASSIFICATION

Morphologically, breast cancer can be classified into clinically relevant subgroups based on histological types. [15]

"Histological type" refers to the growth pattern of the tumour. [16] At a cellular level, normal breast lobules comprise cells with regular, rounded nuclei organized in glands or tubule with a few proliferating cells. The tumour structure can range from stroma-enriched pattern with a glandular frame with minimal atypia, to highly atypical carcinoma cells growing as solid sheets or tumour characterized by a mixture of atypical cells with stroma, pre-invasive lesions, and normal breast tissue. [17] A traditional pathology-driven classification by the World Health Organization (WHO) identifies two common types of breast carcinoma: *non-invasive (or in situ)* and *invasive breast carcinoma*. Two major *carcinomas in situ,* which are both precursors of the invasive counterpart, have been identified: the *ductal* and the less common *lobular carcinoma in situ*. [18] Due to their distinct clinical behaviours, different treatment recommendations have been formulated. [19] Additionally, according to the most recent breast cancer staging systematized by the American Joint Committee on Cancer (AJCC) lobular carcinoma in situ is no longer considered cancer in situ but rather a benign entity and risk factor for cancer without metastatic potential. [20] The most common breast *invasive carcinoma,* accounting for the 70-75% of this morphological category, is the carcinoma *of no special type (NST),* previously defined as *ductal carcinoma not otherwise specified (IDC-NOS)*. [18] This is a diagnosis of exclusion and refers to those carcinomas that do not show sufficient characteristics to warrant their categorization in one of the special types. The special types account for about 25% of all breast cancers and comprise at least 17 distinct histological variants. [16,18] Among them, *invasive lobular carcinoma* is the most common and includes, besides the classical form, many other subclasses defined by peculiar morphological, clinical and biological features. [18] Due to relatively rare prevalence, lack of standardized diagnosis criteria and low inter-observer reproducibility, special types of carcinoma have not been systematically investigated in microarray-based gene expression studies and precise indications on tailored treatments have not been proposed, to date. [16]

Finally, in addition to carcinomas, mesenchymal tumours (including sarcoma), which originate from the connective and fat tissue surrounding the breast gland, are described.

1.2.2 IMMUNOHISTOCHEMICAL CLASSIFICATION

Currently, four immunohistochemical biomarkers are used in routine clinical practice: ER, progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki67.

1.2.2.1 Hormone receptors

Overall, more than 75% of breast carcinomas express the hormone receptors ER and/or PR. The percentage of cancer cells stained for those biomarkers has valuable prognostic and predictive information. [21] ER is an intracellular protein mostly expressed in breast, endometrium, ovarian stroma and hypothalamus. PR is also an intracellular protein and its gene is transcriptionally activated by ER by binding to ER binding sites, so-called ERE, present upstream to PR gene. [22] The expression of PR, thus, correlates to that of ER, and, for this reason, the existence of ER-negative/PR-positive breast cancers is highly controversial. ER and PR are currently measured by immunohistochemistry (IHC), which replaced the ligand-binding assay in the US in the early to mid-90s. IHC, which uses a monoclonal antibody-based biochemical method to identify specific sequences on the receptor gene, faces limitations mainly related to inter-laboratory as well as inter-observer discrepancies. [17] In order to make ER and PR assessment more homogeneous, in 2010 the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) set the new cut-off to distinguish positive from negative cases at the clinically significant level of ≥1%. [21] In Sweden, where IHC definitely replaced the cytosol assay in 2003, a cut-off of 10% is currently recommended [23], and whether endocrine therapy should be administered when ER expression levels is comprised between 1% and 9% is still debated. A Swedish study, in which the IHC cut-off was set to ≥10%, demonstrated that, among ER-negative tumours, just a few falls in the 1-9% range and that no benefit was derived from tamoxifen given when ER was ≤10%. [24]

1.2.2.2 Human epidermal growth factor receptor 2

The clinical significance of HER2 in breast cancer has evolved from a marker of poor prognosis to a marker of response to treatment with therapies targeting the receptor. [25] HER2, also known as HER2/neu or ErbB-2, is a trans-membrane receptor member of the Epidermal Growth Factor (EGF) Receptor Tyrosine Kinase (RTK) family. It is encoded by the ERBB2 gene located on the long arm of the chromosome 17 (17q21-q22). HER2, which normally regulates cell growth, differentiation and survival, is overexpressed in 15-20% of invasive breast cancers and correlates with more aggressive cancer features. [26,27] HER2 receptor, which has no high-affinity ligand, is activated for homodimerization or heretodimerization with other HER receptors and, possibly, for auto-cleavage of the extra-cellular domain. The binding to HER3 receptor generates a dimer of high-signalling potency. [25] HER2 content is routinely analysed either by HER2 protein quantity measurement by IHC or determining gene amplification by fluorescence in situ hybridization (FISH). Updated recommendations on HER2 testing have been published in 2013. [28]

1.2.2.3 Ki67

Ungoverned cellular proliferation is a hallmark of cancer. [29] Ki67, currently the most commonly used biomarker of proliferation in routine clinical practice, is a non-histonic nuclear protein expressed at crescent levels during all the active phases of the cell cycle. [30] Ki67 acts as surfactant by generating a steric and electrostatic charge barrier on the chromosomal periphery, which prevents chromosome collapse into a mass after nuclear envelope disassembly during cell division. In this manner, Ki67 enables the motility of chromosomes and their interaction with the mitotic spindle. [31]

Tumour proliferation rate is generally assessed as the number of cell nuclei positively stained for Ki67 antibody, among the whole number of scored malignant cells. Several monoclonal antibodies against the Ki67 antigen have been developed. The original antibody presented by Gerdes and coll. in 1983 was only for use in frozen tumour sections whereas more recent antibodies can be used on paraffin embedded specimens. Of those, Mib-1 is the antibody routinely employed. [32]

The majority of the studies consistently appointed Ki67 as an independent prognostic factor of disease-free survival in early breast cancer. [33] Nonetheless, despite efforts put in the implementation of quality assurance schemes, the reproducibility of Ki67 measurements between laboratories, although improved, is still controversial and clinical useful thresholds for Ki67 categorization are not unanimously defined, to date. Given these (pre-) analytic caveats, an International Ki67 in Breast Cancer Working Group discouraged Ki67-driven clinical decisions. [34]

1.2.2.4 Immunohistochemical subtypes

Based upon the abovementioned biomarkers, breast cancer has traditionally been classified into four IHC subtypes, which partially recapitulate the intrinsic subtyping defined by gene expression profiles (See chapter 1.2.3 hereunder) [8,9]: Luminal A and B, HER2-positive and triple negative (TNBC) subtypes. Luminal A subgroup is characterized by high levels of hormone receptors and low levels of Ki67, while Luminal B tumours show higher proliferation rate and low hormone-receptor expression. [35] A 20% cut-off for PR has shown ability to improve the identification of good outcome Luminal A breast cancers. [36] HER2-positive subtype is highly heterogeneous and comprises both clinically hormone receptor positive and negative tumours. Cancers overexpressing HER2 are generally highly proliferative and show low levels of luminal and basal gene clusters. [37] Finally, TNBCs are typically hormone receptor negative/HER2-negative and highly proliferative. An expanded immune-panel including cytokeratin 5/6 and the epidermal growth factor receptor (EGFR), provides a more refined and clinically relevant characterization of TNBC, on the top of ER, PR and HER2. [38] TNBC subtype is unanimously recognized as holding the worst prognosis and should be conceived as a biologically and clinically distinct entity. [39-41] In addition to that, breast cancer subtypes are also characterized by different response to systemic therapies. Indeed, HER2-positive and TNBC are more sensitive to chemotherapy than Luminal tumours, in particular Luminal A. [42]

1.2.3 INTRINSIC SUBTYPES

Almost two decades ago, hierarchical clustering based upon the unsupervised analysis of 9000 differentially expressed "intrinsic" genes between tumour samples segregated breast carcinoma in two main clusters, mostly dominated by ER expression, and five sub-clusters, referred as "intrinsic subtypes" (Figure 4A). [8,9] The subtypes were successively validated in several independent cohorts. [43-48] The ER-positive cluster is the most represented and can be further separated into two subtypes, Luminal A and B, identified by the expression of genes reminiscent of the luminal breast epithelial cells, such as ER, GATA3, XBP1, FOXA1 and low weight cytokeratin 8/18, among others. Luminal B is biologically characterized by higher expression of genes related to cell proliferation (i.e. MKI67 and AURKA) compared to the Luminal A subgroup, which has been shown to have higher expression levels of ER-activated genes (Figure 4C and F). [8,9,43,49,50] In contrast, basal-like breast cancers are distinguished by the expression of genes of the myoepithelial/basal epithelial cells (such as KRT5/6A, ID4, FOXC1) (Figure 4D). Basal-like tumours represent a unique molecular entity and are largely but not completely captured by TNBCs. [37,51] Indeed, this subgroup presents the highest intrinsic diversity depending on the complex genomic landscape. [9,40,41,52] TP53 is frequently mutated and a vast variety of copy number alterations (CNAs) as well as gene mutations have been described. [53] Additionally, basal-like subtype is associated with BRCA1 germline mutations. [43] In fact, the phenotypic features of basal-like breast cancers recapitulate those of tumours arising in BRCA1 germline mutated carriers and there is increasing evidence suggesting a dysfunctional BRCA1 pathway in sporadic basal-like tumours. [54] Similarly, HER2-enriched subtype, defined by the amplification of genes associated with HER2 pathway and/or HER2 amplicon on 17q12 (i.e. GRB7), displays a high grade of internal diversity, including a number of subsets with distinctive ER, CNAs and mutational patterns. [42,49,55-57] HER2-enriched tumours are highly proliferative and show lack of expression of genes within the luminal and basal cluster (Figure 4B). [37] Clinically, 70% of the tumours classified as HER2-enriched by gene expression profiles are also HER2-positive, as well as many HER2-amplified/ER-positive cancer are rather classified as Luminal B. [42,49] This incomplete overlap might be explained by similar functional events, such as the mutation of HER2 gene or components of the downstream pathways, which mimic HER2 amplification but are not translated into the HER2 overexpression. However, increasing evidence suggest that HER2-enriched intrinsic subtype may identify a subgroup within HER2-positive tumours more likely to achieve high response rate to dual HER2 blockade. [57-61] Together basal-like,

HER2-enriched breast tumours and Luminal B have a more aggressive behaviour compared to Luminal A subtype. [9] Lastly, a category showing gene expression features usually expressed by the adipose tissue and clustering with fibroadenoma and normal breast tissue, the so-called normal-breast like subtype has been identified (Figure 4E). However, the clinical relevance of this subtype is still unclear and many consider it as a mere artifact, likely attributable to a specimen contamination by normal tissue. [17,49,62]

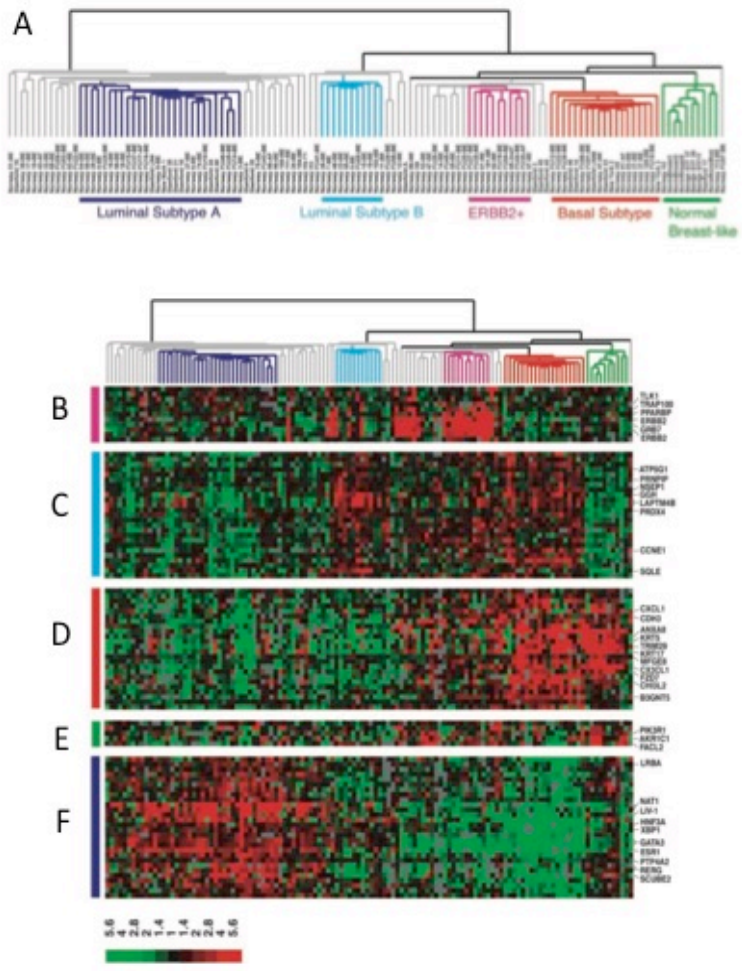


Figure 4. *Hierarchical clustering of 115 tumours and 7 non-malignant breast tissues using the "intrinsic" gene set. In the upper part of the figure: dendogram showing tumor clustering into 5 subgroups (A). In the lower part of the figure: gene clusters associated with the ERBB2 oncogene and other co-expressed genes (B); the luminal B subtype (C); the basal-like subtype (D); the normal breast-like group (E); the estrogen receptor (ESR1) highly expressed in luminal subtype A tumors (F) (Modified from Sorlie el al., PNAS 2003)*

Recently, the US Food and Drug Administration approved the Prosigna test based on a reduced version of the original intrinsic subtyping, referred as PAM50 because it contains 50 genes among those into the "intrinsic list"[8] and is constructed according to the Prediction of Microarray algorithm (PAM). PAM50 classifies breast tumour in Luminal A and B, HER2-enriched, basal-like and normal-like subtypes and provides a score that is predictive of risk of relapse (ROR) in ER-positive tumours. [49,63,64]

Less common intrinsic subtypes have been described, such as claudin-low and the molecular apocrine subtype. [65,66] The classical "intrinsic gene" sets [8,9,48] do not identify them and are, thus, not further discussed here.

1.2.4 CORRELATION BETWEEN IMMUNOHISTOCHEMICAL AND MOLECULAR SUBTYPES

Even though IHC and gene expression based intrinsic subtypes moderately correlate to each other, they are not synonymous. [67] Intrinsic subtypes are, in fact, represented in each IHC-based subgroup [68] and their identification has demonstrated clinical value. [58] Indeed, HER2-enriched subgroup includes approximately 35% of HER2-negative cancers as defined by IHC, and only 52% of the tumours are ER-negative /HER2-positive. A moderate inconsistence has also been demonstrated between TNBC IHC-surrogate and basal-like subtype. TNBC is a highly diverse group composed of many cancer subtypes among whom basal-like tumours predominate (~70% of the cases, when claudin-low are ignored). Within basal-like category, approximately 85% of the cases are classified as TNBC, whereas ER-positive as well as HER2-positive subtype is also significantly represented. Of interest, TNBCs and non-TNBCs within basal-like tumours show a nearly complete overlap in the pattern of expressed genes, which strengthen the notion of their unique biology. [51] Global gene expression analysis has revealed the presence of at least 7 subtypes among TNBCs with potential therapeutic implications. [69]

1.2.5 PATTERNS OF GENE MUTATIONS IN BREAST CANCER IN RELATION TO INTRINSIC SUBTYPES

Massive parallel DNA sequencing in breast cancer has identified a wide range of DNA mutations, such as base substitutions, small insertions, deletions, structural rearrangements as well as CNAs. The most frequently mutated genes are TP53, PIK3CA, MYC, CCND1, PTEN, FGFR1, GATA3, RB1, ERBB2, and MAP3KI. [70] Large-scale mutation data series have provided the evidence that distinct molecular subtypes have different repertoire of mutations, but no mutation or gene is subtype-specific (Figure 5). In the ATLAS study, genomic drivers were correlated to clinical and pathologic features. For instance, PIK3CA, GATA3, MAP3KI, KMT2C, CBFB were more significantly mutated (>5%) in ER-positive cancers. However, while the repertoire of genes mutated in Luminal greatly varies in comparison with basal-like tumours, there is no highly recurrent mutation or highly recurrent mutated gene that defines Luminal A or Luminal B (Figure 5). [71] Indeed, recurrently mutated genes in Luminal B tumours are, among the others, PIK3CA, GATA3, PTEN and TP53, recapitulating at high grade those mutated in Luminal A subtype. In contrast, luminal B has a greater genomic complexity, have more CNAs and higher number of mutations. [71] Furthermore, TP53 is mutated in high-grade ER-positive and is the only significantly mutated gene (>10%) in basal-like cancer, whereas CDH1 and HER2 are rarely mutated in ER-negative subtype. [71,72] Interestingly, from a mutational perspective, the basal-like subgroup forms a unique group distinct from other breast cancers but with similarities with ovarian cancer as well as squamous lung and head and neck cancers. [51,73] Moreover, the molecular heterogeneity of HER2 subtype is reflected at the gene mutational level. In general, HER2-subtype has the highest nucleotide mutation rate but reduced list of recurrently mutated genes. Mutations typical of ER-negative, such as TP53, and of ER-positive cancers, namely PIK3CA and GATA3, are frequently found also in HER2-negative/ER-negative and HER2-negative/ER-positive subgroups, respectively (Figure 5). [71,72] However, mutations in PIK3CA are less common in HER2-positive than HER2-negative/ER-positive and equally represented in HER2-positive/ER-positive and HER2-positive/ER-negative tumours, which has important clinical implications in terms of therapy resistance prediction in HER2-positive malignancies. [74]

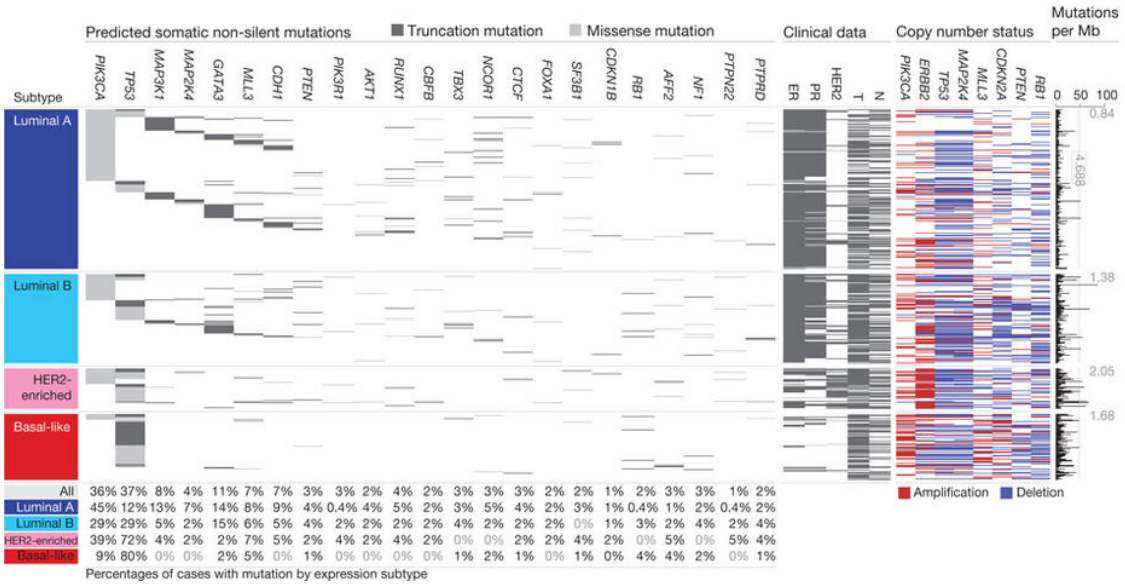


Figure 5. *Significantly mutated genes and correlations with molecular subtypes (Cancer Genome Atlas Network, Nature 2012)*

1.3 PROGNOSTIC AND PREDICTIVE FACTORS

1.3.1 INTRODUCTION

By definition, a prognostic factor is a clinical or biologic characteristic that is objectively measurable and provides information on clinical outcome at diagnosis, independently of the treatment. In cancer, prognostic markers are usually indicators of growth, invasion and metastatic potential. A predictive factor is a clinical or biologic characteristic capable to provide information on the likelihood of response to a given therapy and may serve to identify subpopulations of patients with a higher probability to benefit from a certain treatment. Such markers can be represented by the treatment target or by modulators of the expression and/or function of the target. Some factors in breast cancer function both as prognostic and predictive markers (e.g. HER2). [75]

Several genetic and genomic biomarkers are emerging in breast cancer field, beyond the traditional clinical and pathological factors. However, markers need to demonstrate three characteristics in order to be recommended for use in clinical practice [76,77]:

1) Analytic validity: it refers to the marker ability to precisely measure the molecular event of interest and focuses on the technical aspects of the assay including accuracy, reproducibility, dependence of pre-analytic issues and reliability.
2) Clinical validity: it assesses the strength of the association between assay result and clinical outcomes, such as recurrence-free survival and overall survival. It is the ability to accurately divide the population in two or more groups that differ biologically and clinically. Measuring strength of this association is of primary importance in order to later proceed to the assessment of clinical utility of the marker. There are several methods to quantify the association, such as receiver operator characteristic (ROC) curves, the area under the curve of ROC analysis, sensitivity and specificity, as well as several study designs, which mainly depends on the study sample size (resampling methods for smaller cohorts; identification of a "validation" and a "test" set for larger cohorts). Reporting of the results from studies evaluating new biomarkers should adhere to the Reporting Recommendations for Tumour Markers Prognostic Studies (REMARK). [78]
3) Clinical utility: a clinically useful biomarker is a marker that impacts clinical decision-making and patient outcomes when compared with a clinical situation in which it is not used. Proven analytic and clinical validity do not imply clinical utility. This is the case, for instance, of a biomarker that does not show to be independent from predictors already in use in clinical practice, despite an outstanding clinical validity. High-quality data are required to prove the clinical utility of a biomarker. Retrospective analysis of prospectively collected material, ideally from randomized trials, is a time- and cost-effective strategy. [77,79]

Additionally, a good candidate marker should be feasible, reproducible, widely available, readily interpretable, and not consume tissue needed for other tests. [80]

1.3.2 EARLY STAGE DISEASE

1.3.2.1 Prognostic factors

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

- **Clinical factors:** age; race.

- **Pathologic factors:** primary tumour size; axillary lymph nodes involvement; stage; tumour morphology; histologic grade; peritumoural lymph-vascular invasion; hormone receptors and HER2 overexpression.
- **Markers of proliferation:** Ki67 (addressed in more detail in a separate section)
- **Genomic profiles:** PAM50; 21-gene Recurrence Score (RS); 70-gene signature; EndoPredict; Genomic Grade Index (GGI); Breast Cancer Index (BCI)
- **Emerging biomarkers:** tumour infiltrating lymphocytes (TILs)

Clinical factors

Age Both younger and older age is associated with poorer prognosis. [81] Patients aged 35 years or younger at diagnosis have a worse absolute 5-year survival (74.7 vs. 83.8 to 88.3 percent for women aged 35 to 69 years), even after adjustment for tumour stage, histopathologic characteristics and given treatments, indicating an intrinsic aggressive biology. [82,83] Women >65 years diagnosed with breast cancer have an increased mortality mainly due to later stage at diagnosis, comorbidities and less aggressive therapies. [84-86] Notably, in HER2-positive tumours age is not a factor significantly associated with prognosis both in women untreated and in those receiving trastuzumab. [87] An analysis on approximately 17.500 patients revealed that women ≤40 years diagnosed with luminal A or B breast cancer, but not those with HER2-positive tumours, had a higher risk of dying compared to older women. [88]

Race Breast malignancy in less common in black that in white women in the USA. However despite a general decrease in death rates from breast cancer, mortality in black women is still higher and racial disparities in breast cancer are likely to continue, at least for the next few years, given the increasing incidence rates among black women. [89] Racial disparities may be attributable in part to socio-economic factors (lower access to health care system and screening, delay in treatment start) and in part to higher frequency of biologically aggressive basal-like tumours among African American women. [90] Currently, there is no indication for a distinct systemic management of early breast cancer in this group. A nation-wide study of women followed between 1961 and 2007 in Sweden revealed that, although breast cancer incidence has increased over time, it is lower among immigrants (especially those from Asia and Latin-America) but not among immigrants' daughters when compared to native Swedes. Globally, mortality has decreased both for native Swedes and immigrants, whereas 20% higher case fatality among immigrants diagnosed with breast cancer in the most recent years was described. Higher socio-economic level was associated to higher incidence and lower

mortality. These disparities highlight the importance of targeting interventions on those women who are less likely to participate in screening programs and to adhere to prescribed therapies. [91]

Pathologic factors

Primary tumour size is defined as the largest diameter of the primary tumour. The 5-year survival decreases from 91% for cancer <2 cm to 63% for those >5 cm. [92] Tumour size correlates with the risk of developing metastases in axillary lymph nodes but the two factors are prognostically independent to each other. The correlation between tumour size and nodal involvement as well as risk of death is weaker in ER-negative tumours. [93]

Axillary lymph nodes involvement and number of metastatic lymph nodes is a strong and independent prognostic factor. [92] The 5-year survival rate for tumours localized to the breast vs. tumours that spread to the regional lymph nodes is 99% and 85%, respectively [94], independently of tumour size. [92] In addition, the presence of micro-metastasis (<2mm) in the examined axillary nodes is associated with worse prognosis in comparison with no metastasis whereas no difference in survival emerged between node negative patients and those with isolated tumour cells. [95,96] Occult metastases when retrospectively identified in lymph node initially judged free of metastasis have a negative impact on survival. [96,97]

Stage Combined primary tumour size (T), axillary lymph node status (N) and presence of distant metastasis (M) contribute to breast cancer staging according to the TNM system. [20] TNM is finally combined to provide an overall stage including 5 levels (or stages), from 0 to IV with decreasing rates of survival at 5 years. [98] Interestingly, the last update of AJCC staging suggestes an integrated model combining the anatomical staging of breast tumour with the molecular biomarkers for a more refined prognostic classification that recognizes intrinsic tumour biology. The proposed bio-score need to be validated on larger independent cohorts.

Tumour morphology Lobular carcinoma is associated to a lower risk of recurrence compared to ductal carcinoma in the first 6 years after diagnosis but confers a significantly higher risk after six years. [99] Tubular, papillary, mucinous, medullary and adenoid cystic carcinomas have a better prognosis while micro-papillary and metaplastic are associated with shorter survival. [75]

Histologic grade is a prognostic marker that allows risk stratification within a given tumour stage. [100] The most widely used grading system is the Nottingham histological grading, also called Elston and Ellis grading, and represents an evolution of the Bloom-Richardson Grade

system. It assesses the degree of tumour differentiation (tubule formation and nuclear pleomorphism) and proliferative activity (mitotic index) by giving a score to each of these features and then deriving a final score. Based on this final score, tumours are further divided in three groups, of whom group 1 corresponds to the well-differentiated cancers with the best prognosis and group 3 to the undifferentiated cancers with the least favourable outcome. [101,102] The clinical importance of grade 2 tumours has been largely debated. The gene expression profile of grade 2 tumours, in fact, recapitulates features of grade 1 and grade 3 tumours rather than showing a specific genomic pattern. [103] Despite concerns related to the low inter-observer reproducibility, histologic grade has been incorporated in validated prognostic algorithms such as the Nottingham Prognostic Index and Adjuvant!Online [104] and microarray-based genomic signatures for grade have been developed. [50,103,105,106] The prognostic role of histologic grade has been demonstrated in the original report [101] and subsequently validated. [107] Given concerns related to low inter-observer agreement and lack of a well-characterized clinical value for grade 2 tumours, histologic grade is not presently included in the revised TNM staging system, although it is contained in the previously discussed bio-score. [20]

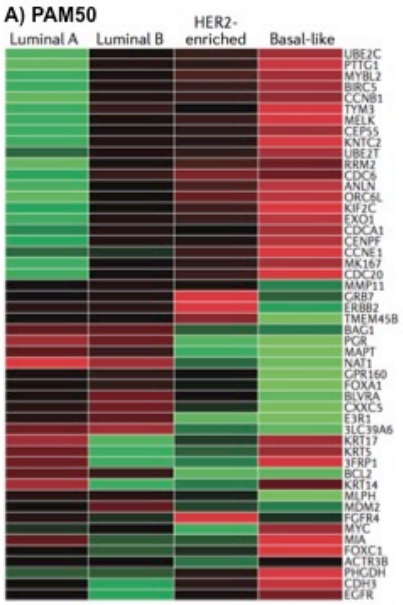
Peritumoural lymph-vascular invasion Traditionally considered a poor prognostic factor [108], particularly in higher-grade tumours, according to more recent evidences it is significantly associated to other prognostic factors and its clinical utility is to be determined. [109,110]

Hormone receptors The prognostic relevance of ER and PR has been a matter of debate for many years. Recently, an analysis on 4000 patients enrolled in four clinical trials with a follow-up of 24 years described that ER-positive tumours have a lower annual hazard of recurrence compared to ER-negative tumours during the first 5 years (9.9% vs. 11.5, p 0.01). Beyond 5 years, hazards in ER-positive cancers are higher and remain fairly stable after 10 years from primary diagnosis, regardless lymph node status. [111] PR is a well-known prognostic factor of time to recurrence and overall survival [112-114] and adds prognostic value to the IHC definition of breast cancer subtypes refining the identification of good outcome Luminal A tumours. [36] Furthermore, PR was found prognostic of survival after relapse in a retrospective cohort. [115]

HER2 In the absence of systemic therapy, HER2 overexpression is associated with poorer prognosis regardless of the axillary lymph node involvement. [116-118] HER2 retains a negative prognostic effect even in tumours ≤1cm with negative lymph nodes. [119]

Genomic profiles

Gene expression-based assays provide prognostic information beyond classical clinical and pathological variables, namely hormone receptor and HER2 status, stage, and grade. Unsupervised analyses have identified signatures associated to biological features, such as ER signaling or proliferation that led to identification of molecular "intrinsic" subtypes. Supervised analyses allowed the development of signatures prognostic of survival. Figure 6 presents a list of the genes used in the commercially available multigene assays.



B)

Recurrence Score					
Reference	**Proliferation**	**Estrogen ER**	**Invasion**	**HER2**	**GSTM1**
β-actin	Ki-67	PR	Stromelysin 3	GRB7	**BAG1**
GAPDH	STK15	Bcl2	Cathepsin L2	HER2	**CD68**
RPLPO	Survivin	SCUBE2			
GUS	Cyclin B1				
TFRC	MYBL2				

C)

70- gene signature	
Cancer cell phenotype/function	**N of genes**
Cell-cycle dysregulation	15
Angiogenesis	12
Proliferation and oncogenic transformation	11
Invasion and metastasis	8
Growth factor signal transduction	6
Resistance to apoptosis	2
Miscellaneous/unknown function	16

D)

Breast Cancer Index	
HOXB13	NEK2
IL17BR	RACGAP1
BUB1B	RRM2
CENPA	H/I

E)

EndoPredict	
Reference genes	**BIRC5**
CALM2	**RBBP8**
OAZ1	**UBE2C**
RPL37A	**IL6ST**
	AZGP1
	DHCR7
	MGP
	STC2

Figure 6. *Genes comprised within the multigene assays: A) PAM50; B) 21-gene Recurrence Score; C) 70-gene signature; D) Breast Cancer Index; E) EndoPredict (Modified from Kwa M. et al, Nature Reviews Clinical Oncology 2017)*

For most of these assays, clinical utility has been demonstrated. Table 1 summarizes the main features of commercially available gene signatures and provides an overview of the studies used in order to train and validate these gene arrays.

Gene array	Genes (n)	Source	Platform	Training set	Initial validation set	Output data	Clinical application
PAM50	55*	FFPE/FF	qRT-PCR/ microarray / nCounter	189 ER+/-, LN +/- tumour samples and 29 non-malignant breast tumour tissue samples	786 ER+/- tumour samples from L+/- disease	Lumina A Luminal B HER2E Basal- like Normal - like Risk of Relapse score (low-medium-high)	Prognosis in post-menopausal women with ER+, LN+/- disease of stage 1 or 2
21-gene signature	21	FFPE	qRT-PCR	447 ER+/-, LN+/- tumour samples from three randomized trials	Largely ER+/ LN- tumours in the tamoxifen arm of the NSABP B-14 trial (including samples from the training set)	Recurrence Score (low-intermediate-high)	Recurrence risk in patients with ER+, LN- tumours
70-gene signature	70	FFPE/FF	Microarray	78 ER+/-, LN- tumours, <5 cm from patients aged <55 years	295 ER+/-, LN+/- tumours, <5 cm from patients <53 years of age (including samples from the training set)	Continuous variable (good-bad prognosis)	Distant relapse free survival prediction in ER+/-, LN- tumours
Breast Cancer Index	7	FFPE	qRT-PCR	60 ER+ tumours samples from patients previously treated with tamoxifen	588 patients with ER+, LN- tumours enrolled in the Stockholm trial	Continuous variable (low-intermediate-high)	Distant relapse free survival prediction in ER+/-, LN- tumours. Prediction of benefit from extended adjuvant
EndoPredict	11	FFPE	qRT-PCR	964 ER+, LN+/- tumours from patients treated with tamoxifen	378 ER+/LN +/- tumours from the tamoxifen arm of the ABCSG-6 trial and 1,324 patients from the ABCSG-8 trial	EPclin continuous score (low- high)	Risk of recurrence at 10- years in ER+, LN+/- tumours. Risk of recurrence after 5-10 years of endocrine therapy
Genomic Grade Index	97	FF	Microarray	64 ER+ tumours of histological grade 1-3	125 ER+/- tumours; histological grade 1-3, LN- tumours	Continuous variable (low-high)	Prognosis and risk stratification based on histological grade

*5 genes for expression normalization
Abbreviations: FFPE, formalin-fixed parrafin- embedded; FF, formalin-fixed; ER, estrogen receptor; LN, axillary lymph nodes; qRT-PRC, quantitative reverse transcription polymerase chain reaction.

Table 1. *Overview of the commercially available gene expression signatures in breast cancer*

PAM50, introduced in 2009 by Parker and coll., characterizes individual tumours by intrinsic subtypes using a set of 50 genes (Figure 6A). [49] PAM50 provides a continuous ROR-score (ROR-S), which ranges from 0 to 100 and stratify patients with ER-positive disease in low, medium and high-risk subgroups on the basis of the 10-year risk of recurrence. The test is performed on formalin-fixed, paraffin-embedded (FFPE) samples with high degree of analytic validity. [63] PAM50 was developed using microarray and quantitative reverse transcription polymerase chain reaction (RT-PCR) data from a set of approximately 190 ER-positive and negative/lymph node positive and negative prototypes [49] and validated in a set of over 700 samples. [63] In this cohort ROR combined to tumour size (ROR-T) showed prognostic ability

beyond classical clinicopathologic markers including Ki67, PR and grade. A ROR model weighted for 11 proliferation genes (ROR-P), among the overall 50 genes, further enhanced the prognostic value of ROR score. The clinical utility of PAM50 and ROR has been validated in several cohorts. [63,120-123] Using data from two separated trials, PAM50 and ROR added prognostic information to that derived by clinical factors. In an analysis in patients treated with adjuvant anastrazole or tamoxifen, ROR showed a continuous relationship with risk of distant recurrence at 10 years, regardless of nodal involvement. [121] The findings were confirmed in a cohort of post-menopausal women enrolled into the ABCSG-8 trial. [123] Two subsequent analyses in the same cohort revealed that PAM50 and ROR are also predictive of local recurrence [124] and accurately differentiate patients on the basis of the risk of relapse beyond 5 years from primary diagnosis. [125,126] Importantly, PAM50 may identify patients, within those who are diagnosed with HER2-postive tumours, who express genes of the basal-like pattern and are less likely to respond to trastuzumab [127] as well as those who are classified as HER2-enriched and benefit the most from dual HER2-blockade. [58] Currently, the Nanostring's technology used for quantification in the Prosigna assay has been validated using FFPE breast tumour samples across multiple laboratories [128] and has been approved in 2013 for use in post-menopausal women with hormone receptor positive lymph node positive/negative disease.

The **21-gene RS** is among the earliest and best-validated prognostic assays in early breast cancer. Presently, there is strong evidence supporting the use of RS for recurrence prediction in ER-positive, HER2-negative, node negative cases and to guide decision regarding adjuvant chemotherapy. [129] Based upon 16 tumour-associated and 5 controls genes (Figure 6B), the signatures provide a continuous RS computed with mathematical algorithms. The score, which ranges from 0 to 100, categorizes patients into 3 risk categories, as follows: low-risk, RS <18; intermediate-risk, RS 18-30; high-risk, RS ≥31. [130] The 21-gene RS was developed by identifying the 250 most promising candidate genes in the original training set, which included 447 samples from patients enrolled in three separated trials, as described by Paik and coll. [130] A RT-PCR method was used in order to quantify the expression levels of the candidate genes. The higher expression of genes in the ER-pathway, GSTM1, BAG1 is associated with favourable prognosis and results in low RS, whereas expression of proliferation related genes, such as Ki67 and cyclin B1, genes within the HER2 and invasion pathway produce higher RS score. RS has been validated as prognostic tool to identify very low-risk patients among those with ER-positive, HER2-negative, node negative tumours, which could be safely spared from chemotherapy. [131] Presently, the RS lowest cut-off

supporting clinical decisions on chemotherapy remains unclear due to different proposed thresholds. Indeed, the most of the "prospective-retrospective" analyses appointed <18 as a cut-off to separate low and intermediate-risk cancers [130,132-134], while Sparano et al. in TAILORx study prospectively validated the ≤10 threshold. [131] The TAILORx study was designed to prospectively validate the 21-gene RS in a population of patients with ER-positive, HER2-negative, node negative tumours for whom adjuvant chemotherapy was indicated based on clinicopathologic features (tumour size >1.1 cm or 0.6-1.0 cm but intermediate-high histologic grade). The first results of the TAILORx trial indicated that patients with RS ≤10, appointing a very low-risk of relapse, may forgo adjuvant chemotherapy and receive endocrine therapy alone. In fact, in this group risk of distant relapse was less than 1%, of any relapse was in the range of 2-5% and overall survival rate 98% at 5-year follow-up. In an exploratory analysis following the publication of the TAILORx study, we retrospectively identified patients (n=908) in the Stockholm regional registry diagnosed during 2005-2006 with ER-positive, HER2-negative, lymph node negative tumours that were treated with adjuvant endocrine therapy alone. We found that Ki67 ≤10% identified patients whose outcome is in the same range as that in the population by Sparano et al., and might represent a surrogate to RS in identifying patients to be treated with adjuvant hormonal treatment alone. [135] Additionally, Plan B trial demonstrated that high-risk patients according to clinical factors but low-risk according to RS ≤11 and not receiving adjuvant chemotherapy had the same outcome as tumours classified as intermediate-risk cancers (RS 12-25) treated with chemotherapy and better prognosis than high-risk cancers (RS>25) receiving chemotherapy. The trial also highlighted the need of integration of gene assay and clinicopathologic factors given a substantial discordance between each other. [136] Whether RS is of aid in the decision-making on adjuvant treatment in node positive tumours is matter of on-going debate. In fact, although there is evidence in favour of the use of RS in predicting benefit from adjuvant chemotherapy [137-139], especially in presence of limited nodal disease [136], data are not conclusive and a higher degree of caution in presence of node positive tumours is required in the light of the higher absolute risk of relapse. The results from the RxPonder trial will more exhaustively clarify on the topic. [140] Finally, the use of 21-gene RS signature in hormone receptor negative tumours is not supported. [129]

The **70-gene assay** was the first multigene test approved by FDA in 2007. Initially developed by Agendia (Amsterdam, The Netherlands) based on Agilent microarray-based platform (Agilent Technologies, Santa Clara, CA) using unfixed fresh-frozen tissue, it has now been adapted and validated for use on FFPE tissue. [141,142] On the basis of a supervised DNA-

analysis, the 70 genes comprised in this expression signature were selected among 25.000 genes from a training set of 78 patients all aged <55, with no nodal involvement and not receiving systemic adjuvant treatment. [143] These genes are mainly related to proliferation, invasion and angiogenesis and are associated to tumour progression and metastasis (Figure 6C). A mathematical formula allows separate patients into two risk groups according to the likelihood of developing distant metastasis at 5 years. The clinical validity of this signature has been demonstrated in several cohorts, some of them including tumours with lymph node metastasis. [144-150] The RASTER study was an observational study that provided the first prospective validation of 70-gene assay clinical utility. Patients who were classified as low-risk according to the 70-gene signature and omitted chemotherapy had an excellent prognosis at 10-years, regardless the clinical-risk. [151] Recently, the MINDACT trial suggested that the 70-gene signature may identify patients with low-risk of distant recurrence among those with high clinical risk. Risk assessment was made on the basis of Adjuvant!Online and the 70-gene assay. Women with discordant risk prediction were randomly assigned to adjuvant chemotherapy or endocrine therapy. Approximately 80% of the enrolled patients had negative axillary lymph nodes. In the discordant group, women with high clinical but low genomic risk who received chemotherapy had 95.9% rate of metastasis-free survival at 5 years vs. 94.7% for those treated with endocrine treatment alone. However, the study was not powered to exclude a benefit from chemotherapy and did not demonstrate a clinical usefulness in demonstrating efficacy of chemotherapy in the small subset of women diagnosed with clinical low-risk/genomic high-risk tumours. [152] Based on these results, ASCO guidelines support the 70-gene signature as a tool for decision on withholding chemotherapy in high-clinical/low-genomic risk malignancies that are hormone-receptor positive, HER2-negative and have no or limited nodal disease. [153]

Breast Cancer Index (BCI) derives from the combination of two profiles, the HOXB13-to-IL17BR expression ratio (H:I ratio) and the Molecular Grade Index (Figure 6D). Using genome-wide microarray analysis, risk of recurrence in ER-positive tumours treated with tamoxifen was associated with the differential expression of 3 genes: the anti-apoptotic homeobox B13 (HOXB13, overexpressed in recurrent cancer treated with tamoxifen), and interleukin 17B receptor (IL17BR) and EST AI240933 (both overexpressed in non-recurrent cancers treated with tamoxifen). The H:I ratio correlated with clinical outcome, disregarding other established clinical prognostic factors. [154] The Molecular Grade Index evaluates the expression of 5 gene related to histological grade and tumour progression (Figure 6D). The combination of H:I ratio and Molecular Grade Index has more predictive value than the two

of them separately. [155] An analysis on 588 patients from the Stockholm trial, which compared the efficacy of tamoxifen vs. placebo, demonstrated the prognostic utility of H:I ratio and Molecular Grade Index. A BCI continuous score was derived and patients separated in 3 risk-subgroups accordingly. Overall >50% were assigned to the low-risk group and <3% of them experienced a relapsed over 10 years. [156] BCI might predict endocrine responsiveness and identify patients more likely to benefit from extended adjuvant endocrine treatment. [157-159]

EndoPredict Based upon the expression of 8 cancer-related genes and 3 reference genes quantified by RT-PCR (Figure 6E), EndoPredict was initially validated in two trials (ABCSG-6 and ABCSG-8), which demonstrated that this assay has independent value regardless classical prognostic factors. The gene signatures combined with tumour size and nodal status provides a risk score, EPclin, that stratifies patients into two categories on the basis on the recurrence risk at 10-years. [160] EndoPredict showed ability in identifying very low-risk tumours among those that are ER-positive and HER2-negative and is predictive of late recurrence. [161] Interestingly, EPclin identifies a subset of patients with very low risk of recurrence after 5-10 years of endocrine therapy and those who might be spared from extended adjuvant endocrine therapy. [161] The test performed in FFPE samples from core biopsies or surgical specimens produces reproducible and accurate results. [162,163] In the GEICAM 9906 trial, EndoPredict was able to independently predict the risk of relapse in low- and high-risk categories according to EPclin but did not demonstrate ability to predict the benefit deriving from adding paclitaxel to antracyclines. [164] Furthermore, EndoPredict and EPclin were highly prognostic of recurrence after endocrine therapy. EPclin was more prognostic than RS and this is in part, but not entirely, attributable to the fact that EPclin integrates molecular and clinical information from tumour size and nodal status. [165]

Genomic grade index (GGI) refers to a list of 97 genes that showed ability in distinguishing histological grade 1 from histological grade 3. [103,166] While in the initial report it was described that GGI reclassified grade 2 tumours improving the prognostic value of tumour grade [103], in later validation cohorts of untreated or tamoxifen-treated patients GGI was found to perform independently and equally well as RS as a prognostic marker [166], and to outperform histological grade and proliferation biomarker including Ki67. [167] However, grade 2 tumours may be also accurately separated in risk categories by the cost-effective Ki67 [168] and, thus, further studies are required to prove the clinical utility of GGI. High expression levels of the genes within the GGI assay are strongly associated to high histological grade and

ER-negative status as well as to higher response rate to neoadjuvant chemotherapy but also to poorer prognosis after systemic chemotherapy. [169]

Emerging biomarkers

Tumour infiltrating lymphocytes (TILs) Level 1b evidence suggests that TILs are prognostic in the context of adjuvant chemotherapy in TNBCs [170,171] but cannot be used for withholding chemotherapy. The analytic validity of TILs has been matter of debate due to low inter-observer reproducibility and the lack of an established clinically relevant cut-off. [170] However, reproducibility of TILs assessment appears feasible and recommendations have been published in order to strengthen the clinical relevance of this immunologic biomarker. [172,173]

1.3.2.2 Predictive factors

Predictive factors enable treatment tailoring by providing tools for the identification of subjects with higher or lower likelihood to respond to a certain treatment. In this way an enhancement in response to treatment may be achieved and non-responsive patients may be spared from unnecessary therapies. The results of a metanalysis of over 100.000 patients included in 123 trials revealed that benefit from adjuvant chemotherapy is independent of age, ER status, grade, tumour size, nodal involvement and adjuvant tamoxifen. [174]

The only well-established predictive markers in early breast cancer are ER and HER2.

ER The predictive role of ER has been extensively described in a metanalysis of over 20.000 patients from 20 trials of adjuvant tamoxifen vs. no tamoxifen. In ER-positive tumours, tamoxifen was related to 39% and 30% decrease in the risk of recurrence and death at 15 years, respectively. The results were independent of PR, age, nodal status and use of chemotherapy. By contrast, tamoxifen did not affect survival in patients with ER-negative disease. PR-positive tumours have a better prognosis when treated with tamoxifen. However, the expression of PR is dependent on ER and the predictive role of PR is thus unclear, especially when ER status is known. [175]

HER2 is a predictive factor of response to HER2-targeted therapies and the benefit from trastuzumab in HER2-positive tumours has been well described both in the early [176-178] and in the advanced setting. [179-181]

In addition to these findings, some evidence of the predictive role of genomic signatures and Ki67 (see section 1.4 Markers of proliferation) is available.

Genomic profiles may predict benefit from chemotherapy but cannot guide the choice of which type of cytotoxic drug should de administered. These findings have been discussed in the previous section. Emerging evidence from a phase II neoadjuvant trial revealed that a PAM50-based chemo-endocrine score predict response to chemotherapy in hormone receptor-positive cancers. [182]

1.3.3 ADVANCED BREAST CANCER

Metastatic breast cancer (MBC) is treated with palliative intention. Median overall survival in metastatic breast cancer approximates two years but can range from few months to many years. [14] The identification of prognostic factors of survival and predictive markers of response to therapy in this setting may contribute to a deeper understanding of the large variability in clinical outcome and aid the selection of more specific treatments.

1.3.3.1 Prognostic factors

Relapse free interval Patients who experienced a disease recurrence ≤2 years from primary diagnosis have a poorer prognosis after relapse in terms of time to relapse and overall survival. [14,183-185]

Age The effect of age on survival after recurrence in not clearly established. However, older age at the diagnosis of MBC might be prognostic of poorer survival [14] likely due to comorbidities, poorer performance status and less intensive anti-cancer treatment. [85]

Site of metastasis Chest-wall, skeletal and lymph node metastases are associated to a better post-relapse prognosis in comparison with visceral metastases. [14,183,184,186]

Adjuvant systemic treatment Disseminated use of adjuvant systemic therapies may be associated with worse survival after relapse, likely due to the selection of cellular clones with more aggressive features and resistance to treatment. [14]

Hormone receptor and HER2-status Hormone receptor positive primary breast cancers have a longer survival in the advanced setting. Tumours overexpressing both ER and PR have a better prognosis than those expressing either ER or PR. [187] HER2-positive tumours in pre-trastuzumab era, and TNBCs have poorer prognosis. [185,188,189] When clinically feasible, re-assessment of hormone receptor status and HER2 should be performed at least once in metastatic setting (level of evidence 1b, see paragraph 1.6 for a detailed discussion). [190,191] Change in ER status from positive to negative in primary tumour tissue and metastasis has been associated to higher risk of death compared with stable ER status. [192] The discordance in HER2 status was also related to shorter post–relapse survival. [193,194]

Genomic profiles Early evidence suggests that PAM50 and the 21-gene RS assay may be useful for prognostication in MBC.

Intrinsic subtypes have prognostic relevance in the advanced setting. A retrospective analysis from the Swedish multicentre randomized TEX study, in which 111 patients had a biopsy from loco-regional or distant relapse, described a shorter survival for basal-like and HER2-enriched subtypes when compared with Luminal A. [195] Similarly, the Luminal B, basal-like and HER2-enriched tumours, as assessed from primary tumour or metastasis tissue, were associated with worse progression-free survival and overall survival in post-menopausal women treated with letrozole with or without lapatinib as first line treatment for MBC. [196] Moreover, the 21-gene RS was found prognostic of progression-free survival and overall survival in a prospective registry study on de novo stage IV breast cancer. [197]

In order to define the prognostic role of IHC markers and gene expression signatures of the primary tumour in MBC, we performed a population-based analysis and compared the prognostic performance in term of post-relapse survival of different prognosticators in a cohort of women diagnosed with systemic disease and for whom tissue from primary tumour was available for IHC and RNA analysis. (Paper II: Falato C et al., Mol Oncol 2015).

1.3.3.2 Predictive factors

Hormone receptor and HER2 status Overall, 14-32% and 10% discordance rate in hormone receptors and HER2, respectively, between primary tumour and corresponding metastasis has been estimated. [192,194] Beyond a proved prognostic role, the new expression pattern of ER, PR and HER2 may be of predictive value and according to expert opinions treatment should be guided by the phenotype of the metastasis rather than that of the primary tumour. [190]

Genomic profiles Retrospective evidence from a prospective trial suggested a predictive role for HER2-enriched subtype in ER-positive/HER2-positive tumours treated with lapatinib. [196] Prospective evaluation of the findings is needed.

Emerging biomarkers

Androgen receptor TNBC is a highly heterogeneous entity that comprises multiple molecular subtypes and oncogenic drivers. Among these, the Luminal Androgen Receptor (AR) subtype, which well recapitulates the non-basal group within TNBCs, may be driven by AR-signalling and appear to have a more indolent behavior. [69,198] Enzalutamide, a potent inhibitor of AR approved for the treatment of prostate cancer, has been tested in a single arm phase II trial leading to an improvement in terms of progression free survival and overall survival, especially in AR-positive tumours by IHC. [199] However, phase III trials testing enzalutamide in breast cancer have been put on hold since enzalutamide development in breast cancer has been suspended.

Germline BRCA mutations Among patients with HER2-negative metastatic breast cancer, a germline BRCA mutation may predict benefit from olaparib, a PARP-inhibitor, as compared with standard chemotherapy. [200] Interestingly, the benefit from olaparib was superior in TNBCs suggesting that the combination of two or more biomarkers can enhance the identification of the best target population. Moreover, evidence suggests that platinum agents are particularly active in TNBCs with germline BRCA 1/2 mutation. [201,202]

PI3K-AKT-mTOR pathway PIK3CA gene is mutated is one third of breast cancers and the most of the mutations are activating. [71] The efficacy of buparlisib, a pan-PI3K inhibitor, in combination with fulvestrant, a selective ER degrader, has been demonstrated in advanced breast cancer but not further investigated due to unacceptable toxicity. [203] PI3Kα inhibitors,

alpelisib and taselisib, combined with fulvestrant, have shown clinical activity, especially in PI3K-mutant tumours, and better tolerability than buparlisib in previous phase I and II trials [204,205], and are now under investigation in Phase III trials. [206,207]

Estrogen-receptor 1 (ESR1) Pre-clinical and clinical evidence suggests that mutations of ESR1 gene promote ligand-independent activation of ERα in breast cancer conferring resistance to current anti-hormonal agents that cause oestradiol deprivation, namely aromatase inhibitors, but not to fulvestrant. The mutation, which is very rare in primary tumours but mutated in approximately one third of patients treated with an aromatase inhibitor, is associated with a more aggressive phenotype. Besides metastatic tumours tissue, ESR1 mutation can be detected in plasma through circulating-tumour DNA-sequencing. [208-210] Furthermore, retrospective analysis showed that ERS1-mutation is polyclonal and may be also found in cellular clones before treatment with aromatase inhibitors, indicating its potential use as a predictive factor for selection of hormonal therapies. [211-213]

Somatic mutations in HER2 TK-domain Prevalently activating, mutations of the HER2 TK-domain are heterogeneous and particularly frequent in invasive lobular carcinomas. These mutations are associated with poor survival in HER2-negative breast tumours [214,215] and might represent a target for irreversible TK inhibitors, such as neratinib. [216,217] Studies specifically investigating how distinct mutations in HER2 TK-domain may predict response to therapy are needed in order to account for the high heterogeneity in the mutation pattern.

1.3.4 LOCO-REGIONALLY RECURRENT BREAST CANCER

Isolated local recurrence, referred as reappearance of cancer in the ipsilateral preserved breast or chest-wall after mastectomy, and loco-regional recurrence involving also ipsilateral regional lymph nodes (more frequently axillary and supraclavicular but also infraclavicular and/or internal mammary), is a negative prognostic factor, leading to a higher risk of distant relapse and poorer overall survival. [218,219] Overall, a multidisciplinary therapeutic approach of the loco-regional disease is recommended and has been related to a better post-relapse outcome. [220] After primary breast conserving surgery and radiotherapy, mastectomy is the standard surgical approach in case of isolated local relapse and may provide long-term survival. [221,222] Although similar prognosis has been described in case of loco-regional tumour reappearance after breast-conserving surgery and after mastectomy [223], post-mastectomy

recurrences tend to occur earlier and more often involve regional lymph nodes. These factors are both recognized as negative prognostic factors of post-relapse survival. [223-225]

1.3.4.1 Prognostic factors

Regardless of the type of primary surgery, established prognostic factors after a loco-regional reappearance of breast cancer are:

- **Invasive disease** Invasive local recurrence is associated with a higher likelihood of developing a second local relapse and/or distant relapse as well as poorer post-relapse survival compared to a non-invasive relapse. [226,227]
- **Skin involvement** A recurrence involving skin is associated with a worse post-relapse prognosis in comparison with a recurrence not including skin. [228]
- **Extensive local recurrence** A local recurrence discovered by physical examination, and thus more extended, has a worse outcome in comparison with a recurrence diagnosed by mammography alone. [229]
- **Disease free interval** Patients who experience a loco-regional relapse within 2 years from primary surgery have a poorer 5-year overall survival compared with those who recurre more than 2 years after the primary diagnosis. [218]
- **Age** Younger age at primary diagnosis (≤35 year) was associated with poorer outcome. [230]
- **Larger primary tumour size, axillary lymph node positive status, ER-negative primary tumour, adjuvant chemotherapy** are related to shorter post-relapse survival. [218,219,231,232]

1.3.4.2 Predictive factors

There are no specific predictive factors to guide treatment after curative surgery of a local recurrence of breast cancer or after occurrence of a loco-regional recurrence. All ER-positive tumours should receive post-operative endocrine treatment [233], while greater benefit from chemotherapy has been demonstrated for ER-negative in comparison with ER-positive tumours according to a single randomized trial. [234] All HER2-positive tumours should receive

HER2-targeted therapies. The reassessment of hormone receptor and HER2 is strongly endorsed and the systemic therapeutic approach should be guided by the pathologic characteristic of the recurrence. [234] However, recommendations on a standardized therapeutic approach after surgery for an isolated local recurrence are presently still awaited.

Survival after isolated local recurrence or loco-regional recurrence of breast cancer and how prognostic factors modulate prognosis has been matter of intense research. Several retrospective analyses from prospective trials have been published but information from population-based cohorts is scarce. . In Paper IV we explored how prognosis after loco-regionally relapsed disease has evolved over the past three decades at the population level and whether trends in survival exist. (Paper IV: Falato C et al., manuscript)

1.4 MARKERS OF PROLIFERATION

Proliferation rate in breast cancer can be assessed by several methods, including mitotic counts, S-phase fraction by flow-cytometry, and IHC using monoclonal antibodies direct against antigens expressed in proliferating cells, such as cyclin A and Ki67. The most commonly used method to determine the rate of proliferative breast cancer cells is to evaluate the percentage of nuclei stained with the monoclonal murine antibody Mib-1, which detects the protein Ki67 with higher sensitivity than other comparable antibodies in FFPE samples. [32] Many studies demonstrated a strong correlation between Ki67 and other well-known proliferation markers. [235,236]

1.4.1 KI67

The prognostic value of Ki67 has been extensively investigated. [33] Despite inconsistencies in assessment methods and cut-points, two large meta-analyses consistently recognized Ki67 as a prognostic factor in early breast cancer, regardless of axillary lymph node status. [237,238] However, retrospective nature of the majority of the studies exploring Ki67, variability in used antibodies, techniques and scoring protocols prevent a harmonious data interpretation. More recently, a retrospective analysis of clinical trials with centralized assessment of Ki67

described an independent value of Ki67 as a prognostic marker of disease-free survival, while only 1 out of 5 trials confirmed an independent association with overall survival. [33]

An IHC4 surrogate composed by centrally assessed ER, PR, HER2 and Ki67, which are combined in a weighted equation used to derive a IHC4 score, has been proposed as a cost-effective alternative to PAM50. [239] Some studies have showed good performance of IHC4 in separating Luminal A and B [35] as well as equivalence with RS in survival prognostication [239], while other studies reported worse results for IHC4 in comparison with PAM50. [63] At present, ASCO and IMPAKT working group do not recommend Ki67 and IHC4 to assess prognosis in breast cancer. [129,240]

Ki67 as a predictive factor in the adjuvant and neo-adjuvant setting is more controversial with conflicting results from different studies. Indeed, some analyses showed that Ki67 could be predictive of benefit from taxanes [241,242] whereas other studies revealed that Ki67 might predict response to chemotherapy but not specifically to taxanes. [243] In addition, Ki67 was not predictive of response either to anthracyclines vs. first generation chemotherapy [244] or first generation chemotherapy plus endocrine therapy vs. endocrine therapy alone. [245] However, Ki67 was significantly associated to benefit from earlier treatment with aromatase inhibitors compared to their delayed administration after 2-3 years of tamoxifen. [246] In the neoadjuvant setting, no evidence indicating an association between pre-treatment Ki67 and response to treatment emerged from clinical trials testing chemotherapy, endocrine therapy or their combination. [33] Change in Ki67 during neoadjuvant endocrine treatment generated greater interest for its potential use as a marker of therapy benefit. In particular, early change in Ki67 after two weeks of hormonal treatment has been demonstrated to precede and significantly correlate with clinical and pathological response and to predict long-term outcome. [247-249] Of interest, change in Ki67 levels was also shown during neoadjuvant therapy with everolimus and letrozole vs. placebo and letrozole, indicating anti-proliferative effect of everolimus. [250]

In the metastatic setting, high Ki67 from primary tumour tissue or locally recurrent tumours was associated to shorter post-relapse survival and to response to first line treatment with aromatase inhibitors. [251,252]

1.4.1.1 Cut-off value of Ki67 for clinical use

Ki67 is a continuous variable expressed as the percentage of positively stained cell nuclei within the scored area. Ki67 follows a lognormal distribution and the log-transformed value for Ki67 should be used for statistical purposes. [253] Many methods have been used to derive the best cut-off(s) for Ki67 categorization. [254] Although most of the studies recognized the prognostic value of Ki67, the comparability between them is low due to several limitations such as the wide range of the used cut-points. [235] Interestingly, in a prospective cohort Ki67 has been found prognostic over a wide range of cut-points suggesting the data-derived cut-point optimization (i.e. based on ROC curves, minimum sensitivity or specificity, significance of correlation with survival variables by log-rank test) may be suboptimal. [255] Recently, a panel of experts has recommended the use of Ki67 at very low (<10%) or very high >25% cut-off for prognostication purposes, in combination with other established prognostic determinants in breast cancer. [256]

Whilst the clinical validity of Ki67 as a prognostic marker in early breast cancer has been widely demonstrated and the instability of hormone receptors and HER2 expression between paired primary tumour and metastasis well recognized, the prognostic implications of Ki67 in the metastatic setting as well as the pattern of variation in Ki67 index during disease progression is not well characterized. In Paper I we sought to address the prognostic role of Ki67 as assessed in metastatic tissue and the clinical relevance of its change during metastatic progression. (Paper I: Falato C et al., Breast Cancer Res Treat 2014)

1.5 IDENTIFICATION OF LOW-RISK CANCERS

In the era of precision medicine to guide therapy decisions, a clinically relevant question is the identification, based on histopathological surrogates, of very low-risk primary cancers that could be safely spared from chemotherapy in the adjuvant setting. Very low-risk tumours have been identified exclusively in the Luminal A category by gene expression profiles [49,257] and have shown to be relatively resistant to chemotherapy. [42] Notably, the most of the gene expression signatures concordantly identify a subset of Luminal A tumours with negative axillary lymph nodes, which could safely receive adjuvant endocrine therapy alone. [258]

Biologically, the most distinguishing features between luminal A and B subtypes is the higher expression of proliferation-related genes in the latter. [35,63] Luminal B, that accounts for about 20% of all Luminal tumours, have also higher RS as assessed by the 21-gene assay and poor 70-gene prognostic signatures. [257] In a pivotal analysis, we have demonstrated that a threshold of 10% for Ki67 performs as good as the 21-gene RS signature in appointing very low-risk tumours within the ER-positive/HER2-negative/axillary lymph node negative cancers treated with adjuvant endocrine therapy alone.[135] Moreover, PAM50 ROR has been shown to provide more information, beyond clinical prognostic factors, than RS but at least as much information as the standardized IHC surrogate (IHC4), using 14% as a cut-off for Ki67.[121] Nonetheless, the optimal Ki67 cut-point is presently still widely debated due to contrasting evidence in favor [35,259] or against the 14% threshold. [260] We described that Ki67, at different cut-points, and gene signatures do not harmoniously stratify patients in good or poor prognosis subgroups. [261] Thus, given the extreme biologic heterogeneity of luminal B tumours and the controversial reproducibility of the IHC biomarkers, especially for Ki67, the clinical issue of the identification of a strong histopathological surrogate to PAM50 in the characterization of Luminal A and Luminal B tumours remains to be solved.

Based on this evidence, we explored whether IHC biomarkers and gene expression profiles might improve long-term prognostication when combined to each other rather than when used separately. The findings of our study are detailed in the section "Results" (Paper III: Lundberg A et al., CCR 2017).

1.6 Phenotypic and Genomic Heterogeneity between primary tumour and metastasis

Two prospective trials and a numbers of retrospective analyses showed that ER, PR and HER2 are discordant across primary tumour and metastasis in a consistent proportion of patients. [192,194,262-264] Change from ER-positive to ER-negative as well as discordance in HER2 status in primary tumour and corresponding metastasis has been associated to a worse post-relapse prognosis as compared with stable receptor levels. [192-194] Moreover, results from prospective trials revealed that the change in biomarker expression during tumour progression led to a change in disease management in 1 every seven patients undergoing a relapse biopsy.

[265] More recently, the instability in molecular subtypes has been studied and a wide range in the rate of subtype conversion has been demonstrated, from 0% for basal-like tumours to 55% for Luminal A tumours. [266] Luminal A tumours tend to turn into Luminal B and, less frequently, into HER2-enriched subtypes reflecting tumour evolution and acquisition of oestrogen independence. Of interest, compared to primary tumours, metastases appear to be more enriched in proliferation and migration-related genes while depletion of luminal-related genes has been described in metastasis compared to matched primary tumours. [266]

The discrepancy in receptor pattern between primary tumour and relapse can be attributed to several factors and, although technical artefacts may account among them [267], a biological basis is present in many cases. Traditionally, primary breast cancers exhibit high intra-tumour heterogeneity with varying metastatic potential. [268] Cancer biology, in particular its intrinsic genomic instability, selective pressure by adjuvant therapy driving to the selection of resistant and more aggressive cellular clones and undetected small cellular sub-clones at the initial diagnosis are among the hypothesized mechanisms potentially generating alterations in tumour phenotype during disease progression. [268-271] Recent studies on next generation sequencing shed new light on the heterogeneity between primary tumour and metastasis and reinforced the hypothesis of a clonal genomic evolution at the bottom of the inconsistency in receptor expression pattern. Indeed, while initial studies identified very few differences between primary tumours and their metastatic deposit from a mutational perspective [272-274], higher degree of heterogeneity has later emerged with a more comprehensive genomic characterization. Primary tumour and metastasis are known to share mutations prevalently in frequently mutated genes, such as mutations in TP53, PIK3CA, GATA3, among others. However, in many distant relapses driver mutations private to metastasis have been found. On average, metastases contain an additional 63% mutational load compared to the primary tumour. Driver mutations acquired late encompass a wider range of cancer genes, are more likely the result of selection pressure from previous treatments and may more closely reflect metastatic environment and immune response. [275-278] Prototypical example of standard treatment pressure is represented by the mutation of ESR1 gene in metastasis of patients previously treated with an aromatase inhibitor. [275] Potential therapeutic implications deriving from this mutation have been discussed above.

AIM OF THE THESIS

The general aim of the thesis was to gain insights into the prognostic role of tumour proliferation and its interplay with other prognostic factors in breast cancer.

More specifically, the studies included in the thesis investigated:

-The prognostic role in terms of post-relapse overall survival of Ki67 as assessed in the first site of distant metastasis. Whether Ki67 rate changed from primary tumour and matched metastasis with an impact on survival was also investigated (Paper I).

-The prognostic value after diagnosis of systemic disease of IHC-and gene expression-based subtypes as well as genomic profiles from the primary tumour with a focus on the PAM50 proliferation signature, beyond classical clinical and pathological prognostic determinants in breast cancer (Paper II).

-Whether the addition of Ki67, on its own or as integrated to ER, PR and HER2, to genomic signatures and vice-versa may increase the prognostic performance in terms of breast cancer specific survival in early breast cancer (Paper III).

-How survival after diagnosis of locally and loco-regionally recurrent breast cancer has evolved over three decades. Age-related trends in survival were also explored (Paper IV).

PATIENTS AND METHODS

3.1 DATA SOURCE

Since 1958 cancer registration is statutory in Sweden and all incident tumours must be reported to the Swedish Cancer Registry, whose rate of completeness is over 96%. [279] The National Cancer Registry was founded with the aim to provide a database, which may allow mapping the occurrence of cancer, monitoring changes in incidence, survival and mortality, facilitating clinical and epidemiological research as well as international comparisons. [280] National cancer registration is based on data collected regionally through six Regional Cancer Centers. The Stockholm Breast Cancer Registry (SBCR) was established in 1976. All new cases of breast cancer in Stockholm County have been registered since then and the registry is continuously updated with the clinical follow-up and treatment of each patient. The vital status is instantly updated by cross-linking to the national registry. The Regional Cancer Centers host 28-quality registers, which are decentralised registers administered via a common platform, INCA (Information Network for Cancer Care) that is jointly maintained by the Regional Cancer Centers and replaced the regional registries in 2007. SBCR has 99% completeness for women 75 years or younger at breast cancer diagnosis. [281,282] The quality registries are often used for population-based epidemiologic studies.

Each individual in Sweden is provided with a personal Swedish identification number, a unique twelve-digit number instituted in 1947 and assigned to all individuals at birth or when migrating to Sweden. Data from the population and health registers in Sweden are linked by the Swedish identification number that was used for our studies in order to identify patients within the SBCR.

For all projects included in this thesis, registry data collection and analysis was approved by the Ethics Committee at Karolinska Institutet.

3.2 STUDY POPULATION

Paper I

The study cohort consisted of patients diagnosed with metastatic breast cancer (MBC) between January 1, 1998 and December 31, 2009 and retrospectively identified within the SBCR. Among those, 210 patients who had a histologic/cytologic confirmation of relapse and for whom Ki67 from the first loco-regional or distant recurrence (mKi67) was available were selected for the analysis. Information on mKi67 was manually retrieved from the database of the Department of Pathology at Karolinska University Hospital. Besides the absence of Ki67 performed on first metastasis, other exclusion criteria were: diagnosis of a second invasive primary tumour; previous breast cancer diagnosed within five years; and bilateral synchronous breast cancer. All patients were diagnosed and treated for MBC at Karolinska University Hospital (Stockholm, Sweden). Patient demographics as well as clinical and pathological disease characteristics were extracted from the registry or manually collected from hospital records. In addition to mKi67, Ki67 was analysed in 131 primary tumours (pKi67). When available, pKi67 was obtained from the surgical specimen. In 43 patients who received neo-adjuvant treatment, pKi67 was taken from pre-treatment core-biopsies, while pKi67 analysed in tissue micro-arrays (TMAs) for the purpose of another study, the Merck study, was used in 29 cases. Merck study is a nested case-control study that aims to explore genomic drivers for metastatic dissemination of breast cancer (more details are provided below).

Paper II

The 220 patients included in this study were diagnosed and treated for primary breast tumour and distant recurrence at Karolinska University Hospital between January 1997 and September 2006 and identified within the Merck study. This study is a larger nested-case control study (controls are non-systemically relapsed tumours in the period 1997-2006 matched to the cases by adjuvant therapy, age and calendar period at diagnosis) based on SBCR and comprises of 768 female study subjects (621 individuals including two with bilateral breast cancer) aged 75 or younger and diagnosed with primary breast cancer in the years 1997-2005. Fresh frozen primary tumour tissue was available in all patients for RNA isolation. Most cases had three controls each (88.4%); 15 cases (7.9%) had four controls and 7

cases (3.7%) had two controls. All patients were diagnosed with primary tumour between January 1997 and December 2005. Tumour stage IV at the time of initial diagnosis was a pre-defined exclusion criterion. For the purpose of the analysis in Paper II, only the 220 patients with relapse of their cancer were selected. Clinical and pathological tumour characteristics as well as treatment information were collected using patient charts and pathology reports. As the cohort was comprised of only breast cancers that subsequently metastasized, clinically high-risk tumours (axillary lymph node positive, ER-negative, HER2-positive, grade 3, high proliferation) were more frequently represented compared with a typical early breast cancer population.

Paper III

The Merck study was also used as "cohort 1" in study III but the nested case-control design was not used here either. Overall, 621 individual patients were taken regardless of relapse occurrence and breast cancer specific survival (BCSS) was analysed from the time of primary tumour diagnosis. The final cohort resulted in 379 subjects after exclusion of bilateral tumours as well as all cases in which one or more among ER, PR, HER2 or Ki67 status was missing. Cohort 2, referred as to the "Uppsala cohort", consists of 484 subjects with systemically treated or untreated primary cancer in the Uppsala County, Sweden between 1987-1989. Quality controlled RNA gene expression profiles were available in 253 subjects. After exclusion of missing gene expression profiles and unclassified tumours, 209 subjects were available for the analysis in cohort 2. Clinical and pathological disease information as well as patient follow-up was extracted from the SBCR and the Swedish National Board of Health and Welfare for cohort 1 and 2, respectively.

Paper IV

For the purpose of the study IV, 3898 women aged 18-75 and diagnosed with invasive loco-regional failure (LRF) of breast cancer between January 1, 1980 and December 31, 2016 were identified within the SBCR. Only histologically verified invasive LRFs with no evidence of previous or simultaneous (within 60 days after LRF occurrence) distant metastasis were included. Moreover, since a quality control of SBCR included years until 2014, women diagnosed with a later relapse were excluded. Using these criteria, 2698 patients were selected for the analysis. LRFs referred to either isolated local recurrences or loco-regional

recurrences. Data on demographics and primary tumour were obtained from the SBCR while information about treatment after relapse was manually retrieved from hospital records. Three cohorts according to the years of LRF diagnosis were derived as follows: 1980-1989; 1990-1999; 2000-1014. Clinical follow-up and vital status were updated as of December 31,2014. Patients were also separated into two subgroups based on the age at relapse (≤60 years; >60 years) and survival analysed accordingly.

3.3 METHODS

3.3.1 IMMUNOHISTOCHEMISTRY

ER and PR

Evaluation of the expression of ER and PR was centralized and performed at Karolinska Hospital laboratory using monoclonal antibody based biochemical methods (with cut-off ≥0.05 fmol/μg DNA as positive) or by IHC/Immunocytochemistry (ICC) (with cut-off ≥10% as positive).[23] IHC/ICC method was introduced in Sweden in 2001 and definitely replaced the biochemical assay in 2003. The ER and PR value that was chosen for the purpose of this study when results from several methods in the same patient were present, was selected in accordance with the following priority order: 1) IHC; 2) ICC; 3) biochemical assay.

In paper III cohort 2, ER and PR were determined by the ligand-binding assay using the same cut-off as the one used at the Karolinska laboratory.

Biochemical method and IHC/ICC provide similar prognostic and predictive information [283,284] and the ≥0.05 fmol/μg cut-off for the ligand-binding assay has been demonstrated to be analogous to the ≥10% IHC cut-point. [24]

HER2

For the paper II and III (cohort 1), HER2 was assessed in TMA sections using Chromogenic in situ Hybridization (CISH), in the same tumour sample used for RNA isolation. CISH test was performed at University of Tampere, Finland and more comprehensive assay description is provided by Tanner et al. [285] For the scoring, unaltered gene copy number by CISH was defined as one to five signals per nucleus. Low-level amplification was defined as six to ten

signals per nucleus in >50% of cancer cells, while HER2 was considered as amplified when a large copy cluster in >50% of scored cells or >10 gene copies was detected. [285]

CISH is a reproducible validated methodology for HER2 testing and a viable alternative to fluorescence in situ hybridization (FISH) and IHC. A good agreement between CISH and FISH has been demonstrated by Tanner and coll. (kappa coefficient 0.81, 95% confidence intervals 0.69-0.92) [285] as well as other independent laboratories. [286,287] A good concordance between CISH and IHC has also been shown. [285,287]

In paper III cohort 2, HER2 was assessed on whole sections using the HER2/neu antibody (CB11, 1:300, NovoCastra Laboratories Ltd.)

Ki67

Ki67 is routinely analysed in primary and metastatic tumour tissue from patients with breast cancer at Karolinska University Hospital since the early 1990. When applying national guidelines, highly reproducible results are obtained in Ki67 assessment between Swedish pathology departments. [288]

In **Paper I**, mKi67 was analyzed both as a continuous and categorical variable dichotomized according to 20% cut-off (low ≤20%; >20% high), in agreement with local [23] as well as international guidelines. [289] Overall, mKi67 was analysed in 35 core-biopsies and 172 fine-needle aspiration (FNAC) samples (biopsy source was unknown in 3 cases). FNAC and core-biopsy are established methods for the diagnosis of primary and metastatic tumour lesions. [290,253] A previous analysis from our group revealed low concordance of biomarkers between IHC and ICC, especially for Ki67, and suggested that specific cut-points should be separately defined for ICC. [291] Scoring variability between different methods has been ascribed to heterogeneity in Ki67 staining due to the presence of two biological patterns of proliferative activity, the tumour invasive edge and hot-spots. [253,292] Indeed, unlike core biopsy the main technical limitation of FNAC is the inability to accurately evaluate the abovementioned proliferation patterns. Furthermore, site of biopsy is an acknowledgeable source of heterogeneity, especially in bone biopsies, in which decalcification causes reduction in IHC biomarker staining. [293-295] Bone was the principal site of metastasis biopsy (28%) in paper I.

Moreover, pKi67 was analyzed in 59 whole-section surgical specimens, 43 pre-surgical core-biopsies and 29 TMA samples. Blocks for the analysis of TMA sections were initially

Score	Mib-1
1	0-1%
2	2-10%
3	11-15%
4	16-20%
5	21-30%
6	31-50%
7	51-100%

Table 2. *Ki67 scoring system in TMA sections*

constructed at Karolinska Institutet and scored by a breast pathologist at Central Manchester University Hospitals NHS Foundation Trust, Manchester, United Kingdom for the purpose of the Merck study. Mib-1 antibody (1:100 diluition, Dako) was used for Ki67 staining. For each tumour, two blocks were obtained and each block was given two scores. Overall, 4 scores for each tumour were available. Ki67 scoring is presented in Table 2. The highest of the 4 scores was chosen and dichotomization performed as follows: low Ki67 score ≤4; high Ki67 score >5. In 20 cases pKi67 was available both in surgical samples and in TMAs with an 80% concordance rate of dichotomized Ki67 between the two methods.

Paper II and III For the purpose of these papers, Ki67 from primary tumour as assessed on TMAs within Merck study was also used. While in paper II the same algorithm for dichotomization as that used in study I was followed, in paper III 16% median cut-off was chosen (and applied also to cohort 2) and dichotomization performed as follows: low Ki67 score ≤3; high Ki67 score >3.

ER, PR, HER2 and Ki67 were used for deriving IHC-based subtypes in paper II and III.

3.3.2 GENE EXPRESSION PROFILING

In the Merck study, surgical specimens were divided in two parts with half being used for ER, PR, HER2, Ki67 and grade determination and half fresh frozen for gene expression profiling. RNA extraction from frozen tumours was performed using the Qiagen RNeasy Mini Kit (Qiagen, Germany). Hybridization of tumour samples was done using the HRSTA-2.0 custom human Affymetrix array, whose details can be found at NCBI Gene Expression Omnibus (GEO) with accession number GPL10379. Array data can be retrieved using GSE48091 as NCBI GEO identifier. For the purpose of paper II and III, the tumours were profiled using the aroma.affymetrix package of the open source R software.

In paper II, research versions of the commercially available PAM50 Prosigna, 21-gene Oncotype DX RS and 70-gene Mammaprint genomic signatures were analysed. Since more aggressive tumours were overrepresented in this cohort, a normalisation using the whole nested case-control population was carried out.

For the purpose of paper III, research versions of GGI, MammaPrint, cell–cycle score (CCS), Oncotype DX RS and Prosigna were applied to cohort 1 and 2. Accession number for array of cohort 2 is available at NBCI GEO with reference GSE3494. CCS was obtained from METABRIC dataset in order to derive cut-offs for cell cycle activity. [296] In total, 463 cell cycle relevant genes were derived from 3 cohorts [296-298] and applied to 1992 cancers within the METABRIC dataset. A continuous score was obtained by adding all the expression values of these genes from each tumour. The score was then divided by tertiles (33% and 66%) in order to classify the tumours in low, intermediate, and high cell cycle activity. A continuous score was obtained in the same manner in cohort 1 and scaled to match the METABRIC score before applying the tertiles.

3.4 STATISTICAL ANALYSIS

Survival analysis is a time-to-event (or survival probability) analysis used to study the time to occurrence of some event of interest (or time to failure). The event of interest in our cancer population was death, whereas paper IV used also time to disease progression as an end-point. Survival was measured at the last follow-up date and analysis was right-censored. In our studies, median survival, that is time beyond which 50% of subjects in the population are expected to survive, was employed as the summary variable.

The population of interest in paper I and II was composed of women diagnosed with MBC. We chose post-relapse OS, defined as the time interval between first distant relapse occurrence and death irrespective of the cause, as the primary end-point assuming that the majority of deaths were related to breast cancer. In paper IV, post-relapse event-free survival (EFS) was calculated as the time between LRF occurrence and disease progression, consisting in a second loco-regional failure, distant relapse or death. Estimates of survival were from Kaplan-Meier method, a non – parametric statistical test used when survival is on a continuous scale and particularly useful in presence of right censoring. Comparisons between groups were made by the log-rank non-parametric test. [299] Unadjusted Cox regression analyses were fitted for hazard ratio (HR) and confidence intervals (CI) estimation. In order to test for the effect modification caused by potential confounding factors, multivariate Cox proportional regression models were implemented. The Cox model is a semi-parametric model that allows for comparison of effect of different exposures on a time to event outcome. [300] The clinical variables selected for multivariate model adjustment were tumour size, hormone receptor

status, Ki67 and grade of the primary tumour, axillary lymph node status, age at relapse, primary tumour surgery (paper IV), recurrence free interval (RFI), site of first metastasis (visceral vs. non-visceral), treatments at the initial diagnosis, first line treatment for the advanced disease (paper II).

Net survival is the proportion of patient that survives in the hypothetical scenario where the cancer of interest is the only possible cause of death. In paper IV, relative survival was used to estimate net survival. Relative survival is the ratio of the observed survivors in the study cohort to the expected survivors in the general population, which is comparable for age, sex and calendar period to the study cohort but free of the cancer of interest. The estimate of relative survival is the excess mortality ratio (EMR), which measures the mortality the patients experience in excess of what would be expected in absence of the cancer of interest. [301] Survival estimates in the general population were extrapolated from The Human Mortality Database that contains population life tables from 37 countries. [302] Relative survival has the advantage of not requiring cause-specific mortality thus circumventing limitations related to incorrect reporting or lack of death certificates. A Poisson regression model was fitted for estimating breast cancer EMRs for clinically relevant prognostic factors. [303]

Likelihood ratio statistics In paper II and III, the likelihood ratio (LR) statistics was applied to test the goodness-of-fit of a model that implies estimating whether an expanded model provides improved fits as compared to a reduced one. [304] In detail, paper II studied the additional prognostic contribution in terms of post-relapse OS prediction of the PAM50 ROR weighted for an 11-gene proliferation index (ROR-P) when added to a clinical model alone or in presence of the other explored tumour prognosticators. Breast cancer specific survival (BCSS) was the clinical end-point for paper III, which analyzed a cohort of primary breast cancers. Here, the LR statistics (LR-χ^2) was used to study whether genomic signatures carry additional prognostic significance when added on the top of Ki67/IHC-based subtypes and whether Ki67 alone or integrated into IHC-subtypes may confer more prognostic information when added to these signatures. A concordance index (c-index), which measures the predictive discrimination (probability of concordance) between predicted and observed outcome between variables or models, was also applied in paper III. [304]

Furthermore, associations between baseline characteristics and variables of interest were explored by using the Pearson's chi-square test in all the papers (the Mann-Whitney test was also used in Paper III) while the k-coefficient was estimated to measure to degree of

agreement between IHC and genomic prognosticators in paper II. [305] In paper I, the McNemar's test, a test that investigates whether there is marginal homogeneity between paired data, was used to explore changes in dichotomized pKi67 and mKi67 in matched primary tumours and metastasis. [306]

Two-sided tests were used with 5% significance level. All analyses were performed and results reported in accordance with the "REMARK" guidelines. [78]

RESULTS

Paper I – Prognostic role of Ki67 assessed in metastatic tissue of patients with advanced breast cancer.

In this study, the prognostic role of Ki67 from metastasis biopsies as well as the change in Ki67 expression rate in first relapse compared with corresponding primary tumour and its clinical implications has been determined.

Overall, 125 (59.5%) and 85 (40.5%) patients had low and high mKi67, respectively. As the cohort was exclusively composed of MBCs, primary tumours carrying more aggressive features were overrepresented in the study cohort as compared with the general breast cancer population. In detail, there was an excess of larger (>2 cm) primary tumours (60%) with axillary lymph nodes metastatic involvement (66%) as well as ER-negative (36%) and undifferentiated (44%) primary cancers. However, this metastatic breast cancer cohort was fairly representative of the overall metastatic breast cancer cohort from the SBCR in terms of baseline characteristics as well as post-relapse OS (median OS 15 months). [14] Interestingly, mKi67 rate was highly associated with primary tumour grade (Pearson's chi-square p 0.01) and 73% of patients with low mKi67 had RFI ≥24 months. The most frequent site of biopsy was bone (28%) and liver (23%). In 15 patients mKi67 was analysed in at least two relapse sites at time of first recurrence. In 14 cases FNAC was the method used to assess Ki67 in multiple first relapses from the same subject with 100% and 50% agreement between two or more first relapses when classifying mKi67 as low or high, respectively.

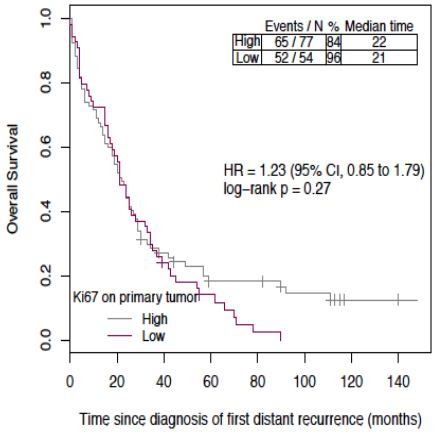


Figure 7. Kaplan-Meier estimates of OS grouped by levels of Ki67 on primary tumour

A total number of 186 deaths were registered, 109 in the low-mKi67 and 77 in the high-mKi67 group. Low mKi67 levels were significantly associated with longer median post-relapse OS (25 vs. 17 months; HR 0.69, 95% CI 0.51-0.92; p 0.01). Conversely, pKi67 was not significantly prognostic of survival after relapse

(Figure 7). When a regression Cox model stratified by RFI was adjusted for clinical prognostic variables statistically significant in univariate models, namely axillary lymph node status, RFI, primary tumour ER and site of metastasis, mKi67 did not retain an independent association with OS (HR 0.85, 95% CI 0.62-1.16, p 0.30). Absence of metastasis in the axillary lymph nodes at primary diagnosis (p 0.005) and non-visceral metastasis (p 0.01) were independently prognostic of longer post-relapse OS. However, in a bivariate Cox regression model mKi67 significantly correlated with OS regardless of pKi67 (HR 0.56, 95% CI 0.38-0.81, p 0.002). The prognostic value of Ki67 was also explored in subgroups of tumours defined by axillary lymph node status and ER (Table 3). Low-mKi67 was associated with longer OS in the group of negative lymph node status (p 0.016) whereas no statistically significant association emerged from the analysis of the positive axillary lymph node group (p 0.24) as well as in ER-positive (p 0.37) and negative (p 0.13) subgroups.

				OS within subgroup		OS between subgroups	
	N	*N* of events	Median (months)	HR (95% CI)	*P*	HR (95% CI)	*P*
Axillary lymph node negative status of primary tumor	70	57	25			0.72 (0.53-0.97)	0,029
mKi67 low (≤20%)	43	35	46	0.51 (0.29-0.89)	0,016		
mKi67 high (>20%)[a]	27	22	19				
Axillary lymph node positive status of primary tumor[b]	139	128	18				
mKi67 low (≤20%)	81	73	20	0.81 (0.57-1.16)	0,243		
mKi67 high (>20%)[a]	58	55	16				
ER positve status of primary tumor	127	113	24			0.78 (0.58-1.06)	0,108
mKi67 low (≤20%)	83	74	28	0.84 (0.57-1.24)	0,374		
mKi67 high (>20%)[a]	44	39	21				
ER negative status of primary tumor[b]	76	67	14				
mKi67 low (≤20%)	38	32	15	0.69 (0.43-1.13)	0,132		
mKi67 high (>20%)[a]	38	35	11				

Abbreviations: OS, overall survival; *N,* number of patients; HR, hazard ratio; 95% CI, 95% confidence intervals; *P,* p-value; mKi67, metastatic Ki67; ER, estrogen receptor.

[a]Reference for the overall survival comparison between low and high metastatic Ki67 within the subgroup.

[b]Reference for the overall survival comparison between low and high metastatic Ki67in axilary lymph node positive vs. negative and in ER positive vs. negative tumor subgroups.

Table 3. *Cox regression analysis for OS depending on subgroups defined by axillary lymph node status and ER status at primary diagnosis (Modified from Falato C et al., Breast Cancer Res Treat 2014)*

When the analysis was restricted to mKi67 assessed in FNAC, thus accounting for a potential source of heterogeneity related to different techniques, the results were substantially unchanged.

Moreover, the prognostic role of the continuous Ki67 was explored showing that increasing levels of mKi67 correlated with increasing death rate at 2 years (HR 1.09, 95% CI 1.04-1.14, p 0.001).

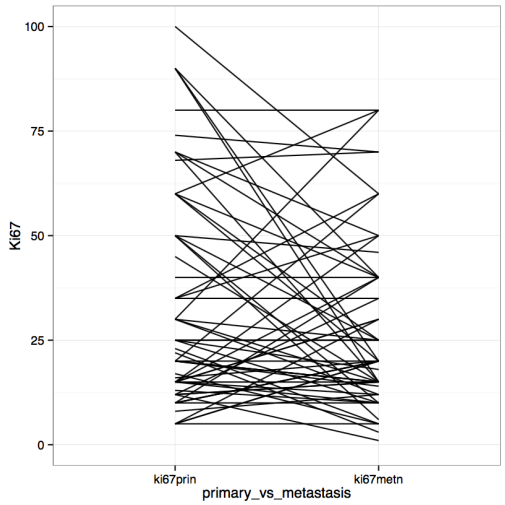


Figure 8. *Continuous Ki67 in primary tumour and metastasis*

Dichotomized Ki67 was available in 131 primary tumours and changed in 54 cases (41%) (p 0.02 by McNemar's test), from low in the primary tumour to high in the metastasis in 18 (14%) and from high to low in 36% (28%) cases. Continuous Ki67 was available in 67 matched primary tumours and metastases (Figure 8). Although the difference was not statistically significant (p 0.8 by t-test for paired samples), the mean Ki67 values tended to be lower in metastasis compared to primary tumours (23 vs. 32).

Furthermore, the prognostic value of the change in dichotomized Ki67 was addressed. Compared to the stable Ki67 category, the change in Ki67 from high to low levels was associated to a better post-relapse OS (HR 0.48, 95% CI 0.31-0.76, p 0.002) while no difference was seen when Ki67 varied from low in primary tumour to high in correspondent metastasis (HR 1.06, 95% CI 0.62-1.80, p 0.83). When the stable group was separated in stable low or stable high, the change of Ki67 levels from high to low was still associated with better survival (p 0.005), suggesting that the decrease in Ki67 rather than Ki67 rate in metastasis may be prognostically relevant (Figure 9).

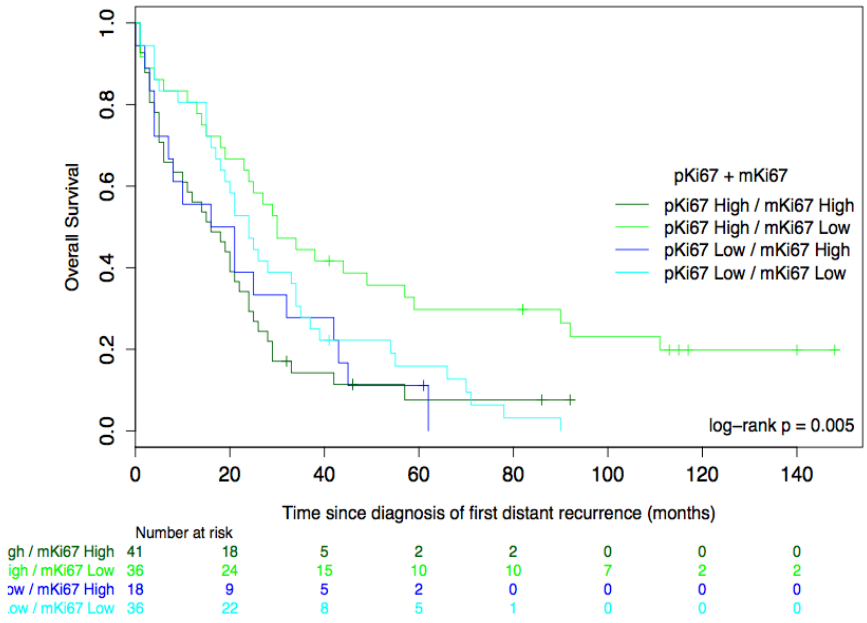


Figure 9. *Survival in relation to Ki67 in primary tumour and metastasis*

In a further exploration, the association between the variation in Ki67 and clinicopathological factors, including neo/adjuvant treatments, was investigated. Only RFI >24 months significantly correlated with variation in proliferation rate with Ki67 changing to low in 24 (59%) out of 41 patients with initially high pKi67.

Paper II – Post-relapse prognosis according to immunohistochemical subtypes and genomic signatures from primary tumour in patients diagnosed with metastatic breast cancer.

This study explored the prognostic value in terms of post-relapse OS of breast cancer subtypes and genomic signatures from primary tumour, beyond classical clinical and pathological prognostic factors.

	Overall cohort	ER positive
Variable	**N (%)**	**N (%)**
IHC classifier		–
luminal A	49 (27)	
luminal B	45 (24)	
HER2 positive	67 (36)	
TNBC	23 (13)	
Total	184 (100)	
PAM50		–
luminal A	41 (22)	
luminal B	44 (24)	
HER2 enriched	49 (26)	
Basal-like	41 (22)	
Normal breast-like	12 (6)	
Total	187 (100)	
ROR-S		–
low	34 (18)	
medium	67 (36)	
high	86 (46)	
Total	187 (100)	
Recurrence Score		
low	11 (6)	11 (9)
intermediate	17 (9)	15 (12)
high	159 (85)	98 (79)
Total	187 (100)	124 (100)
70 gene signature		
good	62 (33)	54 (44)
poor	125 (67)	70 (56)
Total	187 (100)	124 (100)

Table 4. *Classification based on IHC biomarkers and genomic profiles*

Table 4 summarizes tumour classification according to IHC-based subtypes and genomic profiles. Notably, since the cohort is composed of advanced breast cancers 85% and 67% of the tumours in the overall population were classified as high-risk and poor prognosis based upon the 21-gene RS and the 70-gene classifiers, respectively. RS categorized ER-positive tumours as high-risk in the 79% of the cases.

As a prove of concept, all the tested classifiers were explored for their association with survival from primary diagnosis and the results were in line with the main findings of the study. More specifically, a statistically significant association between IHC-as well as PAM50-based intrinsic subtypes and overall survival from primary tumour was shown (p 0.007 and p 0.003 by log-rank test, respectively). Moreover, ROR-S and the 70-gene profile were prognostic of overall survival ($p < 0.001$ and p 0.007 by log-rank test, respectively) while no statistically significant association emerged between RS and survival from diagnosis of primary tumour (p 0.179).

Intrinsic subtypes as assessed by IHC-biomarkers and PAM50 were assessed. Only a moderate degree of agreement between the two classifiers was present (kappa score = 0.51), as Figure 10 shows. In fact, PAM50 molecular subtypes are represented in each IHC subgroup and vice-versa with highest discordance in the Basal-like group. In this study cohort, Luminal B category by IHC was defined as ER-positive and/or PR-positive, HER2-negative and Ki67 ≥20%, while all cancers overexpressing HER2 were categorized as HER2-positive irrespective of hormone receptor status.

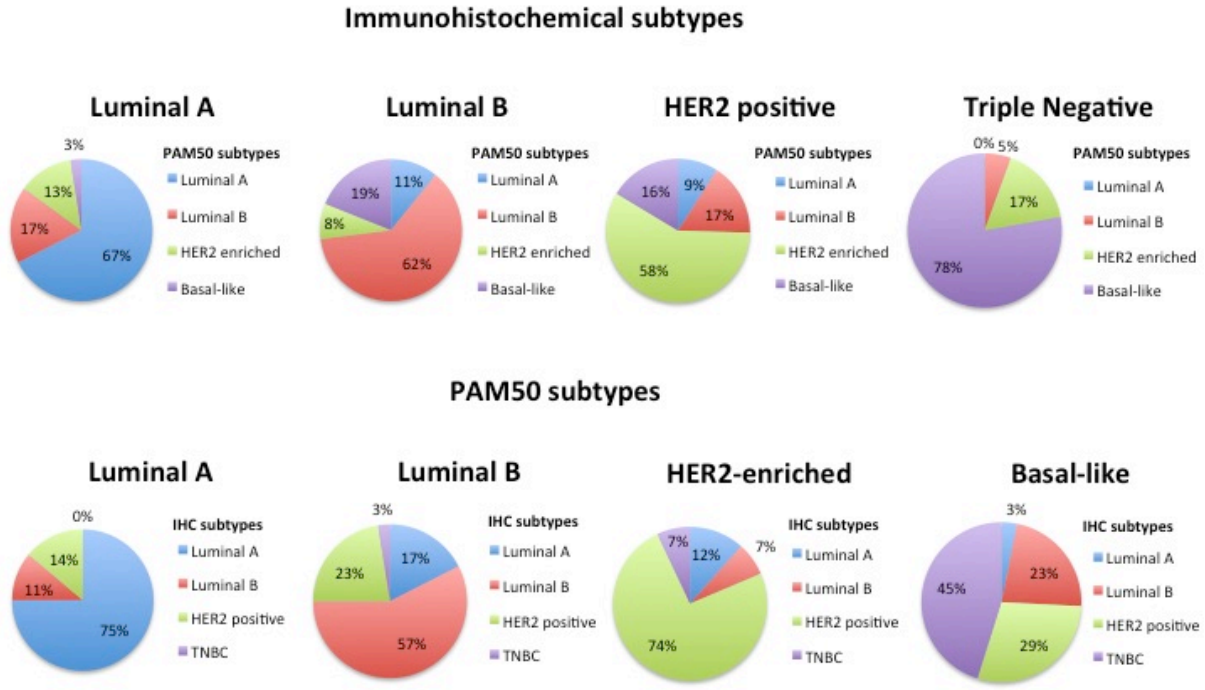


Figure 10. *Intrinsic subtype distribution within IHC subtypes and vice-versa*

First, IHC and PAM50 subtypes were found significantly associated with post-relapse OS (p 0.05 and p 0.03, respectively). Unlike primary breast cancer, no significantly different survival was described in the comparison of Luminal B with Luminal A cancers (p 0.99 and p 0.45 by IHC and PAM50). Neither IHC-subtypes (p 0.64) nor PAM50 (p 0.58) retained an independent prognostic performance when a Cox multivariate model was implemented to

account for the effect of routinely employed clinical and pathological factors. As an exploratory analysis, HER2-positive tumours were further separated in hormone receptor-positive/HER2-positive and hormone receptor-negative/HER2-positive and post-relapse OS estimated by the Kaplan-Meier method. Notably, the hormone receptor-positive/HER2-positive group was associated with a significantly shorter survival (p 0.02) in comparison with Luminal and hormone receptor-negative/HER2-positive tumours. The ability of Ki67 at 15% cut-off as well as continuous Ki67 in separating Luminal tumours was also tested with consistent results in terms of survival prediction.

Furthermore, a good agreement was seen between PAM50-based ROR (ROR-S), RS and the 70-gene good and poor prognosis categories, although more tumours were classified as high risk according to RS. These signatures were tested for association with survival both in the overall cohort and in ER-positive tumours. A significant correlation with longer post-relapse OS was described only for low and medium ROR-S categories and 70-gene poor and good categories in the overall but not in ER-positive group. RS was not prognostic and, thus, not further investigated in the multivariable model. ROS-S (low-risk HR 0.59, 95% CIs 0.34-1.01; medium-risk HR 0.58, 95% CIs 0.37-0.91; p 0.04) but not the 70-gene classifier (good prognosis category HR 0.90, 95% CIs 0.57-1.40; p 0.64) was independently associated with survival in a Cox regression model adjusted for significant clinical and pathological variables. However, the association between low-risk ROS-S and better survival was borderline, likely due to the low number of events. Results were unchanged when grade was removed from the model.

An 11-proliferation gene signature derived from PAM50 was also tested and a significant correlation with post-relapse OS was seen in univariate (HR 1.62, 95 CIs 1.09-2.41, p 0.02) as well as multivariable Cox regression models (HR 1.74, 95% CIs 1.09-2.78, p 0.02).

Additionally, ROR-S weighted for the proliferation score (ROR-P) was investigated. ROR-P provided additional prognostic information when added to a clinical model including primary tumour size and grade, RFI and first systemic relapse site alone ($p < 0.001$) or in presence of the RS ($p < 0.001$), 70-gene signature ($p < 0.001$), ROR-S (p 0.01) and IHC subtypes (p 0.01).

Finally, survival in subgroups of patients identified according to the first line treatment for metastatic disease was tested. Trastuzumab was administered in 48 out of 67 HER2-positive tumours. Low-risk ROR-S was associated with a clinically relevant and statistically significant longer survival in ER-positive tumours treated with endocrine treatment (p 0.002)

but not in those treated with chemotherapy (p 0.097). Moreover, PAM50 better identified Luminal A tumours that could benefit from first line endocrine therapy in comparison with IHC, although the difference in survival between Luminal A and B did not reach the significance level in any of the subgroups (endocrine treated and chemotherapy treated patients). However, the analysis was purely explorative and not intended to provide treatment indications.

Paper III – Evaluation of the additional prognostic role in early breast cancer of genomic signatures and immunohistochemical subtypes when combined to each other.

This study investigated whether Ki67 alone or incorporated into IHC subtypes as combined with genomic signatures, and vice-versa, might provide more prognostic information than each classifier alone in early breast cancer setting.

For both cohort 1 and cohort 2, all analyses were performed in the whole patient population as well as in subgroups defined as follows: ER-positive/lymph node negative, ER-positive/lymph node positive, ER-negative. Cohort 1 was derived from a nested-case control study in which cases developed a relapse and controls did not during the same time frame (Merck study, see Methods above for details). This led to an overrepresentation of tumours with more aggressive features, in particular more histologic grade 3 and highly proliferative tumours with larger size and lymph node metastases at primary diagnosis in patients with younger age, in cohort 1 in comparison with cohort 2. Additionally, in cohort 2 fewer patients received adjuvant systemic treatment and 65% of patients with ER-positive tumours were not treated with endocrine therapy.

In both cohorts, the rate of discordance between Ki67 at a 16% cut-point and the other genomic classifiers was in the range between 14% and 22%, as expected from previous studies (Table 5).

Cross-table of concordance between Ki67 immunohistochemical staining and gene expression signature classifications in the Cohort 1 and 2								
	Number of patients grouped by Ki67 status in the Cohort 1 and 2							
	Cohort 1 (n=379)				Cohort 2 (n=209)			
Characteristics	Ki67 < 16 (n=184) n (%)	Ki67 ≥ 16 (n=195) n (%)	nC n (%)	nD n (%)	Ki67 < 16 (n=140) n (%)	Ki67 ≥ 16 (n=69) n (%)	nC n (%)	nD n (%)
Gene expression signatures								
GGI								
Grade 1	120 (65)	14 (7)	301 (79)	78 (21)	119 (85)	20 (29)	168 (80)	41 (20)
Grade 3	64 (35)	181 (93)			21 (15)	49 (71)		
70-Gene								
Good	142 (77)	32 (16)	305 (80)	74 (19)	102 (73)	8 (12)	163 (78)	46 (22)
Poor	42 (23)	163 (84)			38 (27)	61 (88)		
Recurrence score								
Low	71 (39)	5 (3)			70 (50)	8 (12)		
Intermediate	35 (19)	10 (5)	296 (78)	83 (22)	35 (25)	5 (7)	166 (79)	43 (21)
High	78 (42)	180 (92)			35 (25)	56 (81)		
Cell Cycle score								
Low	70 (38)	3 (1)			103 (74)	11 (16)		
Intermediate	64 (35)	16 (8)	326 (86)	53 (14)	26 (19)	22 (32)	187 (89)	22 (11)
High	50 (27)	176 (91)			11 (8)	36 (52)		
PAM50								
Luminal A	111 (60)	15 (7)			54 (39)	3 (4)		
Luminal B	24 (13)	54 (28)			27 (19)	18 (27)		
HER2-enriched	12 (6)	41 (21)	307 (81)	72 (19)	18 (13)	30 (43)	150 (72)	59 (28)
Basal-Like	14 (8)	78 (40)			8 (6)	15 (22)		
Normal-Like	23 (12)	7 (4)			33 (24)	3 (4)		

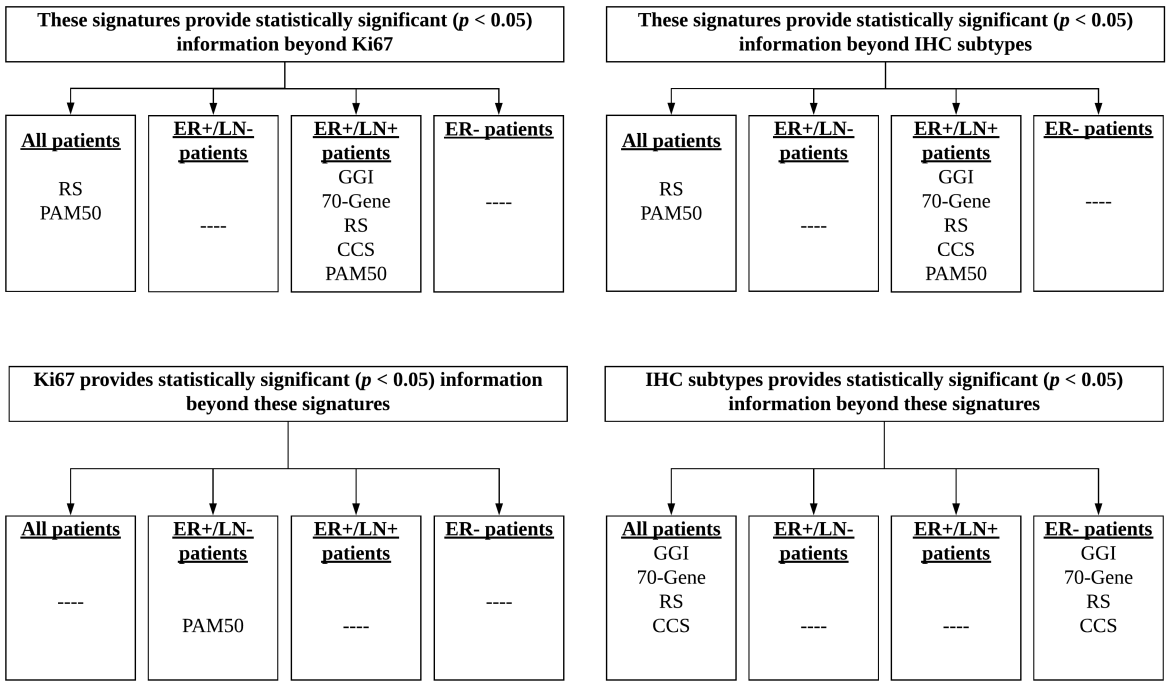
GGI = Genomic grade index, nC = Number of concordant cases, nD = Number of discordant cases

Numbers in red = Cases in which Ki67 and gene expression signatures are not in agreement.

Table 5. Concordance between Ki67 subgroups and gene expression signature classification *(Modified from Lundberg A et al., CCR 2017)*

Among 379 patients included in the analysis in cohort 1, 104, 167, and 103 were categorized in the group of ER-positive/lymph node negative, ER-positive/lymph node positive and ER-negative tumours, respectively. The 21-gene RS and PAM50, but not the other genomic profiles, provided statistically significant additional prognostic information on top of Ki67 (RS: LR-χ^2 test p 0.001; PAM50: LR-χ^2 test p < 0.001) and IHC subtypes (RS: LR-χ^2 test p 0.001; PAM50: LR-χ^2 test p0.020) in the overall cohort (Figure 11). None of the signatures conferred additional prognostic value in ER-positive/lymph node negative, while all were associated to an increased prognostic performance when added to Ki67 and IHC subtypes in ER-positive/lymph node positive group, suggesting a superior prognostic ability for gene signatures as compared to the IHC biomarkers in this specific subset (Figure 11). Gene expression signatures, which are notably driven by proliferation genes, did not show any extra prognostic capacity in ER-negative tumours, except for the 21-gene RS for which a trend towards an improved prognostication was demonstrated (LR-χ^2 test p 0.058). Furthermore, the additional prognostic performance of Ki67/IHC subtype when added to genomic profiles was assessed in the same subgroups. Ki67 did not add any prognostic information in any patient

subgroup while IHC subtypes were associated to an improved prognostication when added to all signatures in the overall and ER-negative subset, except for PAM50 that showed more robust prognostic capacity in comparison with IHC classifiers (Figure 11). Analysis of the c-index led to consistent results in all subgroups.



Flowchart showing simplified results from Cohort 1 based on likelihood ratio test (Δ LR-χ2).
GGI = Genomic grade index, RS = Recurrence score, CCS = Cell cycle score, ER+/- = Estrogen receptor positive/negative, LN+/- = Lymph node metastasis/no lymph node metastasis

Figure 11. Representation of the additional prognostic information deriving from adding A) genomic signatures to Ki67/IHC-subtypes and B) Ki67/IHC subtypes to genomic signature.*(Modified from Lundberg A et al., CCR 2017)*

Cohort 2 included 209 patients. Of these, 115 had ER-positive/lymph node negative, 65 had ER-positive/lymph node positive and 24 had ER-negative tumours. In general, the results were consistent with those from cohort 1 despite the smaller sample size of cohort 2, particularly in ER-positive/lymph node positive and ER-negative subgroup. An exception was that all gene expression signatures when combined to Ki67 and IHC subtypes performed better here than in cohort 1 in the overall population but not in the ER-positive/lymph node positive group, likely due to the lower sample size. Probably due to the same reason, IHC did not provide additional prognostic information beyond PAM50. Strikingly, both Ki67 and IHC

subtype were significantly prognostic in ER-negative tumours ($p < 0.001$) on the top of the 21-gene signature.

Paper IV – Prognosis after loco-regional failure of breast cancer: 34 years longitudinal data from the Stockholm-Gotland cancer registry

The study investigated survival in patients diagnosed with LRF in Stockholm County and explored potential survival trends over the past three decades.

Overall, 1922 and 996 patients received a diagnosis of isolated local relapse and loco-regional relapse, respectively. Median follow-up time was 13 years. More local and less loco-regional relapses were diagnosed over time, likely as a result of the wider use of breast conserving surgery and the better loco-regional control obtained with modern radiotherapy in the most recent decades. In addition, a trend towards smaller primary tumours, lower rate of axillary lymph node metastasis but also increasing use of neoadjuvant/adjuvant chemotherapy was shown over time (Mantel-Haentzel test for trend $p < 0.001$). No trend over time was demonstrated for ER status (Mantel-Haentzel test for trend p 0.75).

Information on relapse treatment was available only for the last cohort. Surgery was performed in 76% and 65% of the isolated local and regional recurrences diagnosed from 2000 and onward. Radiotherapy and chemotherapy were more frequently administered after a loco-regional relapse (20% and 28% vs. 57% and 51% in local relapse group) while the percentage of endocrine treated patients after relapse was similar between the groups (54% vs. 58% in local and loco-regional relapse, respectively).

Overall, 1032 (54%) out of 1922 diagnosed with a local relapse experienced disease progression, and of those 12% recurred loco-regionally, 51% systemically while 37% died without a second relapse. Among women diagnosed with loco-regional relapse, 11% further relapsed loco-regionally, 65% systemically and 24% died without documented progressive disease. In total 931 (48%) and 522 (67%) deaths were registered in the group of local and of loco-regional relapses, respectively. A significant improvement over time in post-relapse EFS and OS was described in both isolated local and loco-regional relapse groups.

Age-related trends were observed in the group of loco-regional but not in that of local recurrences. In fact, in locally relapsed tumours survival changed independently of age at recurrence (≤60 years; >60 years) while in loco-regional recurrence group a significant change was described for the overall cohort and for younger but not for older women. Moreover, in multivariate models stratified by RFI, cohort by year of relapse was associated with improved EFS and OS independently of other clinical prognostic factors only in the overall cohort and younger women within the loco-regional relapse group but not in older patients and in the group of isolated local recurrences (Table 6).

	Isolated local recurrence*						Loco-regional recurrence**					
	Overall cohort		Patients ≤60 years		Patients >60 years		Overall cohort		Patients ≤60 years		Patients >60 years	
Variables	**HR (95% CIs)**	***P***	**HR (95% CIs)**	***P***	**HR (95% CIs)**	***P***	**HR (95% CIs)**	***P***	**HR (95% CIs)**	***P***	**HR (95% CIs)**	***P***
Event-free survival												
Cohort												
1980-1989	1.14 (0.93-1.39)	0.19	1.31 (0.96-1.77)	0.08	1.02 (0.78-1.33)	0.88	1.35 (1.07-1.7)	**0.01**	1.85 (1.32-2.59)	**< .001**	0.9 (0.65 -1.25)	0.54
1990-1999	0.94 (0.80-1.10)	0.43	1.04 (0.83-1.32)	0.71	0.82 (0.66-1.02)	0.08	1.28 (1.05-1.58)	**0.02**	1.59 (1.19-2.13)	**0.002**	1.03 (0.76-1.39)	0.86
2000-2014[a]	1		1		1		1		1		1	
Overall Survival												
Cohort												
1980-1989	1.06 (0.86-1.31)	0.59	1.25 (0.91-1.73)	0.17	0.98 (0.75-1.31)	0.93	1.27 (1.01-1.62)	**0.05**	1.75 (1.23-2.48)	**0.002**	0.91 (0.65-1.28)	0.59
1990-1999	0.90 (0.76-1.07)	0.23	0.95 (0.73-1.23)	0.69	0.88 (0.70-1.10)	0.26	1.20 (0.97-1.49)	0.09	1.56 (1.15-2.12)	**0.005**	0.98 (0.72-1.34)	0.9
2000-2014[a]	1		1		1		1		1			

[a] *reference*

HR hazard ratio, CIs confidence intervals, P p- value

**The model is stratified for recurrence free interval and adjusted for type of primary surgery, primary tumor size and estrogen receptor, axillary lymph node status at time of primary diagnosis, adjuvant/neoadjuvant chemotherapy, age at diagnosis of local relapse (only overall cohort analysis).*

***The model is stratified for recurrence free interval adjusted for primary tumor size and axillary lymph node status at time of primary diagnosis.*

Table 6. *Multivariate analysis for post-relapse survival in patients with loco-regional failure of breast cancer*

Results were essentially unchanged when the analysis was restricted to women aged ≤70 years at relapse accounting for the heterogeneity with respect to survival when older patients are included into the study population.

Relative survival and EMSs were in accordance with post-relapse EFS and OS and relative hazard estimation. Relative survival curves are illustrated in Figure 12.

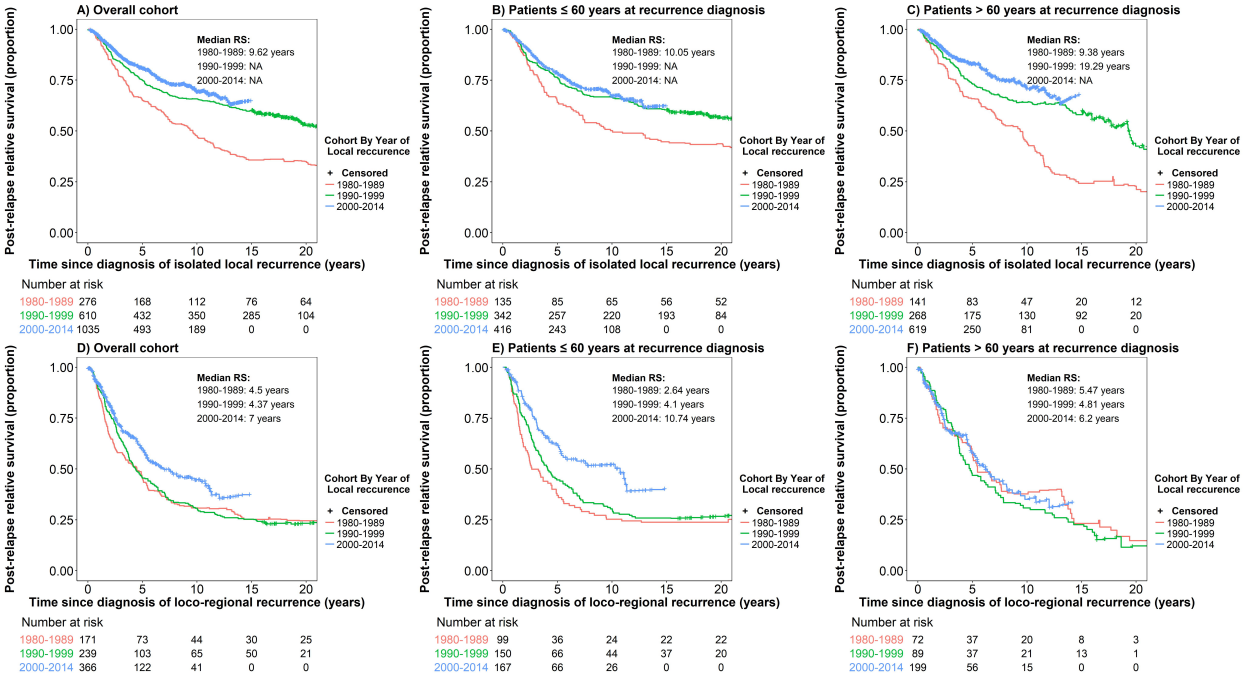


Figure 12. *Relative survival curves according to type of loco-regional failure and age at relapse*

All the analyses were unchanged when restricted to the years 1980 through 2009.

In an exploratory analysis, the 2000-2014 cohort was further divided in two sub-cohorts (2000-2005; 2006-2014) and survival was compared between these groups, aiming to further dissect this period that was characterized by the introduction of new compounds in breast cancer management (especially anti-HER2 therapies). Surprisingly, a better post-relapse EFS (p 0.002) and OS (p 0.03) was shown for older patients in the loco-regional relapse group but not for the other subgroups. Figure 13 and Figure 14 present post-relapse EFS and post-relapse OS curves, respectively. The improvement in survival remained significant for the older patients with loco-regional relapse diagnosis in Cox multivariate models adjusted for clinical confounding factors (EFS, p 0.003; OS, p 0.03). In the same patient subgroup, a clear trend towards an improved relative survival was also demonstrated (p 0.06).

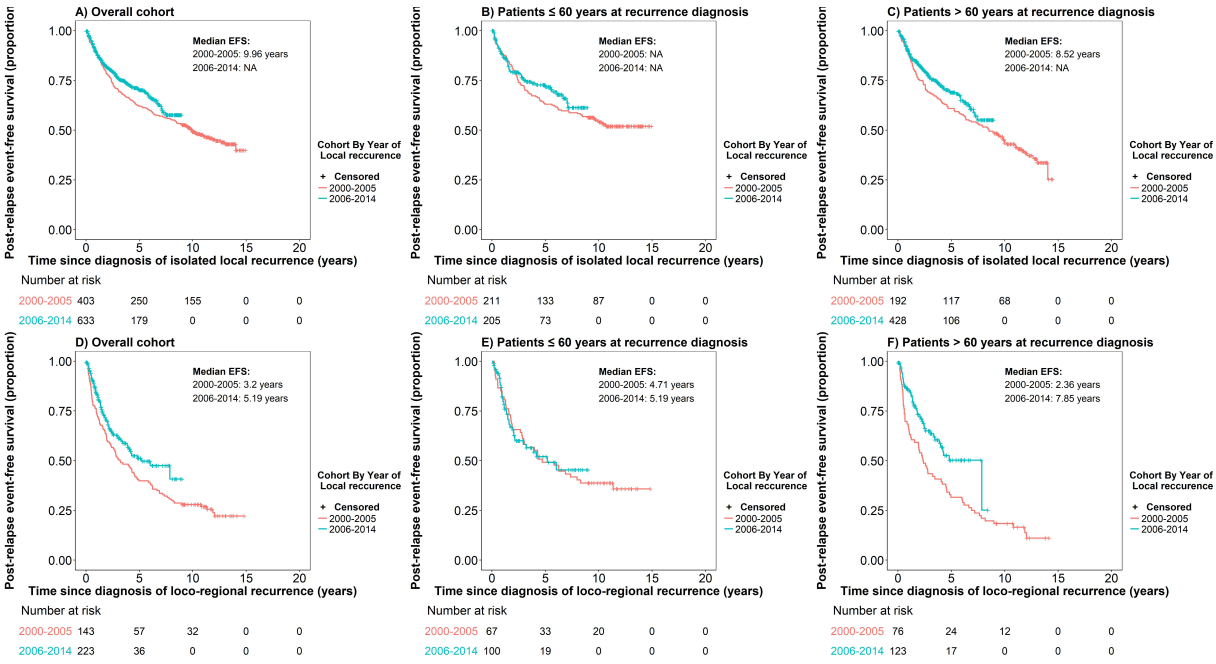


Figure 13. *Post-relapse event-free survival curves according to type of loco-regional failure and age at relapse in the cohort 2000 -2014*

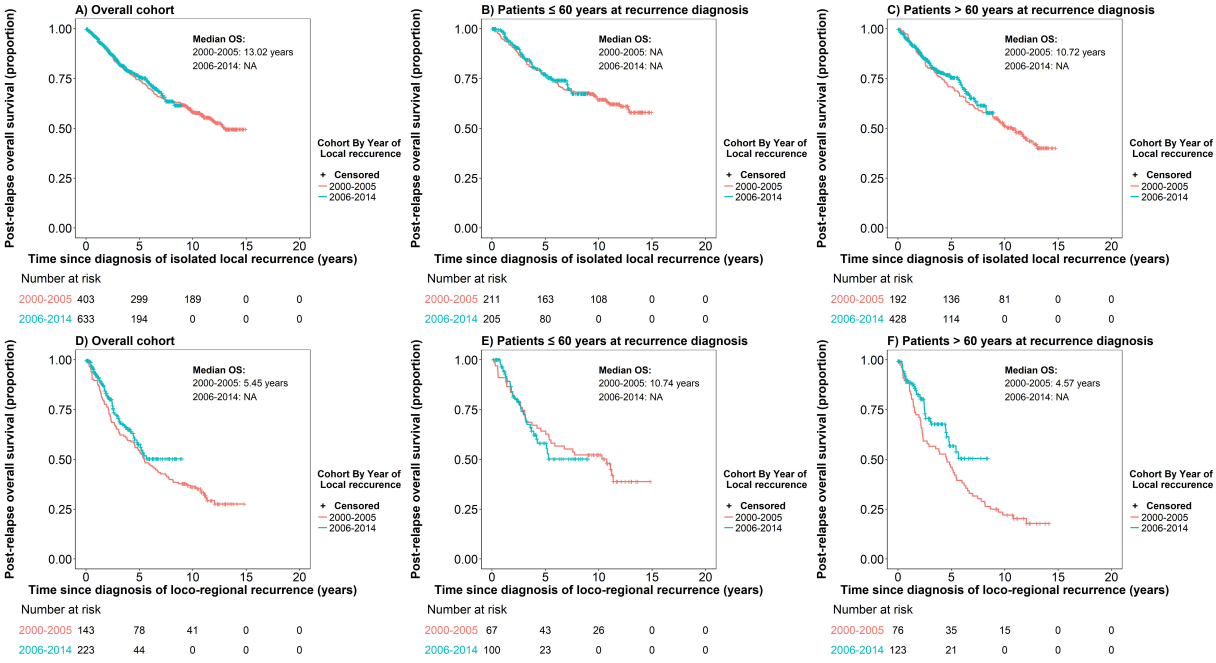


Figure 14. *Post-relapse overall survival curves according to type of loco-regional failure and age at relapse in the cohort 2000 -2014*

CONCLUSIONS

A clinical useful prognostic biomarker has to show the ability to separate a population into clinically relevant subgroups characterized by different prognosis and must add relevant information to the prognostic factors already used in clinical practice. The combination of clinical and pathological markers with newer genomic tools has the potential to optimize the identification of prognostic groups compared to each of these factors alone. The reason for that may be ascribed to their differential biological rationale so as each factor may rather refine and enhance the information carried from another biomarker, instead of replacing it. Population-based studies represent an extreme valuable tool for their ability to picture the variation of survival in a real scenario over decades and on a long-term follow-up. Discovering trends in survival may help, for example, to pinpoint those patient categories that benefited less of the improved therapies and to canalize research efforts towards a more personalized selection of the candidates to certain treatments. More specifically:

- In paper I, Ki67 from metastasis outperformed Ki67 of the primary tumour as a prognostic factor of survival after relapse but its role was not independent from the classical and well-established prognosticators. The change in Ki67 rate during tumour progression revealed the existence of potential biological implications in determining survival in the advanced setting.
- In paper II, ROR score from primary tumour, particularly when combined to proliferation genes, may act as a prognosticator of survival after recurrence beyond clinical prognostic determinants. It supports the utility of the combination of different prognosticators in routine practice and offers an alternative when the biopsy of the relapse is not feasible.
- In paper III, genomic signatures demonstrated to compete well with IHC subtypes on a long-term prognostication in early breast cancer and that the use of several biomarkers may improve the ability to predict survival compared with a single biomarker.
- In paper IV, a significant improvement of survival after local and loco-regional relapse has been described over three decades. An improved local control after primary surgery plays an important role leading to later and less extended local relapses with a higher chance to be radically operated. However, older women benefited less of the advances in breast cancer treatments, likely due to less aggressive therapeutic approaches. Therapy after relapse excision needs standardization to achieve further enhancements.

DISCUSSION

Breast cancer is a multifaceted disease whose variegate phenotype only partially recapitulates the underlying biological complexity. Treatment choices in routine management principally rely on the clinical and pathological characteristics of the disease, although molecular classification currently offers information alongside that provided by clinical and pathological examination. [121,239] Breast cancer phenotype continuously evolves during tumour progression and, while methodological issues might in part explain discrepancy in biomarker expression between primary tumour and metastasis, the contribution of innate and treatment-induced genomic instability is well demonstrated. [191-194,262,263,265,269] Loss of ER and PR in metastasis as compared to primary tumour is associated to poorer post- relapse survival in contrast to stable receptor expression. The use of adjuvant endocrine therapy alone or in combination to chemotherapy has been significantly associated to higher proportion of tumours losing hormone receptors during disease progression in comparison with no adjuvant treatment. [191,192] Indeed, it has been shown that metastases are enriched for proliferation and migration genes as compared to primary tumours and the pattern of genomic heterogeneity between primary tumour and metastasis reflects a pattern of acquired oestrogen independence. [266] The studies in this thesis focused on the prognostic role of proliferation in advanced breast cancer, its interaction with other known clinicopathological and molecular prognosticators and the investigation of their combination for the purpose of refined breast cancer stratification.

Sustained proliferation is essential in cancer growth and progression. [29] Paper I provided the first evidence of the prognostic value of proliferation in advanced disease and revealed a substantial instability in proliferation rate between primary tumour and metastasis. It is not surprising that Ki67 did not retain an independent association with post-relapse survival when the effect of other prognostic variables was examined. This is in line with most studies in early breast cancer, especially when lymph node positive and, thus, more aggressive tumours were analysed. Moreover, ER-negative tumours, which are notably characterized and driven by higher proliferation rates, were overrepresented in this study cohort in comparison with a general breast cancer cohort (36%), leading to an overlap of biological and prognostic information. Variation in Ki67 rate during endocrine treatment and predictive ability of this change has been shown in prospective cohorts in the neoadjuvant setting. [247,248] In this study, a clear association between treatment as well as most of the explored clinical variables and change in Ki67 was not demonstrated. Despite that, the significantly better survival associated

to lower rate of Ki67 in metastasis suggest an undiscovered underlying biological rationale worth further explorations.

Clinical management of metastatic breast cancer may be improved by the reassessment of biomarkers in the relapse tissue. Molecular subtypes vary in metastasis as compared with primary tumour and breast cancer subtypes in the relapse showed a prognostic value in advanced disease. [195,266] However, metastasis biopsy, although frequently feasible, could turn out in the collection of insufficient material for gene expression analysis. Paper II provided the evidence that ROR, as assessed in primary tumour tissue, has an independent prognostic value in terms of post-relapse survival and might allow tailored prognostication in advanced setting. This paper provided also a further evidence of the independent prognostic relevance of proliferation after relapse occurrence and to its ability to enhance the prognostic performance of ROR on the top of other clinical and molecular prognosticators.

In line with the increasing evidence of the reciprocal contribution of IHC markers and gene signatures in refining breast cancer stratification and prognostication, Paper III revealed that the 21-gene array and PAM50 provided additional prognostic information beyond Ki67 and IHC subtypes on a long-term follow-up. Additionally, all the investigated signatures added significant information in the lymph node positive subgroup, in which the value of genomic signatures is notably still controversial. Ki67 alone did not outperform any gene expression array, most of which are driven by proliferation. However, all the IHC biomarkers together added significant prognostic information compared to all gene arrays except PAM50, confirming its strong biological basis. None of the genomic signatures provided relevant prognostic contribution in ER-negative tumours further indicating their limited value in this subgroup. The results were confirmed in a second and not comparable cohort highlighting the strength and generalizability of these findings.

During the past two decades, new drugs have been introduced in breast cancer treatment. Whether these new compounds have led to an improved survival in the general population outside clinical trials has been matter of debate. In this sense, population-based studies from cancer registries represent an invaluable tool for survival trends explorations. We demonstrated a survival improvement after local and loco-regional relapse of breast cancer over 34 years. However, survival in locally relapsed tumours was not improved in the years 2000-2014 compared with the years 1990-1999. Although interpreting these results is complicated by the increasing use of breast conserving surgery followed by radiotherapy in the last 20 years, these findings suggest the need of more standardized therapeutic approaches after relapse excision. The last two decades have been also characterized by a more extensive

use of adjuvant systemic therapies, which have been found to independently correlate with worse post-relapse survival. [14] Selection of more aggressive and resistant cell clones, with acquired oestrogen-independence especially in endocrine-treated tumours, could explain the lack of improvement in survival in the years 2000-2014 compared to the previous decade. Moreover, survival did not improve over time in the older population diagnosed with more extended relapses. Less intensive treatments due to comorbidities as well as age- related decreased functional reserve of multiple organs, physiological pharmacokinetic modifications, and use of concomitant medications might contribute to the reduced benefit from cancer treatments and, consequently, unchanged survival.

The abovementioned considerations leave some unanswered questions and open future research perspectives. In particular:

- Exploration of the biological grounds of the change in Ki67 during tumour progression and its interaction with clinical factors, particularly given treatments;
- Prospective investigation of Ki67 prognostic and predictive value in advanced disease and its contribution as a component of a prognostic and predictive algorithm in metastatic breast cancer;
- Identification of low-risk patients who could benefit mostly of first line endocrine treatment for metastatic disease (e.g. as identified by PAM50 ROR);
- Promoting clinical trials investigating drugs in highly selected population of patients identified by multilevel integration of stratification biomarkers (e.g. fraction of HER2-positive tumours with unaltered PI3KCA pathway and normal levels of PTEN within the HER2-enriched tumours);
- Identification and improvement of therapeutic strategies based on the evidence of potential treatment-induced clonal selection and genomic modifications;
- Canalizing research efforts with the aim to find efficacious treatment options for those groups of patients usually not included in clinical trials and who benefit less of the therapeutic advancements (e.g. older patients);
- Standardizing treatment protocols using more tailored patient risk categorization based on clinical, molecular and new genomic stratification markers.

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