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**PROGNOSTIC AND PREDICTIVE
FACTORS IN VIETNAMESE BREAST
CANCER: A COMPARISON WITH
SWEDISH PATIENTS AND EFFECT
ON SURVIVAL**

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ABSTRACT

Breast cancer is a leading cause of cancer death among women in Western and Asian countries. Previous studies showed that Asian and African-American patients more often had poor tumor prognostic factors than Caucasian patients. There are however only few reports on tumor prognostic factors and survival in Vietnamese breast cancer patients. The aim of this study was to investigate prognostic/predictive factors in Vietnamese operable breast cancer which were compared with those in Swedish breast cancer patients and to estimate survival. Primary breast cancer tissues were collected randomly for analysis of hormone receptors, *HER2* status and cell proliferation. Clinical information, pathology report and treatment protocols were obtained from the files in the National Cancer Hospital, Vietnam.

The hormone receptor content in tumors from Vietnam was analyzed by immunohistochemistry (IHC) using an automated slide stainer (Bench MarkXT, Ventana). Tumors with $\geq 10\%$ stained nuclei were considered as receptor positive. Tumors from Sweden were analysed with an enzyme immunoassay (EIA) with a cut-off point of ≥ 0.10 fmol/ μ g DNA as positive. We found that differences of ER/PgR positivity between Vietnamese and Swedish breast cancer patients. The ER(+) rate was higher in premenopausal but lower in postmenopausal Vietnamese patients as compared to Swedish patients (71.1% vs. 58.4%, 44.7% vs. 71.6%, respectively). The PgR(+) tumors were found in 57.8% of pre- and 24.7% of postmenopausal Vietnamese patients. The corresponding figure for Swedish patients was 72.9% and 65.6%, respectively.

We used IHC and silver *in situ* hybridization (SISH) technique to assess the *HER2* status for Vietnamese and compared to Swedish series with tumors analyzed by IHC and FISH. It was found that tumors from Vietnamese patients with strong, intermediate and low levels of *HER2* protein expression were 39%, 11% and 50%, respectively. The concordance between IHC and SISH was 87%. Postmenopausal women were amplified in 55% as compared to 36% in premenopausal women. *HER2* gene amplification occurred more often in ER(-), PgR(-) tumors and in ductal carcinomas. *HER2* gene amplified rate was present in 41% of Vietnamese breast cancers and 13% in a series of Swedish breast cancers.

We chose the samples from age-matched patients treated in Stockholm, Sweden. Cell proliferation in the two series was stained by anti-Ki67 antigen with an automated procedure. Ki67 index was calculated by counting stained cell nuclei in a total of 400 cells in intermediate area. No difference in distribution and mean of Ki67 indices was seen between the two series, 27.7% ($\pm 17.1\%$) vs. 26.9% ($\pm 23.1\%$) or with respect to age, tumor size and lymph node status. Swedish patients with poor prognosticators had significantly higher Ki67 indices than Vietnamese patients, 52.8% vs. 31.9% in ER(-), 39.6% vs. 30.7% in PgR(-) and 40.1% vs. 28.3% in *HER2*-amplified tumors.

We estimated survival by using the life-table method. The Cox model was used to determine the relationship between survival and prognostic factors and treatment. The disease-free survival rate, overall survival rate and cancer-specific survival rate in Vietnamese patients was 75.8%, 80.6%, and 86.4%, respectively at 5 years; 62.3%, 68.1%, 78.9%, respectively at 9 years. Women with poor prognostic factors had worse survival. Postmenopausal women had significantly lower survival as compared to premenopausal women as analyzed by univariate analysis (HR=0.6, 95% CI: 0.38-0.95, $p=0.029$), however, not by multivariate analysis (HR=0.67, 95% CI: 0.41-1.08, $p=0.1$). Premenopausal women had more benefit than postmenopausal patients from either endocrine treatment or chemotherapy.

This thesis suggests that Vietnamese breast cancers have different tumor cell characteristics to those reported for Caucasian patients in general.

Keywords: Breast cancer, Vietnamese women, Swedish women, comparative study, prognostic factors, predictive factors and survival

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- II. **Thang VH**, Tani E, Van TT, Krawiec K, Skoog L: HER2 status in operable breast cancers from Vietnamese women: Analysis by immunohistochemistry (IHC) and automated silver enhanced in situ hybridization (SISH). *Acta Oncol.* 2011 Apr; 50 (3): 360-6. Epub 2011 Feb 21.
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LIST OF ABBREVIATIONS

| | |
|-----------------------|--|
| ASR | Age standardization rate |
| ELISA | Enzyme-linked immunosorbent assay |
| LBA | Ligand binding assay |
| EIA | Enzyme immunoassay |
| IHC | Immunohistochemistry |
| RT-PCR | Real time reverse transcription-polymerase chain reaction |
| HRs | Hormone receptors |
| ER α , β | Estrogen receptor α , β |
| ER(+)/(-) | Estrogen receptor (positive)/(negative) |
| PgR(+)/(-) | Progesterone receptor (positive)/(negative) |
| HER2(+)/(-) | Human epidermal growth factor receptor-2 (positive/negative) |
| SISH | Silver <i>in situ</i> hybridization |
| FISH | Fluorescence <i>in situ</i> hybridization |
| Ki67 | Cell proliferation specific nuclear antigen |
| VEGF | Vascular endothelial growth factor |
| DTCs | Disseminated tumor cells |
| CTCs | Circulating tumor cells |
| ALND | Axillary lymph node dissection |
| RT | Radiotherapy |
| RS | Recurrence score |
| OS | Overall survival |
| BCSS | Breast cancer-specific survival |
| DFS | Disease-free survival |
| EBCTCG | Early Breast Cancer Trialists' Collaborative Group |
| VS. | Versus |
| OR | Odds ratio |
| HR | Hazard ratio |
| CI | Confidence intervals |
| NPI | Nottingham prognostic index |
| WHO | World Health Organization |
| UICC | Union for International Cancer Control |

1 INTRODUCTION

1.1 EPIDEMIOLOGY OF BREAST CANCER

1.1.1 Incidence

Breast cancer is one of the most common cancer among women in both developed and developing regions [1]. There are reports that the incidence varies among ethnicities and is low in Asian and African women, but higher in Caucasian women [2]. The incidence of breast cancer in women living in Hanoi, Vietnam (17.5/100,000) is much lower than reported for Vietnamese (36.6/100,000) and Caucasian women living in the US (98.7/100,000) [3]. Another report on breast cancer incidence for Vietnamese women living in the United States found an incidence rate of 55.5/100,000 and this was lowest among Asian and much lower than non-Hispanic White women [4].

The incidence in Sweden was 145.2/100,000 in 2009 (www.socialstyrelsen.se). So far, all published data have suggested that Vietnamese women have a considerably lower incidence of breast cancer than Swedish women. A most recent study has shown that immigrant Vietnamese in Sweden had half the incidence of breast cancer compared with native Swedish [5]. This difference in incidence has been attributed to lifestyle and genetic factors [2]. Breast cancer incidence has been reported to have increased gradually in Asian populations in recent years [2]. There are no data on the incidence of cancer nationwide in Vietnam, but in a recent report of cancers from different regions of Vietnam it was shown that the incidence for all cancers increased continuously over time and that breast cancer was the most common cancer in females, 24.55/100,000 [6, 7]. This increasing incidence of breast cancer is probably due to changes in risk factors such as childbearing, exogenous hormone exposure and reduced physical activity [2]. It seems likely that breast cancer will become a big burden for healthcare in many developing countries.

1.1.2 Breast cancer mortality

Breast cancer is still the major cause of cancer death, although mammographic screening has resulted in reduced mortality by as much as 30% [2, 8, 9]. The mortality rates in European countries declined by 6.9% between 2002 and 2006, from 17.9 to 16.7/100,000 [10]. Breast cancer survival has improved markedly in UK, Denmark as compared to Canada, Norway and Sweden [11-13]. Breast cancer mortality after implementation of mammography screening in Sweden decreased 11% for women

between 40 and 79 years [14]. This is also likely to have resulted from more advanced treatment [15]. Multidisciplinary management and adjuvant therapy should also be accounted for the increased survival [8].

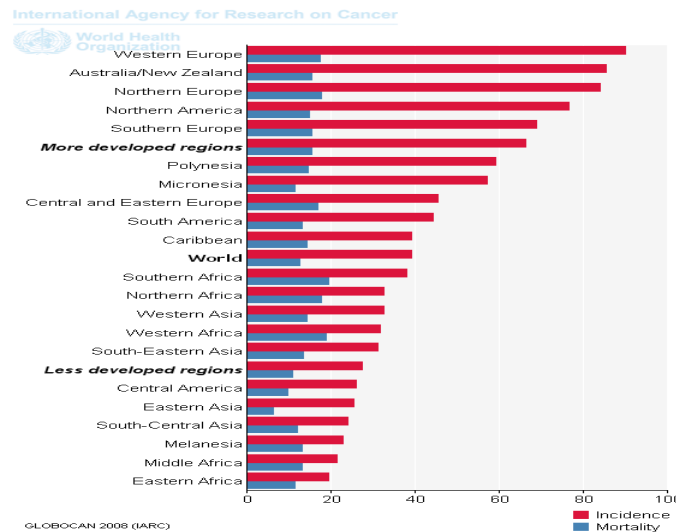


Fig. 1: Incidence and mortality in different regions worldwide. Estimated ASR per 100,000 [16]

Variation in breast cancer survival was seen in different subpopulations [9, 17] or ethnicities [4, 17]. The mortality is higher in Caucasian, as compared to African-American [18], which can result from various tumor cell characteristics which influence the outcome of treatment [19-21]. Socioeconomic status is also defined as an additional factor that influences breast cancer outcome [19, 22]. In recent years, several studies have shown difference in cancer mortality among Asian women. Interestingly, Vietnamese breast cancer patients had the lowest mortality rate among Asian women. In addition, it is also of interest that Vietnamese women living in the US, but born outside of the country had a risk of mortality close to four times as high as USA-born Vietnamese, while US-born Vietnamese women, who had a substantially lower risk of mortality (HR=0.3; 95% CI=0.1, 0.9) as compared with other ethnicities [17]. As mentioned above, although breast cancer is the most common cancer in Vietnamese women, breast cancer death has been reported to be 5.69% of all cancers in women nationwide [23]. Different biomarker frequencies in various populations may partly explain the variety of treatment outcome [19].

1.2 PROGNOSTIC AND PREDICTIVE FACTORS

Breast cancer treatment has been more individualized over the last decades due to the discovery of prognostic and predictive biomarkers. Prognostic and predictive factors are often related to various tumor cell characteristics while tumor size and lymph node status more likely reflect the duration of disease [24]. A prognostic factor is capable of providing information on clinical outcome at the time of diagnosis, which is usually independent of therapy and is often an indicator of growth, invasion, and metastatic potential [25, 26]. The most important role is that which helps to discriminate between a group of patients with good prognosis who do not require adjuvant systemic therapy after local surgery and a group with a poor prognosis for whom additional treatment is indicated. A predictive factor is capable of providing information on the likelihood of response to a given therapeutic modality, for example, hormone receptors which so far have been the best predictive tumor markers [25]. For example, lymph node status is an important prognostic factor, but does not supply information on the likelihood of response to therapy. There are, however, some makers which have both predictive and prognostic values. Of those, HER2 expression is a predictive factor since it indicates the likelihood of response to targeting therapy and is also a prognostic factor signaling poor prognosis. Recently, it has been described that markers such as cell proliferation, circulating tumor cells as well as circulating cell-free DNA and micro RNA and multigene signatures can be of value in the management of breast cancer patients [27]. Today, several factors in breast cancer are used in clinical practice for aiding choice of appropriate treatment [28].

1.2.1 Tumor size

Tumor size is as an independent prognostic factor and larger tumors have been found to have negative effects on breast cancer-specific survival [29]. Tumor size predicts both relapse and distant relapse in non-operable stage [30]. Patients had a relapse-free survival rate of 91% at 10 years and 87% at 20 years for tumors <1 cm compared to 73% and 68% for tumors greater than 1cm [31]. Variation in tumor size predicts 10-year distant metastasis risk ranging from below 10% for tumors less than 10 mm to 90% for tumors larger 30 mm [32]. An increased mortality rate was associated with larger tumor size (11-20 mm tumors vs. 1-10 mm tumors, standard morality ratios =1.42) in hormone receptor-positive breast cancer patients [33]. The prognostic impact of tumor size is partly related to the fact that tumor size is capable of predicting incidence of axillary lymph node metastasis, 10% in tumors less than 1 cm and 35% for

a tumor diameter of 1.6-2cm [34]. An increase in tumor size has been associated with a significant risk of lymph node metastasis in stage I [35, 36]. Tumor size is still an apparent independent factor for long-term survival and patients with larger tumors had lower survival rate [37, 38].

1.2.2 Axillary lymph node

As the axillary lymph nodes receive 85 percent of the lymphatic drainage from all quadrants of the breast, histologic examination of removed axillary lymph nodes is the most accurate method for assessing spread of disease [28].

Axillary lymph node status is one of the most important prognostic factors in women with early stage breast cancer [32, 39]. Lymph node metastasis has been assessed as a strong independent factor for both overall survival (OS) and disease free survival (DFS) [40, 41]. In addition, 5-year specific survival and disease-free survival rates were lower for patients with micrometastasis (pN1mi) than for those with node-negative disease (pN0) [42]. In multivariate analysis, the presence of occult metastasis in the lymph nodes was found to have a prognostic impact on survival as assessed by a 25-year of follow-up [37]. Postmenopausal women with more than 3-node metastasis have ten times higher mortality rate than those without node involvement [36]. The extent of node metastasis is also an important significant prognostic factor. The overall survival in patients with node metastasis was significantly lower in patients with extra-capsular tumor growth as compared to those with intra-capsular growth [43]. Even in advanced tumor stages, clinical node involvement was an independent factor for predicting relapse [30]. Thus, the number of metastatic nodes today is used as a guide to recommend adjuvant systemic therapy. Endocrine therapy alone is indicated for node negative patients with hormone receptor-positive tumors and adjuvant chemotherapy often being applied in those cases of patients with equal to or above 4 metastatic nodes [44]. Patients who with one to three positive axillary nodes have additional risk factors are considered, and patients with four or more positive axillary nodes is always recommended for adjuvant radiotherapy [28].

1.2.3 Histologic type

Histological examination of cancer cell morphology and architectural patterns are of importance in defining tumor subtype. Invasive ductal carcinoma is the most frequent subtype and presents two thirds of all breast cancers. This cancer is aggressive and typically metastasizes to bone, lung and liver. The lobular subtype is found in

approximately 10% of patients and a better survival is expected if patients receive endocrine therapy as compared with patients with invasive ductal carcinoma [45]. Lobular carcinomas are more often low grade and patients have a better prognosis than those with ductal carcinomas [46]. The medullary type is typically hormone receptor-negative, HER2(-) and p53(+) positive with an aggressive clinical behavior [47]. It is reported that about 5-7% of all breast cancers are of this type. Mucinous carcinoma is found in 3% of patients and tends to have a rather good prognosis. Papillary carcinoma represents 1-2% of all breast cancers and is in a majority of cases ER(+) and has a good prognosis. However, patients with a ductal or lobular infiltrating histological type had a poor prognosis compared with those with other subtypes [48].

1.2.4 Histologic grade

Assessment of histologic grade: Tumor grade is defined as prognostic factor in breast cancer [49]. Invasive carcinomas are today graded according to Scarff-Bloom-Richardson (SBR) or Elston-Ellis system; they are strongly correlated to overall and recurrent free survival [28, 50, 51]. The grading is based on the sum of scores assigned three histological features: degree of ductal differentiation, pleomorphism, and mitotic index.

Prognostic value: A comparative report from France showed that both these two histological grade systems were strongly predictive for overall and disease-free survival. Assessment of 1,831 patients with operable breast cancer showed that patients with grade I tumors had a significantly better survival than those with grade II and III tumors [52]. Patients with low grade tumors had survival higher than high grade tumors, 9% and 20% in disease stage I and II, respectively [46]. Tumor grading was found to be the strongest independent prognostic factor for both OS and DFS in Malaysian [40] or breast cancer-specific survival in both Caucasian and African-American population sampled [29]. Postmenopausal women with high grade tumors have eight times higher mortality as compared to those with low grade tumors [36]. A similar correlation was also seen for 10-year disease free survival in untreated young patients [53]. The tumor grade was also confirmed as an independent marker of long-term survival in patients with lymph node negative disease [37]. High tumor grade was likely to predict regional metastasis and a 2.69 times increased risk of node metastasis was observed for high-grade tumors as compared with low-grade tumors [35]. In operable breast cancer, histologic grade was an independent predictor of both BCSS and DFS [54].

Predictive value: It has also been described that SBR tumor grade is a marker of chemosensitivity in invasive ductal breast carcinomas. Thus, grade III tumors responded better to neoadjuvant treatment than SBR grade I tumors, independently of type of chemotherapeutic regimens [50]. In patients treated with radiotherapy alone, histological grade was important for prediction of local and distant disease control [30]. High tumor grade was a significant predictor of distant recurrence in patients with node-negative and tamoxifen-treated breast cancer [55].

1.2.5 Estrogen receptor and progesterone receptor

There are two kinds of hormone receptors, estrogen receptor (ER) and progesterone receptor (PgR), which are members of a nuclear hormone receptors superfamily that is located in the cytosol for operation of ligand-dependent transcription factors. There are two types of ER, (ER_{α} and ER_{β}) of which ER_{β} is more widely distributed in the body than ER_{α} , which is expressed mostly in the uterus and mammary gland [56]. The molecular weight for ER_{α} is 65,000 Daltons and 54,000 Daltons for ER_{β} . Both proteins have five functional domains which include a DNA- and a ligand-binding domain. The ligand binding domain in ER_{α} and ER_{β} shows 58% homology which may explain the different responses to various hormones and anti-hormones. Upon ligand binding, ER_{α} and ER_{β} may form homo-dimers or heterodimers which after binding to DNA either activate or suppress genes. The role of ER_{α} - and ER_{β} -driven pathways might change during breast tumorigenesis [57].

Most reports on ER as a predictive or a prognostic factor are related to ER_{α} content which is in excess over ER_{β} [58]. It has been reported that 62% of the tumors are ER_{α} positive and 65% ER_{β} positive. ER_{α} correlated positively with ER_{β} ($p=0.001$) [59]. Both forms are ER-regulated and mediate the effect of progesterone both in normal breast epithelium and breast cancer cells [60].

Analysis of hormone receptors: Several techniques have been used to assess the hormone receptor content in breast cancer tissues [56]. There have been two major techniques for quantitation of ER and PR in clinical practice and they either involve the binding of a radiolabeled steroid ligand to the receptor, or the recognition of the receptor protein by specific antibodies.

The ligand binding assay (LBA) was the first technique developed for analysis of ER and later PgR in tumor cytosolic fraction which was correlated with response to endocrine treatment [61]. The assay is based on binding of radioactive estrogen to the receptor protein in homogenized fresh tumor tissue. It measures the ER or content of

both epithelial, stromal cells in tumors. This quantitative technique allows analysis of many samples in a few hours and is very sensitive. The ligand binding assay has been the first method to evaluate the receptor content in clinical samples of breast cancer. The advantage of this technique was that it gave a quantitative receptor value with good reproducibility. There were, however, some disadvantages with the ligand binding assay. In the first place it requires fresh or frozen tumor tissue in quantity (at least 0.5-1.0 cm) and fixed in liquid nitrogen at -70°C and shipped on dry ice [62]. Moreover, this technique does not discriminate between tumor cells and benign cells which may result in low or false-negative results because of dilution effects. In addition, it is labour intensive and involves the use of radioactive material that is difficult to assess in less developed countries. The production of specific antibodies to the ER and PgR later led therefore to the development of new assay techniques.

The enzyme immunoassay (EIA) is an assay based on specific anti-ER and anti-PgR antibodies [63]. The epitope of the receptor is recognized by the antibody which is linked to an enzyme. The intensity of the color is quantified spectrophotometrically and the receptor level is determined by comparison to a standard curve. This technique measures the receptor protein and requires fresh or frozen tumors with a size between 0.5-1 cm in diameter. This technique has been used in Sweden since 1988 with the hormone receptor values defined as fmole of receptor normalized to DNA content as measured by the Burton method. In adjuvant therapy, this method was validated in patients administered long term treatment of tamoxifen [64].

Immunohistochemistry (IHC) has rapidly become the predominant method for measuring ER and PR in clinical practice and it can be performed on a variety of samples including fine needle aspirates, core biopsies, fresh or frozen tissue and paraffin-embedded archival tissue. After evaluation in the light microscope, a semi-quantitative receptor value is calculated based on staining intensity and percentage of stained cells. A distinct advantage is that the IHC assay only measures the ER and PgR content in cancer cells. Moreover, this method is today robust, cheap and easy to use in clinical routine work. Thus, IHC is used in most laboratories for measuring ER and PgR in clinical routine pathology [27, 28]. However, the definition of receptor positivity varies between different laboratories and several cut-off points have been suggested [63]. Thus, cut-off points between 1% and 25% have been suggested [56]. However, there is a trend to accept 10% as cut-off between negative and positive tumors.

An excellent agreement for positivity as determined between EIA (cut-off at 0.10fmol/ μ g DNA) and IHC (cut-off at $\geq 10\%$ cell stained) has been published [62, 63]. Newer techniques have been applied to assess hormone receptor status such as Real-time polymerase chain reaction (RT-PCR) and ER messenger RNA or northern blot assay. RT-PCR has high concordance with IHC for ER and PgR status in surgical specimens [65]. However, these procedures are not yet recommended in daily clinic routine [28, 66].

Prognostic factor: The prognostic capability of ER had been described in early studies before ER β was discovered. It can therefore be assumed that the prognostic value of ER is confined to ER α since this isoform is present in excess over ER β in breast cancer cells [57]. In long-term follow-up, hormone receptor status has been identified as an independent prognosticator of outcome [38, 67-69] and an independent prognostic factor for both OS and DFS in Asia patients [40]. However, another cohort study from Sweden failed to demonstrate a significantly prognostic value of ER at 5 years after diagnosis, in spite of lower survival in patients with ER negative tumors [39] or at 10-years post-diagnosis in untreated young patients [53]. Patients with double hormone receptor positive tumors had the best breast cancer-specific survival with a 50% of risk reduction of breast cancer death compared to those with ER/PgR(-) tumors. Hormone receptor status was identified therefore as an independent prognostic factor of outcome [67], which was also observed for postmenopausal women [36]. Although patients with hormone receptor-negative tumors received greater benefit from neoadjuvant chemotherapy in terms of pathologic response, they have worse outcomes in terms of recurrence and survival as compared to hormone receptor-positive patients [70]. However, PgR status is reported as a prognostic factor for survival but not ER with 25-year of follow-up [37]. Today, it is accepted that the prognostic information by ER is not sustained at long-term follow-up. These conflicting results may be explained by small patient series, short follow-up, various techniques for ER analysis and the lack of standard cut-off points between receptor positivity and negativity. The prognostic value of PgR alone is less well documented. A report from Sweden showed that PgR level was an independent factor in patients without systemic adjuvant treatment [32].

Predictive value: while the prognostic role is still a controversial issue, the predictive value of hormone receptor status is well defined. Receptor positivity will predict response to endocrine treatment in 75-80% of patients with ER(+) tumors as compared with less than 10% of those with ER(-) tumors [71]. The predictive value of tumor

receptor content has been verified for both premenopausal and postmenopausal patients and in both advanced and early stage breast cancer [27, 28]. In a recent review of randomized studies, it was concluded that patients with ER(+) tumors had a 31% death risk reduction if treated with tamoxifen for 5 years, but had no effect on survival in ER(-) patients as reported from the Early Breast Cancer Trial' Collaborative Group [68]. Hormone receptor status has also been described as a predictive factor for chemotherapy responsiveness: thus, both total and pathologic complete response rates were higher in hormone receptor negative patients (26% and 32%, respectively) than in hormone receptor-positive patients (4% and 7%, respectively; $p < 0.001$) [70]. ER(-) tumors are better severed by chemotherapy. In patients treated with chemotherapy, the 5-year disease free survival rate was 23% for patients in the ER(-) tumors category vs. 7% for patients whose tumors were ER(+) [69]. The predictive value of PgR is less clear. In patients with inflammatory cancer, ER(+) tumors are more sensitive to anthracycline-based chemotherapy [72].

Knowledge about ER/PgR status helps to select appropriate treatments in clinical practice. Therefore, it should be measured on primary breast cancer tissue and metastatic lesion if possible [27]. Tumors with expression of ER and/or PgR in as low as 1% of the tumor cells may respond to endocrine treatment but some studies suggest that 10% should be used as a cut-off. Notably, if patients whose tumors contain low levels of ER by IHC as classified as weak positive (1% to 10%) should be discussed before a decision of treatment is made [66]. Patients with uncertain or hormone receptor-negative tumors are often recommended to be administered adjuvant chemotherapy [28].

1.2.6 HER2 status

The HER2 oncogene is located in chromosome 17 that encodes for a 185 KD transmembrane glycoprotein receptor belonging to the epidermal growth factor receptor (EGFR) family including HER1/EGFR, HER2, HER3 and HER4 [73, 74], which are crucial in the activation of subcellular signal transduction pathways controlling epithelial cell growth, regulation of cell proliferation, differentiation and survival. Epithelial cells express around 20,000 HER2 receptors on their cell membranes. A fraction of breast cancers overexpress the HER2 receptor as a result of gene amplification which results in a 100-fold increase of the protein on cell membranes [26, 74]. So far, no specific ligand has been identified for HER2 but

specific antibodies to the receptors have been produced. Such antibodies suppress HER2- stimulated growth both *in vitro* and *in vivo* [75].

Assays for HER2: Procedures for measurement of HER2 protein are Western blotting, enzyme linked immunosorbent assay (ELISA) and immunohistochemistry (IHC). In IHC, the membrane staining patterns are identified as negative (0 or 1+) or positive (3+) and equivocal (2+). There are several methods to assess amplification of the *HER2* oncogene such as fluorescence *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH), silver enhanced *in situ* hybridization (SISH) and polymerase chain reaction (PCR) [76, 77]. In recent years, SISH has emerged as a new method which uses light microscopy and allows assessment of *HER2* gene status with a high concordance with FISH [77, 78]. Today, it is generally accepted that *in situ* hybridization or PCR better define *HER2* amplification than various protein measurements. The level of discordance in HER2 status paired primary tumor and metastasis is noteworthy [79]. It seems reasonable to reanalyze HER2 in tissue from recurrent lesions; particular if the primary tumor is negative or weakly positive.

Prognostic value: women with *HER2* gene amplification have poor prognosis. *HER2* amplification has been identified as an independent prognostic factor in breast cancer with lymph node metastasis and is also associated with other poor prognostic factors [80, 81]. It also predicted shorter disease free and cancer-specific survival in ER(+) patients but not in ER(-) cases [53, 82]. Overexpression of HER2 protein also influences in outcome of treatment in patient with ER(+) tumors treated with adjuvant endocrine therapy [82], which was also a negative prognostic factor in untreated patients with ER(+) tumors .

Amplification of *HER2* oncogene in tumors and metastatic lymph nodes may be a useful independent marker of poor prognosis and correlated with tumor recurrence and shorter survival in early stage [38, 83]. The *HER2* oncogene was an independent prognostic factor for DFS in premenopausal women with node negative disease [84]. However, HER2 overexpression did not adversely influence response to adjuvant oophorectomy plus tamoxifen treatment in patients with estrogen receptor-positive tumors [85] and notably, in a population from Asia [40]. It has been suggested that patients with *HER2* positive early breast cancer should be treated with adjuvant therapy with trastuzumab and chemotherapy [28].

Predictive value: women with a high level of HER2 protein expression or an amplified *HER2* gene benefit from treatment by HER2 antibody such as trastuzumab (Herceptin) and lapatinib, both in the adjuvant and metastatic disease settings [27, 86]. Addition of neoadjuvant trastuzumab for HER2(+) tumors results in a superior response irrespective of hormone receptor status [70]. Furthermore, HER2 status is also likely to predict sensitivity to chemotherapeutic agents. Thus, tumors with HER2 overexpression potentially have good response to anthracycline-based regimen [27, 74, 87] and little resistance to paclitaxel treatment alone, but get more benefit if in combination with AC regimen [88] and have improved survival with CMF regimen [89]. In contrast, HER2 negative inflammatory cancers were reported more sensitive to anthracycline-based chemotherapy [72]. The role of HER2 status to predict endocrine therapy is still conflicting, while an inverse correlation to tamoxifen response has been described [71, 90, 91]. This correlation does not seem to exist in premenopausal patients [53, 85]. It is considered nowadays to be indicated with trastuzumab both in the adjuvant and metastatic disease settings for patients with *HER2*-amplified tumors. It has been described that the HER2 status can show disagreement between primary and recurrent tumors which likely influence response to therapy [79]. Recently, the panel of the St Gallen international breast cancer conference strongly supports that HER2, together with hormone receptor status, is useful in defining subtypes of breast cancer [92].

1.2.7 Cell proliferation

The proliferative rate of breast cancer has been assessed by various methods, including mitotic index, thymidine labeling index, bromodeoxyuridine labeling, S-phase fraction determined by flow cytometry and IHC using monoclonal antibodies to antigens associated with proliferation of cells, for instance Ki67 [93, 94]. Most recent studies have however assessed cell proliferation by determining S-phase fraction using flow cytometry or Ki67 staining. The Ki67 protein is a nuclear protein doublet, 345-395 kDa, playing a pivotal role in maintaining cell proliferation. Ki67 is present in all non-G0 phases of the cell cycle. Beginning in the mid-G1, the level increases through S and G2 to reach a peak in M. In the end of M phase, it is rapidly catabolized. The Ki67 labelling index is defined as the percentage of cells in a tissue with nuclear staining for Ki67 with different antibodies, such as MIB-1 and K-2 [94-96]. The median value for

MIB-1 staining shows large variations in breast cancers with figures of less than 10% up to values above 20% [97].

Prognostic value: The rate of cancer cell proliferation measured by Ki67 has also been shown to be a good prognostic factor. Ki67 index showed a significant relation with survival in node-positive patients [38]. Klintman et al found that a proliferation rate over 20% was correlated to a worse prognosis in premenopausal patients with node-negative breast cancer [84]. The usefulness of Ki67 as a prognostic marker in early breast cancer has been shown in a recent meta-analysis involving 12,155 patients [98]. Another study found that the rate of proliferation was only an independent prognosticator if other factors such as age, tumor size, histological grade and nodal status were co-analyzed [99]. Recent findings confirmed that Ki67 expression in metastatic lymph nodes was an independent factor for disease-free survival [38] and this marker could be considered to add prognostic information on the risk stratification with the use of the St. Gallen consensus guideline [100]. Thus, it is presently recommended to assess cell proliferation as a part of the pathological routine work-up [28].

Predictive value: The rate of cell proliferation as measured by Ki67 staining is an important parameter *in vitro* for the selection of treatment strategies aimed at inhibiting cell proliferation [94]. Recent studies on the Ki67 index as a biomarker for response to chemotherapy have shown that it is an important marker for both neoadjuvant therapy and metastatic settings [24, 101]. Patients with a Ki67 index of less than 15% benefited from endocrine therapy in contrast to those with higher Ki67 indices [102]. Recently, the St Gallen international breast cancer conference recommended the use of proliferation, such as Ki67 staining to select optimum treatment for early breast cancers [44, 92]. Though other markers of cell proliferation, such as MI, TLI and S-phase determination by DNA flow cytometry, have a significant correlation with other clinicopathologic factors they do not appear useful in routine clinical work. The American Society of Clinical Oncology (ASCO) tumor marker expert panel did not recommend the use of S-phase or other flow cytometry techniques to assign patients to prognostic groupings [27]. Today, these techniques have however, to a large extent, been replaced by immunohistochemical analysis of Ki67. Guidelines for diagnosis and treatment recommend the use of Ki67 staining for pathologic work-up in clinical practice to guide systemic treatment [28, 44].

1.2.8 Internationally approved combination of prognostic and predictive factors

1.2.8.1 TNM grouping system

Table 1: Anatomic stage/prognostic groups [28]

| |
|---|
| Stage 0 — $T_{is} N_0 M_0$ |
| Stage I — $T_1 N_0 M_0$ (including T_{1mic}) |
| Stage IIA — $T_0 N_1 M_0$; $T_1 N_1 M_0$ (including T_{1mic}); $T_2 N_0 M_0$ |
| Stage IIB — $T_2 N_1 M_0$; $T_3 N_0 M_0$ |
| Stage IIIA — $T_0 N_2 M_0$; $T_1 N_2 M_0$ (including T_{1mic}); $T_2 N_2 M_0$; $T_3 N_1 M_0$; $T_3 N_2 M_0$ |
| Stage IIIB — $T_4 N_{0-2} M_0$ |
| Stage IIIC — Any T $N_3 M_0$ |
| Stage IV — Any T Any N M_1 |

Definition: T-tumor size (cm), N-lymph node, M-distant metastasis.

Clinical and pathologic stages consist of factors which reflect on extent of disease which is strongly prognostic. The TNM stage is apparent to add prognostic factors at 5-year relative survival. The 5-year relative survival rates were 96% for Stage I, 86% for Stage II, 59% for Stage III and only 26% for Stage IV [46].

1.2.8.2 Nottingham prognostic index

The Nottingham prognostic index (NPI) which combines nodal status, tumor size and histological grade, has been validated by further studies in Nottingham and by associated studies in several other countries [103]. $NPI = \text{Lymph node Stage (1-3)} + \text{histologic grade (I-III)} + \text{tumor size (cm)} \times 0.2$, if $NPI < 3.4$: good prognosis, 3.4-5.4: moderate prognosis and > 5.4 : good prognosis. One study from a Nottingham hospital reported that 15-year survival was related to NPI-good prognosis, moderate prognosis and poor prognosis, as 80%, 42% and 13%, respectively, [104]. Similarly, this index was confirmed as an independent prognostic factor 10-year survival reduced from 88% in the good prognostic group to 40% in poor prognostic group [48]. In recent years, NPI has been shown to be a reliable prognostic tool in triple negative breast cancer [105].

1.2.8.3 Prognostic factors as markers for selecting treatment modalities

Based on all approved prognostic and predictive factors, the expert panel of the St. Gallen Consensus Conference agreed to recommend a guideline for treatment practice [44].

Table 2: Threshold for treatment modalities based on prognosticators

| Clinicopathological features | Relative indications for chemoendocrine therapy | Factors not useful for decision | Relative indications for endocrine therapy alone |
|------------------------------|---|------------------------------------|--|
| ER and PgR | Lower ER and PgR level | | Higher ER and PgR level |
| Histological grade | Grade III | Grade II | Grade I |
| Proliferation | High* | Intermediate* | Low* |
| Nodes | Node positive (≥ 4 involved nodes) | Node positive (1-3 involved nodes) | Node negative |
| PVI | Presence of extensive PVI | | Absence of extensive PVI |
| pT size | >5 cm | 2.1-5 cm | ≤ 2 cm |
| Patient preference | Use all available treatments | | Avoid chemotherapy-related side effects |
| Multigene assays | | | |
| Gene signature | High score | Intermediate score | Low score |

*Conventional measures of proliferation include assessment of Ki67 labeling index (e.g. low, $\leq 15\%$; intermediate, 16-30%; high, $>30\%$)

PVI: peritumoral vascular invasion.

1.2.8.4 Adjuvant online (www.adjuvantonline.com)

Adjuvant online, which was developed in the United States, is an internet-based computer program providing 10-year prognosis predictions for early breast cancer patients. When information is entered of patient's age, ER status (positive, negative, undefined), tumor grade (I,II,III, undefined), tumor size (0.1-1 cm, 1.1-2 cm, 2.1-3 cm, 3.1-5 cm, >5 cm), and the number of positive nodes (0, 1-3, 4-9, >9), predictions are obtained of 10-year OS, BCSS, and DFS. According to a cohort study, predictions of adjuvant for all three outcomes were significantly greater than the observed outcomes. The difference between predicted and observed was 5.54% ($P<0.001$), for BCSS, 4.53% ($P<0.001$), and for DFS, 3.51% ($P=0.001$) [106]. But this model predicted and observed outcomes were within 2% for most demographic, pathologic, and treatment-defined subgroups, except in women younger than age 35 years [107]. However, adjuvant online needs to be updated to adjust overoptimistic results in young and high

grade patients, and should consider new predictors such as Ki67, HER2 and mitotic index [108]. Again, 10-year mortality predictions according to adjuvant online are most sensitive to comorbidity levels, particularly among older women from the US [109]. Thus, this model should be based upon validation in other ethnic populations worldwide.

1.2.9 Other prognostic factors

1.2.9.1 Oncotype DX

Oncotype DX or recurrence score (RS) can be performed on formalin-fixed tissues, and is a RT-PCR based assay. The levels of expression of 16 cancer-related genes and five reference genes were used in a prospectively defined algorithm to calculate a recurrence score and to determine a risk group (either low, intermediate, or high) for each patient, which so far is among the best prognostic assays [110, 111].

Prognostic value: Initially, it was found that RS could predict prognosis in node negative patients treated with tamoxifen alone. Thus, 93% patients with low RS (<18) were free of distant disease as compared with only 70% of those with high RS (>31) [55]. This marker is recommended by the ASCO for use in practice in women with node-negative, ER(+) breast cancer [27]. The recurrence score (RS) was an independent significant predictor of recurrence along with age and type of initial treatment. Recurrence was 4% for patients with a low RS (<18), 7.2% for those with intermediate RS (18-30), and 15.8% for those with a high RS (>30) after 10 years of tamoxifen treatment. It is recommended to determine RS for loco-regional radiotherapy in node-negative and ER(+) patients [110]. Together with a prognostic value, it has also been reported that Oncotype DX could predict chemotherapy response.

Predictive value: The Recurrence Score was positively associated with the likelihood of response of neo-adjuvant paclitaxel and doxorubicin in advanced stages [112]. Gene expression also predicts benefit from chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. Patients with high-RS (≥ 31) tumors (i.e., high risk of recurrence) had a large benefit from chemotherapy [111]. The Panel from St the Gallen conference agreed that this predictive factor could be used for selecting chemo-endocrine therapy in patients with ER-positive, HER2-negative early stage breast cancer [44], whereas the role of RS in hormone receptor-negative and HER2-positive breast cancer is less clear.

1.2.9.2 Mammaprint

Mammaprint uses the expression of 70 genes analyzed to identify patients with poor (high risk) or good prognostic (low risk) signatures. Gene expression was a strong independent factor for survival of early breast cancer patients and was particularly strongly associated with outcome in patients with node positive disease [113]. In Stage II-III patients, poor prognostic signatures cancer had 20% completed response to neoadjuvant chemotherapy whereas no patient with good prognostic signatures achieved. Tumors with a poor prognostic signature are more sensitive to chemotherapy [114]. The 70-gene signature is an apparently independent prognosticator for clinicopathologic risk assessment in patients with early breast cancer since this also predicts responsiveness to conventional chemotherapeutic regimens [115]. Despite concerns as to validity, Mammaprint has been cleared as a tool to assess the risk of disease recurrence in women with node-negative breast cancer. The expert panel from ASCO concluded that the clinical utility and appropriate application of Mammaprint need to be established. Thus, until now this marker has not been sufficiently established and trials are required to clarify this role [27, 92].

1.2.9.3 Other markers

Several other factors are defined as prognosticators. In early history, p53 protein accumulation was assessed as an independent prognostic factor and associated with metastasis-free and overall survival [116]. Vascular endothelial growth factor (VEGF) is valuable to predict both general DFS ($P=0.0289$), OS ($P=0.0004$) and in patients who received adjuvant endocrine therapy (DFS, $P=0.0238$; OS, $P=0.0121$). This factor can also contribute to predict metastatic sites [117]. The level of cyclin E expression by IHC was an independent prognostic factor for relapse-free survival (DFS, HR 1.72) and for breast cancer-specific survival (HR 2.86) but not for DFS [118]. Detection of disseminated tumor cells (DTCs) in bone marrow was an independent prognostic factor for DFS and OS in patients with Stage I to III breast cancer [119]. Furthermore, circulating tumor cells (CTCs) were found to be an independent prognostic factor for early relapse but did not predict treatment response [120]. However, measurement of CTCs should not be used to decide treatment in early stage or metastatic disease [27]. The markers described above and other markers such as cyclin D, p27, p21, thymidine kinase or topoisomerase II, DNA content (ploidy) and S-phase are at present not recommended in routine clinic [27, 28]. Their

clinical role has, however, not yet been defined in larger series of patients but it seems likely that they will be of importance in the future.

1.3 TREATMENT FOR BREAST CARCINOMA

Methods for treatment are today dependent on several factors such as stage of disease, patient and tumor characteristics [28].

1.3.1 Surgery

Surgery is crucial in management of breast cancer patients. Modified radical mastectomy refers to the complete removal of the breast and the underlying fascia of the pectoralis major muscle along with the removal of the level I and II axillary lymph nodes. Breast cancer surgery has changed dramatically over the last 20 years. Breast conservative therapy (BCT) is defined as removal of the tumor without removing excessive amounts of normal breast tissue which leads to a cosmetically acceptable result with a low rate of local recurrence. Breast cancer survival after breast conservative surgery is equal to total mastectomy [121]. Traditionally, an axillary lymph node dissection (ALND) was recommended in every patient with invasive breast cancer. Currently, ALND remains the standard approach for patients who have clinically palpable axillary nodes. Completion of ALND is also indicative in patients who have positive sentinel lymph node biopsies since there is a risk that additional nodes are involved [28]. Sentinel lymph node biopsy (SLNB) is indicated for patients with clinically axillary node-negative early breast cancer. With remarkably improved screening techniques and the introduction of sentinel node biopsy techniques, the indications for axillary dissection have diminished in European countries [122]. Sentinel lymph node biopsy results lower morbidity and better quality of life as compared with standard axillary treatment [123]. Notably, the overall false-negative rate for sentinel lymph node biopsy was reported as 7.7% in 20 hospitals in Sweden which often was due to multifocal tumors. However, it was shown to be a reliable method for axillary staging of unifocal breast tumors [124]. Sentinel lymph node biopsy is currently accepted as the standard of care for axillary staging in early breast cancer unless axillary node involvement is suspected clinically or on ultrasound [28].

Surgical oophorectomy, either with open or laparoscopic procedures, causes an immediate and permanent drop in ovarian steroid production for premenopausal patients with hormone receptor-positive tumors. In women with a known or suspected breast cancer susceptibility gene (*BRCA*) mutation, it may be a component of adjuvant

treatment. Breast reconstruction following mastectomy improves psychological health and quality of life [125]. Breast reconstruction can be performed at the time of the mastectomy or at a subsequent operation. There are two general types of reconstructive options: implant-based techniques and autologous tissue reconstructions with tissue flaps.

1.3.2 Radiotherapy

Postoperative radiotherapy not only decreases the risk of loco-regional recurrence including axilla, supraclavicular and internal mammary nodes but also improved survival in high-risk postmenopausal breast cancer patients after mastectomy. The absolute reduction of loco-regional recurrence was 27% after 123 months of follow-up [126]. However, in a premenopausal population, post-mastectomy radiotherapy reduced loco-regional recurrences but did not reduce mortality with 20 years of follow-up [127]. Postoperative radiation reduces local recurrence after lumpectomy from 39% to 14% at 20 years [128]. The addition of radiotherapy to surgery resulted in a rate of local recurrence that was three times lower than surgery alone [129]. In the subset of patients with T3N0 disease, adjuvant radiotherapy improved OS from 33% to 40% and increased the 10-year DFS rate to 15% [126]. The benefit of adjuvant radiotherapy was also seen after 5 years in patients with Stage I-II lymph node negative breast cancer and the disease-free survival (DFS) was significantly higher after postoperative radiotherapy, 88% vs. 77% [130]. One overview of randomized trials reported that radiation therapy reduces 5-year local recurrence with 17-19% and 15-year breast cancer mortality by 5.4% in both node negative and node positive disease [121]. Post-mastectomy radiation therapy was strongly indicated for patients with four or more axillary lymph nodes metastasis and for patients younger than 45 years with 1-3 positive nodes as well as patients at any age with extensive vascular invasion in two or more blocks in conjunction with 1-3 positive nodes. Whole breast radiotherapy reduces the risk of local recurrence by two-thirds and an additional boost gives a further 50% risk reduction. Additionally, radiotherapy has a beneficial effect on survival. The recommendation is at present 45-50 Gy for entire breast treatment for use of eradicating microscopic residual foci of breast carcinoma, and an additional boost up to 60-65Gy to the tumor bed. Notably, this method of treatment is not indicated for elderly patients with good prognosis. Recently, partial breast irradiation (PBI) is a new approach that early results are promising. However, long term follow-up will be needed to confirm these results before partial breast radiotherapy is

accepted as a routine procedure. The Panel of the 12th International Breast Cancer Conference generally accepted PBI as an alternative to conventional external beam boost to the tumor bed [28].

1.3.3 Chemotherapy

Adjuvant chemotherapy: following primary surgery for breast cancer the elimination of possible clinically occult micrometastasis is attempted. Systemic adjuvant chemotherapy plays a major role in improving survival of breast cancer patients [68]. It is of importance to estimate the risk for recurrence or presence of metastasis in individual patients and the likelihood that they will benefit from adjuvant chemotherapy. Until now, clinicopathologic prognostic factors such as patient age, tumor size, histologic grade, lymphovascular invasion, axillary lymph node status, hormone receptor status, and HER2 expressions have all been used to aid treatment decision.

Table 3: Table of selected current adjuvant chemotherapy regimens, adapted from ESMO guidelines 2011 [28].

| Regimen | No. of cycles | Duration of cycles (weeks) |
|----------------------------|---------------|----------------------------|
| AC | 4 | 3 |
| CMF (oral or IV. days 1+8) | 6 | 4 |
| FE ₁₀₀ C | 6 | 3 |
| CE _{1,8} F | 6 | 4 |
| A (or E)→CMF | 4→4 (→8) | 3→4 |
| AP→CMF | 4→4 | 3→4 |
| DC | 4 | 3 |
| AC→P(H) qwk | 4→4 | 3→3 |
| AC→D(H) | 4→4 | 3→3 |
| DCarboH | 6 | 3 |
| ddAC→ddP (G-CSF) | 4→4 | 2→2 |
| DAC | 6 | 3 |
| FEC ₁₀₀ →D | 3→3 | 3→3 |

A, doxorubicin; C, cyclophosphamide; D, docetaxel; E, epirubicin; F, fluorouracil; G-CSF, granulocyte colony-stimulating factor, e.g. filgrastim; M, methotrexate; P, paclitaxel; Carbo, carboplatin; H, trastuzumab, may be given with a taxane; qwk, weekly; dd, dose-dense; →, followed by.

One study showed that women younger than 50 years of age treated with six months of adjuvant anthracycline-based chemotherapy, irrespective of hormone receptor status, reduced the 15-year probability of recurrence (from 54% to 41%) and breast cancer mortality (from 42% to 32%). For women aged 50 to 69 years, treatment reduced the 15-year probability of recurrence (from 58% to 53%) and breast cancer mortality (from 50% to 47%). The absolute benefit of chemotherapy is thus about three times greater for young as compared to old women [68]. Adjuvant systemic therapy appears to provide additional local tumor control after conservative surgery. At present, anthracycline is recommended for most patients and especially for patients with HER2(+) tumors except for elderly patients or those with heart problems [74]. Clinicopathologic features which convey a relative indication for chemotherapy include ER negativity, grade III tumors, elevated cell proliferation determined by Ki67 staining, four or more lymph nodes positive, peritumoral vascular invasion, tumors greater than 5 cm and patient preference [131].

Neoadjuvant therapy: Neoadjuvant chemotherapy is normally indicated for locally advanced breast cancers (Stages IIIa-c) or inflammatory breast cancer (Stage IIId) and for large or inoperable tumours for reducing tumour size in order to perform BCS [132]. One advantage is that response can be monitored, thereby allowing the continuation of effective therapy or change to other regimens when indicated. Moreover, patients with loco-regional tumor control are likely to have a higher DFS rate [133]. In addition, preoperative chemotherapy reduces disease stage [134]. This study showed that tumor size was reduced in 80% of patients after a preoperative AC regimen and 36% had a clinical complete response. In women with tumors ≥ 5 cm, preoperative chemotherapy made breast-conserving surgery possible in 20% of patients who were not initially eligible. Together, clinical positive node responded in 89% of cases in which 44% of those had a pathological completely remission. Clinical measurement of breast masses is often used to assess the response to neoadjuvant chemotherapy according to WHO (World Health Organization)/UICC (Union for International Cancer Control) criteria.

1.3.4 Endocrine therapy

Over the last 35 years, the selective estrogen receptor modulator tamoxifen became the standard of care in the Western world for metastatic hormone receptor-positive

tumors in both premenopausal and postmenopausal women due to its more favourable safety profile.

In patients treated with breast conservative surgery with node-negative breast cancer, the Stockholm Breast Cancer Study Group found that the ten-year rate of local recurrence was significantly less in women receiving tamoxifen (3% vs. 12%) [135]. For patients with ER(+) tumors, five years of adjuvant tamoxifen reduced the 15-year probability of recurrence (from 45% to 33%) and breast cancer mortality (from 35% to 26%) [68]. While the absolute risk reduction after five years of tamoxifen was similar for women younger than 50 years and women above 50 years (10% vs. 12%), it was more pronounced for those with node-positive than node-negative disease (16% vs. 9%). Relative-risk of relapse in ER(+) patients treated with tamoxifen was lower than those with ER(-) (HR 0.77, CI 0.63-0.93) [71]. Tamoxifen treatment even only 2 years improved survival in ER(+) premenopausal patients [53]. Using tamoxifen therapy for 5 years instead of 2 years was found to be beneficial for patients with ER(+) and PgR(+) early stage, invasive breast cancer [136]. Today tamoxifen with a standard dose of 20mg per day is a current appropriate first-line agent in premenopausal women who have never received tamoxifen or who relapse at least 12 months after completion of adjuvant tamoxifen [28].

However, tamoxifen resistance is a problem which related to reduced CYP2D6 activity [137]. Aromatase inhibitors including anastrozole, letrozole, or exemestane should be currently administered for patients who had contraindication to tamoxifen. One multicentre clinical trial claimed that early breast cancer postmenopausal patients treated with anastrozole had higher DFS compared to tamoxifen and also associated with a prolonged time to recurrence (absolute difference of 2.8% at five years, and 4.8% at 9 years), reduced distant metastases, and fewer contralateral breast cancers but not significant difference in deaths after recurrence [138]. Duration of tamoxifen or aromatase inhibitors, alone or sequentially, is currently used for at least 5 years. Most premenopausal women with early stage hormone receptor-positive breast cancer can be treated with ovarian suppression/ablation, alone or combined with tamoxifen that is clearly superior. The choice of treatment is individualized according to patient preference, whereas, ovarian suppression/ablation in combination with aromatase inhibitor is at present not recommended [131].

1.3.5 Targeted therapy

Patients with either HER2 protein overexpression determined by IHC staining or *HER2* gene amplification by FISH/CISH/SISH are treated with targeted therapy such as trastuzumab, both in the adjuvant and metastatic disease setting [27, 28]. Trastuzumab (Herceptin®) is a human monoclonal antibody (MoAb) that binds to a specific epitope of the HER2 protein on the cell surface. This interaction inhibits signal transduction induced by other peptide growth factors interacting with their own receptors [74]. The consequence is inhibition of cancer cell growth. Additionally, it has also been shown to enhance the TNF-alpha sensitivity of breast tumor cells that overexpress this proto-oncogene [139]. Trastuzumab is initially indicated for metastatic disease with strong (3+) IHC for HER2 protein product or *HER2* gene amplification by FISH and is now the first-line choice for these patients in practice.

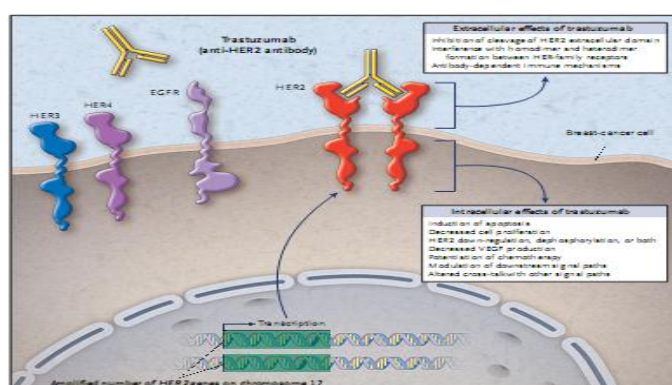


Fig. 2: Interactions between trastuzumab and cancer cells, adapted from [74]

The overall response rate was seen in 35% of patient with (3+) but was not in patients with (2+) tumors. The use of trastuzumab in patients with HER2(+) breast cancer can lead to increased survival. In HER2(+) advanced stage, neoadjuvant trastuzumab results in pathologic complete response as compared to those without HER2 positivity (37% vs. 17%; $p=0.02$) in breast, (47% versus 23%; $p=0.05$) in lymph nodes [70]. Furthermore, lower recurrence rates (5% vs. 42%; $p<0.001$) and increased overall survival (97% vs. 68%; $p<0.001$) were also observed. In HER2(+) metastatic breast cancers, chemotherapy plus Trastuzumab is recommended rather than trastuzumab alone. However, this treatment is not usually indicated for patients with HER2(-) [140].

In patients with HER2(+) locally advanced or inflammatory breast cancer, the combination of trastuzumab to neoadjuvant chemotherapy improved disease-free survival, survival, and clinical and pathological tumour responses [141]. In early stage disease, the international multicentre randomized (HERA) trial stated that Trastuzumab improved in 3-year DFS up to 11.3% and reduced death risk with 36% vs. without Trastuzumab. Additionally, trastuzumab reduced the risk of relapse [142]. The DFS and OS rates at 5 years were higher among those receiving anthracycline-cyclophosphamide plus taxane in combination with trastuzumab as compared to those without (84% vs. 75%; 92% vs. 87%, respectively). Trastuzumab thus improves survival in either combination with anthracycline or taxane [143]. Trastuzumab can be administered concomitantly with taxane or vinorelbine [144]. The recommendation for one year treatment with Trastuzumab can be considered in combination with polychemotherapy [142, 143]. Anthracycline-containing regimen plus trastuzumab may have cardiotoxic effects. It is important to avoid trastuzumab in patients with low left ventricular ejection fraction (LVEF, <50%–55%) and in patients whose cardiac function deteriorates during therapy [28].

Lapatinib is an orally active tyrosine kinase inhibitor that blocks HER2 as well as other members of this receptor family which was approved to use for women who progressed on trastuzumab and were chemorefractory [145]. A combined regimen of lapatinib plus capecitabine had an improved overall response rate (24% vs. 14%) but showed no significant effect on survival [146]. Lapatinib plus letrozole as first line treatment in HER2(+) patients with metastatic disease failed to improve overall survival despite improvement of median progression-free survival [147] and Lapatinib seems better than Trastuzumab in patients with brain metastasis. Lapatinib is currently being evaluated in both the adjuvant and metastatic setting and was recently approved by the United States Food and Drug Administration, in combination with capecitabine, for the treatment of women with HER2-positive, pretreated, metastatic breast cancer [148].

Another targeting agent named Avastin is a monoclonal antibody (MoAb) against vascular endothelial growth factor (VEGF), arguably the most important regulator of tumor angiogenesis of the EGFR family. However, this agent has been revoked the approval for metastatic breast cancer in the United States (<http://www.fda.gov>) because of failure in efficacy.

2 RATIONALS FOR THIS THESIS

Previous reports on breast cancer from pathology departments from various hospitals in Vietnam reported ER, PgR positivity and HER2 overexpression in the ranges 55.2-59.1%, 39.2-51.4% and 35.1-44.2%, respectively. However, none of these studies analyzed the correlation to clinical characteristics [149, 150]. In one study it was found that 26% of Vietnamese premenopausal patients with hormone receptor-positive tumors had HER2 protein overexpression [85].

Several studies on breast cancer in women living in the USA found that Vietnamese as well as Asian women had different tumor cell characteristics as compared with women of Caucasian or Hispanic origin [4, 7, 17, 151]. Similarly, a number of studies indicated that breast cancer from Afro-Americans more often had poor prognostic factors than had Caucasian-American and that tumor characteristics vary among Asian ethnicities [18, 152]. One comparative study stated that breast cancers from Korean patients more often had unfavorable factors compared to other American patients [153]. A recent report from the USA showed different treatment outcome because of different characteristics of breast cancers among ethnicities [152]. One study performed in the National cancer hospital revealed that Vietnamese breast cancer patients had the lowest frequency of *BRCA* mutations worldwide [154]. Thus, several studies indicated that tumor cell characteristics of breast cancer varied between patients of different ethnicities. Up until this study, there is no report describing prognostic and predictive factors for Vietnamese women with breast cancer. From the above cited reports, it seemed likely that Vietnamese breast cancer patients might have different profiles for prognostic and predictive factors as compared to Swedish patients. If so, the question is to what degree they are different and if there were differences how would this affect the choice of treatment and clinical outcome.

3 OVERALL AIMS

This study aimed to investigate prognostic and predictive markers such as hormone receptors, HER2 expression and cell proliferation in operable breast cancers sampled from Vietnamese women and then correlated them with clinicopathologic factors and outcomes. A comparison of these factors was also made with a counterpart sample of operable breast cancers in Swedish women treated in Karolinska University Hospital and in Stockholm region.

SPECIFIC AIMS:

1. To analyze the estrogen and progesterone receptor content in operable breast cancers from Vietnamese women and make a direct comparison with the receptor profiles in Swedish patients treated over the same period of time (Study I).
2. To assess the HER2 status in pre- and postmenopausal Vietnamese women with immunohistochemistry and silver *in situ* hybridization in operable breast cancers and compare the *HER2* status in a series of Swedish patients (Study II).
3. To analyze cell proliferation as determined by Ki67 staining in breast cancers from Vietnam. The results were correlated with predictive and prognostic factors in breast cancers and compared with a series of Swedish breast cancer patients (Study III).
4. To examine the correlation between prognostic factors, treatment and survival in Vietnamese women with breast cancers treated at the National Cancer Hospital, Hanoi, Vietnam (Study IV).

4 PATIENTS AND METHODS

4.1 PATIENTS AND SAMPLES

In the National Cancer Hospital, Hanoi, Vietnam there is at present no computerized patients file or records. All patients in this study were registered with medical records at the breast cancer unit which takes care of treatment and follow-up. Therefore, a total of 338 breast cancer paraffin blocks randomly selected from thousands of breast cancer samples which were stored disorderliness in the tumor bank at the department of clinical pathology. At first, blocks were picked out randomly and checked the tumor tissue in them and tumors equal to or above 0.5 cm in dimension were chosen for detailed study. Name of the patients, pathology report and medical record were identified then checked for proper clinical information such as age at diagnosis, menopausal status, clinical stage, tumor size and axillary lymph node status, histological subtypes (World Health Organization criteria) and tumor grade (Scarfbloom-Richardson). Patients with neoadjuvant chemotherapy or/and inflammatory cancer or incomplete medical record were excluded. Finally, we contacted each patient via telephone for informed crosscut and to allow follow-up. After review, invasive carcinoma was selected for staining for the biomarkers. Two hundred and fifty six patients were eligible and enrolled in the study. Other cases were out of the scope of the current study because they were not adequate with our requisition. Moreover, the required sample size for the Vietnamese series was calculated to be two hundred and forty four. All clinicopathologic variables of these patients were stored in SPSS software as a questionnaire profile.

4.1.1 Study I

The hormone receptor levels for estrogen and progesterone were analyzed using immunohistochemistry in a series of 249 Vietnamese women with operable primary breast cancer treated the years 2002-2004 at the National Cancer Hospital, Hanoi, Vietnam since seven cases of immunostaining failed for technical reasons. The results obtained from the Vietnamese samples were compared to those obtained from 1,257 Swedish breast cancers patients treated in the Stockholm region between the years 2002 and 2003. The tumor receptor content in the Swedish series was analyzed by the enzyme immunoassay. We primarily compared the frequencies of hormone receptor

positivity in the two series and correlated the receptor status to clinical and pathological parameters.

4.1.2 Study II

The rates of HER2 protein overexpression and gene amplification were analyzed in the same series of operable primary breast cancer tissues as analyzed previously for hormone receptor content.

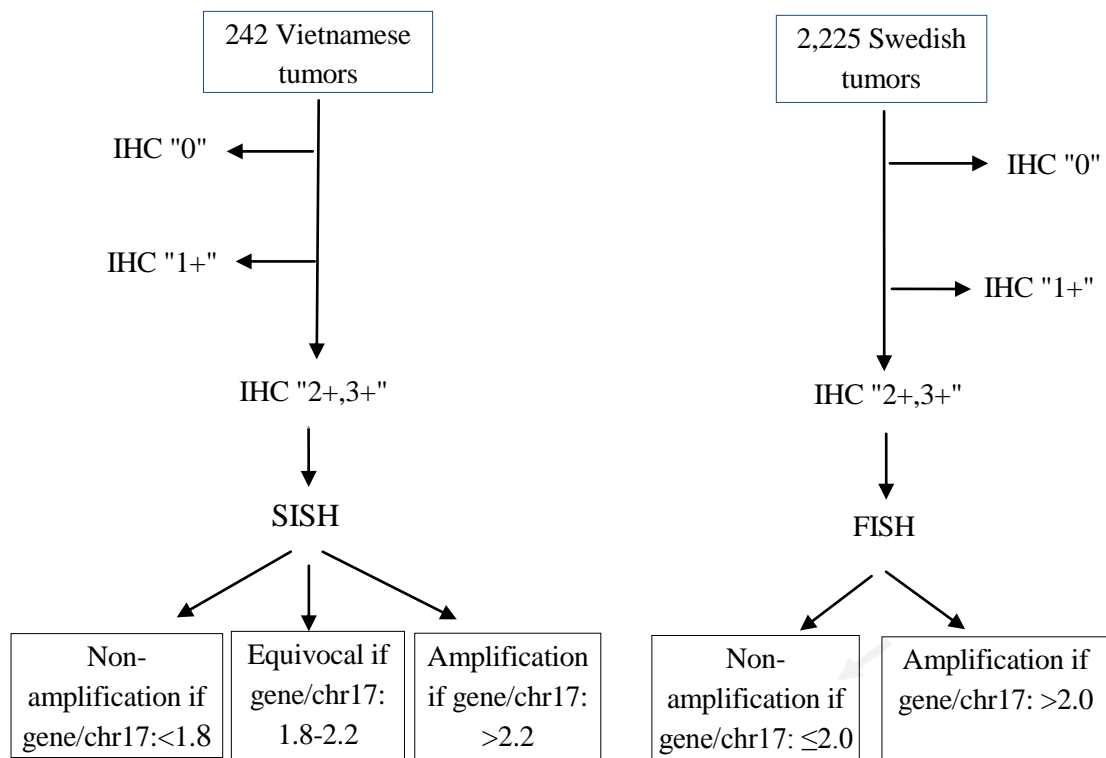


Fig. 3: Steps to assess *HER2* gene status in the two sample series

A total of 242 patients were included and seven slides, as mentioned above, were not available for technique reasons. Moreover, the rate of protein overexpression by immunohistochemistry was compared with that of gene amplification as assessed by SISH staining. The rates of gene amplification in Vietnamese breast cancer materials were compared to those found in a series of 2,225 Swedish patients operated between the years 2007 and 2008 for invasive breast cancer in the Stockholm Region, Sweden. The rate of *HER2* gene amplification was also assessed in correlation with clinicopathologic parameters for the Vietnamese patients.

4.1.3 Study III

Of the invasive primary breast cancers in Vietnamese patients operated on during the years 2002-2003, as mentioned above, 237 tumors were analyzed for cell proliferation by staining for the anti-Ki67 antigen. Twelve cases were excluded because of illegible count. In this study, all tumor tissues were sectioned to stain Hematoxylin-Eosin for classification of grade according to Elston-Ellis criteria. Results were compared to 237 age-matched Swedish women with breast carcinomas from the Department of Pathology of the Karolinska University Hospital, Solna who were operated upon in the period 2007 and 2008. These samples were also analyzed by staining for the Ki67 antigen. The rate of cell proliferation in the two populations were compared and correlated to parameters such as patient age, clinical stage, tumor size, and the numbers of metastatic axillary lymph nodes.

4.1.4 Study IV

This study investigated the clinical outcome of the 248 Vietnamese patients described in the previous three studies. The patients were treated with either modified radical mastectomy or conservative surgery and axillary node sampling with a median of 10 nodes (range 6-35). Classification of histological type was done according to WHO criteria. Tumor grade was assessed by the Scarff-Bloom-Richardson (SBR) as well as Elston-Ellis grading [51]. Informed consent was obtained from all patients before treatment. Patients operated on with modified radical mastectomy were treated with adjuvant radiotherapy for tumor ≥ 3 cm at a dose of 50 Gy to the chest wall and 50 Gy to the axillary area if node positive. Patients operated with breast conservative mastectomy were given 50 Gy to the entire breast and a boost to 60-65 Gy to the tumor bed. Patients with lymph node metastasis received adjuvant chemotherapy with anthracycline or taxane regimens. Of 123 premenopausal patients with hormone receptor-positive tumors, 104 (84.5%) received endocrine therapy including 74 patients who were castrated with radiotherapy at a dose of 15 Gy. A total of 11 patients entered menopause after chemotherapy and 19 patients were treated with tamoxifen. Postmenopausal women with hormone receptor-positive tumors were treated with tamoxifen at a dose of 20 mg oral daily for at least 2 years but often for 5 years. In the first years, all patients normally were followed up with physical examination, blood tests for CA15.3 levels, chest x-rays and abdominal ultrasound examinations. Patients with symptoms suggesting metastasis were examined with CT/MRI scans or bone scans. The majority of the patients were continuously followed up

by examination at the National Cancer Hospital, Hanoi but some patients living outside of the city were contacted by telephone. The last day of follow-up was July 31, 2011, with a 99-month median follow-up (range 4-108 months). Patients who were alive after the last day of follow-up were censored.

4.2 TISSUE MARKER ASSESSMENT

Tissue handling for Vietnamese women with breast cancer in the Vietnam National Cancer Hospital was as follow. Five 4 µm-thick sections of each tissue paraffin block were produced. The tumor sections were stored at 2-8°C before immunohistochemical staining. Positive and negative slide controls were included with every staining set.

All of slides from Vietnam were stained at the Cancer Centre Karolinska (CCK), Karolinska Institute, Stockholm using the Ventana HX automatic system BenchMark (Ventana Medical Systems, SA, Illkirch Cedex, France), with the antibodies anti-ER (clone SP1) and anti-PR (clone 1E2). ER and PgR were defined as positive if 10% or more of the cell nuclei were stained. Enzyme immunoassay (EIA) utilizing the EIA monoclonal kits (ER-EIA and PgR-EIA, Abbott Laboratories, Abbot Park, IL, USA) was used for assessing hormone receptor status in Swedish samples and a cut-off point was positive at 0.10 fmol/µg DNA (Study I).

In Study II, Vietnamese tumor samples were stained using an anti-HER2 antibody (clone 4B5) for detecting protein expression. Tumors with 2(+) and 3(+) protein expression were stained with SISH (silver *in situ* hybridization). A ratio of *HER2* gene copies/chromosome17 above 2.2 was considered as amplified. Determination of HER2 protein expression for Vietnamese and Swedish breast cancer series were applied as recommended [155]. In the series of samples from Swedish women, FISH (Fluorescence *in situ* hybridization) analysis was carried out as a part of histological routine using PatVision, the cut-off level being set at ≥ 2.0 as positive [79].

The rate of tumor cell proliferation was measured by Ki67 immunohistochemistry staining by rabbit monoclonal antibody clone 30-9 with an automated machine of the Ventana Medical System. The tissue sections from the Swedish patients were stained using the automated Bond Max system. The antibody with Ki67 used in the Swedish series was the clone M7240 from the Dako Company. All of slides from both series were counted independently by two investigators under a light microscope. Four hundred cancer cells in each slide were evaluated in area with intermediate frequency of stained cells. The Ki67 index was measured as percent stained cells of total cancer cells (Study III). On the basis of biomarker profiles, the breast cancers in the study

population were also grouped into four phenotypic subtypes: luminal A [ER(+) or/and PgR(+) and *HER2*(-)], luminal B [ER(+) or/and PgR(+) and *HER2*(+)], *HER2* enriched tumors [ER(-), PgR(-) and *HER2* (+)] and triple negative [ER(-), PgR(-) and *HER2*(-)] (study III,IV).

4.3 STATISTICAL ANALYSIS

SPSS statistical program (version 15.0 for paper I/II and version 19.0 for paper III/IV, IBM. Chicago, IL, USA) was used to analyze the data. The Mann-Whitney test was used to compare the different categorical variables such as age at diagnosis, clinical stage, number of lymph nodes and tumor size between two populations (Study I). The odds ratio (OR) with 95% confidence interval (95% CI) and the Pearson's chi-square test (or Fisher's exact test when appropriate) used in relation with the fraction was calculated to measure the correlation or difference between variables of the two groups (all four studies). Kappa statistic was analyzed (Study II) for agreement between two procedures [156]. T-test was used for comparing the means with 95% CI, Wilcoxon rank-sum test for the median (Study III). Disease-free survival (DFS) was defined as an interval from the date of operation to the date of first detection of metastasis or contralateral breast cancer. Breast cancer specific survival (BCSS) was calculated from the date of operation to the date of death caused by cancer. Overall survival (OS) was defined as the date of operation to date of death of any cause or the last day of follow-up. The survival rates were estimated by using Kaplan-Meier method. Log-rank test was used to compare DFS and OS between two groups for each parameter. Univariate and multivariate cox regression were used to determine the relationship between breast cancer deaths and prognostic and treatment factors (Study IV). All tests were two-sided and $p \leq 0.05$ value was used as the significant level in all four studies.

4.4 ETHICAL ASPECTS

All four studies were approved by the Hanoi Medical University Review Board (HMURB) No 38/HMURB and No 95/HMURB (extension), Ministry of Health (Vietnam) and ethical permits: Dnr 03-630/2003-12-01, Dnr 2007/1366-32 and 2011/2033-32 from the Karolinska Institutet (Sweden). Vietnamese patients treated were voluntary to come back to the National Cancer Hospital for check-ups and to be interviewed via telephone about treatment aspects as well as symptoms. If a patient could not be contacted, the family members were interviewed to clarify the reasons for this failure. All collected information was handled anonymously.

5 RESULTS

5.1 PROGNOSTIC CLINICOPATHOLOGIC CHARACTERISTICS

In the current study, Vietnamese patients were younger than their Swedish counterparts at diagnosis ($p<0.001$). As seen in Table 4, sixty four percent of the Vietnamese patients were premenopausal while the corresponding figure was 25% for Swedish patients (Paper I) and 28% (Paper II).

Table 4: Patients and tumor characteristics

| Variable | Vietnamese (n=249) n (%) | Swedish (n=1,257) n (%) |
|--------------------------|--------------------------------|-------------------------------|
| Age (years) | | |
| Mean (SD) | 47.5 (9.1) | 60.7 (13.3) |
| Menopausal status | | |
| Premenopause | 159 (64) | 310 (25) |
| Postmenopause | 85 (34) | 864 (69) |
| unknown | 5 (2) | 83 (6) |
| Tumor size (mm) | | |
| Mean (\pm SD) | 38.7 (\pm 16.3) | 23.7 (\pm 14.9) |
| Missing data | 0 (0) | 16 (1) |
| Histologic type | | |
| Ductal carcinoma | 217 (86) | 867 (69) |
| Others | 32 (14) | 390 (31) |
| DIS component | 17 (7) | 21 (2) |
| Axillary node dissection | | |
| Mean (\pm SD) | 11 (\pm 3.3) | 9.4 (\pm 5.2) |
| Missing data | 0 (0) | 55 (4) |
| Lymph node involvement | | |
| Negative | 141 (57) | 692 (55) |
| Positive | 108 (43) | 508 (40) |
| Missing data | 0 (0) | 57 (5) |
| Clinical stage | | |
| I | 26 (11) | 655 (52) |
| II | 175 (70) | 553 (44) |
| III | 48 (19) | 49 (4) |

The mean tumor size from Vietnamese patients, as given in the histopathologic reports, was 38.7 mm compared with 23.7 mm for the Swedish patients (Study I). A similar distribution was also seen in Study III. The mean number of removed axillary lymph nodes was 11.0 for the Vietnamese patients, whereas 12% of Swedish patients underwent sentinel lymph node biopsy with maximum three lymph nodes recorded. If

these patients were excluded from calculations of the mean number excised lymph nodes which was 10.4 (SD=4.8) for Swedish patients. The percentage of patients with metastatic lymph nodes was similar in both groups; 43.4% for the Vietnamese vs. 40.4% for the Swedish patients. Vietnamese patients had later Stages at treatment compared with Swedish patients. For Vietnamese women, clinical Stages I and II were reported in 10% and 70% of the population studied, respectively. The corresponding figure for the Swedish patients was 52% and 44%, respectively, $p<0.001$ (Table 4).

5.2 HORMONE RECEPTORS

The frequency of ER(+) tumors in Vietnamese patients (i.e. 61.8%) was lower than that for Swedish patients (OR, 95% CI, 0.58-1.02) (Table 5). This difference did not, however, reach statistical significance ($p=0.060$). Tumors with ER(+) were found in 67.8% (Paper I) and 81.4% (Paper III) of the Swedish series. Even when it was used a cut-off point at 1% as positive, only one more Vietnamese patient was found to be classified as receptor positive. The ER(+) rate in Vietnamese patients increased from 61.8% to 62.2%, which is still lower than that found for Swedish patients (data not shown).

Table 5: Comparison of hormone receptor status between Vietnamese and Swedish patients with respect to menopausal status

| | | ER | | P value | OR* (95% CI) | PgR | | P value | OR* (95% CI) |
|---------------|-----|------------------------|---------------------|------------|-------------------------|------------------------|---------------------|------------|-------------------------|
| | | Vietnamese n (%) | Swedish n (%) | | | Vietnamese n (%) | Swedish n (%) | | |
| All patients | (+) | 151 (61.8) | 800 (67.8) | 0.060 | 0.76 (0.58- 1.02) | 113 (46.4) | 793 (67.6) | <0.001 | 0.41 (0.31- 0.55) |
| | (-) | 93 (38.2) | 374 (32.2) | | | 131 (53.6) | 381 (32.4) | | |
| Premenopause | (+) | 113 (71.1) | 181 (58.4) | 0.007 | 1.75 (1.16- 2.64) | 92 (57.8) | 226 (72.9) | 0.001 | 0.51 (0.34- 0.76) |
| | (-) | 46 (28.9) | 129 (41.6) | | | 67 (42.2) | 84 (26.1) | | |
| Postmenopause | (+) | 38 (44.7) | 619 (71.6) | <0.001 | 0.32 (0.20- 0.50) | 21 (24.7) | 567 (65.6) | <0.001 | 0.17 (0.10- 0.29) |
| | (-) | 47 (55.3) | 245 (28.4) | | | 64 (75.3) | 297 (34.4) | | |

The ER(+) tumors were found in 71.1% of the premenopausal Vietnamese patients as compared to 58.4% of Swedish patients (Table 5). This difference in ER content between premenopausal Vietnamese and Swedish patients was statistically significant ($p=0.007$). In contrast, 44.7% of postmenopausal Vietnamese patients had ER(+)

tumors, which were much lower than the 71.6% observed for postmenopausal Swedish patients ($p<0.001$). The frequency of ER positivity was then calculated for clinical Stages I-II-III, for which the rates were observed as 70.5%, 66.7%, 44.9% for Swedish patients and 57.7%, 62.0%, 58.3% for Vietnamese patients, respectively. Thus, it appears that the rate of ER(+) tumors decreased with advanced stage in Swedish patients but not in Vietnamese patients. PgR(+) tumors were observed in 46.4% of Vietnamese and 67.6% of Swedish patients (OR, 99% CI 0.31-0.55) (Paper I) and 65.7% (Paper III). Even when 1% was used as a cut-off point for Vietnamese tumors, there were 7 cases with expression at 5%. The PgR(+) rate increased for 2.8% (46.4% to 49.2%), which still was significantly lower than that (67.6%) for counterpart Swedish tumors, $p<0.001$ (data not shown).

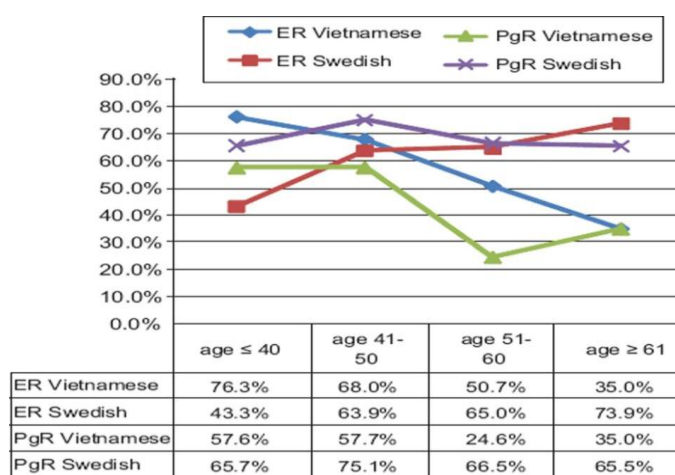


Fig. 4: The trends of ER and PgR positivity in relation to age of patients

The frequency of ER positivity and PgR positivity in different age groups is presented in Fig. 4. The frequency of ER(+) was 76.3% for Vietnamese patients younger than 41 years of age (Fig. 4). This frequency then gradually decreased with increasing age and was 35.0% for patients older than 60 years. For Swedish patients, an opposite pattern was observed and patients younger than 41 years were ER(+) in 43.3%. In the oldest age group, >60 years, the rate of ER(+) tumors increased to 73.9%.

No significant difference in PgR positivity was found between Vietnamese and Swedish patients who were ≤40 years. However for patients >40 years, the PgR positivity of Vietnamese patients was much lower than that found for Swedish patients. The maximal difference was observed among patients over 60 years. In this age group, the Vietnamese and Swedish patients were PgR(+) in 35.0% and 65.5% of

cases, respectively. Our findings also show that the rates of PgR(+) tumors in Vietnamese patients were significantly lower than those for both counterpart pre- and postmenopausal Swedish patients.

5.3 HER2 STATUS

The information about the HER2 status in Vietnamese breast cancer patients is still limited. Immunohistochemistry (IHC) was used to analyze the HER2 protein expressed and it was found in 38.9% of the tumors with strong expression (3+). An intermediate level (2+) of the protein was found in 11.5% while 49.6% showed no or low (0/1+) levels.

Table 6: Association of *HER2* gene amplification with clinicopathologic variables in Vietnamese breast cancer patients

| | | SISH | | OR (P value) |
|---------------------|---------------------|-------------------|-------------------|--------------|
| Parameters | | Negative n (%) | Positive n (%) | |
| Age (yrs.) | ≤50 | 97 (64.7) | 53 (35.3) | 2.1 (0.006) |
| | >50 | 41 (46.6) | 47 (53.4) | |
| Menopause | Pre | 98 (64) | 55 (36) | 2.2 (0.005) |
| | Post | 37 (45) | 45 (55) | |
| Tumor stage | ≤T1 | 21 (54.9) | 17 (45.1) | 0.93 (0.84) |
| | >T1 | 110 (57) | 83 (43) | |
| Histo- Pathology | Ductal carcinoma | 120 (56.1) | 94 (43.9) | 2.3 (0.07) |
| | Others | 18 (75) | 6 (25) | |
| Tumor grade | I, II | 97 (53.6) | 84 (46.4) | 0.68 (0.36) |
| | III | 17 (63) | 10 (37) | |
| Lymph node | (+) | 59 (57.3) | 44 (42.7) | 1.0 (0.85) |
| | (-) | 79 (58.5) | 56 (41.5) | |
| ER status | ER(-) | 38 (43.2) | 50 (56.8) | 2.6 (0.0004) |
| | ER(+) | 100 (66.7) | 50 (33.7) | |
| PR status | PgR(-) | 60 (47.2) | 67 (52.8) | 2.6 (0.0004) |
| | PgR(+) | 77 (70) | 33 (30) | |

The unexpectedly high frequency of tumors with protein overexpression motivated a further study using silver *in situ* hybridization (SISH) with which to analyze *HER2* gene amplification. An excellent correlation was found between protein overexpression and gene amplification (paper 2). *HER2* gene amplification was found in 40.9% of the tumors and the concordance between the two techniques was 87%. An investigation was then undertaken to examine the correlation between *HER2* gene

status and other prognostic factors (Table 6). It was clear that *HER2* gene amplification was more frequent in old patients, ductal carcinoma, high grade tumors, hormone receptor-negative tumors, but no difference was observed in various disease Stages in Vietnamese patients.

As can be seen from Table 7, *HER2* gene amplification in Swedish patients was observed in 13% of patients as analyzed by FISH. This result was then confirmed in study III. With age-matched patients, *HER2*(+) was found in 16% of Swedish patients (which was much lower than that of Vietnamese patients). In the counterpart Swedish series from Study II, premenopausal patients had frequency of *HER2* gene amplification compared with postmenopausal patients of 16% vs.12%.

Table 7: *HER2* gene amplification in breast cancer samples taken from Vietnamese and Swedish women (%)

| | Vietnamese (n=242) | Swedish (n=2,225) | P value |
|----------------|-----------------------|----------------------|---------|
| All patients | 41 | 13 | <0.001 |
| Premenopausal | 36 | 16 | <0.001 |
| Postmenopausal | 55 | 12 | <0.001 |
| ER positive | 33 | 9 | <0.001 |
| ER negative | 57 | 31 | <0.001 |

5.4 CELL PROLIFERATION

The rate of cell proliferation was analyzed in the same series of Vietnamese breast cancer tissues as used for analysis of hormone receptors and *HER2*, using immunohistochemistry with monoclonal antibody against Ki67 antigen. The number of Vietnamese samples with MIB-1 antibody was tested. Concordance between the two different antibodies used in the Vietnamese and Swedish counterpart series of tissue samples was high as tested in a limited series of tumors (data not shown).

The rate of proliferation varied between 3% and 90% (Fig. 5). From this Figure, it can be seen that a majority of the tumors had a proliferation in the interval between 10% and 45%, with a median of 24%. In an age-matched counterpart series of Swedish patients, the cell proliferation varied between 1% and 95%, with a majority in the range 4-50%.

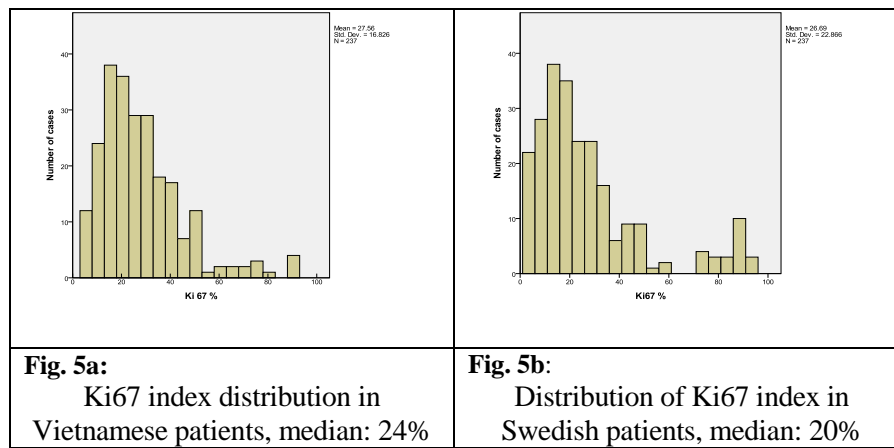


Fig. 5: Vietnamese (A) and Swedish (B) patient group

Based on the findings of the hormone receptors and *HER2* status studies described above, the tumors in both series were classified into either luminal A, luminal B, triple negative or *HER2* enriched. It can be seen that a high rate (>30%) of proliferation was observed in a minority of luminal A patients: 22.8% and 15.6%, for Vietnamese and Swedish patients, respectively. In contrast, the triple-negative and *HER2*-enriched subtypes were dominated by highly (>30%) proliferative tumors in both series of patients.

Table 8: Comparison of Ki67 index and luminal status between breast cancer tumors in Vietnamese and Swedish patients (%)

| Variables | | Frequency | ≤15% | 16-30% | >30% |
|----------------------|------------|-----------|------|--------|------|
| Luminal A | Vietnamese | 44.1 | 30.7 | 46.5 | 22.8 |
| | Swedish | 73.0 | 46.2 | 38.2 | 15.6 |
| Luminal B | Vietnamese | 22.3 | 31.4 | 37.2 | 31.4 |
| | Swedish | 8.9 | 14.2 | 42.9 | 42.9 |
| Triple negative | Vietnamese | 14.4 | 18.2 | 39.4 | 42.4 |
| | Swedish | 10.5 | 12.0 | 12.0 | 76.0 |
| <i>HER2</i> enriched | Vietnamese | 19.2 | 9.1 | 40.9 | 50.0 |
| | Swedish | 7.6 | 11.1 | 27.8 | 61.1 |

5.5 SURVIVAL IN OPERABLE PRIMARY BREAST CANCER PATIENTS

The DFS, OS and CSS rates of Vietnamese women with operable breast cancers were 75.8%, 80.6% and 86.4% at 5 years, respectively; and 62.3%, 68.1%, 78.9% at 9 years. Lung was the most common site of metastasis in this population, followed by liver and bone (Paper IV). Also, those with favorable pathologic tumor factors including grade

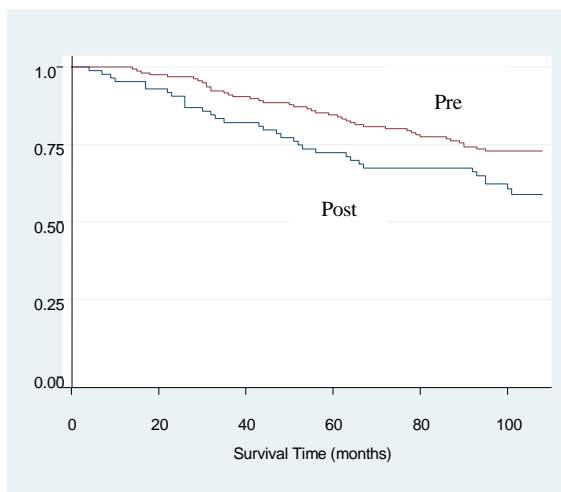
I/II, negative axillary node and Stages I/II had significantly better survival ($p=0.029$, $P<0.0001$, and $p<0.0001$, respectively).

Table 9: Results of univariate and multivariate analysis of crude survival rate

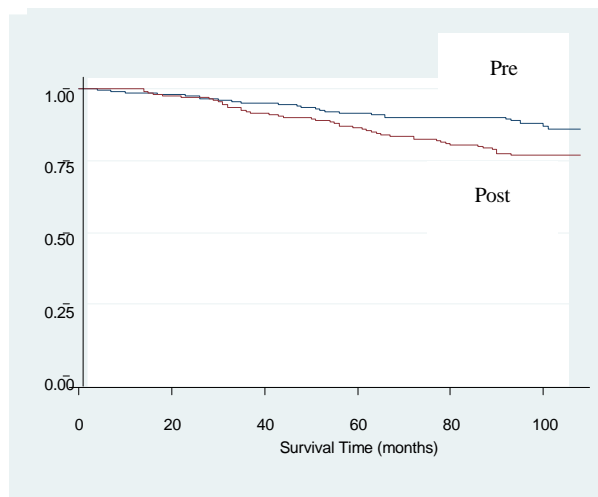
| Variable | Univariate model | | Multivariable model | |
|-------------------------|------------------|-------------------|---------------------|--------------------|
| | HR (95% CI) | P value | HR (95% CI) | P value |
| Menopause | | | | |
| Post | Ref. | | Ref. | |
| Pre | 0.61 (0.38-0.95) | 0.031 | 0.67 (0.41-1.08) | 0.10 |
| Clinical stage | | | | |
| I or II | Ref. | | Ref. | |
| III | 2.55 (1.56-4.17) | $<0.0001^*$ | 2.45 (1.49-4.02) | $<0.0001^*$ |
| ER status | | | | |
| (+) | Ref. | | Ref. | |
| (-) | 1.23 (0.77-1.94) | 0.39 | 0.76 (0.44-1.33) | 0.34 |
| PgR status | | | | |
| (+) | Ref. | | Ref. | |
| (-) | 1.78 (1.11-2.85) | 0.02 [*] | 1.77 (1.01-3.11) | 0.045 [*] |
| <i>HER2</i> gene status | | | | |
| (-) | Ref. | | Ref. | |
| (+) | 1.34 (0.85-2.11) | 0.20 | 1.07 (0.66-1.73) | 0.78 |

The OS rates at 5 years and at 9 years were significantly higher in premenopausal patients as compared to postmenopausal ones: 84.5% vs. 72.3%; and 72.7% vs. 58.9%, respectively ($p=0.03$). Patients with ER(-) or PgR(-) tumors had worse OS after 5 years and after 9 years compared to those with receptor-positive tumors. In contrast, patients with *HER2*-amplified tumors also had lower survival as compared to those with non-amplified tumors. Similarly, patients with ER(+) tumors showed a better survival than those with PgR negative ones. The ER and *HER2* status of the tumors had little effect on the crude survival rate.

The overall survival was different for pre- and postmenopausal patients in univariate as well as multivariate analysis (Table 9 and Fig. 6). Thus, premenopausal patients had a higher survival rate than postmenopausal patients.



Univariate analysis



Multivariate analysis (after adjusted stage, hormone receptors, *HER2* status)

Fig. 6: Overall survival of operable breast cancers by menopausal status

6 DISCUSSION

6.1 HORMONE RECEPTORS

Hormone receptors are biomarkers used in clinical routine since they are useful for selecting adjuvant therapy and as prognosticators. The previous findings in ethnical differences showed that tumors from Asian patients expressed low levels of hormone receptors compared to Caucasian patients. The ER(+), PgR(+) rates in Vietnamese breast cancer patients living in the Greater San Francisco Bay Area (the USA) were 55.5%, 48.1%, respectively [151]. ER/PgR(-) tumors from Vietnamese women were more frequent than those from non-Hispanic White patients in the USA [152]. Similarly, frequencies of hormone receptor positivity were low in breast cancers from Japanese women [157]. The PgR(+) rate in Asian patients was lower than for their Western counterparts [21]. It was found in the current study that both ER(+) and PgR(+) rates in Vietnamese were lower than those in Swedish women, 61.8% vs. 67.8% and 46.4% vs. 67.6%, respectively. Tissue samples studied which had 1-9% of stained cells had low intensity. Although 1% was used as a cut-off, the hormone receptors from Vietnamese patients were still lower than those found in their Swedish counterpart. Patients with 1% stained cells probably are acceptable for endocrine therapy as this method has low toxicity: however those with tumors in which 1-9% cells were stained should be considered before treatment. Thus, tumors with low percentage expressed receptor normally showed a weak staining intensity [66]. That was the reason 10% was chosen 10% as cut-off point in the current studies for discriminate negative or positive assessment for tumor samples originating from Vietnamese patients.

It is interesting that the frequency of ER(+) in premenopausal Vietnamese patients seemed significantly higher than that of premenopausal Swedish patients. Conversely, ER(+) rates of postmenopausal Vietnamese breast cancer patients were much lower than those of their counterpart postmenopausal Swedish patients. Notably Vietnamese women were diagnosed with breast cancer at a younger age and a majority of patients were diagnosed at or before menopause. This is in agreement with the previous findings which showed that Vietnamese patients and other Asian women at diagnosis of breast cancer were younger than non-Hispanic White patients [151, 152, 158]. In contrast, Swedish postmenopausal women had a higher incidence of ER(+) tumors.

From the data obtained in the current study, it is obvious that trend of ER(+) frequency decreased gradually for each 10-year group of Vietnamese women, from 76.3% to 35.0%. But, interestingly, there was an opposite trend for Swedish women in whom the frequencies of ER(+) tumors were lower among patients 40 years or younger and high in patients above 61 years, increasing from 43.3% to 73.9%. Our findings are thus in general agreement with previous studies which showed a low frequency of ER(+) breast cancers in women from developing countries [159, 160]. The difference in ER(+) between the two counterpart series of samples used in the current studies varied in different age groups of patients, while the PgR(+) rates of Vietnamese patients were lower than those of Swedish women in all age groups. PgR(+) frequency in young Korean was also lower than that for Caucasian counterpart, 42.4% vs. 52.6% [153]. However, the trend of this marker by age has not been documented to date that needs further study for this issue.

Risk factors such as age at first birth, postmenopausal obesity, and menopausal hormone therapy have been correlated to hormone receptor positivity in breast cancer [161]. The differences have also been partly explained by alcohol drinking, especially among those women who use postmenopausal hormone replacement [162]. One study from Sweden by *Rosenberg et al* showed that patients who had received long-term menopausal hormone therapy more often than not had hormone receptor-positive tumors [36]. Although the reason(s) for differences in hormone receptor status between races is unknown, true genetic differences are likely to be a major contributory factor. In addition, several factors such as difference in age at diagnosis and Stage present may be contributory. Lifestyle differences have also been suggested: women exposed to alcohol or contraceptive pills more often than not had ER(+) tumors [162, 163]. Reporting cut-off point is also one reason for producing different frequencies in hormone receptor assessments [56]. The low frequencies of hormone receptor-positive tumors indicate that few Vietnamese postmenopausal patients would likely benefit from endocrine therapy. In contrast, the high frequency of young patients with hormone receptor-positive tumors suggests that these patients could benefit from endocrine treatment.

6.2 HER2 STATUS

A previous clinical trial showed that HER2 protein overexpression was observed in 26% from Vietnamese and Chinese premenopausal women in ER(+) tumors [85]. Another cohort study with 1,359 cases reported that 35.1% of breast cancers in

Vietnamese patients had HER2 protein overexpression which is considerably higher compared to results from Western countries [149]. The true frequency of *HER2* gene amplification in Vietnamese breast cancer patients is, however, unclear because *in situ* hybridization had not been used prior to the current study. Using IHC to analyze HER2 protein expression on Stage I-II-III breast cancer tumors, it was found that 38.9% of patients had overexpression and 11.5% of intermediate levels of protein expression and 49.6% had no or low levels of expression. Using SISH it was found that 40.9% of Vietnamese patients had tumors with *HER2* gene amplification. Concordance between the IHC and SISH detections was 87%. These results are in agreement with reports from Asia that 31.5% of breast cancers had HER2 protein overexpression in northern Malaysia [164]. A protein overexpression of HER2 was detected in 32% and gene amplification in 37.5% of cases reported in breast cancers from Thailand [41, 165]. Similarly, high rates (32-65%) of *HER2* gene amplification were reported for large samples of Chinese breast cancer patients [166-168]. A most recent report from Southern Vietnam showed that *HER2* gene amplification assessed by FISH was 36% [169]. In comparison with the counterpart series of tissues from breast cancer patients in Sweden, it was found that *HER2* amplification in tumors from Vietnamese patients was significantly higher than that of their Swedish counterparts (40.9% vs. 15%), irrespective of other markers or menopausal status.

Conflicting results concerning *HER2* amplification and menopausal status both for Asian and Western women have been reported. With a comparison of five biomarkers and *HER2* gene amplification between Caucasian and Korean patients younger than 45 years, only *HER2* gene amplification was significantly higher in Korean patients (47.5% vs. 15.8%), whereas other markers were not different [153]. *HER2* gene status in this study was investigated in correlation with other prognostic markers. It was found that a high frequency of *HER2* gene amplification was seen in postmenopausal patients. In a very recent study, overexpression of HER2 protein in two hundred patients in northern Malaysia was not significantly associated with age but that a high percentage (i.e. 75%) of overexpression was noted in the age group 81-85 years [164]. Higher rates of HER2 overexpression in postmenopausal women were also seen in breast cancer patients in the south of Switzerland [170].

Overexpression of HER2 protein was correlated with unfavorable factors such as lymph node positivity and large tumor size [40, 41, 74]. HER2 positivity was also significantly associated with the Nottingham histological grade III ($p < 0.001$), ER negativity ($p < 0.001$), PR negativity ($p < 0.001$) [53, 164]. The *HER2* gene amplification

was more frequent in tumors with hormone receptor negativity, high grade, and high cell proliferation, but not related to age and tumor size [74, 171]. Despite these recent findings, the etiology of *HER2* gene amplification today remains unclear. Differences in *HER2* gene amplification may partly be explained by hormone replacement therapy, oral contraceptive and body mass index $>27.3 \text{ kg/m}^2$ all of which were associated with a lower frequency of *HER2*(+). Interestingly, patients who had been breastfeeding for ≥ 12 months were more often than not found to have *HER2*(+) tumors [172].

Trastuzumab is used for patients with *HER2*-amplified tumors but for Vietnamese patients the use of this agent is at present limited because of restrictive budgets. Nevertheless, our findings show that a high number of Vietnamese with breast cancer could have benefited from anthracycline-based chemotherapy and trastuzumab treatment.

The luminal-A subtype is the most common phenotypic subgroup and was found in 44.1% and 73% of Vietnamese and Swedish patients, respectively. Luminal-B, triple-negative and *HER2*-enriched tumors were all more common in Vietnamese than in their Swedish patient counterparts.

6.3 CELL PROLIFERATION MEASURED BY KI67 STAINING

The rate of cell proliferation was analyzed in Vietnamese operable breast cancer patients who had a Ki67 index in the range of 3-90% (Fig. 5) with a majority above 15%. Previous reports on Ki67 index in breast cancer showed discrepant results. The findings of the current study were similar to those obtained by *Nishimura et al* from Japanese patients who had a proliferation indices over 15% in 68% of the cases [101]. In contrast, a majority of Korean patients (78%) had a low cell proliferation index (under 14%) [173]. However, one study claimed that only 54% of breast cancer in Japanese women had a proliferation above 1% [174]. In the current study with age-matched Vietnamese and Swedish patients, no significant statistical difference could be found in mean, median or range of cell proliferation between these two study populations. Findings for Swedish patients were consistent with those of other reports from Western patients in recent years [96, 97]. The variation in Ki67 index among various reports may reflect true variations but methodological differences such as those influenced by various methods of fixation, staining and evaluation: these could have had a major impact on results of Ki67. In addition, differences in the study cohorts and cut-off levels may contribute to the variations [175].

The Ki67 index is classified as low ($\leq 15\%$) and high ($>15\%$), it was found that the frequency of high Ki67 index tumors from Vietnamese patients was higher than those indices obtained from their Swedish counterparts: 75.9% vs. 62.9%, $P=0.011$, (data not shown). This is in agreement with a report that the Ki67 index was higher in tumors from African-American as compared to Caucasian patients living in the USA [176].

During the current study, the mean Ki67 indices were compared between two series according to various clinicopathologic parameters. The means of Ki67 indices of breast tumors in Swedish patients were higher than those of counterpart Vietnamese patients according to poor prognostic factor subsets, such as hormone receptor negativity and *HER2* gene amplification. Contrastingly, in tumors with good prognostic factors, the mean values were higher in tumors present in Vietnamese patients. It was obvious that mean Ki67 indices in tumors with poor clinicopathological factors were higher than those with favorable factors in both series. These findings may partly support the notion that chemotherapy should be indicated for patients who have unfavorable factors (results and evidence presented in Paper III).

Until recently, there has been no consensus on reliable cut-off point for low and high Ki67 indices. However, two recent reports from the St Gallen Conference addressed this issue. It has been suggested that breast carcinoma should be classified into three groups: low ($\leq 15\%$), intermediate (16-30%) and high ($>30\%$) rates of proliferation that aid selection of a type of adjuvant therapy [44]. In the present study, the Ki67 levels which had been divided into three intervals were also associated with prognostic factors. It was found that Ki67 levels were significantly associated with factors such as histologic subtype, tumor grade, ER, PgR and *HER2* gene amplification. This means that Ki67 level was not independent of both pathologic and biomarker characteristics, according to suggestions from other researchers [84, 101, 171].

In this study, frequencies of Ki67 indices were compared according to biomarker subtype. The findings support previous reports that the good prognosis luminal A has few tumors with a high proliferation. In contrast, triple-negative and *HER2*-enriched tumors from Vietnamese and Swedish patients were more often than not highly proliferative ($>30\%$). Furthermore, the rate of cell proliferation among the four subtypes showed less variation in the Vietnamese series as compared to tumors from Swedish patients. A comprehensive search of the literature showed that this is the first comparative report on the rate proliferation in the immunohistochemical subtypes in

tumors from Asian and Western patient populations. It will be of interest to study the prognostic value of this subtyping in Vietnamese patients, as well as of the proliferation rates, as a potential independent prognostic factor. Furthermore, the results of the current study provide a baseline cohort for comparative studies on breast cancers of other ethnicities and especially of Vietnamese who have migrated over varying periods of time to Western countries.

6.4 BIOMARKER PHENOTYPES AND SURVIVAL

Little is known about the clinical outcome of Vietnamese breast cancer patients. In the current study, the disease-free survival (DFS), breast cancer-specific survival (BCSS) and overall survival (OS) in the series of breast cancer patients which were characterized for clinicopathologic and biomarker profiles (Studies I-III). The 5-year overall survival in the present study was 80.6% for the series of Vietnamese patients, which was comparable to reports from Cote d'Or region of France (74%) and from the Eastern region of England (78%) [177, 178]. Also, the 5-year survival of young Vietnamese women treated in the Central Cancer Hospital in Hanoi (81%) is consist to that reported in the Eastern region of England [178], but was lower than the figure from a recent Wishart- study in England at 9 years after diagnosis. It is considered that the OS levels reported for Vietnamese breast cancer patients were comparable to the English patients since they had been given similar treatment. The guidelines for diagnosis and treatment of breast cancer have been improved resulting from a collaborative program between the National Cancer Hospital and the University of Wisconsin since 1993 [179]. National Cancer Hospital is the largest cancer center in Vietnam treated for patients from Northern and middle Vietnam; therefore most advanced treatment applied. A recent report from Malaysia and Singapore showed that the 5-year survival for Stages II and III was lower than that found in the current study, which may be explained by patient recruitment from different hospitals with various therapeutic approaches and variations in tumor prognostic factors [180]. Breast cancer survival has been shown to be different for pre- and postmenopausal patients [170]. In a very recent observation for survival of breast cancer patients in Sweden, risk of mortality in addition to traditional risk factors depends on the age of immigrant races [5]. One population-based study in Hong Kong showed that DFS of Chinese patients was higher than the finding of this study for Vietnamese patients (81.2% vs. 75.8%), but no difference was observed for breast cancer-specific survival (85.2% vs. 86.4%)

[181]. Breast cancer survival observed in the current study was comparable to that reported in other Asian and Western countries.

As described above, Vietnamese postmenopausal patients had tumors that more often than not had poor prognostic factors. In univariate analysis, it was found that postmenopausal patients had lower survival (Fig. 6), but this difference was not significant in a parallel multivariate analysis (Table 9). It seems likely that the different survival in pre- and postmenopausal women was seen because of the various proportions of prognostic factors [46]. On the other hand, the breast cancer patients after diagnosis may also have died from causes other than breast cancer in the different age groups [39].

In general, patients with favorable prognosticators experience better survival. Lymph node status was the strongest factor for prediction of survival [40]. This can probably be explained by the fact that lymph node status reflects disease extent [32]. Tumor grade was found to be an important factor in the current study. Risk of mortality was increased from low grade to high grade tumors (19%, 50.4%, respectively) after 9 years. Patients with ER(+) had general a better survival as compared to those with ER(-), but this difference was not statistically significant. This may, however, be partly explained by adjuvant chemotherapy given to patients with ER(-) tumors. Thus, 40.9% of patients with ER(-) tumors received chemotherapy as compared to 34% of those with ER(+) (data not shown). In contrast, PgR status was an independent significant factor for patient survival, according to both univariate and multivariate analyses. Although *HER2* status in the current study did not relate statistically to survival, it seems that patients with *HER2*-amplified tumors have poor survival (Table 9). When combining data of the hormone receptor and *HER2* statuses, so as to subdivide the tumors into four phenotypic subtypes, patients with luminal A had the best survival, whilst the worst survival was observed for patients with triple-negative tumors. These findings are consistent with previous reports based on random samples from either Asian or Western women with breast cancer [39, 41, 157, 178, 182]. Available data suggest that Asian patients more often than not have high grade, ER(-) tumors which contribute to a lower survival rate than is the case for Western patients. It should, however, be pointed out that the survival of patients suffering from breast cancer is not only dependent on clinicopathological features but also on the availability of treatment [178].

Most of the Asian countries are low- and middle-income countries where the late detection and available access to care, survival of women with breast cancer in Asia

is likely to be adversely affected in comparison with Western countries. In most Asian countries, improvement of breast health care remains a challenge that should be overcome with collaboration from multiple hospitals, both public and private.

6.5 METHODOLOGICAL ASPECTS

Today prognostic and predictive biomarkers play an important role in the management of breast cancer patients. A prerequisite for their application in clinical routine is that they can be reliably and reproducibly analyzed. The commonly analyzed biomarkers such as hormone receptors, *HER2* status and cell proliferation have all been assessed by several different techniques. Some of these today are accepted as gold standards in clinical routine work. In the current study of such biomarkers in breast cancers from Vietnamese patients, the most recent and well-documented methods for their analysis were used. This approach allowed the current investigations to make reliable comparisons with series of breast cancers present in a population of Swedish patients.

Hormone receptors: Immunohistochemistry was used for the analysis of the receptor content in sections from Vietnamese breast cancers. An automated platform reproducibly stained the slides which were assessed by counting 400 tumor cells in each sample. Ten percent stained nuclei was chosen as a cut-off point between receptor-negative and receptor-positive tumors. In a series of Swedish breast cancer patients operated on between 2002 and 2003, it was found that 342 cases had been stained with both IHC and EIA. However, these cases had been stained at different hospitals with various antibodies and different staining methods. At this time, hormone receptor analyses were also carried out in a reference laboratory which still used the EIA technique and participated in a nationwide quality control program [64]. This allowed a comparison of receptor positivity as defined by EIA technique in 342 cases. This resulted in a kappa value of 0.56. This relatively poor level of concordance was most likely caused by the use of various techniques for the IHC analysis. Therefore, it was decided to use the results from the EIA assessment, although the definition of receptor positivity was based on a biochemical measurement instead of IHC staining. In spite of these methodological differences, it was believed that a comparison between hormone receptor content in the two series in the end gave reliable results.

HER2 analysis: In the Stockholm Region during 2007-2008, IHC and FISH were used to define the *HER2* status of breast cancers. The tumors were stained with IHC and

tumors with 2+ and 3+ protein expressions were further analyzed for *HER2* amplification by FISH. A similar approach was used for testing *HER2* amplification in the cases of counterpart Vietnamese breast cancers. In the first place IHC was used to identify tumors with protein overexpression (2+ and 3+). These tumors were then analyzed for gene amplification using SISH. SISH approach was chosen because the slides could be evaluated using light microscopy which also allowed for a morphologic control to be made. The reliability of SISH is high and a concordance of 96% with FISH data has previously been described [183-185]. In addition, SISH has been approved by the American Food and Drug Administration for analysis of *HER2* gene amplification. It was therefore considered that the comparison between *HER2* status in Vietnamese and Swedish breast cancer was valid for the purposes of the current investigations.

Cell proliferation: Today the most accepted and used technique for analysis of growth rate in tumors is evaluation of IHC staining of Ki67. The technique is robust and reliable but it should be stressed that the evaluation can be controversial. This is because the staining intensity of samples can vary between the different growth phases and may therefore lead to an underestimation of the growth fraction. In the series of Vietnamese breast cancers applied in the current study, automated staining was used an anti-Ki67 (30-9) rabbit monoclonal antibody. Each sample was evaluated by counting 400 tumor cells in an area of tissue sections showing intermediate proliferation. The counterpart tumor samples from Swedish patients were also stained in an automated platform using the MIB-1 antibody. The staining property was compared with the two antibodies used also in a series of Vietnamese breast cancers and was found to give a good correlation. The Swedish samples were also counted to standardize the evaluations since they had been originally scored by several pathologists that made the primary results less reproducible. It was therefore considered that the current comparison of this marker of breast cancer samples from counterpart materials of Vietnamese and Swedish was valid.

Survival: As previously pointed out, clinical follow-up of Vietnamese breast cancers patients is hampered by lack of computerized patient files and frequent drop-out of patients, mostly caused by economy. All available patient records were read and relevant clinical information computerized. In patients with incomplete records, a telephone contact was made with either the patient or a relative to obtain relevant

information. Patients who had metastasis diagnosed and later died were registered as dead from disease. In none of these cases was the cause of death verified by autopsy since this is not performed by tradition in Vietnam. Findings of the current study indicate however that breast cancer survival of women treated at the National Cancer Hospital, Hanoi, Vietnam have a prognosis comparable to that of breast cancer patients in Western countries.

7 CONCLUSIONS AND PERSPECTIVES

Both marked differences and similarities were found between operable breast cancer patients from Vietnam and Sweden. Vietnamese patients were younger at diagnosis and had more often advanced stages of the disease. The tumor biomarker profiles showed a higher frequency of ER(+) tumors in premenopausal- and lower frequency in postmenopausal patients. *HER2*-amplified tumors were more common in both among Vietnamese pre- and postmenopausal patients.

The rate of cell proliferation in Vietnamese breast cancer patients showed a similar distribution to that of Swedish patients. Moreover, the breast cancer survival rate for Vietnamese patients was similar to that for Western patients.

The first study for Vietnamese patients living in Northern and middle Vietnam also suggests that Vietnamese breast cancers have different tumor cell characteristics to those reported for Caucasian patients in general. The results provide a basis for further comparative studies with breast cancer patients of other ethnical background.

Today there are no nationwide screening and treatment programs for breast cancer in Vietnam. In addition, biomarkers are only analysed in a few sufficiently equipped pathology laboratories. These are challenging matters for the future. Economical restraints are at present difficult to overcome and this will have an impact on the introduction of new more precise techniques for early detection of breast cancer as well as the introduction of new therapeutic agents which could be used for a more individualized therapy if all tumors could be analysed for the biomarkers described in this study.

The contribution of *BRCA* mutations to breast cancer development seems low in Asian patients. Identification of other genetic factors and their correlation to treatment response is accordingly a most challenging field for future research.

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