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LIBYAN BREAST CANCER: HEALTH SERVICES AND BIOLOGY.

Diagnosis delay and prognostic value of DNA ploidy, S-phase fraction, and Ki-67 and Bcl-2 immunohistochemistry

by

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ERAMAH ELARABI A. ERMIAH: Libyan breast cancer: Health services and biology. Diagnosis delay and prognostic value of DNA ploidy, S-phase fraction, and Ki-67 and Bcl-2 immunohistochemistry**.**

ABSTRACT

The aim of this study was to investigate the diagnosis delay and its impact on the stage of disease. The study also evaluated a nuclear DNA content, immunohistochemical expression of Ki-67 and bcl-2, and the correlation of these biological features with the clinicopathological features and patient outcome.

200 Libyan women, diagnosed during 2008–2009 were interviewed about the period from the first symptoms to the final histological diagnosis of breast cancer. Also retrospective preclinical and clinical data were collected from medical records on a form (questionnaire) in association with the interview. Tumor material of the patients was collected and nuclear DNA content analysed using DNA image cytometry. The expression of Ki-67 and bcl-2 were assessed using immunohistochemistry (IHC).

The studies described in this thesis show that the median of diagnosis time for women with breast cancer was 7.5 months and 56% of patients were diagnosed within a period longer than 6 months. Inappropriate reassurance that the lump was benign was an important reason for prolongation of the diagnosis time. Diagnosis delay was also associated with initial breast symptom(s) that did not include a lump, old age, illiteracy, and history of benign fibrocystic disease. The patients who showed diagnosis delay had bigger tumour size ($p<0.0001$), positive lymph nodes ($p<0.0001$), and high incidence of late clinical stages ($p<0.0001$).

Biologically, 82.7% of tumors were aneuploid and 17.3% were diploid. The median SPF of tumors was 11% while the median positivity of Ki-67 was 27.5%. High Ki-67 expression was found in 76% of patients, and high SPF values in 56% of patients. Positive bcl-2 expression was found in 62.4% of tumors. 72.2% of the bcl-2 positive samples were ER-positive. Patients who had tumor with DNA aneuploidy, high proliferative activity and negative bcl-2 expression were associated with a high grade of malignancy and short survival. The SPF value is useful cell proliferation marker in assessing prognosis, and the decision cut point of 11% for SPF in the Libyan material was clearly significant ($p<0.0001$). Bcl-2 is a powerful prognosticator and an independent predictor of breast cancer outcome in the Libyan material ($p<0.0001$).

Libyan breast cancer was investigated in these studies from two different aspects: health services and biology. The results show that diagnosis delay is a very serious problem in Libya and is associated with complex interactions between many factors leading to advanced stages, and potentially to high mortality. Cytometric DNA variables, proliferative markers (Ki-67 and SPF), and oncoprotein bcl-2 negativity reflect the aggressive behavior of Libyan breast cancer and could be used with traditional factors to predict the outcome of individual patients, and to select appropriate therapy.

ERAMAH ELARABI A. ERMIAH: Rintasyöpä Libyassa: tautiin liittyvät terveyspalvelut ja taudin biologia. Diagnoosiviive ja kasvainsolujen DNA-pitoisuuden, DNA-synteesissä olevien solujen osuuden ja immunohistokemiallisesti todettujen Ki-67 ja bcl-2 merkkiaineiden ennusteellinen arvo.

YHTEENVETO

Työn tarkoituksena oli tutkia diagnoosiviivettä ja sen vaikutusta Libyassa todetun rintasyövän yhteydessä ja sen vaikutusta taudin kliiniseen levinneisyysasteeseen (stage). Tutkimus arvioi myös kasvainsolujen DNA-pitoisuutta, immunohistokemiallisesti arvioitua Ki-67- ja bcl-2- expressiota ja näiden biologisten piirteiden korrelaatiota kliinispatologisiin piirteisiin ja potilaiden ennusteeseen.

Työssä haastateltiin 200 libyalaista naista, joilla oli tai oli ollut rintasyöpä, muistikuvistaan ensimmäisistä oireista lopulliseen rintasyövän diagnoosiin. Myös retrospektiivisiä prekliinisiä ja kliinisiä tietoja kerättiin sairaalatiedostoista kyselylomakkeeseen haastattelun yhteydessä. Kasvainnäytteitä kerättiin ja niistä määritettiin kasvainsolujen DNA-pitoisuus käyttäen staattista sytometriaa. Ki-67- ja bcl-2- ekspressio arvioitiin käyttäen immunohistokemiaa.

Tutkimus osoitti, että diagnoosiaika rintasyöpäpotilailla oli keskimäärin (mediaani) 7,5 kuukautta. 56% potilaista diagnostisoitiin yli 6 kuukauden kuluttua ensi oireista. Liiallinen perusteeton vakuuttelu siitä, että rinnan kyhmy on hyvänlaatuinen oli tärkeä syy siihen, että diagnoosi viivästyi. Diagnoosin viivästymiseen liittyivät myös sellaiset oireet, joihin ei liittynyt kyhmyä, korkea ikä, potilaan lukutaidottomuus ja aiempi rinnan hyvänlaatuinen tauti (mastopatia, fibrocystic disease). Potilailla, joiden tautiin liittyi diagnoosiviivettä oli diagnoosihetkellä keskimääräistä suurempi kasvain ($p<0.0001$), imusolmukemetastaaseja ($p<0.0001$), ja korkea kliininen levinneisyysaste ($p<0.0001$).

Biologisesti arvioiden 82,7 % kasvaimista oli aneuploidisia ja 17,3% diploidisia. Keskimääräinen DNA synteesissä olevien kasvainsolujen osuus (S-phase fraction, SPF) oli 11% (mediaani), ja Ki-67-positiivisuus 27,5 %. Ki-67-ekspressio todettiin 76%:lla potilaista. Bcl-2-positiivisista kasvaimista 72,2% oli myös estrogeenireseptori (ER)-positiivisia. Potilailla, joiden kasvaimissa oli aneuploidisia soluja korkea proliferatiivinen aktiviteetti ja negatiivinen bcl-2-ekspressio liittyivät korkeaan histologiseen erilaistumisasteeseen (gradus, grade) ja lyhentyneeseen elinaikaan. SPF-arvo on käyttökelpoinen huonon ennusteen arviointiin ja yli 11%:n arvot liittyivät huonoon ennusteeseen ($p<0.0001$). Bcl-2 on myös vahva muista tekijöistä riippumaton ennustetekijä tässä potilasaineistossa ($p<0.0001$).

Libyalaisten naisten rintasyöpää arvioitiin tässä tutkimuksessa kahdesta näkökulmasta: terveyspalvelujen ja biologian kannalta. Tulokset osoittavat, että diagnoosiviive on libyalaisessa rintasyövässä vakava ongelma ja se liittyy moniin tekijöihin, jotka ovat omiaan tehostamaan kasvaimen etenemistä ja lisäämään potilaan kuoleman riskiä. Sytometriset muuttujat, proliferaatiota mittaavat muuttujat (Ki-67, SPF), ja bcl-2-negatiivisuus liittyvät libyalaisen rintasyövän agressiiviseen käyttäytymiseen. Niitä voidaan käyttää perinteisten ennustetekijöiden ohessa potilaiden ennusteen arviointiin ja hoitolinjan valintaan.

ABBREVIATIONS

ACS	American cancer society
ACOG	American college of obstetricians and gynecologists
Ais	Aromatase inhibitors
BSE	Breast self examination
BRCA	Breast cancer gene
Bcl-2	B-cell lymphoma 2
CBE	Clinical breast examination
CISH	Chromogenic in situ hybridization
DNA	Deoxy ribonucleic acid
DAP	Diaminobenzidine
DCIS	Ductal carcinoma in situ
EBCTCG	Early breast cancer trialists collaborative group
ER	Estrogen receptor
FDA	Food and drug administration
FNAB	Fine needle aspiration biopsy
FISH	Fluorescence in situ hybridization
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
IBC	Inflammatory breast cancer
LN	Lymph node
LCIS	Lobular carcinoma in situ
LHRH	Luteinizing hormone releasing hormone
MRI	Magnetic resonance imaging
MPC	Metaplastic carcinoma of breast
NCCN	National comprehensive cancer network
NGS	Nottingham grading system
PCR	Polymerase chain reaction
pCR	Pathological complete response
PLD	Pegylated liposomal doxorubicin
PCNA	Proliferating cell nuclear antigen
PR	Progesterone receptor
SPF	S phase fraction
TOP2A	Topoisomerase 2-alpha
TP	Thymidine phosphorylase
TNBC	Triple negative breast cancer
US	Ultrasonography
USPSTF	United States preventive services task force

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications

I. Ermiah E, Abdalla F, Buhmeida A, Larbesh E, Pyrhönen S, Collan Y. Diagnosis delay in Libyan female breast cancer. BMC Research Notes 2012; 5:452. (8 pages)

II. Ermiah E, Abdalla F, Buhmeida A, Alshrad M, Salem N, Pyrhönen S, Collan Y. Prognostic significance of DNA Image Cytometry in Libyan Breast Cancer. Oncology 2012; 83: 165-176.

III. Ermiah E, Buhmeida A, Abdalla F, Khaled B, Salem N, Pyrhönen S, Collan Y. Prognostic Value of Proliferation Markers: Immunohistochemical Ki-67 Expression and Cytometric S-Phase Fraction of Women with Breast Cancer in Libya. J Cancer 2012; 3:421-431.

IV. Ermiah E, Buhmeida A, Khaled B, Abdalla F, Salem N, Pyrhönen S, Collan Y. Prognostic value of bcl-2 expression among women with breast cancer in Libya. Tumor Biol 2013; 34:1569-1578.

1. INTRODUCTION

Breast cancer is an important public health problem worldwide; it is one of the most prevalent malignancies in the world, and comprises 23% of all cancers, with over one million new cases diagnosed annually [American Cancer Society: Cancer Facts and Figures 2010, Sabratha Cancer Registry 2008, Jemal et al. 2011]. There are about 4.4 million women living with breast cancer and over 400,000 die from the disease yearly. Breast cancer accounts for 14% of all cancer deaths. It is the most common cause of female death in industrialized countries, the second most common cause in the world, and the third most common in developing countries [Williams et al. 2006, Jemal et al. 2011, American Cancer Society: Cancer Facts and Figures 2011].When detected early, breast cancer is highly treatable and curable. Despite development in treatment strategies, advanced breast cancer remains incurable and the goals of therapy range from symptom palliation to extending survival.

In Libya and in developing countries, management of breast cancer forms a big medical, social and economic issue. In most developing countries, breast cancer patients often present with advanced stage, dominant presence in young age, premenopausal status, have early disease recurrence, and are associated with high mortality [Ikpatt et al. 2002, Williams et al. 2006, El Mistiri et al. 2007, Sabratha Cancer Registry 2008]. In spite of advances in treatments, the mortality rate is still high. Therefore, it is important to secure good cancer control through two different strategies, first by improvements in early detection, and second to find prognostic factors, which applied with traditional factors can predict the outcome of the individual patient, and allow selection of appropriate therapy [Ikpatt et al. 2002].

As to the first, we can improve the early detection through learning more about the extent and reasons behind diagnosis delay in Libyan breast cancer. In order to improve breast cancer outcome and survival, early detection remains the cornerstone of breast cancer control. To improve breast cancer care better understanding of the predicting factors and causes for treatment delay are important issues [Montazeri et al. 2003]. Early detection of cancer is important because delay is preventable and earlier treatment can lead to improved patient outcome. Late detection has been associated with advanced stages and low survival as well as negative implication on cost and choice of treatment [Velikova et al. 2004, Stapleton et al. 2011, Chagpar et al. 2011, Kim et al. 2012].

As to the second, because breast cancer is heterogeneous in respect to genetics, and variable in biological and clinical features, the identification of prognostic and predictive markers is clinically important. Biological markers have an important role as independent prognostic parameters in relation to the traditional clinicopathological variables which lead to the determination of tumour prognosis.

Cytometric quantitation of nuclear DNA content can assist in the diagnosis and grading of malignant tumors [Bocking et al. 1995]. Several studies report that the analysis of nuclear DNA content using flow cytometry or static cytometry has a significant value in prognosis of breast cancer [Yildirim-Assaf et al. 2007, Karra et al. 2012].

The proliferation biomarker Ki-67 is considered to be a prognostic factor for breast cancer, and its high expression was associated with an increased risk of recurrence, and with short survival [Urrticoechea et al. 2005, Jalava et al. 2006, Yerushalmi et al. 2010, Nishimura et al. 2010].

Bcl-2 is the most important antiapoptotic protein and it is expressed in many types of normal tissue [Arun et al. 2003, Petros et al. 2004]. Bcl-2 expression in human tumors could be expected to be associated with an aggressive phenotype and to confer resistance to cytotoxic drugs inducing cell death. This is actually true for some human tumor types such as non-Hodgkin's lymphoma [Hermine et al. 1996]. However, in other human tumors including breast carcinoma, bcl-2 expression has been mainly associated with favorable prognosis [Divito et al. 2004, Callagy et al. 2006, Treré et al. 2007, Callagy et al. 2008, Nadler et al. 2008, Dawson et al. 2010].

This work investigates the diagnosis delay and its impacts on stage of disease; analysis of nuclear content of DNA by image cytometry, and immunohistochemical analysis of Ki-67 and bcl-2. Many molecular markers are available for better evaluation of breast cancer tumorigenesis, disease progression and treatment choice [Fillmore and Kuperwasser 2008, Casadei et al. 2011]. Covering studies on Libyan breast cancer are few, and the tumor phenotype alterations in Libyan population are not well known. In Libya, the follow-up of patients is very variable due to the fact that patients are often partly or fully treated outside the hospital which made the histopathological diagnosis. This may explains the limited understanding of the clinical and pathologic prognostic factors among women with breast cancer in Libya.

2. REVIEW OF LITERATURE

2. 1 Clinical background

2. 1. 1 Epidemiology

Breast cancers is an important public health problem worldwide; it is the most frequently diagnosed cancer and the leading cause of cancer death among woman, and comprise 23 % of all cancer cases and 14% of cancer deaths [Jemal et al. 2011]. According to American Cancer Society (ACS), nearly 1.4 million new cases of breast cancer occurred among women worldwide in 2008. When detected early, breast cancer is highly treatable and curable.

Globally, the incidence of breast cancer is increasing, but varies from areas of high incidence (United States, Western Europe, Northern Europe and Australia) to areas of low incidence (Japan and other Asia, Latin America, and African countries). In the USA there were less than 0.9 new cases per 1000 women in 1990s, and more than 1.4 new cases per 1000 women in 2006 [McCracken et al. 2007, American Cancer Society: Cancer Facts and Figures 2010]. In Finland the incidence also increased from 0.63 per 1000 women in 1987 to 0.94 in 2010 [Finnish cancer registry report 2010]. The incidence in UK has increased from 0.75 per 1000 in 1977 to 1.2 per 1000 in 2006 [Stasistacal information UK team 2009]. Breast cancer is clearly less common in Asia and Africa. Many studies show that American women of African origin have lower breast cancer incidence but higher breast cancer mortality than white women [American Cancer Society: Cancer Facts and Figures for African American 2008, American Cancer Society: Breast cancer survival rates by stages 2012, McBride et al. 2007]. In Africa the incidence of breast cancer appears to be rising. In Nigeria for example, incidence rate has increased from 0.14 per 1000 in the 1970s to 0.33 per 1000 in 1990s [Williams et al. 2006, Adsunkaammi et al. 2006].

In the Arabic countries the studies are not fully covering. In Libya, the incidence of breast cancer is 0.18 new cases per 1000 women per year, and the majority of patients present in advanced stage [El Mistiri et al. 2007]. In Egypt about 35% of all female cancer is breast cancer [Nadia et al. 2007].The background of Arabic breast cancer patients may be more related to the genetics of African population than to the genetics of European breast cancer patients. However, also other demographic and environmental features may be important [Boder et al. 2011].

The characteristics of breast cancer in North Africa may be associated with multiple factors, including geographic variation, racial/ethnic background, genetic differences, lifestyle, utilization of screening mammography, and availability of appropriate care [Hortobagyi et al. 2005]. These features stress the importance for investigating prevention and treatment strategies among different subgroups of the breast cancer populations in North Africa.

The incidence rate is rising globally, but annual mortality rates from breast cancer have decreased over the last decade (1.8 percent per year from 1998 to 2007) [Kohler et al. 2011]. A significant frequency of the decline in mortality is attributable to the impact of screening mammography, which permits diagnosis and treatment at an early stage of the disease [Berry et al. 2005].

2. 1. 2 Risk factors

There is no single identified specific cause for breast cancer, but there are many established risk factors that increase the likelihood that a woman will develop breast cancer [McPherson et al. 2000]. The potential risk factors include the age of the patient, family and/or personal history, proliferative breast disease, and increased estrogen exposure. The most common risk factors and their relative risk are listed in Table 1(next page).

Age is a major risk factor for sporadic breast cancer. About 66% of all invasive breast cancer is diagnosed in women at age 55 or older, while about 12% are diagnosed at younger age than 45 [American Cancer Society: Breast cancer survival rates by stages 2012]. At age 75 to 80, risk stabilizes, decreasing slightly thereafter [Costanza and Chen 2012]. In Africa and Asia, breast cancer incidence peaks among women in their forties, whereas in the United Status and Europe, it peaks among women in their sixties [Green and Raina 2008, Boder et al. 2011].

In 5-10% of breast cancer cases, there is strong inherited familial risk for mutations. Two autosomal dominant genes BRCA1 and BRCA2 account for most of the cases of familial breast cancer [Schwartz et al. 2008]. These mutations are present in far less than 1% of the general population, but occur more often in certain ethnic groups such as those of Ashkenazi (Eastern European) [Schwartz et al. 2008].Women who carry a harmful BRCA1 mutation have a 44-78% risk for developing breast cancer by 70 years of age in their lifetime; the corresponding risk for BRCA2 mutations is 31-56% [Antoniou et al. 2003].
There are other genes conferring an increased risk of breast cancer involved in hereditary cancer syndromes have been identified, such as p53 and PALB2. p53 is constitutionally mutated in the Li-Fraumeni [Gasco et al. 2003]. This gene is also more commonly altered in BRCA1 and BRCA2 related breast cancer in comparison with non-BRCA related breast [Holstege et al. 2009].

Table 1. The most common risk factors and their relative risk for breast cancer [modified from American Cancer Society: Breast Cancer Facts and Figures 2012].

Relative risk	Factor
>4.0	Advanced age Atypical hyperplasia BRCA1and/or BRCA2 gene mutation Mammography: dense breasts Family history of breast cancer (≥ 3 relatives with breast cancer)
2.1-4.0	High endogenous estrogen level High bone density (postmenopausal) Two first-degree relatives with breast cancer
1.1-2.0	Alcohol consumption Early menarche (<12 years) Tall patient Lack of exercise Radiation to chest or face before age 30 High socioeconomic status Late age at first pregnancy (>30 years) Late menopause Never breastfed a child No full-term pregnancies Obesity Personal history of endometrium , ovary or colon cancer Long-term use of menopausal hormone therapy with estrogen and progesterone analogs Recent oral contraceptive use

Inherited mutation in the BRCA2-interacting protein PALB2 (Partner and Localizer of BRCA2) are also associated with increased risks of developing breast cancer [Casadei et al. 2011].

Women with a family history of breast cancer (cancer in a first-degree female relative like mother, sister, or daughter) are at increased risk of developing breast cancer. About 15 -20% of women with breast cancer report a positive family history in a first degree relative [Costanza and Chen 2012].Compared to women without a family history, risk of breast cancer is 1.8 times higher for women with one first-degree female relative diagnosed with breast cancer, nearly 3 times higher for women with two relatives, and nearly 4 times higher for women with three or more relatives [Collaborative group on hormonal factors in breast cancer 2001].

2. 1 3 Clinico-pathological classification

2. 1. 3. 1 Screening

Early detection of breast cancer remains the primary way available to patients in preventing the development of life-threatening disease.

Breast tumors that are smaller or nonpalpable are more treatable when detected, and thus are associated with a more favorable prognosis. Breast cancer screening refers to the medical detecting of asymptomatic disease among apparently healthy women. But, the screening of large population is associated with anxiety and negative psychological effect.
The life style and cultural attitude have essential role in compliancy of screening programs in some countries. Introduction of the health education at schools, in the media and in the primary health care centers of some countries may well be a successful option to change the attitudes toward acceptance of breast screening programs [Smith 2006].

A number of tests have been employed in screening programs including: breast self examination (BSE), clinical breast examination (CBE), mammography, ultrasonography (US), magnetic Resonance imaging (MRI), and genetic screening.

- **Screening guidelines**

Early detection is recommended by organizations that issue guidelines for breast cancer care. Guidelines may vary, through. The most important differences are related to patient age. Questions include at what age to start screening and how frequently it should be done, and how to organize screening among women with a high risk for developing breast cancer.

The ACS recommends that annual screening mammography begins at age 40, and for women younger than 40 years monthly BSE and CBE every 3 years, beginning at age 20 years. Women with high risk for developing breast cancer may benefit from earlier initiation of screening, screening at shorter intervals, and screening with additional method such as ultrasonography or magnetic resonance imaging [Saslow et al. 2007]. However, the United States Preventive Services Task Force (USPSTF) recommends that screening mammography begin at age >50. The USPSTF states that the decision to start mammography screening before the age of 50 should take into account the patient's situation, including her values regarding the benefits and hazards of screening [United States Preventive Services Task Force 2010]. The USPSTF also recommends against teaching BSE, because studies suggest that it did not reduce the mortality. Rather, imaging procedures and biopsies were recommended. On the other hand, the American College of Obstetricians and Gynecologists (ACOG) continues to recommend BSE. The ACOG suggests screening mammography every 1-2 years for women aged 40-49 years and every year for women 50 years or older [American College of Obstetricians and Gynecollgists 2010].

- **Mammography**

Mammography is currently the best available population-based method to detect breast cancer at an early stage, when the treatment is most effective.

The USPSTF reports that mammography reduces mortality by 30% among women aged 50-75 years, and 17% among women aged 40-49 years [American College of Radiology 2011]. The sensitivity of mammography declines significantly with increased breast density. False-positive results may arise when benign microcalcifications are regarded as malignant.
Many women experience psychological distress because of false positive findings. 50% of suspicious findings are not going to lead to malignancy, or will subside over time [Gøtzsche and Nielsen 2006]. On the other hand, according to data from the Breast Cancer Detection Demonstration Project (BCDDP), the false-negative rate of mammography is approximately 8-10%. A negative mammogram and a negative sonogram may still be associated with cancer. Birdwell et al [2001] found in mammograms with missed cancers that 30% showed calcification, of which 49% were clustered or pleomorphic.

- **Breast self examination (BSE) and Clinical breast examination (CBE)**

BSE and CBE are inexpensive and noninvasive procedures for the regular examination of breasts. Evidence supporting the effectiveness of these tests is controversial. Even with appropriate training, there is no association between BSE and mortality [Gaskie and Nashelsky 2005]. Anyhow, with improvements in treatment regimes for early disease, BSE and CBE, particularly among women younger than 40 years, continues to be recommended [American College of Obstetricians and Gynecollgists 2010].

- **Ultrasonography (US)**

Ultrasonography has become widely available and useful adjunct to mammography in the clinical setting. In the screening setting, ultrasound is limited by a number of factors, most notably the failure to detect microcalcifications and poorer specificity. Ultrasonography is relatively inexpensive and effective method to discriminate breast cystic lesions from solid lesions that usually need biopsy. This imaging technique is also useful in the guidance of biopsies and therapeutic procedures; research is currently under way to estimates its role in cancer screening. When performed carefully, ultrasonography may be useful in detecting occult cancer in dense breasts [Buchberger et al. 2000]. Recently, new technique called the somo-v Automated Breast Ultrasound System (ABUS) was approved by the Food and Drug Administration (FDA) for breast cancer screening specifically in women with dense breast tissue [Food and Drug Adminstration 2012]. ABUS is indicated as an adjunct to standard mammography for women with a negative mammogram, no breast cancer symptoms, and no previous surgical intervention.

- **Magnetic resonance imaging (MRI)**

MRI has been explored as a modality for detecting breast cancer in women at high risk and in younger women.

An MRI technique has been found to be highly sensitive for detection of malignant changes in the breast. MRI is an important adjunct screening tool for women with BRCA1 or BRCA2 mutations, identifying cancers at earlier stages.
Annual MRI-based screening among BRCA1 and BRCA2 carriers resulted in a significant 70% reduction in hazard of late stage compared to BRCA1 and BRCA2 carriers not undergoing annual MRI [Warner et al. 2011]. However, breast MRI has limited use as a general screening tool, with a 10-fold higher cost than mammography and poor specificity, resulting in significantly false-positive results that create more diagnostic costs and procedures. The ACS recommends MRI screening for certain high-risk groups, including women with BRCA1 or BRCA2 mutations, women with the first degree relatives of BRCA carrier, and women with greater than 20% lifetime risk [Saslow et al. 2007].

- **Genetic testing**

Genetic testing does not detect cancers, but may reveal a propensity to develop cancer. A woman with higher risk of developing breast cancer usually needs aggressive screening. Clinical practice guidelines by USPSTF recommend against routine referral for genetic counseling or routine testing for BRCA mutation, in consideration the hazards over the benefits [United Status Preventive Services Task Force 2011]. It also encourages a referral for counseling and testing of women with a family history that shows they have an increased risk of a BRCA mutation.

2. 1. 3. 2 Diagnosis

Evaluation of breast symptom or abnormal finding begins with a thorough medical history and clinical examination, followed by imaging tests and biopsy (triple assessment). There is a variation in diagnostic evaluation of women suspected to have breast cancer. Patterns of referral vary dramatically, and the work up may differ, depending upon which physician is seen first. The breast cancer diagnostic algorithm provides detailed guidelines for the work-up of a new palpable mass and abnormal screening mammogram for accurate assessment of any suspicious finding in the breasts. Furthermore, a suspicion of breast cancer requires coordination among physicians in several specialties. The coordination between radiologists and breast surgeons can reduce unneeded biopsies and expedite diagnosis for women who receive a diagnosis of breast cancer. In addition, since the diagnosis of cancer is made interdisciplinary coordination between the breast surgeons, medical oncologists, radiologists and pathologist are needed in treatment planning [Chang et al. 2001].

- **Clinical examination**

The main symptom of early breast cancer is a painless lump in the breast.

Other less common clinical presenting features of breast cancer include: asymmetrical breast (shape and size), nipple or skin retraction, blood-stained discharge from the nipple, areolar eczema, ulceration and palpable regional lymph node.
Systemic complains include generalized fatigability, cough, anemia, ascities, jaundice, or skeletal pain, especially in advanced disease.

- **Imaging tests**

The radiological imaging tool including mammography, ultrsonography and magnetic resonance imaging are very successful in screening programs and clinical diagnosis of breast lesions.

Mammography

The majority of breast cancers are diagnosed as a result of an abnormal finding on mammogram, but not all breast cancer will be detected on a mammogram. Less than 10 percent of breast cancers were detected by physical examination and above 90 percent were identified mammographically [Smart et al. 1993]. Women who have abnormal findings on mammogram often need further diagnostic evaluation. The most aggressive cancers may emerge between normal screening mammograms (interval cancers) [Lin et al. 2009]. Younger women may be present with large tumor prior to the age at which screening is recommended. So, when women present with a suspicious new mass, diagnostic mammograms should be advised as part of initial diagnostic workup. Diagnostic mammography is associated with higher sensitivity but lower specificity as compared to screening mammography. The sensitivity and specificity of diagnostic mammograms decreases with breast density and younger age [Mandelson et al. 2000].

Ultrasonography (US)

Breast ultrasound (US) is an important diagnostic addition to mammography and is used for discrimination between solid and cystic masses and for providing guidance for interventional procedures [Berg et al. 2004, Harvey et al. 2009].

Magnetic resonance imaging (MRI)

Breast MRI is highly sensitive, and can identify foci of cancer that are not shows on physical examination, mammogram, or ultrasound [Peters et al. 2008]. Because of limited specificity, the use of breast MRI increases the number of unneeded biopsies, delays definitive treatment, increases the number of patients undergoing surgery [Bleicher et al. 2009]. Histopathological confirmation is required for all suspicious findings on MRI.

- **Breast biopsy**

In the patient with an abnormal finding on mammogram or a palpable breast mass, the mandatory diagnostic technique is biopsy. A number of biopsy techniques are used:

- Fine needle aspiration biopsy (FNAB)
- Core biopsy/true-cut biopsy
- Open biopsy

Surgical biopsy should not be utilized as a diagnostic tool unless percutaneous palpation-guided or image-guided biopsy is not practical [Silverstein et al. 2009].

2. 1. 3. 3 Tumor type

The major component of the breast is the mammary gland. Each mammary gland is made of multiple lobules connected to ducts and surrounding connective tissue and fat which include the blood vessels. Malignant tumors may arise from any of these structures. After diagnosis of breast cancer they should be classified histologically. Carcinomas can be non-invasive (confined to the ducts or lobules) or invasive tumors (extending into surrounding stroma).

- **Non-invasive tumors**

Ductal carcinoma in situ (DCIS)

DCIS is a non-invasive breast cancer, and consists of malignant epithelial cells confined to the mammary duct, without evidence of invasion to the surrounding tissue. There are various histologic subtypes of DCIS, namely comedo, micropapillary, cribriform, papillary, mixed and solid. The comedo subtype is associated with high nuclear grade, and overexpression of the HER2/neu oncogene [Pinder 2010]. DCIS is of non-invasive type, but when untreated about 20 to 30% of DCIS will progress to invasive type [Morrow and Harris 2010].

DCIS is usually treated surgically with or without radiation and hormonal therapy. Lumpectomy for DCIS followed by radiotherapy reduces the risk of invasive breast cancer and DCIS relapse [Zujewski et al. 2011, Wapnir et al. 2011]. Patient with small tumors may be planned for lumpectomy without radiotherapy [Hughes et al. 2009]. Overall survival appears to be the same for patient with DCIS who have lumpectomy with or without radiotherapy [Correa et al. 2010]. The National Comprehensive Cancer Network (NCCN) recommended that the patients who are treated with lumpectomy plus radiotherapy for estrogen receptor-positive DCIS should take tamoxifen for five years [National Comrehensive Cancer Network 2012].

Tamoxifen can decrease the risk of relapse of DCIS and the risk of invasive cancer in the affected breast, and the risk for breast cancer in the opposite breast [Cuzick et al. 2011].

Lobular carcinoma in situ (LCIS)

LCIS is a non-invasive tumor, and consists of abnormal cells growing inside the lobules of the breast, but have not spread to surrounding tissue. LCIS increases the risk of invasive tumor. Women who have LCIS are more likely to develop invasive cancer in either breast than women without LCIS [Kibride and Newman 2010]. The NCCN and the ACS recommended special breast cancer screening guidelines for women with LCIS breast cancer [American Cancer Society: Cancer Prevention and Early Detection Facts & Figures 2011, National Comrehensive Cancer Network 2012]. Hormonal therapy (tamoxifen) reduces the risk of invasive breast cancer for both pre-and post menopausal women with LCIS [Vagel et al. 2010].

- **Invasive tumors**

Invasive tumors spread from the original site (either milk duct or lobules) into surrounding tissue of the breast and may spread to lymph nodes and/or other parts of body. Table 2 lists the major histological types of invasive tumors, along with their frequency and estimated 5 year overall survival [Rosen 2009, Dillon et al. 2010, Merajver et al. 2010]. Invasive ductal carcinoma which is the most common type comprises 50-75% of all breast cancers. Invasive lobular carcinoma is the next most common type and comprises about 10-15% of cases [Dillon et al. 2010].There are less common types of invasive breast carcinomas that tend to have a better prognosis. They are mixed tubulolobular carcinoma, medullary carcinoma, mucinous (colloid) carcinoma, papillary carcinoma and tubular carcinoma [Dillon et al. 2010, Merajver et al. 2010].

- **Other types of breast tumor**

Inflammatory breast cancer (IBC)

IBC is an aggressive type of breast cancer with symptoms that differ from other types of breast cancer. IBC comprise 1-3% of all breast cancers and 7% of breast cancer mortality [Hance et al. 2005, Merajver et al. 2010]. At the time of diagnosis, most of women with IBC will have positive lymph nodes, and about 25% have distant metastasis [Merajver et al. 2010]. IBC tumors are estrogen receptor-negative, HER2/neu-positive, and tend to have a higher proliferative activity than other types of breast cancer [Merajver et al. 2010]. With treatment, the overall survival rate is about 50% [Ellis et al. 2011].

Table 2. The major histological types of invasive breast tumor, along with their frequency and 5 year overall survival [modified from Tavassoli and Devilee 2003, Dillon 2010, Merajver 2010, and Rosen 2009].

Histological type	Frequency (%)	5-year-survival rate (%)
Invasive ductal carcinoma	50-75	80-90
Invasive lobular carcinoma	10-15	85
Mixed tubulolobular carcinoma	6	>85
Medullary carcinoma	3-10	>80
Mucinous (colloid) carcinoma	2	90-100
Papillary carcinoma	1-5	90-100
Tubular carcinoma	1-5	90-100
Cribriform carcinoma	3-4	90-100

Paget's disease of the breast

Paget's disease of the breast is a rare type of cancer involving the skin of the nipple or in the skin closely surrounding the nipple. Paget's disease of the breast can be found with invasive breast cancer or DCIS in the same breast [Caliskan et al. 2008]. The average age at diagnosis is 60 years, but the disease has been found also in younger patients [Kanitakis 2007]. The symptoms of Paget's disease are often confused with benign skin conditions such as dermatitis, and it may be undiagnosed at first [Caliskan et al. 2008]. Surgical treatment (mastectomy with or without axillary lymph node dissection), is regarded as a suitable therapy for Paget's disease of the breast [Kanitakis 2007]. In patients with both Paget's disease of the breast and invasive cancer in the same breast, the 5-year survival rate declined with increasing stage of the cancer [Chen et al. 2006].

Metaplastic carcinoma of the breast (MPC)

MPC is an uncommon form of breast cancer comprising less than 5% of breast cancer patients [Dillon 2010]. The pathological classification of MPC is variable, MPCs are differentiated into 5 subtypes: spindle cell, squamous cell, carcinosarcoma, matrix-producing, and MPC with oseoclastic gaint cells [Lee et al. 2012]. Compared to other types, metaplastic tumors tend to be larger, are poorly differentiated and are often not associated with estrogen and progesterone receptors, or HER2 expression [Ayar et al. 2010, Lee et al. 2012]. Lymph node involvement at the time of presentation accounts for 8% to 40% [Park et al. 2010]. The risk of recurrence and poor prognosis with MPC is high compared with IDC or ILC [Shin et al. 2007]. MPC should be differentiated from IDC or ILC and primary breast carcinoma in order to determine appropriate management planning and patient outcome.

Phyllodes tumors of the breast

Phyllodes tumors of the breast are rare fibroepithelial lesions that comprise less than 1% of all breast tumors [Paker et al. 2001]. The importance of phyllodes tumors lies in the need to discriminate them from other benign breast lesions, especially fibroadenoma [Yohe et al. 2008]. About 85-90% of phyllodes tumors are benign and approximately 10-15% are malignant [Jones et al. 2008]. Diagnosis of phyllodes tumors is suspected in women, often over age of the 35 years, who present with a rapidly growing breast lump. Wide complete excision with accurate histological examination and regular follow up care is the best way to treat phyllodes tumors [Guillot et al. 2011]. Some studies suggest that total mastectomy is more effective than breast conserving-surgery [Belkacemi et al. 2007]. Local recurrence occurs approximately in 15% of patients and is more common after incomplete excision, and with lesion that display malignant histology [Pezner et al. 2008]. Distant metastasis is typically observed in the lung, mediastinum, and bone [Abe et al. 2011]. The role of chemotherapy, radiotherapy and hormonal manipulation in the treatment of phyllodes tumors remain to be defined.

2. 1. 3. 4 Grading

Histological tumor grade is based on the degree of differentiation of the tumor tissue, and classifies the cancer as well differentiated (low grade), moderately differentiated (intermediate grade), and poorly differentiated (high grade), reflecting progressively less normal appearing cells in line with worsening prognosis.

Grading in breast cancer refers to semi-quantitative evaluation of morphological characteristics and is a relatively simple low-cost method, requiring only adequately prepared hematoxylin-eosin-stained tumor tissue sections to be assessed by an appropriately trained pathologist using standard protocol. The Nottingham Grading System (NGS) is the grading system recommended by various professional bodies internationally [Pathology Report of Breast Disease 2005, National Comprehensive Cancer Network guidelines 2011].

Multiple independent studies have shown that histological grade has value that is equivalent to that of lymph node status and greater than that of tumor size [Galea et al. 1992, Walker et al. 2003].

Nottingham group demonstrated that histological grade is an important determinant of breast cancer outcome and complementary to LN stage through the ability to influence the outcome of patients in different LN stage categories [Rakha et al. 2008].

2. 1. 3. 5 Staging

Staging is the process of determining the growth and extent of the cancer in the body. It is based on whether cancer is invasive or non-invasive, on tumor size, on lymph node status, and on spread to other parts of the body. Staging of breast cancer is the most important component of traditional classification methods in determining prognosis and treatment options. Other components include estrogen and progesterone receptor levels in the tumor tissue, the human epidermal growth factor receptor 2 (HER2/neu) status, menopausal status, and the general health of the patient [Edge et al. 2010].The stage of a breast cancer is determined by information gathered from the staging process. Clinical staging takes place prior to surgery and is based upon information obtained from physical examination and imaging tests. Pathological staging occurs after operation and is done from resected breast tissue, and includes both clinical information and findings from microscopically examined tissue. Pathological staging is the standard way to stage breast cancer. Accurate staging can help in estimating prognosis and in selecting treatment options. It is also critical in evaluating the overall results of treatment.

The American Joint Committee (AJC) on cancer has designed the TNM classification staging system that was proposed by the International Union Against cancer (IUAC) and based upon the size of tumor (T), degree of spread to lymph nodes (N) and systemic metastasis (M) at time of diagnosis. The staging system runs from stage 0 (DCIS or LCIS) to stage IV. Table 3 shows breast cancer staging and TNM classification (next page). The data are modified from Edge et al [2010].

Staging is regarded as the most important prognostic factor. As stage increases the prognosis worsens. For example; the 5 year survival in stage I breast cancer is more than 90% while patients with stage IV disease have very poor prognosis and a 5 years survival is less than 30% [Edge et al. 2010, American Cancer Society: Breast cancer survival rates by stages 2012]. Considering all races, 5-year relative survival is 90% for localized disease and 23% for advanced stage disease [Howlader et al. 2012].

Table 3. Breast cancer staging and TNM classification [modified from Edge 2010]

Anatomical staging and prognostic groups			
Stage 0 (DCIS)	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IB	T0	N1	M0
	T1	N1	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Furthermore, cancer staging is the most basic method to prognostic evaluation of any type of cancer, including breast cancer, and is used to assess survival at the population level. Over the past three decades, the distribution of breast cancer stage at diagnosis has changed. The diagnosis of stage 0 (DCIS), and very early stage breast cancer (stage I) have increased. On the other hand, the diagnosis of stage II, III, IV breast cancer has either subsided or stayed about the same. Large increases have been show in diagnosis DCIS (stage 0) and stage I breast cancer in women under age 50 year [Ries et al. 2001]. These changes are attributable to the higher screening rates and better screening tools, which permits diagnosis at an earlier stage of disease [Ries et al. 2001].

Although racial differences in stage at diagnosis are decreased, African American women are still more likely than white women to be diagnosed at advanced stage [Adams et al. 2012]. Health service aspects are important, but differences in biology of breast cancer may also be involved. Studies suggest that African American women are more likely to have breast cancers with factors linked to poorer prognosis such as high grade, hormone receptor negativity and HER/2neu positivity [Millikan et al. 2008, Tamimi et al. 2012].

2. 1. 3. 6 Hormonal status (estrogen and progesterone)

Estrogen and progesterone are hormones that regulate the sex hormone-dependent organs such as the female reproductive system and the breast. Steroid hormones with growth factors drive the development, growth, and differentiation of breast epithelial tissue following activation of the nuclear estrogen (ER) and progesterone (PR) receptors and are also critical for breast cancer development and progression [Joshi et al. 2010]. Their activations basically through ligand binding or growth factor-induced phosphorylation-engines drive gene networks, and metabolic and cell regulatory pathways, thus supporting cancer cell growth [Frasor et al. 2003]. The removal of estrogen from the environment of the cells containing steroid receptors adversely affects the growth of these cells. This is the basis for endocrine therapy in breast cancer patients. Approximately 70% of breast cancers are ER-positive, and most ER-positive breast cancers are also PR-positive [Freedman and Winer 2010].Whereas the PR is induced by an active estrogen-ER pathway, the expression of hormonal receptors depends on many factors: histological type and grade, HER-2 expression, age at diagnosis, BRCA-1/2 mutation, and body mass index [Montemurro et al. 2012].

Two types of ER have been identified: ER-alpha and ER-beta. These are transcription factors that mediate estrogen signaling and define the hormone-responsive phenotype of breast cancer. The two receptors can be found co-expressed and play specific, often adverse roles, with ER-beta being able to modulate the effects of ER-alpha on gene transcription and cell proliferation [Ordóñez-Morán et al. 2009]. ER-beta is frequency lost in breast cancer, where it is found generally associated with good prognosis of the disease [Sugiura et al. 2007].

In the past, ER and PR were assessed using biochemical ligand binding assays (LBA), giving a quantitative result. The production of antibodies directly targeting ER and/or PR, and retrospective studies suggest that semi-quantitative IHC (immunohistochemistry) analysis of hormone receptor expression was superior for prognostic and predictive purposes which led to replacement of biochemical assays by IHC [Bartlett et al. 2011]. IHC is simple, cheaper, specific, and usable for evaluating cytology, fresh frozen tissue, and paraffin embedded tissue. IHC also allows the microscopists to allow differentiation between malignant and benign cells.

A lack of standardized protocols in the pre-analytical stage, different assessment methods and availability of diverse antibodies remain critical issues leading to inaccurate results in many centers [Allred et al. 2010, Bartlett et al. 2011, Hammond et al. 2010]. Since these tests guide the use of endocrine therapy in patients with breast cancer, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) developed recommendations for tumor marker testing in breast cancer to improve the accuracy and reproducibility of these tests [Hammond et al. 2010].

Many semi-quantitative scoring systems for ER and PR IHC have been developed. Presently, the most established score is the Allred score [Allred et al. 2010]. Standardized cut-offs for determining hormone receptor positivity is very important. Prospective studies show that knowledge of optimal IHC cut-off for defining positivity for efficient endocrine therapy is lacking. However, a wide range of cutoffs are being used (from 0% to 20%) on stained cells [Putti et al. 2005, Stendahl et al. 2006, Yip et al. 2011]. In view of the great progress in the IHC technique and the introduction of new highly sensitive antibodies and the wide use of 10% as a cut-off for positivity in many centers, classification of tumors showing 1%–9% IHC staining remains problematic. Advanced research at this point is needed to address not only the response rates of these tumors but also their biological features with regard to ER–pathway activation. New reports in this field suggest that almost half of tumors staining from 1% to 9% by IHC have molecular features of the basal-like phenotype [Iwamoto et al. 2012].

Novel approaches such as qRT-PCR (real-time quantitative reverse transcription polymerase chain reaction) and gene expression microarrays further revise the assessment of hormone receptor expression. The Oncotype DX recurrence score using qRT-PCR ER gene information is not only a highly significant prognosticator, but it is also being used as a predictive tool to estimate the benefit of adjuvant therapy [Badve et al. 2008]. Possibly future integration of these new molecular techniques with more traditional morphological methods will pave the way to fully accurate treatment for breast cancer patients.

The presence of hormone receptors within a tumor indicates that tumor cells have a higher degree of functional differentiation. Importantly, they are more likely to benefit from endocrine therapy. Tumors with intense IHC expression for ER/PR are associated with good prognosis and have maximum response to endocrine therapy [Goldhirsch et al. 2011, Prat et al. 2012]. The semi-quantitative analysis of hormone receptor expression rather than qualitative analysis estimates the benefit neo-adjuvant chemotherapy, and the benefit decreases with increasing hormone receptor expression [Colleoni et al. 2010].

2. 1. 3. 7 HER2 status (HER2/neu)

HER2 is proto-oncogene located on chromosome 17, and it is encoded by the ERBB2 gene, which is a protein of a family of four transmembrane receptor tyrosine kinases that mediate the growth, differentiation and cell survival [Gschwind et al. 2004]. HER2 is a member of the epidermal growth factor receptor (EGFR/ErbB) family with extracellular domain and interacellular tyrosine kinase activity [Ali et al. 2002]. Amplification of this gene has been shown to play an important role in the pathogenesis and progression of certain aggressive types of breast cancer and it has evolved to become an important biomarker and target of therapy for breast cancer [Kaufmann et al. 2011].

Amplification or over-expression of HER2 is present in about 30% of breast cancers. There is amplification of the gene with resultant over-expression of the membrane related protein. Carcinomas which have these abnormalities are strongly associated with aggressive behavior, and have a high grade of malignancy, and show high proliferative rates [Tan and Yu 2007, Yeh et al. 2011].

HER2 protein over-expression can be identified at cell membrane by IHC, while the number of copies of the gene is usually detected by fluorescence in situ hybridisation (FISH) or chromogenic in situ hybridization (CISH). These methods can be performed on paraffin-embedded tissue and are used as an eligibility criterion for HER2 target therapy by the monoclonal antibody trastuzumab (titled as Herceptin), which is effective only in cancers where HER2 is over-expressed. Pertuzumab is another monoclonal antibody, which inhibits dimerization of HER2 and HER3 receptors, and was approved by the FDA for use in combination with trastuzumab in June 2012. Measurement of serum HER2 by enzyme-linked immunosorbent assay (ELISA) is a less invasive method of determining HER2 status than a biopsy, and changes in serum concentrations may be useful in predicting response to anti-HER2 therapy [Ali et al. 2008].

HER2 status is important in predicting prognosis in patients with breast cancer, and over-expression of HER2 protein without trastuzumab treatment is associated with worse prognosis [Tan and Yu 2007, Yeh et al. 2011]. The expression of HER2 is also predictive to many systemic therapies and is thought to have positive predictive value as regards the response of patients with breast cancer to anthracycline-based chemotherapy and negative predictive value to anti-hormonal therapy (tamoxifen) [Pritchard et al. 2006, Villman et al. 2006]. Joensuu et al [2006] observed that the overall therapy response rate was better among patients with HER2 gene amplification or intensive membrane staining by IHC than patients with tumors having normal HER2 expression. Approximately 20% of metastatic breast cancers may have HER2 over-expression. HER2 targeted therapy with other chemotherapy may also slow down or even stop the growth of such cancers [Bilous 2003].

2. 2 Treatment

2. 2. 1 Surgery

Surgery is considered as the primary treatment for breast cancer, many patients with early stage being cured with surgery alone.

The aims of breast surgery include complete resection of the primary tumor, with clear margins to achieve good local control for both the primary tumor and regional lymph nodes, and pathological staging of the tumor and evaluating the status of axillary lymph nodes for providing essential prognostic information. Stage I, II, IIA, and operable IIC breast cancer often requires a multi-modality approach to treatment.
The diagnostic biopsy and the surgical procedure should be performed as two separate procedures. After the diagnosis of malignancy is confirmed, treatment options should be discussed with the patients before the therapeutic option is decided.

Options for surgical management for the primary tumor include:

- Breast conserving surgery
- Mastectomy

Long term randomised follow up trials compared the therapeutic efficacy of breast conserving surgery plus radiotherapy with mastectomy for invasive breast cancers (tumor size <2.5 cm). The results suggested that the survival was similar in the groups, with an increase in local recurrence in the conservation group [Fisher et al. 2002, Veronesi et al. 2002]. Recently, the EBCTCG results reported that the mortality rate and local recurrence decreased in patients treated with conserving surgery plus radiotherapy [Early Breast Cancer Trialists Collaborative Group 2011].

Accurate pre-operative assessment of the size and extent of the tumor is essential for deciding whether breast conservation surgery can be considered an option. The axillary lymph nodes should be staged with an idea to determine prognosis and get guidelines for therapeutic options. Sentinel lymph node is defined as any node that first receives drainage directly from the primary tumor. Determining the status of that lymph node is the initial standard procedure for axillary staging in patient with invasive breast cancer. Studies report 97.5% to 100% conformity between sentinel lymph node biopsy and complete axillary lymph node dissection. Axillary dissection was associated with significant lymphedema of the upper limb [Veronesi et al. 1999].

Preoperative preparation of the patient for breast surgery is important, and should include attention to psychosocial and surgical issues. Patient may have negative concerns regarding risk of recurrence, need for adjuvant radiotherapy or chemotherapy, surveillance, duration of rehabilitation, and particularly the cosmetic result. In introducing the patients to surgical treatment options, it is important to discuss the options of immediate versus delayed reconstruction.

2. 2. 2 Radiotherapy

Radiotherapy continues to have an important role in the management of patients with breast cancer. Radiotherapy is used to kill the cancer cells remaining in the breast, chest wall, or underarm area after breast conserving surgery.

Radiotherapy may also follow mastectomy in patients with tumor size larger than 5 cm and/or positive lymph nodes. The main aim of adjuvant radiotherapy is to eradicate the residual disease thus reducing local recurrence, and improving survival. Additionally, radiotherapy has a major role in the palliation setting.

There are two types of radiotherapy:

- External beam radiation
- Internal radiation therapy (brachytherapy)

The type of radiation therapy depends on the histological type, stage, and location of the tumor. Target radiotherapy has increased dramatically in recent decades, which has greatly diminished side effects and also shortened treatment duration, with promising results [Beitsch et al. 2011]. Clinical trials are also investigating other types of accelerated partial breast irradiation that are designed to give radiotherapy to a small segment of the breast, and over a period of 5 days [Dirbas et al. 2009].

Adjuvant radiotherapy

- **Adjuvant radiotherapy after breast conserving surgery**

Women who are treated with breast-conserving surgery, the most typical site of local recurrence is the conserved breast itself. The risk of relapse in the conserved breast is fundamentally high even in confirmed axillary lymph node negative patients. Thus, whole breast radiation therapy following breast conserving surgery is vital [Eifel et al. 2001]. Many trials assessing the role of radiotherapy after breast conserving surgery have shown reduction in local relapse rate and increased survival [Clarke et al. 2005, Darby et al. 2011].

- **Adjuvant radiotherapy after mastectomy**

Patients who are treated with mastectomy and considered to be at high risk for local-regional failure, adjuvant radiotherapy to chest wall and regional lymph nodes are indicated following surgery [Early Breast Cancer Trialists Collaborative Group 2000]. Patients at highest risk for local relapse include patients with:

- four or more positive axillary lymph nodes
- extracapsular nodal extension
- large primary tumor
- only narrow negative deep margin or positive deep margins [Clarke et al. 2005].

Palliative radiotherapy

Radiotherapy has a major role in the palliation of localized symptomatic metastasis. Radiotherapy may be indicated as a palliative treatment for:

- painful bone metastasis
- unresectable brain, meningeal, and spinal cord metastasis
- fungating/painful breast
- decompression of intracranial or spinal cord metastasis
- fixation of pathological fractures

Toxic effects of radiotherapy, though uncommon, include radiation pneumonitis, cardiac events, arm edema, brachial plexopathy, and the risk of second malignancies [Harris et al. 2006, Mery et al. 2009]. Such toxic effects can be minimized with new radiotherapy techniques and with careful delineation of the target volume.

2. 2. 3 Chemotherapy

2. 2. 3. 1 Adjuvant chemotherapy

Chemotherapy in the adjuvant setting has contributed to the significant progress in management of patients with breast cancer, and remains an important and frequently used treatment option. In the past few decades, we have experienced a pattern shift, from classic CMF drugs (cyclophosphamide, methotrexate and 5-florouracil) to the use of other agents. Early diagnosis and novel chemotherapy strategies have improved the outlook for many patients with breast cancer, and given curative intent of adjuvant therapy. All patients with breast cancer do not need adjuvant chemotherapy. Patients at high risk of cancer relapse should receive adjuvant treatment. Age and lymph node status, and other prognostic and predictive factors should be considered to decide who will benefit from adjuvant treatment [Goldhirsch et al. 2005].

Common adjuvant chemotherapy regimens for breast cancer patients with negative HER2/neu are listed in Table 4. Anti-HER2/neu directed therapy (e.g. trastuzumab) is indicated for use in combination with chemotherapy in patients with HER2/neu-positive disease. Trastuzumab in concurrent combination with adjuvant chemotherapy is more effective than given after chemotherapy [Viani et al. 2007].The EBCTCG (Early Breast Cancer Trialists Collaborative Group) meta-analysis showed that adjuvant chemotherapy significantly reduced the risk of relapse and death from breast cancer, especially in premenopausal patients [Early Breat Cancer Trialists Collaborative Group 2005].

Table 4. Adjuvant chemotherapy regimens for breast cancer patients with negative HER2/neu

Regimens	Number of cycles and repetitions (weeks)	Cyclophosphamide (mg/m²)	Methotrexate (mg/m²)	5-Fluorouracil (mg/m²)	Doxorubicin (D) /Epirubicin (E) (mg/m²)	Paclitaxel (P) /Docetaxel (D) (mg/m²)
First generation						
CMF	8 q 3	600, IV* day 1	40, IV day 1	600mg, IV day 1		
AC	4 q 3	600, IV day 1			D: 60, IV day 1	
Second generation						
FAC	6 q 3	500, IV day 1		500, IV day 1 and 8	D: 50, IV day 1	
TC	4 q 3	600, IV day 1				D: 75, IV day 1
Dose-dense AC-P	4 (AC) q 2 and 4 (T) q 2	600, IV day 1			D: 60, IV day 1	P: 175, IV day 1
Third generation						
AC-P	4 (AC) q 3 and 12 (P) q 1	600, mg IV day 1			D: 60, mg IV day 1	P: 80, IV day 1
TAC	6 q 3	500, IV day 1			D: 50, IV day 1	D: 75, IV day 1
FEC-D	3 q 3	500, IV day 1		500, IV day 1	E: 100, IV day 1	D: 100, IV day 1
FEC-P**	4 (FEC) q 3 and 8 (P) q 1	600, IV day 1		600, IV day 1	E: 90, IV day 1	P: 100, IV day 1

CMF= cyclophosphamide, methotrexate and 5-fluorouracil [Bonadonna 2005]; AC= doxorubicin and cyclophosphamide [Fisher etal. 2001]; FAC= 5-fluorouracil, doxorubicin and cyclophosphamide [Early Breat Cancer Trialists Collaborative Group 2005]; TC= docetaxel and cyclophosphamide [Jones et al. 2006]; AC-P= doxorubicin, cyclophosphamide and paclitaxel [Citron et al. 2003]; TAC= docetaxel, doxorubicin and cyclophosphamide [Swain et al. 2010]; FEC-D= 5- fluorouracil, epirubicin and docetaxel [Roché et al. 2006]; FEC-P= 5- fluorouracil, Epirubicin and Paclitaxel [Matin et al. 2008].

* = Intravenous.

** = 4 cycles of (FEC) every 3 weeks, followed by 3 weeks treatment off, and then start 8 cycles paclitaxel (P) weekly.

Anthracycline based chemotherapy shows an advantage over CMF-based chemotherapy to lower the risk of relapse and death from breast cancer [Early Breat Cancer Trialists Collaborative Group 2005]. The 2006 EBCTCG overview showed that the adjuvant treatment with anthracycline, and taxane-based chemotherapy further reduced the risk of breast cancer relapse compared with anthracycline-based chemotherapy alone [Peto et al. 2007]. Results of individual studies are in agreement with the conclusions of meta-analysis [Fisher et al. 2004]. Lastly, the EBCTCG overview also reported that adjuvant taxane-anthracycline-based regimens significantly reduced breast cancer mortality [Early Breast Cancer Trialists Collaborative Group 2012].

Adjuvant chemotherapy is associated with toxic effects that vary according to the individual drugs used in each regimen. Common short-term toxic effects include nausea and vomiting, myelosuppression, alopecia, and mucositis. Uncommon longterm, but serious toxic effects include heart failure, secondary malignancy, cognitive impairment function, neurotoxicity, and sexual dysfunction [Vardy 2009, Hawkins et al. 2009].

2. 2. 3. 2 Neoadjuvant chemotherapy

Neoadjuvant chemotherapy has been considered a standard option for locally advanced breast cancer, aimed to improve surgical outcomes and facilitate the primary surgical treatment. Also it is currently accepted for patients having operable breast cancer, who desire breast conservation. Additionally neoadjuvant chemotherapy allows for an early evaluation of the effectiveness of systemic therapy, and this may permit to discontinue ineffective treatment [Schwartz et al. 2004].
Neoadjuvant systemic chemotherapy is indicated for all patients with:

- inflammatory breast carcinoma
- a large primary tumor (> 5 cm)
- extension to the skin or chest wall
- ipsilateral supraclavicular adenopathy
- bulky axillary adenopathy

Several chemotherapy regimens are recommended for inoperable locally advanced breast cancer. In patients with HER2/neu-positive disease, concurrent trastuzumab with chemotherapy improve the pathological complete response (pCR) rate [Untch et al. 2010]. Preoperative chemotherapy has been found to be as effective as therapy given after surgery in terms of survival, disease progression, and distant relapse [Mauri et al. 2005, Rastogi et al. 2008].

2. 2. 3. 3 Systemic treatment of advanced breast cancer

2. 2. 3. 3. 1 Anthracyclines

Anthracyclines are anticancer compounds that were originally derived from the pigment-producing Streptomyces peucetius early in 1960s [Brockmann 1963]. They are presently among the most effective anticancer drugs and may be recommended as standard adjuvant regimens in the main breast cancer treatment guidelines.The anti-tumor activity of anthracycline is still not completely clear and multiple pathways may be involved in the cytotoxicity of those drugs:

- **DNA intercalation**

Intercalation into DNA, leading to inhibition of macromolecular synthesis, was the first mechanism described for cytotoxicity of anthracyclines [Marco and Arcamone 1975]. The drug inhibits nuclear DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand and thus prevents the replication of rapidly-growing cancer cells [Takimoto et al. 2008].

- **Interaction with DNA binding proteins**

Regulation of gene expression by inhibiting or promoting the binding transcription factors is considered in anthracycline cytotoxicity with potential involvement of SP-1 transcription factor as a specific target to these drugs [Mansilla 2008]. Biologically, anthracycline inhibit topoisomerase IIα (TOP2A), an enzyme important in the repair of double-strand DNA breaks that occur after treatment with some cytotoxic drugs.

Anthracycline-based chemotherapy regimens have demonstrated significant disease free survival and overall survival improvements in adjuvant setting [Early Breat Cancer Trialists Collaborative Group 2005]. Patients with HER2/neu-positive breast cancer cells are more responsive to anthracycline-based chemotherapy than HER2/neu-negative patients [Jones et al. 2009]. Addition of taxanes further increases the efficacy of anthracyclines as first line treatment in metastatic setting [Piccart-Gebhart et al. 2008].

Anthracyclines have been used in the treatment of early and advanced breast cancer for a long time and up till now. But still there are concerns regarding anthracycline-associated cardiotoxicity and the leukemogenic potential. The EBCTCG suggests that patients who were treated with anthracycline-based regimens were associated with an annual risk of cardiac mortality of 0.08% per year, as compared with 0.06% per year with nonanthracycline-based regimens.
New anthracycline formulations were developed to increase the therapeutic index of conventional anthracyclines by maintaining antitumor efficacy while improving the safety profile. Pegylated liposomal doxorubicin (PLD) is the most widely studied of the liposomal anthracyclines, and seems to have similar anti-tumor activity to conventional doxorubicin, but a better safety profile [O'Brien et al. 2004, Trudeau et al. 2009].

2. 2. 3. 3. 2 Taxanes

The introduction of taxanes in the mid-1990s marked a significant progress in the treatment of breast cancer. In clinical trials, these agents provided improved outcomes for patients with early and advanced disease. Taxanes include paclitaxel and docetaxel. Anti-tumor activity of both paclitaxel and docetaxel is the disruption of microtubule function that promote microtubule polymerization and inhibits depolymerization, which result in cell cycle arrest in G2 and M phase, ending in cell death [Hagiwara and Sunada 2004].

Paclitaxel and docetaxel are the most widely used drugs among the taxane class and have important clinical benefits in the adjuvant and metastatic setting [Ferguson et al. 2007, Ellis et al. 2009, Burnell et al. 2010]. Results from a large number of clinical trials suggest that docetaxel produces higher anti-tumor response rates, progression-free survival and overall survival compared with paclitaxel [Jones et al. 2005].

Resistance to chemotherapy causes 90% of therapy failures in patients of metastatic breast cancer [Longley et al. 2005, Rivera et al. 2010]. Novel classes of anti-tumor agents are developed to overcome tumor resistance mechanisms, which includes eribulin mesylate, ixabepilone and nab-paclitaxel [Gradishar et al. 2005, Morris et al. 2009, Fornier et al. 2011].

2. 2. 3. 3. 3 Capecitabine

Capecitabine is a fluoropyrimidine carbamate, which is supplied for oral administration as a systemic prodrug of 5′-deoxy-5-fluorouridine (5′-DFUR). 5′-DFUR is converted to 5-FU by sequential enzyme activity, and it eventually inhibits DNA synthesis and function [Miwa et al. 1998].

Capecitabine has been studied as a single agent or in combination with other therapeutic agents in patients with advanced breast cancer pretreated with anthracyclines or/and taxanes. Capecitabine has improved time to progression and overall survival in patients with metastatic breast cancer that is resistant to both anthracyclines and taxanes [Lee et al. 2004, Wist et al. 2004, Bajetta et al. 2005].

Capecitabine is generally well tolerated, with low rate of grade 3 or 4 events. The most adverse effects with this drug include fatigue, hand-foot syndrome, and gastrointestinal upsets [Morris et al. 2009].

2. 2. 3. 3. 4 Gemcitabine

Gemcitabine (2’, 2'- difluor-2'-deoxycytidine; dfdC) has shown activity in various solid tumors, including breast cancer [Spielmann et al. 2001

Gemcitabine is used as monotherapy and in combination with anthracyclines and taxanes in patients with advanced breast cancer [Benekli et al. 2007, Albain et al. 2008]. As a single agent, gemcitabine produces overall response rate ranging from 14 to 37% in chemotherapy-native patients and from 12 to 30% in patients previously treated with anthracyclines and/or taxanes [Morris et al. 2009, Benekli et al. 2007].

The most common adverse effects associated with gemcitabine are hematologic, particularly neutropenia and thrombocytopenia [Morris et al. 2009].

2. 2. 3. 3. 5 Vinorelbine

Vinorelbine (5'-noranhydrovinblastin) is a semisynthetic vinca alkaloid. Vinorelbine induces cytotoxic effects by inhibiting polymerization of tubulin dimers into microtubules, disrupting mitotic spindle formation and preventing mitosis, ending in apoptosis [Morris et al. 2009].

Vinorelbine represent both an active and a well tolerated treatment for metastatic breast cancer in patients who display for refractory disease for anthracycline and/or taxane [Zelek et al. 2001, Valero and Hortobagyi 2003, Gil-Delgado et al. 2008, Morris et al. 2009]. Vinorelbine is one of options for treatment of anthracyclin- and taxane-pretreated advanced breast cancer, with availability as oral formulation. Equivalence in bioavailability has been demonstrated between the oral and intravenous formulations [Marty et al. 2001].

2. 2. 4 Hormonal treatment

Hormonal treatment slows or ceases the growth of hormone-dependent tumors by inhibiting ability of the body to yield hormones or by interfering with hormone action. Breast tumors that are hormone-independent do not respond to hormonal treatment. Hormonal treatment includes ovarian ablation, blocking the effects of estrogen, and blocking of estrogen production.

Ovarian ablation

This is to reduce the estrogen levels in premenopausal women by eliminating or suppressing ovarian function. Irreversible ovarian ablation includes surgical oophorectomy and ovarian irradiation. Alternatively, temporal castration can be induced by using luteinizing hormone releasing hormone (LHRH).

Ovarian ablation or suppression reduced the absolute risk of recurrence at 15 years by 4.3% and the risk of mortality from breast cancer by 3.2% [Early Breat Cancer Trialists Collaborative Group 2005].

Blocking the effects of estrogens

Many types of drugs interfere with the ability of estrogens to stimulate the growth of breast cancer cells:

- **Selective estrogen receptor modulators (SERMs)**

Examples of SERMs approved by the FDA are tamoxifen, raloxifene, and toremifene for treatment of breast cancer patients. Tamoxifen has been used for more than 3 decades to treat hormone-dependent breast cancer patients.

Tamoxifen is metabolized by the cytochrome P450 (CYP2D6) into primary active metabolite (endoxifen) [Desta et al. 2004].

SERMs play an important role in the treatment of both premenopausal and post menopausal patients with early and advanced breast cancer as well as for primary prevention in women with high risk of breast cancer.

Tamoxifen has been shown in multiple studies to decrease breast cancer-associated mortality and relapse. A recent study included information on 80,373 women with stage I or stage II breast cancer in 71 trials of adjuvant tamoxifen [Early Breat Cancer Trialists Collaborative Group 2005]. In this analysis, the 15-year and 5-year absolute reduction in recurrence and mortality associated with tamoxifen were 12% and 9%, respectively. The EBCTCG demonstrated that the risk reduction from adjuvant tamoxifen is the same in older and younger women. In addition, the proportional reductions in both recurrence and mortality associated with tamoxifen use were similar in women with either node-negative or node-positive breast cancer, but the absolute improvement in survival at 10 years was higher in the latter group. Tamoxifen is also effective in the treatment of patients with advanced breast cancer. In premenopausal women with advanced breast cancer, tamoxifen in combination with LHRH analogues/ovarian ablation remain a first-line option [Beslija et al. 2009, National Comprehensive Cancer Network 2011].

Tamoxifen has been associated with certain adverse effects. The most important event is the development of endometrial cancer; it is reported to occur at a rate from 2 to 7 times more often than in untreated women [Fisher et al. 2005]. Tamoxifen use is also associated with an increased incidence of deep venous thrombosis and pulmonary emboli, stroke, cataract, and depression [Fisher et al. 2005, Vogel et al. 2006].

- **Selective estrogen-receptor downregulators (SERDs)**

The newest agent for use in hormone-dependent advanced breast cancer is fulvestrant. Fulvestrant is a steroid-based pure ER-antagonist without agonistic activity.

Efficacy of fulvestrant in hormone-dependent advanced breast cancer has been investigated in several studies [Howell 2006, Valachis et al. 2010, Robertson et al. 2009, Di Leo et al. 2010]. High dose fulvestrant significantly improves progression-free survival in patients with advanced breast cancer compared with anstrozole, and the combination fulvestrant-anstrozole gives better result than either agent alone [Robertson et al. 2009, Di Leo et al. 2010].

Fulvestrant is well tolerated, but associated with bone and joint pain, nausea and vomiting, hot flashes, sweating, mild increases liver enzymes, and pain at injection site [Howell 2006, Robertson et al. 2009].

Blocking of estrogen production

Aromatase inhibitors (AIs) can be used to block the activity of an enzyme called aromatase. It is responsible for the conversion of the adrenal androgen substrate and rostenedione to estrogen in peripheral tissue, which is the main source of estrogen in postmenopausal women [Aguas et al. 2005, Burstein et al. 2010, Janni and Hepp 2010]. The third generations of AIs (i.e. anastrozole, letrozole, and exemestane) are the most potent, most selective, least toxic, and their use is approved by the U.S. FAD.

AIs represent both efficacy and low toxicity in the treatment of breast cancer patients in adjuvant, neoadjuvant and metastatic setting, as well as in primary prevention in women with high risk of breast cancer [Thürlimann et al. 2005, Forbes et al. 2008, Gibson et al. 2009, National Comrehensive Cancer Network 2011].

AIs associated with common adverse effects: hot flashes, arthralgia/arthritis, headache, vaginal dryness, and mood changes [Coombes et al. 2007, ATAC Trialists' Group 2008].

2. 2. 5 Targeted therapy

Targeted cancer therapies are drugs that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. These targeted drugs work differently from standard chemotherapy and often have different or less severe side effects.

Targeted cancer therapies that have been approved for use in treatment of patients with breast cancer include the following.

Anti-HER2

Trastuzumab

Trastuzumab (trade name Herceptin) is a monoclonal antibody that interferes with HER2/neu receptor, and was first approved for treatment of metastatic breast cancer in 1998. In 2006 it was included in adjuvant treatment for breast cancer [de Azambuja et al. 2009].

Trastuzumab is effective only in patients with HER2-positive breast cancer, and the American Society of Clinical Oncology/College of American Pathologists guidelines define the positivity of HER2 as 3+ for IHC testing and more than six HER2 gene copies per nucleus for FISH testing, and also recommended the use of a combination of IHC and FISH testing [Wolff et al. 2007]. This can predict the efficacy of treatment with trastuzumab. A patient with erbB oncogene over-expression with no significant pre-existing heart disease is eligible for anti-HER2 therapy [Yu and Hung 2000].

The efficacy of trastuzumab as a single agent or in combination with chemotherapy has been investigated in several breast cancer studies.

In metastatic setting, trastuzumab as a single agent produced an objective response rate up to 26%, and in combination with chemotherapy significantly improved overall response rate, median overall survival, and time to disease progression [O'Shaughnessy et al. 2004, Marty et al. 2005, Kaufman et al. 2009]. Neoadjuvant combination of trastuzumab and different chemotherapy agents induced a high pathological complete response (pCR) rate with minimal toxicity [Untch et al. 2010].

Pertuzumab

Pertuzumab (as trade name Perjeta), an anti-HER2 humanized monoclonal antibody that binds the extracellular domain of HER2 at a different epitope than at which trastuzumab binds, and prevents dimerization of HER2 with other ligand-activated HER receptors, results in slowed tumor growth [Franklin et al. 2004].

Pertuzumab in combination with trastuzumab show activity in patients with HER2-positive metastatic breast cancer and in patients with early breast cancer [Portera et al. 2008, Gianni et al. 2010, Baselga et al. 2012]. Pertuzumab received US FDA approval for the treatment of HER2-positive metastatic breast cancer on June, 2012 [Food and Drug Administration 2012].

Trastuzumab emtansine

Trastuzumab emtansine (T-DM1) is an antibody-drug conjugate consisting of the monoclonal antibody trastuzumab linked to the cytotoxic agent mertansine (DMI) [LorRusso et al. 2011].

T-DM1 significantly prolonged progression-free survival and over all survival with less toxicity than lapatinib plus capecitabine in patients with HER2-positive advanced breast cancer previously treated with trastuzumab and a taxane (paclitaxel or docetaxel) [Verma et al. 2012]. In 2013, the FDA approved T-DM1 for treatment of patients with HER2-postive metastatic breast cancer who have been treated with trastuzumab and a taxane [Pollack 2013].

Lapatinib

Lapatinib is an orally active small molecule with a dual kinase inhibitor which interrupts the HER2/neu and epidermal growth factor receptor (EGFR) pathways [Higa and Abraham 2007].

The clinical activity of lapatinib in combination with other cytotoxic agents has been shown. Lapatinib in combination with capecitabine reduced the risk of disease progression by 51% without increases in toxic side effects, in patients with breast cancer whose cancer have progressed following previous treatments with anthracycline, taxanes and trastuzumab compared to capecitabine alone [Geyer et al. 2006].

On 2007, the FDA approved lapatinib in combination therapy for breast cancer patients already using capecitabine, and in 2010 also for the treatment of postmenopausal women with hormone receptor positive metastatic breast cancer [Higa and Abraham 2007, Pazdur and Richard 2011].

The most common side effects of lapatinib include diarrhea, nausea, vomiting, and hand-foot syndrome [Higa and Abraham 2007]. Diarrhea is a common side effect and can be severe, so it is very important to follow any changes in bowel habits during treatment.

mTOR inhibitor

Everolimus

Everolimus (Afinitor as trade name), an oral medication of targeted therapy, is a sirolimus derivative that inhibits mTOR (mammalian target of rapamycin) through allosteric binding to mTORC1 [Efeyan and Sabatini 2010].

The efficacy and safety of everolimus has been investigates in many studies. In phase II study comparing neoadjuvant everolimus plus letrozole with letrozole alone in newly diagnosed ER-positive breast cancer patients, the response rate was higher for the combination than that for letrozole alone [Baselga et al. 2009].The FDA has approved everolimus in combination with exemestane for treatment of postmenopausal women with advanced, hormone receptor positive and HER2-negative breast cancer [US Food and Drug Administration 2012].

2. 3 Biological prognostic markers

2. 3. 1 Tumor cell DNA content (DNA ploidy and S-phase fraction)

DNA Image Cytometry (DNA-ICM) analysis displays the distribution of DNA content within a sample of cell nuclei and provides useful data on the DNA of single cells, and the fraction of cells in active DNA synthesis (S-phase fraction, SPF). As such, it is a simple objective measure that is likely to play important role in evidence-based cancer pathology. Human neoplasms actively synthesizing DNA replicate through a process similar to that of normal cells in the cell cycle [Ross 1996, Gillett and Barrnes 1998]. Diploid cells are in the resting phase (G0) or in the first gap phase of the cell cycle (G1). During the synthesis phase (S phase), cells increase their DNA content until they reach the tetraploid state with twice the diploid DNA content. The second gap (G2 phase) refer to this tetraploid or premitotic, fraction of cells that undergo mitosis in the M phase to yield 2 diploid G0 cells. A DNA index of 1.0 corresponds to a 2N or 46 chromosome number characteristic of G0 and G1 cells. The G2 and M cells feature a 2.0 DNA index that corresponds to a 4N (or 4c) amount of DNA and chromosome number of 92. The distribution of DNA of a population of cells within the cell cycle generates a pattern known as DNA histogram and represents DNA ploidy [Ross 1996].

The measurements estimate the fraction of cells in the S phase (S-phase fraction, SPF), which reflects also proliferative activity. Cells with disrupted or defective cell cycle will typically show errors in chromosome replication. These cells show abnormal numbers and appearances of chromosomes and are referred to as aneuploid.

Aneuploidy is recognized as a cardinal feature of many human cancers and it seems to have an important role in tumorigenesis and aggressiveness of the tumor [Salisbury et al. 2004, Kronenwett et al. 2005].

DNA content, studied easily by the image analysis system using Feulgen-stained preparations, and DNA ploidy can be measured in both cytological smears and tissue sections [Buhmeida 2006]. In addition, this technique may be more sensitive for detecting aneuploid tumor than flow cytometry when small numbers of tumor cells are present. However, the majority of comparative studies have shown excellent correlation between analysis of DNA content by flow cytometry and static image technique [Linder 1990].

A large number of prognosis-related studies using flow cytometry and static cell image analysis have been performed on breast cancer specimens to measure DNA content and determining the SPF. The first major retrospective flow cytometry study of DNA content as predictor of prognosis in breast cancer by Auer and co-workers [1980] showed that patients with diploid tumor had an excellent prognosis. Results of a variety of studies on the prognostic significance of DNA ploidy and S-phase status have been notably variable, with some studied indicating that DNA ploidy status is an independent and powerful prognosticator in breast cancer [Tsutsui et al. 2001, Tsutsui et al. 2003, Moureau-Zabotto et al. 2005, Yildirim-Assaf et al. 2007]. Others have reported that the association of both ploidy and S-phase fraction status with prognosis was variable and not conclusive [Mandard et al. 2000, Chassevent et al. 2001]. Separate determination of DNA analysis for prognosis prediction for node-negative and node-positive status in patients with breast cancer has been performed. Some studies suggest that the low SPF is associated with an excellent prognosis particularly in LN-negative patients [Jones et al. 2001]. Moureau-Zabotto et al [2005] reported that combination of DNA ploidy and SPF improves the prediction of patient's outcome, especially among LN-negative breast cancer patients. However, DNA aneuploidy and high SPF were associated with high tumor grade, negative hormone receptor status, and amplification of certain molecular markers indicating unfavorable prognosis, such as Her-2/neu and c-myc genes [Bagwell et al. 2001].

2. 3. 2 Cell proliferation markers

Ki-67 (MIB-1)

Ki-67 is a cellular marker for proliferation identified by Gerdes and his group in 1991 as a nuclear non-histone protein that can be detected by IHC [Gerdes et al. 1991].

Ki-67 protein was originally defined by the phenotype monoclonal antibody Ki-67, which was generated by immunizing mice with nuclei of the Hodgkin lymphoma cell line L428 [Gerdes et al. 1983]. The name is derived from the city of origin (Kiel, Germany) and the number of the original clone in the 96-well plate. The half-life of Ki-67 protein has been estimated to be around 60 to 90 minutes, and its expression varies throughout the cell cycle phases [Heidebrecht et al.1996]. Cells express the antigen during the G1, S, G2, and M phases but not during the resting phase. Levels of Ki-67 are low during G1 and early S-phase and progressively increase to reach a maximum during mitosis [Urruticoechea et al. 2005].

Ki-67 and MIB-1 monoclonal antibodies are directed against different epitopes of the same proliferation-related antigen. Whereas the original Ki-67 stained only proliferating cells in unfixed tissues, MIB1 may be used also on formalin-fixed paraffin-embedded samples [Key et al. 1993]. Several antibodies are now commercially available to stain Ki-67 in fresh and paraffin-embedded tissues, and MIB-1 is most often used to assess the Ki-67 labelling index. Ki-67 labelling index is an excellent marker for determining growth fraction of tumor cell populations (growth fraction) and the biological aggressiveness [Urruticoechea et al. 2005]. An index based on the Ki-67/apoptosis ratio has been considered to approximate the contribution that these two factors may make to tumor growth [Urruticoechea et al. 2005].

Ki-67 is expressed in normal breast tissues as well as in breast cancers but at a very low level (< 3% of cells) [Harper-Wynne et al. 2002, de Lima et al. 2003]. Ki-67 shows a strong association with other well-known markers of proliferation such as S-phase fraction, mitotic index, tyrosine kinase, and proliferating cell nuclear antigen [Spyratos et al. 2002, Trihia et al. 2003]. In addition, Ki-67 is positively correlated with apoptosis and epidermal growth factor, p53 and Her-2 expression [Trihia et al. 2003, Urruticoechea et al. 2005]. On the other hand, breast tumor with high rates of ER positivity and bcl-2 expression shows the smallest proliferating status [Spyratos et al. 2002].

The clinical value of Ki-67 in breast cancer has been widely investigated and the results suggested it had some prognostic or predictive role in clinical practice [Viale et al. 2008, Yerushalmi et al. 2010, Goldhirsch et al. 2009, Karanikas et al. 2011].

The clinical utility of Ki-67 as a prognostic marker might be more apparent, high expression of Ki-67 is associated with higher grade of malignancy and defines a high-risk group in terms of prognosis, particularly among patients with ER positive, HER-2 negative, and LN-negative breast cancer [Goldhirsch et al. 2009]. However, the independent prognostic value of Ki-67 is much less than that of mitotic count [Collan et al. 1996, Jalava et al. 2006].

In addition, Ki-67 is an important marker in determining and distinguishing biological subtypes of breast cancer such as luminal A and B tumors. By the IHC, there have been attempt to differentiate between luminal A and B using the protein expression of Ki-67 as possible marker [Cheang et al. 2009]. The luminal A subtype has been associated with low expression of Ki-67, while the luminal B has tumors with high expression of Ki-67.

Ki-67 is a reliable measure of tumor proliferation, and has independent prognostic significance in several large clinical trials of adjuvant endocrine therapy. Because the standardization and detailed guidelines for this marker is very important, efforts are being taken to reach a consensus on how to evaluate. Recently, a set of recommendation was published for assessment of Ki-67 in breast cancer [Dowsett et al. 2011]. In European countries like Finland, Ki-67 is widly used in breast tumors.

AgNOR

Special silver stain has been applied to study another marker of cell proliferation, the argyrophilic nucleolar organizer region (AgNOR). Nucleolar organiser regions (NORs) are loops of deoxyribonucleic acid (DNA) that encode ribosomal ribonucleic acid (rRNA) and are located in the nucleoli of cells and in the chomosomes 13-15, 21, and 22 in associaton with proteins [Sivridis and Sims. 1990]. NORs proteins are argyrophilic and can be detected by using modified silver staining method (AgNOR technique) [Beresford et al. 2006]. The number of silver binding dots is a valuable marker of cellular activity as it reflects the extent of ribosomal biogenesis [Vijaya et al. 2008]. AgNOR is useful for estimating the proliferative activity of neoplasms, and increased AgNOR counts may reflects increased growth activity of the cells.

Prognostic value of AgNOR counts in breast carcinoma is controversial. Some studies have observed that quantitative analysis of AgNORs has a prognostic value especially among the LN-positive groups [Derenzini et al. 2004]. Others did not find any prognostic significance in AgNOR counts for breast cancer [Simha et al. 1996].

PCNA

A non-histone nuclear protein, proliferating cell nuclear antigen (PCNA) is forming a trimeric ring structure around DNA to facilate and control DNA replication and repair [Stoimenov and Helleday 2009, Strzalka and Ziemienowicz 2011].
PCNA is a cofactor for DNA polymerase delta that has ability to promote the activity of DNA polymerase and to interact with p21 in regulation of the cell cycle. PCNA can be detected on formalin-fixed and paraffin-embeded section, and its expression is increased during G1 phase, maximal in S-phase and decreases during G2/M [Linden et al. 1992].

Several studies suggest that PCNA is important for cell replication, and DNA damage will signal for different PCNA modification which may be of importance for the outcome of treatment [Stoimenov and Helleday 2009, Strzalka and Ziemienowicz 2011]. Some studies reported that high PCNA is associated with high histological grade and aneuploidy of breast cancer [Aaltomaa et al. 1993]. Others showed that PCNA has significant prognostic value in breast cancer [Jeziorski et al. 2000], but Aaltomaa and co-workers [1993] showed this only among LN-negative patients.

Cathepsin D (CD)

Cathepsin D is a lysosomal acidic protease that can be found in various forms within the epithelial cells of breast cancer with important function in protein catabolism. The enzyme also has a mitogenic activity and plays an important role in metastatic spread by promoting the destruction of normal tissue arcitecture and in tumor growth by influencing growth factors [Westley and May 1996, Greco et al. 2000].

Prognostic value of Cathepsin D in breast cancer has been observed in some studies [Greco et al. 2000]. Patients with positive Cathepsin D IHC stain often show high recurrence rate, nodal and systemic involvements and poor prognosis [Greco et al. 2000]. However, there were studies that did not observe any prognostic association with Cathepsin Din breast cancer [Ramírez-Ortega et al. 1997].

2. 3. 3 Onco-supressor genes and products

Bcl-2

The disturbance in the homeostatic balance between apoptosis and growth of cells usually results in numerous disease states, including autoimmunity, degenerative disorders, and cancer [Thompson 1995]. Importantly, alterations within the apoptotic pathway contribute to tumorigenesis and confer resistance to not only physiological apoptotic stimuli but therapeutic regimens as well. Tumorigenesis is promoted by both the loss of pro-apoptotic signals and the gain of anti-apoptotic mechanisms [Hanahan et al. 2011]. Bcl-2 protein along with its other family members plays a fundamental role in these processes by integrating the complex pathways incorporating pro-and anti-apoptotic signals at the mitochondrial membrane [Tsujmoto et al. 2002].

The Bcl-2 family comprises a network of related proteins that are defined by their α-helical composition of up to 4 Bcl-2 homology domains (BH). Based on the number of BH, Bcl-2 proteins are grouped into three classes: those that inhibit apoptosis (e.g., Bcl-2 and Bcl-xL); those that promote apoptosis a multi-BH domain group (e.g., Bax and Bak); and pro-apoptotic BH3-only proteins (e.g. Bad and Bik) [Petros et al. 2004].

Bcl-2 (B-cell lymphoma) is the founding member of the Bcl-2 family of apoptosis regulator proteins encoded by the BCL2 gene [Cleary et al. 1986]. Bcl-2 derives its name from B-cell lymphoma 2, as it is the second member of a range of proteins initially described in chromosomal translocation involving chromosomes 14 and 18 in follicular lymphomas.

Bcl-2 is an intracellular membrane associated protein of 24-kilodalton (KDa) [Bozzetti et al.1999, Malamou-Mitsi et al. 2006]. It has been localized in the nuclear envelope, endoplasmic reticulum, outer mitochondrial membranes of hematopoietic and lymphoid cells, neurons, and hormone-influenced glandular epithelium such as the thyroid, prostate, and breast [Bozzetti et al. 1999].

Bcl-2 inhibits apoptosis through the mitochondrial pathway by blocking the release of cytochrome c from the mitochondria, thus preventing the sequence of events that results in compromise of the mitochondrial outer membrane potential, which in turn leads to caspase-9 activation and subsequent apoptosis [Adams et al. 1998]. In addition to anti-apoptotic action of the bcl-2, paradoxically, it has also anti-proliferative effect [Zinkel et al. 2006]. Cells overexpressing the bcl-2 gene product, not only show delayed of apoptosis but also a rapid arrest in G1 phase in cell cycle [Tsutui et al. 2006].
Furthermore, over-expression of Bcl-2 inhibits apoptosis induced by anticancer drugs, radiation, and other DNA-damaging agents [Emi et al. 2005]. Chemotherapy resistance has been reversed in tumor cells treated with Bcl-2 targeting therapy [Emi et al. 2005].
Despite the anti-apoptotic and chemoresistant effects favoring tumor survival, bcl-2 seems to act as both an oncogene and a tumor suppressor gene in different tumor types. For example, high bcl-2 expression was found to be associated with a more aggressive phenotype and with a worse clinical outcome for some tumor types, including acute myeloid leukemia, aggressive non-Hodgkin's lymphoma, prostatic carcinomas, and testicular carcinomas [Hermine et al. 1996, Quinn et al. 2005]. However, in other human tumors including non-small-cell lung carcinoma, melanoma, and breast carcinoma, Bcl-2 expression was associated with a lower grade of malignancy and better survival [Divito et al. 2004, Treré et al. 2007, Nadler et al. 2008]. The association between the bcl-2 expression and favorable prognosis may be explained by the fact that bcl-2 expression creates a restrictive environment for the expansion of genetically unstable and potentially malignant p53-deficient cells, causing a delay in tumor progression and explaining the different prognostic value of bcl-2 and p-53 [Gurova et al. 2002]. Anyhow, bcl-2 is known to be up-regulated by estrogen and down-regulated by p-53 [Teixeira et al. 1995].

In breast cancer, bcl-2 is expressed in about 40-80% of cancers in women with primary tumors, and is strongly correlated with the expression of ER and PR [Natha et al. 2006]. Zhang et al [1997] observed that bcl-2 gradually decreases during the development of breast cancer, i.e. from a normal epithelium (96%) to intraductal carcinoma (79%), and from intraductal to invasive carcinoma (45%). Decreased bcl-2 expression due to abnormal regulation of bcl-2 gene can be associated with increased tumor aggressiveness and presence of chemotherapy resistance [Dawson et al. 2010].

Overexpression of bcl-2 protein detected by IHC stain in patients with ER-positive/PR-positive tumors, small tumors and well differentiated of tumor [Tang et al. 2004, Treré et al. 2007, Nadler et al. 2008]. Many studies concluded that increased Bcl-2 expression is associated with good patient outcome [Callagy et al. 2008, Dawson et al. 2010, Ali et al. 2012]. A multivariate analysis incorporating large published data from 11,212 breast cancer patients strongly supported the independent prognostic value of bcl-2 positivity with improved survival [Dawson et al. 2010]. Expression of bcl-2 has been proven to be an independent indicator of favorable prognosis for all types of early-stage breast cancer [Callagy et al. 2008, Dawson et al. 2010].

P53

P53 was first described in 1979 as a protein that binds to the simian virus (SV40) large T antigen [Lane and Crawford 1979]. In humans,TP53 gene is located on the short arm of chromosome 17p13.1, and encodes for p53 which is a protein that normally regulates cell cycle [Kern et al. 1991 Because of its role in conserving stability by preventing genome mutation p53 is also called the guardian of the genome [Lane 1992, Read et al. 1999].
p53 is activated in response to many of stress types including DNA damage induced by irradiation, chemical agents, and oxidative stress [Han et al. 2008]. When p53 is activated different responses follow, including senescence, cell-cycle arrest and apoptosis [Vousden et al. 2009]. When DNA is damaged, p53 induce growth arrest by interrupting the cell cycle at G1 to S phase and activates DNA repair proteins. Cell with irreparable DNA are directed to apoptosis through activation of the apoptotic genes [Levine 1997].

Mutation of the gene, which can occur though chemicals, radiation or viruses, causes cells to undergo uncontrolled cell division. P53 mutation is the most common genetic change identified in human neoplasia. Mutant p53 proteins lose their ability to bind wild-type responsive elements and to regulate the expression of p53 transcriptional targets, thus losing tumor suppressor activity in continuous cellular growth, which can induce carcinogenesis in many organs including lung, colon, and breast [Levine 1997]. However, cellular preservation of mutated p53 may confer malignant potential such as the capacity to metastasize, through gains of function activities [Oren and Rotter 2010].The substitution of an arginine for a proline at codon position 72 is a common polymorphism involved. A genetic link between this variation and cancer susceptibility has been studied. However, the results were controversial. For example, a meta-analysis from 2009 failed to show a link for cervical cancer [Klug et al. 2009]. Piao and his group [2011] found that TP53 codon 72 polymorphism was associated with an increased risk of lung cancer. A study of Arab women found that proline homozygosity at TP53 codon 72 is associated with a decreased risk for breast cancer [Alawadi et al. 2011].

Mutation of p53 leads to an increased half-life of non-functional p53 proteins, accumulating in tumor cell nuclei. The accumulated p53 protein can be evaluated by IHC [Kim et al. 2010]. Overexpression of p53 protein has been detected in many human cancers including breast cancer [Levine 1997, Kim et al. 2010]. Breast tumors expressing a high amount of p53 were associated with higher grade of malignancy and poor survival [Rolland et al. 2007, Kim et al. 2010]. TP53 mutation, detectable by sequencing is associated with a poor prognosis, predicting poor disease-free survival and overall survival [Olvier et al. 2006].

P21 (WAF1)

P21 also known as cyclin-dependent kinase inhibitor (CDKI) is a protein that in humans is encoded by the CDKNIA gene located on chromosome 6 [Harper et al. 1993].

The p21 protein binds to and inhibits the activity of cyclin-dependent kinases and leads to cell cycle arrest at the G1 phase as a result of inhibiting DNA replication [Abukhdeir et al. 2008]. Despite p21 mediates p53-dependent G1 growth arrest [Skildum et al. 2002], it also acts as an effecter of other tumor suppressor pathways for inducing anti-proliferative activities that are independent of the classical p53 tumor suppressor pathways.
P21 has appeared as a major down-stream target of tumor suppressor genes, including p53, BRCA1, CHK2 FOXP3, Sp1/Sp3, and importantly repressed by oncogenes, such as c-myc and Gfi1[Skildum et al. 2002, Liu et al. 2009]. P21 is down-regulator in many human cancers including breast cancer [Pinto et al. 2005, Tiezzi et al. 2007].

The prognostic value of p21 in breast caners is still unclear [Liu et al. 2009]. Pellikainen et al [2003] could not find a significant prognostic value while Thor et al [2000] show that expression of p21 in the LN-positive breast cancer patients show a weak association with the survival.

P27

P27 is also a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, which arrest progression of the cell cycle with potential tumor suppression function [Chiarle et al. 2001].
Loss or mutated p27 may lead to loss of control over the cell cycle leading to uncontrolled cellular proliferation and neoplasia formation [Chiarle et al. 2001]. Low expression of p27, as determined by IHC, is associated with poor survival in many human cancers, including breast cancer [Colozza et al. 2005] and have potential therapeutic implication [Wander et al. 2011].

The prognostic effect of this protein could depend on the treatment of cancer and response to various anticancer drugs [Nahta et al. 2004]. Furthermore, there is evidence from some studies that the p27 is essential for cell cycle arrest by tamoxifen and other antiestrogenic agents [Donovan et al. 2001]. Filipits et al [2009] concluded that low p27 expression independently predicts early relapse and death in postmenopausal women with early-stage and hormone receptor-positive breast cancer who received tamoxifen for 5 years.

Myc

Myc gene, located on chromosome 8q24, is a proto-oncogene with a central role in proliferation and malignant transformation in human cell [Liao and Dickson 2000]. Myc protein is a transcription factor that critically participates in most aspects of normal cellular function, including replication, proliferation, metabolism, differentiation, and apoptosis [Chen and Olopade 2008]. When Myc gene is mutated, or overexpressed, the Myc protein doesn't bind correctly, and often results in progression of malignant transformation and angiogensis [Chen and Olopade 2008].

In breast cancer, Myc is mutated in 30% of primary cancers and is typically associated with poor prognosis [Naidu et al. 2002].

Some studies observed that Myc amplification may be correlated to breast cancers that have worse prognosis particularly in basal tumor types [Naidu et al. 2002, Xu et al. 2010]. In addition, Myc amplification has been associated with HER2 amplification and is an important predictor of response to HER2-targeted therapies [Park et al. 2005, Chen and Olopade 2008, Perez et al. 2009]. In BRCA1-associated breast cancer Myc is an important in targeting therapy particular in triple-negative breast cancer [Chen and Olopade 2008, Xu et al. 2010].

2. 3. 4 Growth factors

Epidermal growth factor receptor (EGFR)

EGFR is a trans-membrane glycoprotein receptor, member of tyrosine kinase growth factor receptor family that have a well-defined function in cell signaling, controlling cell proliferation and differentiation. EGFR also plays an important role in carcinogenesis of several human tumors including breast cancer [Abd El-Rehim et al. 2004, Sergina and Moasser 2007].

EGFR amplification and/or over-expression have been recognized in breast cancer patients particularly among triple negative breast cancer, and is associated with advanced disease, development of metastasis and poor prognosis [Abd El-Rehim et al. 2004, Pakha et al. 2007, Viale et al. 2009]. EGFR is also an indicator of hormone independence of breast cancer. Estrogen depleted breast cancer cells are more sensitive to the mitogenic effects of epidermal growth factor, and patients with positive EGFR and negative ER had poor clinical outcome [Tsutsui et al. 2002].

EGFR inhibitors are now in use clinically. They recognized as important treatment for breast cancer. A variety of modalities for inhibiting EGFR expression in cancer cells including anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors have been proven effective, particularly when used in combination with other cytotoxic agents [Lu et al. 2009, DiLeo et al. 2008, Johnston et al. 2009, Pal et al. 2011].

Transforming growth factor receptor (TGFR)

Transforming growth factors (TGF) are multifunctional growth factors that show different expression and have produced variable roles during cell proliferation with pleiotrophic effects during carcinogenesis [Ghellal et al. 2001, Baumeister et al. 2009]. TGFs include TGF alpha and beta isoforms (TGFα, TGFβs). Co-expression of EGFR and TGF-α (detected by IHC) is an independent prognostic indicator in many human solid tumors, and also may be helpful for determining the group of breast cancer patients with aggressive phenotype [Ghellal et al. 2001, Baumeister et al. 2009]. TGFβ isoforms play dual role in tumorigenesis, reflected by the two opposing properties of growth inhibition and tumor promotion [Roberts and Wakefield 2003]. TGFβ is very potent inhibitor of primary human epithelial cells, and most human breast cancer cell lines are growth inhibited by TGFβ as well [Hsu et al. 2009].

In later stages of breast cancer, TGFβ may lose this potential and shift to a tumorigenic phenotype [Weserhausen et al. 1991]. TGFβ2 and TGFβ3 may have good prognostic value in patients with breast cancer [Ghellal et al. 2001].

Vascular endothelial growth factor and receptor (VEGF-R)

Neo-vascularisation is the formation of new vascular networks from pre-existing blood vessels in the stroma. There is general agreement that angiogenesis is one of the crucial factors that contributes to breast cancer growth and metastasis [Bergers and Benjamin 2003]. Several growth factors including fibroblast growth factor, platelet-derived growth factor, transforming growth factor- β participate in regulation of this complex process and the most important of these is thought to be VEGF.

Several studies have implicated the important of VEGF in breast cancer and over-expression of endothelial surface receptor for VEGF is associated with a high grade of malignancy and poor survival [Ghosh et al. 2008, Hilmi et al. 2012].

VEGF targeting therapies have shown significant benefits and have been integrated in clinical practice for other types of human cancer. In breast cancer the value of anti-VEGF is highly variable and controversial. Phase III studies were unable to show the benefits of these agents, but increased toxicity [Miles et al. 2009, Robert et al. 2009].

Insulin-like growth factor (IGF)

Insulin-like growth factors (IGFs) are proteins with high sequence similarity to insulin. IGFs are part of a complex system that consists of two cell surface receptors (IGFR1 and IGFR2), two ligands (IGF-1 and IGF-2) and six binding proteins (IGFBP1 to 6). This complex system (IGF axis) plays an important role in the biological activity of the cell. The IGF-1 and IGF-2 are circulating peptide hormones and have mitogenic and apoptotic effect on breast cancer cell lines [Ellis et al. 1998].

The role of IGF system in the progression of breast cancer is still a controversial issue. Some authors suggested that breast cancer with positive IHC stain for IGF has lower grade than breast cancer with negative staining [Eppler et al. 2002]. Others reported that IGFs have important role in the progression of breast cancer [Maor et al. 2007]. The IGF-1R as a target for cancer treatment has been investigated and the results suggest that this type of therapy may have an effect on treatment of patients with breast cancer and other solid tumors [Aleksic et al. 2010].

2. 3. 5 Adhesion molecules

E-cadherin

E-cadherin is a member of the cadherin family of transmembrane proteins that play important roles in the maintenance of tissue architecture [Gonzalez et al. 1999].

E-cadherin is a transmembrane glycoprotein that mediates calcium dependent cell to cell adhesion and recognition in epithelial tissue [Harris et al. 2010]. The E-cadherin gene, located on chromosome 16q22.1, is an important regulator of morphogenesis [Harris et al. 2010]. E-cadherin (epithelia cadherin) is usually strongly expressed in normal epithelial tissue but variably in cancer cells. Reduction or loss of E-cadherin in cancers has been suggested to play a role in alterations that characterize the invasive phenotype, and the data support its role as a tumor suppressor gene [Pierceall et al. 1995].

Studies have shown that aberrant E-cadherin expression is associated with increase in breast cancer infiltration and metastasis [Bukholm et al. 2000, Karray-Chouayekh et al. 2012]. The expression of E-cadherin correlated to histological type and grade. Infiltrating lobular carcinoma (ILC) and lobular carcinoma in situ (LCIS) is usually E-cadherin negative or mostly weak [Bratthauer et al. 2008]. Thus E-cadherin may have a role in histological diagnosis of ambiguous LCIS and ILC [Berx et al. 2001]. E-cadherin expression was associated good prognosis and its prognostic value is particularly prominent among patients with LN+ breast cancer [Abd Elmoneim and Zaghloul 2011, Karray-Chouayekh et al. 2012].

Catenins

Catenins are a family of interacellular proteins (alpha, beta and gamma) found in complexes with cadherin cell adhesion molecules of cells. This interaction is important to stabilization of adhesion effects of E-cadherin [Tripathi et al. 2012]. Any alterations in cytoskeletal organization and adhesion can lead to altered signaling, migration and loss of a contact inhibition that induce cancer development and progression [Tripathi et al. 2012]. Mutation in genes can lead to loss of intracellular catenins to inactivation of cadherin cell adhesion and elimination of contact inhibition allowing cells to proliferate and migrate [Yoshida et al. 2001, Hirohashi and Kanai 2003].

Many studies have suggested that lack of catenins expression may be associated with poor survival in breast cancer [Yoshida et al. 2001, Uchino et al. 2010].

CD 44

CD44 is a family of cell surface glycoproteins with various isoforms and is involved in cell-cell interactions, cell adhesion and migration. CD44 isoforms are expressed in a large number of mammalian cell types and linked to metastatic spread in a variety of tumors [Morris et al. 2001]. In addition, variations in CD44 are reported as cell surface markers for breast cancer stem cells (BCSCs) [Li et al. 2007].

CD44 was observed in 40% of IDC and associated with unfavorable prognostic features such as advanced stage, poor differentiation of the tumor, large tumor size, and lymph node involvement [Bhatavdekar et al. 2000, Uchino et al. 2010]. Some studies suggested that CD44 expression had an independent prognostic value in breast cancer [Horiguchi et al. 2010].

Tempfer et al [1996] suggested that CD44 is not an independent prognosticator in breast cancer. Other studies reported that the expression of CD44 may be a marker for identifying patients with relatively good prognosis only among LN-negative patients [Foekens et al. 1999]. This discrepancy may be explained by the fact that other prognostic variables carry the same idea in respect to prognosis

2. 4 Molecular classification of breast cancer

The conventional clinicopathological parameters such as histological grade, nuclear grade, lymph node involvement, tumor size, hormonal status (ER and PR), and HER2 status all have been successful in terms of predicting prognosis and guiding the treatment of patients with breast carcinoma [Moinfar 2007, Tavassoli 2010].
With regard to the biology of breast cancer, however, the established prognostic factors provide limited information and might not be fully suitable to all clinically progressive elements of the disease [Riley et al. 2003]. The molecular-genetic heterogeneity and the large number of genes included in controlling cell proliferation, differentiation, and apoptosis, strongly underline the importance of studying multiple genetic changes in a variety of phenotypically different breast cancers. The introductions of gene expression profiling technologies determine gene activation through mRNA expression patterns. Significant improvement in bioinformatic analysis has resulted in further accurate prognostic assessment that could improve patient outcome by identifying high-risk individuals who might need aggressive treatment. This could limit the treatment for low-risk individuals [Weigelt et al. 2010]. Furthermore, it may help in advance revaluation of new biological targets which could help in efforts to find novel anti-cancer agents [Colombo et al. 2011].

In 2000, Perou and co-workers demonstrated that the phenotype diversity of breast cancer is accompanied by a corresponding diversity in gene expression patterns and recognized that breast carcinomas could be classified into distinctive subtypes discriminated by characteristic variations in their gene expression patterns. Breast cancer is categorized into several conceptual molecular classes [Prat and Perou 2011] .These are:

- Basal-like class, usually negative for ER, PR and HER2 receptors. This group is also called triple negative breast cancer (TNBC) and most BRCA 1 breast cancers are basal-like TNBCs [Perou 2011].
- Luminal A is the most common subtype, and comprises 50-60% of all breast cancer. It is characterized by the expression of genes activated by the ER transcription factor that is typically expressed in the luminal epithelium lining the mammary ducts. It also associated with low expression of genes related to cell proliferation [Perou et al. 2000, Sorlie et al. 2001].
- Luminal B presents 10% to 20% of all breast cancers. Compared to the luminal A, they have more aggressive phenotype, higher proliferative index, and higher histological grade, and poor prognosis [Parker et al. 2009].

The main biological difference in luminal A and B tumors is increased expression of proliferation genes, such as MKI67 and cyclin B1 in the luminal B subtype which often expresses HER2 and EGFR [Fan et al. 2006, Loi et al. 2007].

- HER2-enriched breast cancers comprise about 15-20% of all breast cancers and they are associated with a high expression of the HER2 gene in the 17q 12 chromosome [Prat and Perou 2011, Fan et al. 2006]. HER2-enriched tumors have poor prognosis and are prone to early recurrence and metastasis without anti-HER2 treatment [Gianni et al. 2011]. This subtype also has a high chemosensitivity with higher response rate than that for luminal A and B types in neoadjuvant setting [Parker et al. 2009]. Women with HER2-enriched tumors appear to be diagnosed at younger age than those with luminal A and B tumors [Voduc et al. 2010].

- Normal breast-like subtypes account for about 5-10% of all breast cancers. They have been grouped into the classification of intrinsic subtypes with fibroadenomas and normal breast samples, and to be enriched for genes usually expressed in adipose tissue [Peppercorn et al. 2008].

- Claudin-low class, a more recently described class which is often negative for ER, PR and HER2, but distinct in that there is low expression of cell-cell junction proteins including claudins 3, 4 and 7, E-cadherin, and ample lymphocyte infiltration [Prat and Perou 2011, Perou 2011].

The differences in gene expression between above types may suggest they originate from different cell types within the breast. These types also have different prognosis and may have different responses to specific therapies [Paik et al. 2006, Geyer et al. 2009, National Comprehensive Cancer Network 2011]. For example, patients with luminal A subtypes are associated with the most favorable prognosis. On the other hand, patients with basal-like type and HER2-enriched tumors are more sensitive to chemotherapy but with poor final outcome [Paik et al. 2006, Ishihara et al. 2009).

3. AIMS OF THE STUDY

1. The study investigates diagnosis delay and its impact on stage of disease in Libyan breast cancer. (I)

2. The study evaluates the significance of DNA image cytometry among women with breast cancer in Libya. Specially, DNA content was compared with clinicopathological variables and patient outcome. (II)

3. The study investigates the association of the immunohistochemical Ki-67 expression, and the S-phase fraction (SPF) with clinicopathological variables and prognosis in Libyan breast cancer. Ki-67 and SPF are compared as proliferation markers for best patient outcome assessment. (III)

4. The study investigates the value of immunohistochemical bcl-2 expression in evaluating the clinicopathological variables and prognosis in Libyan breast cancer. (IV)

4. MATERIAL AND METHODS

4. 1 Patient material

There are two group of patients included in this study:

4. 1. 1 Patient material (I)

Study (I)

Two hundred Libyan female patients with diagnosis of breast cancer presented at the National Oncology Institute (NOI), Sabratha, during the period from Jan 1, 2008 to Dec 31, 2009. During that time 419 patients were registered at the institute. All patients who were diagnosed and registered at the NOI during that period (2008 and 2009) were targeted for this study. Obtainable patients who were treated at the NOI during that period were included. Only the patients who were diagnosed within a period less than 3 months from diagnosis were selected for the interview. All interviews were conducted only after obtaining the consent of the patient for the interview. The patients were asked to be interviewed and the collection of data was stopped after 200 interviews had been completed. Age of patients ranged from 22 to 75 years with a mean of 45 years. Age distribution at diagnosis is shown in Figure 1(next page).

62% of patients were literate. 79% of patients were married, multi-parous, and had their babies. 24.5% of patients had taken oral contraceptive pills, 26% [include single and married women (21% and 5%; respectively)] did not have any children, and 36.5% were post-menopausal. Only 9% had a family history of breast cancer, 9.5% had a history of benign breast disease. 68% of patients with breast carcinoma noted a lump or lumps as an accidental finding, while 2% of patients detected lump(s) during self examination. Family physician noted a lump in 1 case and referred the patient to a proper health care facility. Other symptoms of the breast such as skin changes, nipple discharge or bleeding were reported less frequently (29%). Systemic involvements as the first symptom occurred in 3% of patients.

At time of diagnosis, 34.5 % of patients presented at early stages (1 and 2), and 65.5% at late stages (3 and 4). Clinical stage distribution at diagnosis is shown in Figure 2 (next page).

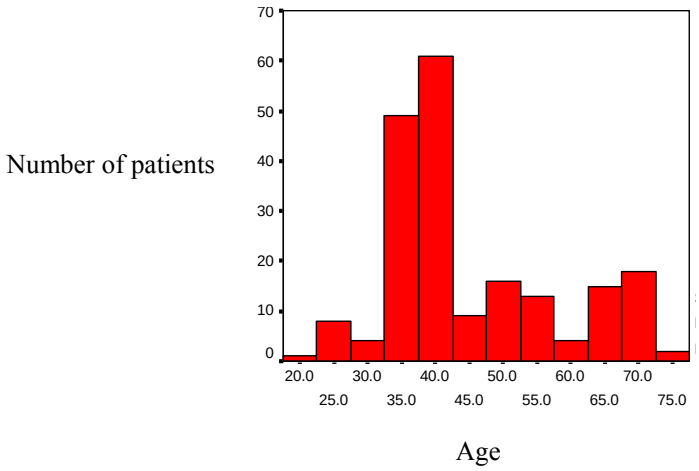

Figure 1. Age distribution at diagnosis of 200 women with breast cancer in Libya (2008-2009).

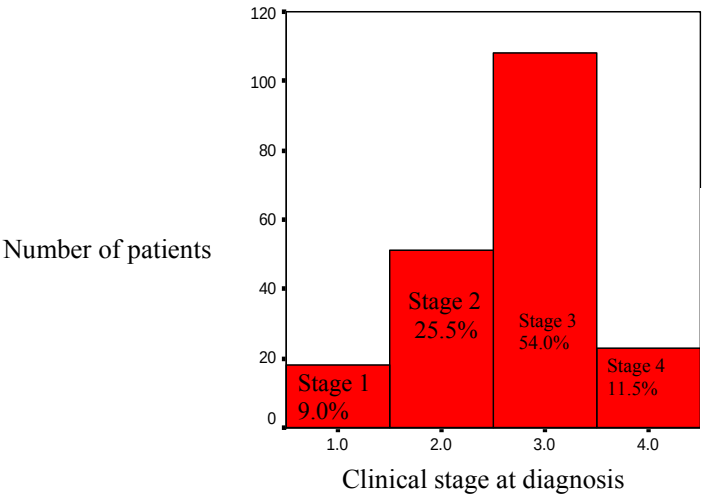

Figure 2. Stage distributions at diagnosis in 200 Libyan breast cancer patients (2008-2009) Study (I).

4. 1. 2 Patient material (II- IV)

Study II and III

The paraffin embedded histological samples of 150 patients with breast cancer, diagnosed between 2000 and 2007 in the African Oncology Institute (presently National Oncology Institute), Sabratha and Tripoli Medical Center, Tripoli, were collected for this retrospective study. 46 paraffin blocks were excluded because the DNA samples contained only a small number of nuclei (<50 nuclei).This left 104 samples that could be used in the study II.
One hundred histological samples out of the above 150 patients were available for both DNA content analysis and Ki-67 IHC staining. Thus 100 samples were used in study III.

Study (IV)

Histological samples from 215 patients with breast cancer diagnosed between years 2000 and 2007 in the National Oncology Institute, Sabratha, and Tripoli Medical Center, Tripoli, Libya were collected for this retrospective study (patient materials were completely different from the study II and III. Patient materials for study II and III were not included in this study because there was no possibility to cut more paraffin blocks).
Immunohistochemical staining could not be evaluated from samples of 45 patients because histological samples were used for diagnostic purposes or sections cut for immunohistochemistry had detached from slides before or during immunohistochemical staining. Recutting of these samples was done again for IHC analysis and the results appear the same (staining sections did not show malignant tissue).Thus 170 samples were left for the study

Clinicopathological data (II, III, IV)

A detailed history and clinicopathological data included: age, menopausal status, family history, hormonal status, histological type, tumor size, lymph node status, tumor stage, histological grade, treatment type, and follow up and survival data, all collected from patient's files. Tumor staging of breast carcinoma was evaluated according to TNM classification [AJCC 1997]. For hormonal receptors assessment, sections were cut serially at 5 μm from formalin-fixed, paraffin-embedded breast tumor tissue for IHC analysis. IHC analysis was carried out using an automatic system (BenchMark XT; Ventana Medical Systems, Inc. Tucson, Arizona, USA). Anti-estrogen receptor rabbit monoclonal antibody (clone: SP1, isotype: IgG; Zymed Laboratories, San Franscisco, CA, USA), and anti-progesterone receptor rabbit monoclonal antibody (clone 1E2, isotype: IgG: Zymed Laboratories) was used. Both antibodies were incubated for 32 min at 37C.

Universal Diaminobenzidine (DAP) detection kit (a biotin-free, Multimer-based detection system for the specific and sensitive of mouse IgG, mouse IgM, and rabbit IgG primary antibodies), was applied. After staining, the sections were dehydrated in ethanol, cleared in xylene, and mounted.

The anti-estrogen receptor and anti-progesterone receptor antibodies react directly with human estrogen and progesterone proteins located in the nuclei. Estrogen and progesterone receptor expression were determined according to Allred score method (Allred et al. 1998]. The proportion of positive cells (scored on scale of 0-5 and staining intensity (scored on scale of 0-3) were evaluated. The proportion and intensity were then summed to produce total scores of 0 through 8. The cutpoint between positive and negative samples is at 3 (<3+ negative, ≥3+ positive).

Treatment and follow-up (study IV as example n=170)

126 (74.1%) patients were treated by modified radical mastectomy and axillary dissection, 21 (12.4 %) patients received neoadjvant chemotherapy with modified radical mastectomy and axillary lymph node dissection. Lumpectomy was done in three patients, and simple mastectomy in one patient. No therapeutic surgical intervention was done for 19 (11.2%) patients with metastasis at time of diagnosis (diagnosis with core biopsy). Adjuvant and neoadjuvant chemotherapy according to the CAF regime (cyclophosphamide 500 mg/m^2, doxorubicin 50 mg/m^2 and 5-FU 500 mg/m^2; intravenously day 1 every 3 weeks for 6 courses) was given to 131 (77.1%) while 27 (17.1%) patients received doxorubicin, at a dose of 50 mg/m^2, and paclitaxel, at a dose of 175 mg/m^2 intravenously every 3 weeks for 4 courses. Hormonal treatment (tamoxifen, at a dose of 20 mg orally every day for 5 years) was given to 126 (74.1%) hormone receptor-positive patients. Axillary radiotherapy was given to node-positive patients (n=130).

The patients were followed-up until death or to the end of the observation period until the end of October 2010. The follow-up ranged from 5 to 125 months, the average being 48 months. The bone isotope scan, and chest, and abdominopelvic CT scan were performed every 6-12 months. At end of the follow-up period, 101 (59.4%) patients were alive, 60 (35.5%) had experienced disease recurrence, and 69 (40.6%) had died.

The clinical characteristics of Libyan patients with breast cancer, in study II and IV are shown in Table 5.

Table 5. The clinicopathological variables of Libyan patients with breast cancer, in studies II and IV.

Clinicopathological variables			
		Study II	Study IV
Number of patients		104	170
Age at diagnosis (years)	Mean (SD*)	43.6 (11.72)	45.1 (12.41)
	Median	42	44
	Range	20-80	21-80
Menopausal status (percent)	Premenopausal	58.7%	62.4%
	Postmenopausal	41.3%	37.6%
Nodal status**	Positive	80.6%	78.8%
	Negative	19.4%	21.2%
Tumor size (cm)	Mean (SD)	5.37 (1.92)	4.82 (2.09)
	Range	1.5-9	1.5-10.5
Duration of follow-up (months)	Mean	48	48
	Range	3-113	5-125

*SD= standard deviation, ** some patients had unknown lymph node status

4. 2 Methods

4. 2. 1 Diagnosis delay in Libyan breast cancer (1)

Preclinical data was collected on a form (questionnaire) during the interview with each patient. Structured face-to-face interviews were arranged either during the first hospitalization due to breast cancer or during follow-up in the outpatient department. 22.5% of all interviews took place within 4 weeks after diagnosis and 77.5% within 8 weeks after diagnosis. The data collection included social and demographic data, medical and obstetric history, symptom-related questions, and consultation-related questions. Dates of the chronological events (first recognition of symptoms, first consultation, referral and first hospital appointment) were included. Data regarding tumour stage relied on histopathological and clinical data including TNM stage [AJCC 1997] and were collected from medical records of each patient.

Diagnosis time and delays were estimated in days. First symptoms included: lump, breast symptoms other than lump, and symptoms not related to the breast. The respondents were questioned about previous use of oral contraceptives, hormone replacement therapy or alternative therapy if these therapies were taken regularly for at least one month. Complementary alternative therapy included any therapy using methods and products not associated with conventional modern medicine.

Diagnosis time was measured from the date of the first symptoms to the date of final breast cancer diagnosis based on histopathological examination (including needle biopsy or excisional biopsy) or on FNAC (fine needle aspiration cytology).

Diagnosis time was categorized into periods: <3 month, 3–6 months, and > 6 months and we used three months as cut-off point of delay [Arndt et al. 2002]. Diagnosis was considered delayed if it took longer than 3 months after symptoms to reach the final diagnosis of breast cancer (diagnosis delay). Consultation time was the time taken to visit the general practitioner after the first symptoms.

4. 2. 2 DNA imaging cytometry method (II and III)

Feulgen staining

Nuclei were isolated from 30 micrometer thick paraffin sections after xylene treatment and digestion with proteolytic enzymes according to the method described by Hedley et al [1985]. Isolated nuclei in solution were centrifuged on microscope slides. Feulgen stain was applied according to the Gaub's et al. [1975] method. Acid hydrolysis of the samples was carried out in 5M hydrochloric acid at 21C° for 1 hour. After washing in distilled water slides were immersed in Schiff's reagent in the dark for 2 hours 45 minutes, rinsed in distilled water many times, washed in fresh aqueous sodium thiosulphate solution and rinsed thoroughly in distilled water. Finally, the samples were rehydrated and mounted. The intensity of the stain in the nuclei represents the amount of nuclear DNA.

Image analysis cytometry

The intensity of Feulgen staining was measured using a computer-assisted image analysis cytometric system AHRENS ICM with a Nikon microscope (Eclipse E 400; Japan) designed and produced by Olaf Ahrens (Messtechnische Beratung, Bargteheide/Hamburg, Germany). The field of view from the CCD camera (JAI DSP surveillance, CV-S 3200/3300) was stored in image memory at a resolution of 736 by 560 pixels. The image was produced by a plan objective (Nikon; ×40, numerical aperture 0.65) and the measurements were made from that image. Prior to each measurement session, the illumination of the microscope was adjusted according to the Köhler method [Bohnhoff 1979]. Several histograms were produced twice, and they were found to be very similar. From the stained slide, 200 nuclei of breast cancer cells were selected, if available. In addition, small lymphocytes were used as internal controls. Laboratory work for measurement of the DNA variables was done at the Department of Pathology, University of Turku, Turku, Finland.

Histogram interpretation

Many different approaches applied in interpreting the of DNA histograms: histogram with 2 gates, S phase fraction (SPF), number of cells >5c, number of cells >9c, number of peaks, and width of the histogram. In the 2 gates approach the diploid region was viewed to be situated within the gate of 1.8c - 2.2c, c being the haploid amount of DNA.

A peak was considered as tetraploid within the range of 3.6 c - 4.4 c, and as aneuploid when the mode of the peak was outside the mentioned gates (1.8c – 2.2c and 3.6c - 4.4c). DNA histogram interpretation was applied according to the ESACP consensus report [Haroske et al. 1998].

The S phase fraction (SPF) - a measure of tumor proliferative activity- was automatically calculated by the Ahrens program as the percentage of cells in the S phase of the cycle, and was recorded for all cases. The median SPF value (11%) was used as cut off point for distinguishing between tumours with high proliferative activity (≥11%) and tumours with low proliferative activity (< 11%), because it provided the best prognosis prediction result in this study.

The amount of aneuploid nuclei (DNA content > 5c) and highly aneuploid nuclei (DNA content > 9c) were calculated for all histograms, the numbers of peaks of DNA histogram were also counted. When the histogram included several aneuploid peaks it was called multiploid. The width of DNA histogram was manually measured for all cases in millimeters. Aneuploid cells were called as aneuploid when nuclei were >5c, and highly aneuploid when they were >9c.

4. 2. 3 Immunohistochemistry method (III and IV)

Study III

Formalin fixed, paraffin embedded tissues were cut into sections of 3 μm. The sections were de-waxed in xylene, rehydrated in graded ethanol. Novocastra Peroxidase (3% hydrogen peroxide) was used to neutralize endogenous peroxidase activity of the samples for 5 minutes. Ki-67 staining was carried out by mouse monoclonal antibody (Anti Ki-67: Clone NCL-L-KI67-MM1, Novocastra Laboratories, Newcastle Upon-Tyne, UK) at a 1:200 dilution and the samples were incubated for 30 minutes at room temperature. To reveal the binding of primary antibody by peroxidase staining, the substrate/chromogen, 3, 3-diaminobenzidine (DAP), prepared from Novocastra DAP Chromogen and NovaLink DAP Substrate Buffer (Polymer) was used and the samples were incubated for 30 minutes. Finally, the sections were counterstained with hematoxylin, dehydrated, cleared, and the sections were mounted for examination.

All slides were evaluated without knowledge of the patient's outcome. The slides were examined by one pathologist (B.K) in a Nikon microscope (Eclipse E 600; Japan). Malignant cells with brown nuclear staining were considered to be positive. Ki-67 expression was counted as a percentage. The percentage was determined by the number of Ki-67 positive cells among the total number of counted tumor cells. A minimum of 500 cells were counted in hot spots per slide. A cut point of 10% was used to distinguish between the categories of low and high proliferative tumors [Molino et al. 1997, Pinto et al. 2001], because it provided the best prognosis prediction result in this study. Laboratory work for Ki-67 immunostaining was done at the Department of Pathology, Salah Azaiz Cancer Institute, Tunis, Tunisia.

Study IV

Formalin fixed, paraffin embedded tissues were sectioned at 3 μm. The sections were de-waxed in xylene, and rehydrated in graded ethanol. Novocastra peroxidase (3% hydrogen peroxide) was used to neutralize endogenous peroxidase activity of the samples for 10 minutes. Bcl-2 staining was carried out by using mouse monoclonal antibody (bcl-2 oncoprotein: Clone NCL-L-bcl-2-486, Novocastra Laboratories, Newcastle Upon-Tyne NE 12 BEW, UK) at a 1:100 dilution and the samples were incubated for 30 minutes at 25°C. To reveal the binding of primary antibody by peroxidase staining, the substrate/chromogen, 3, 3-diaminobenzidine (DAP), prepared from Novocastra DAP Chromogen and NovaLink DAP Substrate Buffer (Polymer) were used. Finally, the sections were counterstained with hematoxylin, dehydrated, cleared with xylene, and the sections were mounted for examination. T-cell lymphocytes were used as positive internal control and fibroblasts as negative internal control.

All slides were evaluated without knowledge of the patient's outcome. The slides were examined by one pathologist (B.K) in a Nikon microscope (Eclipse E 600; Japan). Malignant cells with cytoplasmic staining were considered to be positive. A minimum of 500 cells were evaluated per slide. The evaluation of bcl-2 expression was done according to the following quantification model: bcl-2 negative-no tumor cells stain or there is weak heterogeneous positive stain in less than 10% of tumor cells; bcl-2 positive-more than 10% of tumor cells were stained [Zhang et al. 1997].

In addition to the evaluation of the proportion of stained cells, bcl-2 staining intensity was evaluated as the average intensity of staining: 0= no bcl-2 staining, 1= weak staining intensity, 2= moderate staining intensity, 3= strong staining intensity.

To test the reproducibility of evaluation another investigator evaluated the same slides without knowledge of the first evaluation or other data. The great majority of slides stained for bcl-2 expression (90.4%) were similarly evaluated by both investigators either as the proportion of stained cells (- or +), and staining intensity (0 to 3). For interobserver variability, Spearman correlation =0.91, with p value <0.0001, furthermore the Kappa statistic correlation = 0.82 and also high significance the p value <0.0001. The evaluation of the pathologist (B.K) was used in statistical analysis. Laboratory work for bcl-2 immunostaining was done at the Department of Pathology, Salah Azaiz Cancer Institute, Tunis, Tunisia.

4. 3 Statistical analysis

4. 3. 1 Statistical analysis (I)

Socio-demographic characteristics, as potential determinants of diagnosis delay, included age, education, and employment status.

Health characteristics, evaluated as potentially affecting the duration of diagnostic time, included menopausal status, use of oral contraceptives, breast self examination, history of fibrocystic disease, and family history of breast cancer. Data were analyzed using SPSS for Windows (version 17, SPSS, Inc., Chicago, USA).
The Chi-square test, with likelihood ratio (LR), or Fisher's exact test was used to assess the significance of the association between potential predictor factors and diagnosis delay, and to identify independent determinants of diagnosis delay of 3 to 6 months and more than 6 months versus less than 3 month. Additionally the association between diagnosis delay and clinical stage was examined for all patients. In all tests, the values $p < 0.05$ were regarded statistically significant.

4. 3. 2 Statistical analysis (II-IV)

The variables of the material were grouped into logical classes [which include clinicopathological variables; age, menopausal status, hormonal status, staging, grading, tumor size, and nodal status, as well as biological variables; DNA ploidy, proliferative rate (S-phase fraction and Ki-67), and Bcl-2 expression] and descriptive statistics calculated for the continuous variables using SPSS 19.0 for Windows (SPSS, Inc., Chicago, USA). Frequency tables were analysed using the Chi-square test, with likelihood ratio (LR), or Fisher's exact test to assess the significance of the correlation between the categorical variables. Kaplan- Meier curves were plotted, and differences between the curves analyzed using the log-rank test. Student's t-tests and ANOVA were also used to test differences between the groups. Multivariate survival analysis for the outcome measure [overall survival, disease-specific survival (DSS), and disease-free survival (DFS)] was carried out using Cox's proportional hazards model in a backward stepwise manner with the log-likelihood ratio (L-R) significance test, using the default values for enter and exclusion criteria. The assumption of proportional hazards was controlled by log-minus-log (LML) survival plots. In all tests, values of $p < 0.05$ were regarded as statistically significant.

In study IV, only 151 patients with stage 1, 2, and 3 were included in survival analysis and stage 4 was excluded to justify the comparison with other studies.

5. RESULTS

5. 1 Diagnosis delay (I)

5. 1. 1 Diagnosis time and consultation time

The median of diagnosis time was 7.5 months, 25 months as the maximum. 30% of patients were diagnosed within 3 months after detecting symptoms. 14% of patients were diagnosed within 3–6 months and 56% within a period longer than 6 months.

The median of consultation time was 4 months, 24 months as the maximum. 44.5% of patients had a medical consultation within one month after detecting symptoms, while 15.5% had visited the doctor within 1–6 months after symptoms. 40% of patients had consultation later than 6 months after first symptoms.

The majority of patients (84.5%) were diagnosed within one month after the visit to the general practitioner. 4.5% of patients were diagnosed from 1 to 6 months after the first visit to the general practitioner, 11.0% of patients had waited for more than 6 months for the final diagnosis after the visit to the general practitioner.

5. 1. 2 Diagnosis delay and associated factors

This study shows that diagnosis delay was associated by many factors:

- Symptoms were not considered serious (27% of patients)
- Alternative therapy (13.0% of the patients) was given
- The patient feared cancer diagnosis (10% of patients)
- The patient was ashamed to go to specialist for medical consultation (4.5% of patients)

In addition diagnosis delay was associated with initial breast symptom(s) without a lump ($p<0.0001$), with women who did not report monthly breast self examination ($p<0.0001$), with old age ($p=0.004$), with illiteracy ($p=0.009$), with history of benign fibrocystic disease ($p=0.029$) and with women who had used oral contraceptive pills longer than 5 years ($p=0.043$). Also, inappropriate reassurance that the presenting lump was benign was an important reason for delay.

5. 1. 3 Diagnosis delay and clinical stage

Advanced stage (stage 3 and 4) of breast cancer was found in 65.5% of all patients, 89.3% of these had diagnosis delay >6 months. Diagnosis delay was associated with bigger tumour size ($p<0.0001$), with positive lymph nodes (N2, N3; $p<0.0001$), with high incidence of metastasis ($p<0.0001$).

5. 2 DNA image cytometry (II)

5. 2. 1 Description of DNA content

The mean of DNA ploidy mode for all tumors was 3.43, and 17.3% of tumors were diploid, 24% were tetraploid and 58.7% were aneuploid. The median S-phase fraction (SPF) of the tumours was 11% (the mean 16.64%), ranging from 0 to 62 %. The S-phase fraction was significantly higher in aneuploid tumours than diploid tumours ($p<0.0001$). DNA cytometric variables are shown in Table 6. Examples of DNA diploid histogram, DNA tetraploid histogram, and DNA aneuploid histogram are shown in Figure 3 (next page).

Table 6. Biological variables according to DNA cytometric variables (study II), immunostaining analysis of Ki-67 expression (study III), and immunostaining analysis of bcl-2 expression (study IV) in Libyan breast cancer.

	Threshold	No of patients	Percent
Study II (N=104)			
DNA ploidy	Diploid	18	17.3
	Aneuploid	86	82.7
S-phase fraction	<11%	44	42.3
	≥11%	60	57.7
Number of cells > 5 c	< 5 cells	40	38.5
	≥ 5 cells	64	61.5
Number of cells > 9 c	No cells	65	62.5
	≥ one cell	39	37.5
Number of DNA histogram peaks	One peak	32	30.8
	>one peak	72	69.2
Width of DNA histogram	< 2 c	22	21.2
	≥ 2 c	82	78.8
Study III (n= 100)			
Ki-67 Expression*	Low	24	24
	High	76	76
Study IV (n= 170)			
Bcl-2 expression**	Negative	64	37.5
	Positive	106	62.4
Bcl-2 staining intensity	Weak	37	21.8
	Moderate	35	20.6
	Strong	24	20.0

*= Low Ki-67 expression (< 10% nuclear staining) and high expression (≥ 10% nuclear staining).

** = see page 60.

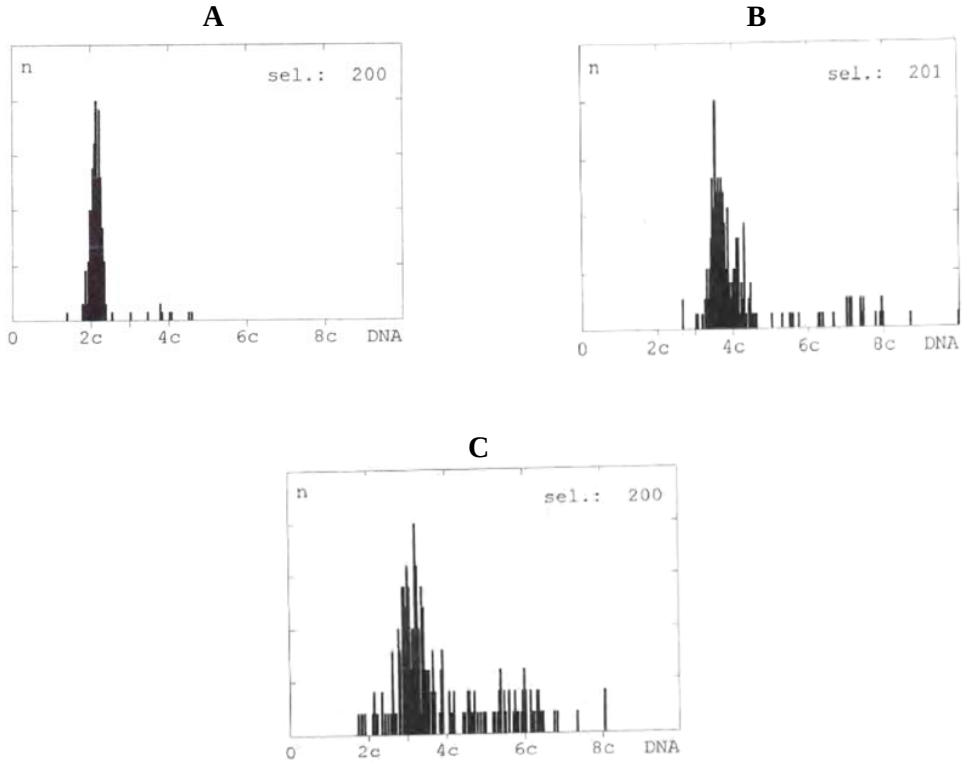

Figure 3. Examples of DNA ploidy histogram: Diploid DNA histogram (**A**), Tetraploid DNA histogram (**B**), and Aneuploid DNA histogram(**C**).

5. 2. 2 Association of nuclear DNA content with clinicopathological variables

Abnormal DNA content was significantly associated with poor differentiation of tumors ($p=0.001$), with distant metastasis ($p=0.006$), and with advanced clinical stage ($p=0.02$). DNA aneuploidy was not found to be significantly associated with age, menopausal status or histology type.

High SPF was more frequent in patients with advanced clinical stage ($p<0.0001$), positive axillary lymph nodes more than three in number ($p<0.0001$), distant metastasis ($p<0.0001$), poor differentiation of tumors ($p=0.009$), distant metastasis ($p=0.006$), and large pathological tumor size ($p=0.02$). Young patients (<50 years) tended to have tumors with higher SPF values than older patients ($p=0.02$).

High number of aneuploid cells of >5c (less than 5 cells / 5 or more cells) associated with premenopausal status and advanced clinical stage ($p=0.06$ and $p=0.08$; respectively).

Aneuploid cells of > 5c were not clearly associated with other clinicopathological features. Aneuploid cells of > 9c (no cells / presence of 1 or more cells > 9c) were significantly associated with high histological grade and with negative hormonal receptor (p=0.017, p=0.045, respectively).

Multiploid histograms were associated with positive axillary lymph nodes more than three in number (p=0.01) and with poor differentiation of tumors (p=0.045). High width of histogram was associated with poor differentiation of tumors and lymph node involvement ((p=0.002, p=0.03, respectively).

5. 2. 3 Survival. Univariate and multivariate analysis (study II)

DNA diploidy was significantly associated with high survival rate (p<0.0001) and the lowest SPF (<11%) values were predictors of longer survival time (p<0.0001). Breast cancer patients with less than 5 aneuploid cells (>5c) showed a significantly longer survival time than the patients with 5 or more aneuploid cells (p= 0.003).

In addition, the analysis using Kaplan Meier curves based on DNA histograms indicated that short survival time was associated with aneuploid histograms, with high SPF tumors, with multiploid DNA histograms, and with more than one highly aneuploid cell (>9c).

The DNA histogram as an independent predictor of overall survival as assessed in a multivariate survival (Cox) analysis containing age, hormonal status, menopausal status, histological grade, tumor size and stage variables (lymph node status excluded from analysis because of incomplete data). With respect to lymph node status, DNA ploidy appeared to have an independent effect on the overall survival (p=0.010), and was associated with age (p=0.038) and clinical stage (p=0.001). DNA ploidy was also an independent predictor of disease-specific survival (p=0.018) and was associated with clinical stage (p=0.001). Overall survival according to DNA histograms using Kaplan Meier curves is shown in Figure 6 A (page 69).

5. 3 Proliferation markers: Ki-67 and S-phase fraction (III)

5. 3. 1 Description of the Ki-67 expression and S-phase fraction

Ki-67 expression and SPF value were assessed in all tumors; median Ki-67 expression was 27.5% (mean 29.6%; range 1%-80%). Ki-67 expression was low (<10%) in 24 samples and high (≥10%) in 76 samples (Figure 4: A and B). Median SPF was 11.0% (mean 16.6%; range 0%-62%). S-phase fraction was low (<11%) in 44 samples and high (≥11%) in 56 samples. The Ki-67 expression was more frequent in tumors with high SPF than low SPF (p<0.0001). Frequency of Ki-67 expression (positive/negative) and SPF values (high/low) are shown in Table 6 (page 63).

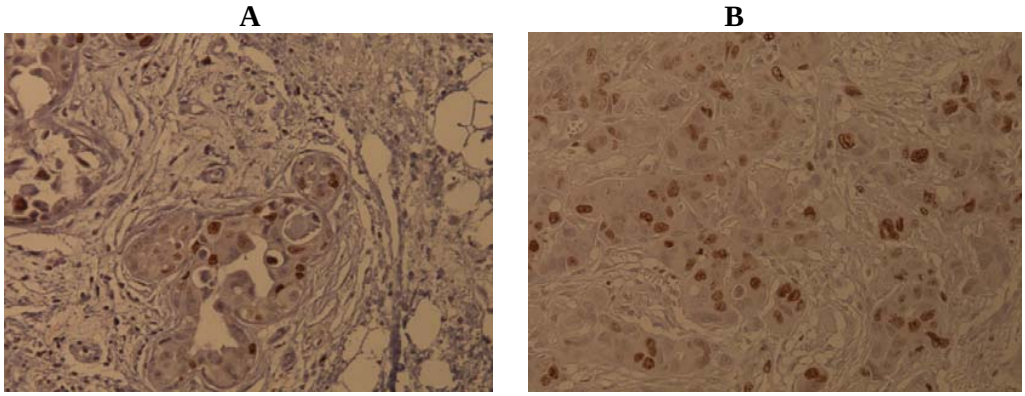

Figure 4. Immunohistochemical staining of Ki-67 expression. **A**. low expression (<10% nuclear staining). **B**. high expression (≥10% nuclear staining).

The S-phase fraction also was significantly higher in aneuploid tumours than diploid tumours ($p<0.0001$).
The Ki-67 expression was more frequent in tumors with high SPF than low SPF ($p<0.0001$). The Ki-67 was also associated with high width of histogram and multiploid histogram ($p=0.005$, $p=0.007$, respectively). DNA ploidy did not have significant association with Ki-67.

5. 3. 2 Association between Ki-67 expression and clinicopathological variables

High Ki-67 expression was significantly associated with hormone receptor negative tumors ($p=0.001$), advanced stages ($p=0.001$), poor differentiation of tumors ($p=0.01$), positive axillary lymph nodes more than three in number ($p=0.01$) and distant metastasis ($p=0.01$). Age at diagnosis, histological subtypes, tumor size or type of surgery did not have significant relationship with Ki-67.

5. 3. 3 Association between SPF and clinicopathological variables

High SPF was significantly associated with positive axillary lymph nodes more than three in number ($p<0.0001$), distant metastasis ($p<0.0001$) and poor differentiation of tumors ($p=0.005$). Young patients (<50 years) tended to have tumors with higher SPF than older patients ($p=0.03$). Hormonal status, histological subtypes, tumor size or treatment did not have significant relationship with SPF level.

5. 3. 4 Survival. Univariate and multivariate analysis (study III)

The survival rate was 64.5% in patients with low Ki-67 expression (p=0.001) and 88.6% in patients with low SPF values (p<0.0001). The analysis using Kaplan Meier curves on immunohistochemical Ki-67 and cytometric S-phase fraction indicated that long survival time was associated with low Ki-67 and low SPF (Figure 6 B and C, page 69). This study also shows that longer survival time was associated with low SPF in hormone receptor positive and negative patients (p=0.004, p=0.005, respectively).

Survival analysis was done according to the Cox model for all breast cancer patients with lymph node status excluded from analysis (98 patients had lymph node status histologically evaluated and 2 patients were unknown lymph node status, so lymph nodes were excluded from this analysis because of incomplete data), and the results shows that high SPF (p= 0.007), hormonal status (p=0.001) and clinical stage (p=0.005) were independent predictors of disease-specific survival. The Ki-67 (p=0.065) in borderline significance proved to be only independent predictor of disease-free survival.

5. 4 Prognostic value of bcl-2 expression (IV)

5. 4. 1 Description of bcl-2 expression

This study shows that positive bcl-2 expression was found in 62.4% of tumors. Of the positive samples, 72.2% were estrogen receptor positive (ER-positive), and 71.8% were progesterone receptor positive (PR-positive).The majority of patients with positive bcl-2 expression demonstrated moderate-to-strong bcl-2 staining intensity. Frequency of bcl-2 expression is shown in Table 6 (see page 63). Examples of positive and negative bcl-2 expressions are shown in Figure 5(next page).

A **B**

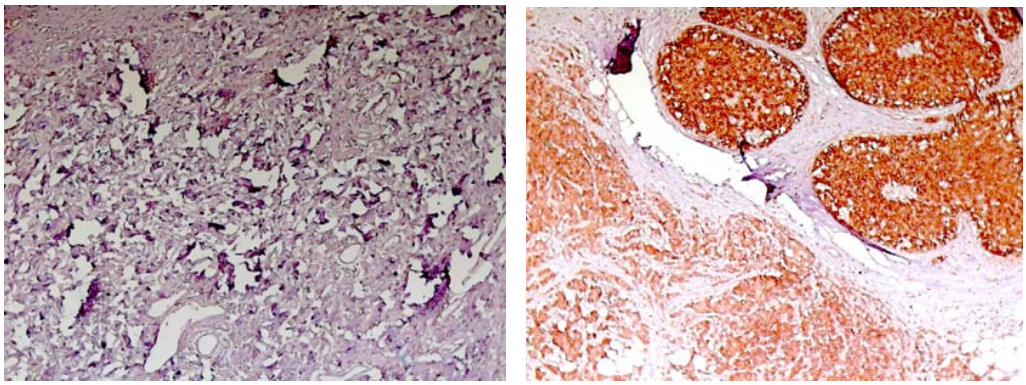

Figure 5. Immunohistochemical evaluation of bcl-2 expression. **A**. Negative bcl-2 expression. **B**. Positive bcl-2 expression

5. 4. 2 Association between bcl-2 expression and clinicopathological variables

Positive expression of bcl-2 was significantly associated with estrogen receptor ($p<0.0001$) and progesterone receptor positive tumors ($p=0.002$), small tumor size ($p<0.0001$), low tumor grade ($p<0.0001$), negative axillary lymph nodes ($p<0.0001$), early stages ($p =0.001$), and low risk of metastasis ($p<0.0001$). Positive expression was also associated with older patients (>50 years) ($p=0.04$). Histological subtypes and family history of breast cancer did not have significant relationship with bcl-2.

5. 4. 3 Survival. Univariate and multivariate analysis (study IV)

In this study, survival analysis includes only the patients with stage 1, 2, and 3 to justify the comparison with other studies.The study shows that bcl-2 appeared to have strong association with the survival.The survival rate was 89.3% in patients with positive expression of bcl-2 and 16.7% in patients with negative expression of bcl-2 ($p<0.0001$). Additionally, a direct association between the intensity of bcl-2 staining and survival was identified in this study. Patients who had tumors with high bcl-2 staining intensity (moderate –to-strong bcl-2 staining intensity) were associated with the best survival, and showed no deaths during the follow up period.

The role of bcl-2 as an independent predictor of disease-specific survival was assessed in a multivariate survival (Cox) analysis, including age, hormonal status, recurrence, histological grade, and clinical stage variables. Bcl-2 ($p<0.0001$) and clinical stage ($p= 0.016$) were independent predictors of disease-specific survival. For analysis of disease-free survival, the same variables were entered to the model and only bcl-2 proved to be an independent predictor ($p=0.002$). Overall survival according to analysis of bcl-2 expression using Kaplan Meier curves is shown in Figure 6 D.

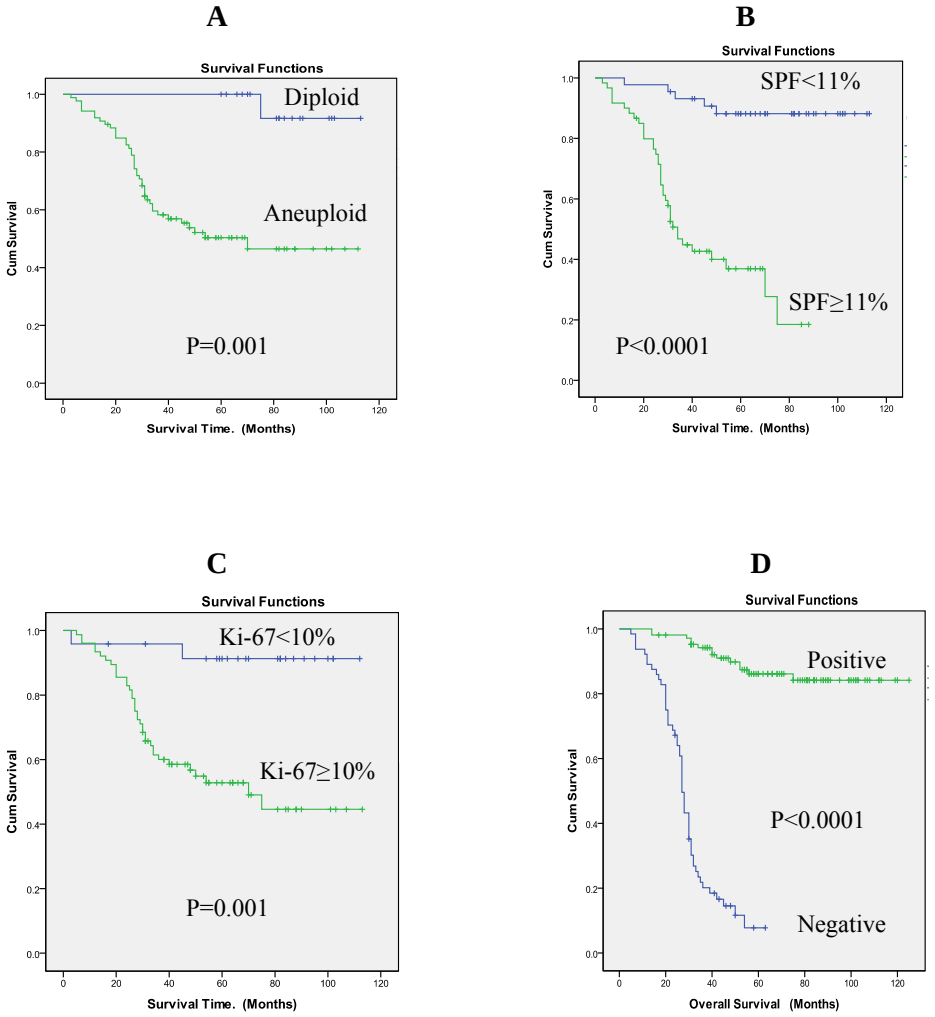

Figure 6. Overall survival in women with breast cancer in Libya evaluated by different biological factors. **A**. DNA histograms evaluated in 2 gates. **B**. Evaluated by SPF at cutoff point of 11%. **C**. Evaluated by immunohistochemical Ki-67 expression at cutoff point of 10%. **D**. By immunohistochemical bcl-2 expression (positive and negative).

This study also observed that shorter survival time was associated with premenopausal women, with negative hormonal status, with positive lymph nodes, with large tumor size, with advanced stage, and with poorly differentiated tumor (Figure 7).

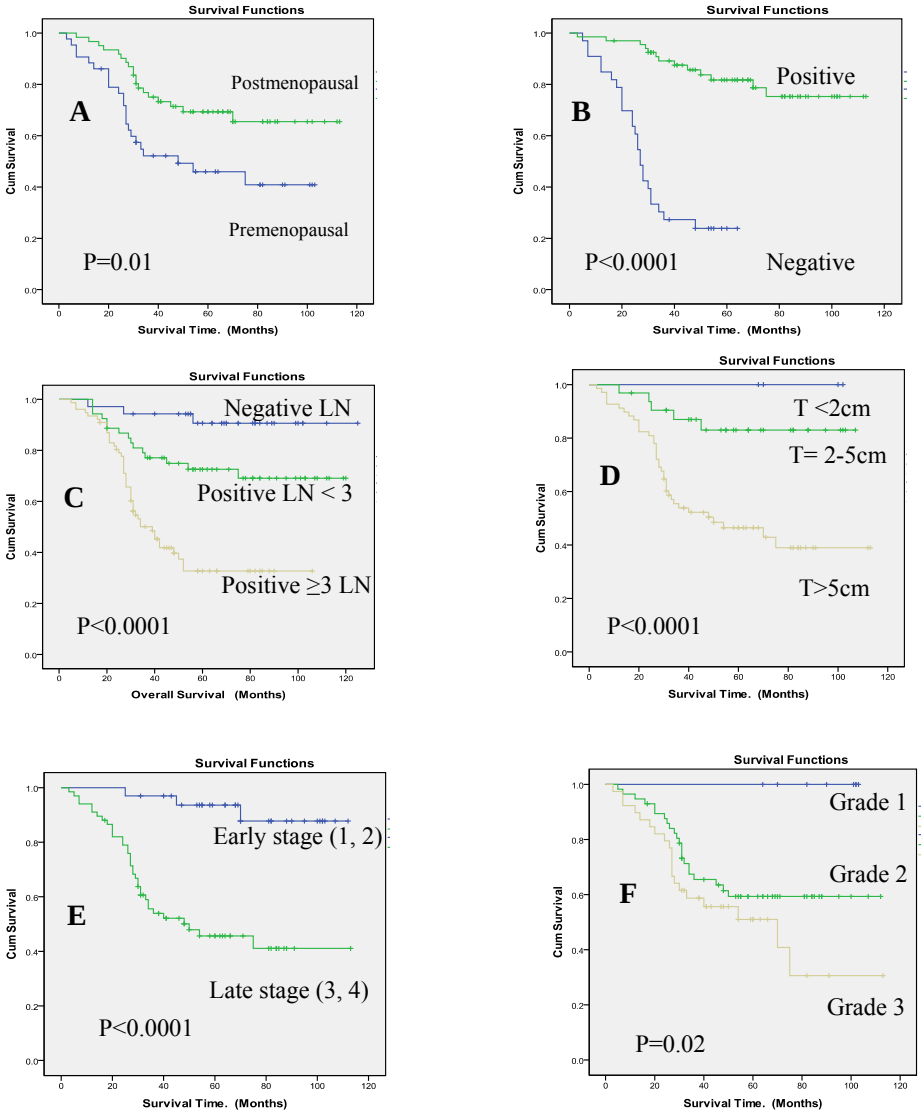

Figure 7. Overall survival using Kaplan Meier curves in patients with breast cancer in Libya based on clinicopathological variables. **A**. Menopausal status. **B**. Hormonal status (ER positive/negative). **C**. LN involvement. **D**. Tumor size. **E**. Clinical stage. The group of patients with early stage had good 5-year survival ($p<0.0001$). **F**. Histological grade.

6. Discussion

6. 1 Diagnosis delay (I)

In Libya breast carcinoma is the most common malignant tumor in women. Patients often present with advanced disease, have early disease recurrence and are associated with high mortality [El Mistiri et al. 2007, Sabratha Cancer Registry 2008]. Because diagnosis delay is preventable and has major effects on patients' prognosis and outcome, it is necessary to investigate diagnosis delay and to understand the causes of delay to reduce delays and to improve early diagnosis. In fact, this is the first study to investigate diagnosis delay among women with breast cancer in Libya. This work shows that the diagnosis delay of breast cancer is a serious problem in Libya.

The average time before medical advice and diagnosis was long, and the diagnosis time was higher than in developed or developing countries [Montella et al. 2001]. Perhaps this trend can be attributed to low awareness of health issues among women, to poor information campaigns, and to the absence of mammographic or other screening programs for early detection of breast cancer in Libya.

A number of factors predicting diagnosis delay were reported in this study, in agreement with other studies [Arndt et al. 2002, Neal et al. 2008]. A relationship between the patient-associated factors (psychology and/or sociology) and delayed presentation of symptoms was important predictor of delay. Some studies suggest delays when the patients assumed that symptoms were benign and would disappear without medical interference [Arndt et al. 2003].We found that patients often considered symptoms as benign and probable to fade without any medical intervention. This was the most important reason for delay in seeking doctors' advice.

Most patients looked for alternative treatment as means to avoid surgery. Some patients believed that there were no effective treatments for breast cancer, or that traditional medicines are more effective than modern drugs. While taking alternative treatments, most patients experienced worsening of symptoms, which eventually led to more advanced stage. In this study, 13% of the respondents had taken alternative therapy instead of visiting the doctor, compared to 41.5% in other studies [Norsa'adah et al. 2011].

This study showed that negative information on breast cancer diagnosis and treatment caused delays. Some patients believed that breast cancer could not be cured, so there was no point of having it diagnosed and treated [Andersen et al. 2009]. Fear of divorce or remarriage of the husband could lead some women to decide not to get their symptoms diagnosed if they suspected breast cancer. Negative information, such as side-effects and expected toxicity of chemotherapy led to fear and refusal of therapy.

Diagnosis delay was also related to a belief that mastectomy causes disfigurement and disability [Grunfeld et al. 2003]. In addition, shame and personal need to keep symptoms secret was one of the reasons for diagnosis delay, particularly among older women > 50 years in this study.

There are certain 'alarm symptoms', important to prediction of cancer diagnosis [Jones et al. 2007]. However, these are not always known by doctors or nurses. An easy way to prevent delay in diagnosis would be for doctors to encourage diagnostic tissues sampling from all palpable breast masses, even through the majority of lumps in young patients are benign. In this work, we found that the respondents were inappropriately reassured after the first visit that a lump can be considered benign without biopsy. This is a false attitude. This attitude was an important reason to the magnitude of the diagnosis time in this study. Similar results were reported by Goodson et al [2002].

One of the important finding in this work showed that the initial breast symptoms that did not include a lump were strongly associated with diagnosis delay. The discovery of a breast lump reduces the patient delay as confirmed in other studies [Stapleton et al. 2011]. The results suggest that doctors and patients need to be educated about different types of breast cancer symptoms.

This study observed that the patients who made self examination monthly were more educated, younger and asked for medical help more rapidly than less educated or older patients. Mammography is a sensitive means for early detection of breast cancer, but both clinical breast examination (CBE) and breast self-examination (BSE) have the potential to advance the diagnosis of breast cancer without the expenses of a mammography facility [Weiss 2003].

This study also founded that older women waited longer than younger women before presenting their symptoms to a physician. Many studies have shown that older age is a predictor of diagnosis delay [Stapleton et al. 2011]. Any intervention program should target older women regarding this issue.

The role of education on breast cancer symptoms has been reported in a number of studies [Velikova et al. 2004, Stapleton et al. 2011]. Lack of knowledge about breast cancer is an important factor in Libya as observed in this work, and there is a need for public educational programs especially for less educated women.

Women with a history of fibrocystic disease were associated with diagnosis delay. Explanation is that former episodes of breast tissue alterations, if benign is also later considered benign by the doctors. Thus, it might be worth while to encourage women with known benign breast disease to present new breast symptoms promptly.

Additionally, doctors should understand that new symptoms should be evaluated as potential new risks for breast cancer.

Diagnosis delay strongly impacts on stage of disease and potentially on survival as reported in this work. Diagnosis delay was also significantly associated with lymph node involvement, with bigger tumor size, with high incidence of advanced clinical stages and with metastatic disease.

The delay studies were done on material that did not include IHC ER, PR, HER2 and Ki-67 expression. Expression associations with delay could not be evaluated. Anyhow, numerous studies suggest that African American women with breast cancer are more likely to presents with advanced stage and more aggressive tumor at time of diagnosis than white women [Adams et al. 2012]. This can reflect the failure of medical care leading in delay of diagnosis and staging or differences in biology of the African American population. The African American women also were more likely to be diagnosed with high grade tumor, ER-negativity, PR-negativity, HER2-positivity, and high proliferative rate [Amend et al. 2006, Adams et al. 2012, Tamimi et al. 2012]. The result suggests that delay of presentation in African American women with breast cancer at diagnosis may be associated with the biologic characteristics of the tumor themselves, which is tends to be more aggressive.

Comparison between this work and others from Europe (Arndt et al. 2002) is interesting in light of the difference in African and European breast cancer. These differences may be related to the health care, or be explained by difference in other aspects such as biology and genetics. The observations in both studies suggest that the diagnosis delay is strongly associated with late stage of disease at diagnosis. But in Europe, late diagnosis was observed among patients with poorly differentiated breast cancer.

In the Libyan patients, the median of consultation time was 120 days and 54.5% patients waited longer than 3 months before seeking a medical advises. Among European patients the median of consultation time was 16 days and only 18 % of patients waited longer than 3 months before seeking medical occultation.

On the other hand, the mean age of Libyan patients was 45.4 years and 65.5% of patients were presents with advanced stage. In comparison, the mean age of European patients was 57.5 years and 51% of patients were at late stage in Europe.

The big difference in these results may reflect the difference in health care and/or be related to other demographic, social, and patient associated factors. These results also suggest that Libyan women with breast cancer are present at lower mean age and advanced stage at time of diagnosis than European women.

With respect to the health care, differences in the biology of breast cancer may also be involved. African American women with breast cancer are more likely to be diagnosed at advances stage and have breast cancer markers of bad prognosis [Tamimi et al. 2012]. Breast cancer in the North Africa may be related to many factors, such as geographic variation, racial and ethnic background, genetic difference, and availability of good health care [Hortobagyi et al. 2005].

One of the important observations in this work is the finding that patients in Libya have low mean age at diagnosis, showing that premenopausal cancers are more common than in Europe. These also confirm results of other studies in Libya [Boder et al. 2011, El Mistiri et al. 2007, Abdalla et al. 2007]. The age pattern is identical with age of breast cancer patients in Africa or Middle and North Africa (MENA) region [Najjar and Easson 2010, Abulkhair et al. 2010, Boder et al. 2011]. These observations suggest that Libyan and other African breast cancer patients are clearly dominantly of premenopausal type. In Europe and US most of patients with breast cancer are postmenopausal at diagnosis. The younger presentation in African patient may partly be associated with the age distribution of population in respective countries, but biological difference may also be present. In this work we also observed that Libyan young women with breast cancer tended to have tumors with higher S-phase fraction and low bcl-2 expression than older women with breast cancer.

The variation in genetic marker distribution between central and north African, and European populations may also be involved [Jopling et al. 2004, Alero and Lisa 2005]. African populations are characterized by African genomic halplotypes, and the premenopausal type of breast cancer may be more common and more aggressive than the postmenopausal type in this population [Alero and Lisa 2005].

In this work we observed that patients in Libya present with advanced stage, have early disease recurrence and are potentially associated with high mortality. Our findings confirm other previous results [Boder et al. 2011]. The large percentage of patients in advanced stages indicate delayed presentation and late diagnosis as noted in this study and also in the study of Ikept et al on Nigerian breast cancer and other studies in North Africa countries [Nadia et al. 2007, Ben Ahmed et al. 2002]. Perhaps this trend can be attributed to low awareness of health issues among women, to poor information campaigns, and to the absence of mammographic or other screening programs for early detection of breast cancer in Libya. Mammography in Libya is not part of screening program, but it can be speculated that the potential of mammography is limited because of difficulties of making early mammographic diagnosis in premenopausal breast cancer [Keen and Keen 2009].

Health service aspect is important, but biological factors as explanation of difference should not be excluded. That African and European breast cancers are different in terms of biology is one of the dominant discussions today. However, in this work we observed that the frequency of aneuploidy and proliferative rate (SPF and KI67) values were higher in Libyan patients with breast cancer than in European patients [Chassevent et al. 2002, Jalava et al. 2006]. The fraction of medullary carcinoma and histological grade are also higher in Libyan patients than in European patients [Boder et al. 2011]. It may be that genetic factors are involved, but so far we have a little evidence that breast cancer genes (BRCA1 and BRCA2) are more often involved in African population [Alero and Lisa 2005]. Other explanation for grade difference may just be the more active cell proliferation in premenopausal type of breast cancer, which is common in Africa.

6. 2 Tumor cell DNA content (II)

Abnormalities in DNA ploidy are seen in many human tumors, and determination of ploidy and proliferative activity has been shown to provide prognostic information in many solid tumors [Bazan et al. 2002, Karra et al. 2012]. DNA content of individual tumor cells, as determined by image cytometry, has been shown to give significant information on breast cancer [Auer et al. 1980, Baldetorp et al. 1992, Yildirim-Assaf et al. 2007]. Chromosomal abnormalities in cancer cells are frequently related to the structure or the number of chromosomes. Aneuploidy is a marker of poor prognosis. Aneuploidy, one of the features of cancer cells that distinguish them from normal cells [Rajagopalan et al. 2004].

In this work we investigated the relation of nuclear DNA content, as determined by static image cytometry, with clinicopathological variables and patient outcome in Libyan material. However, several interesting observations were made, all implicating that the quantitatively measurable DNA content in tumor cells could provide significant information among women with breast cancer in Libya.

The frequency of aneuploidy was, and so were SPF values higher than in most published data, especially from European countries [Collan et al. 1994, Yildirim-Assaf et al. 2007]. Perhaps this trend can be attributed to many explanations: breast cancer in Libya is of more aggressive type, and diagnosis delay of breast cancer may be associated with this. However, biological difference should not be excluded. That African and European women with breast cancers are different in many respects is one of the dominant discussion aspects today.

A statistically significant association between DNA cytometric variables and clinicopathological variables was found in this study. DNA aneuploidy and high SPF (≥11%) were associated with advanced stage, with high grade tumor, and high risk of metastasis. The same findings were reported previously [Wenger et al. 1993, Spiethoff et al. 2000, Tsutsui et al. 2001, Yildirim-Assaf et al. 2007]. In addition high SPF was associated with positive lymph nodes, and with large pathological tumor size. This study also confirmed previous observations of higher proliferative activity in younger women with breast cancer [Wenger et al. 1993].

DNA aneuploidy tended to be more often associated with positive lymph nodes and with hormone receptor negative tumors, but this did not reach to statistical significance.

DNA ploidy measured by image cytometry is associated with markers of cell differentiation [Spiethoff et al. 2000]. Nuclear DNA content was strongly correlated to histological grade of ductal carcinoma and poorly differentiated tumors were more likely to be aneuploid than others [Spiethoff et al. 2000, Yildirim-Assaf et al. 2007]. This work observed that grade 3 tumors often have DNA content of aneuploid nature with high SPF. In this study, 14% of patients had distant metastasis at diagnosis. All metastases showed aneuploid tumors and high SPF and the aneuploidy group in metastatic tumors had higher histological grade. This is in alignment with the study by Kang et al [2000], which analysed the ploidy status of primary tumors and their corresponding metastatic nodes in breast cancer. The study concluded that the ploidy pattern and SPF in metastatic nodes might be considered as risk factors in breast cancer patients with positive axillary node. Moureau-Zabotto et al [2005] suggested that combination of DNA ploidy and SPF predict patient's outcome particular in LN-negative breast cancer patients. So, DNA ploidy results can be combined with other features in efficient evaluation of prognosis.

This work also showed that the tumors with higher DNA content were more often found with the presence of lymph node metastasis. This finding needs further assessment. We can anticipate that tumours with aneuploidy might be more aggressive and more likely to be associated with LN involvement at diagnosis, and were associated with shorter survival. Many observers stated that aneuploid tumors are associated with a poorer prognosis than diploid tumors in LN-negative breast cancer patients [Spiethoff et al. 2000]. However, an adverse correlation was stated by Chassevent et al [2001].

In this study also important observation is the one linking DNA content with the disease outcome i.e. appearance of recurrence and length of survival. This is clinically relevant for several reasons. The patient with breast cancer who present at any stage of disease, it would be of paramount importance to find good prognostic markers that would accurately distinguish those patients, who should be considered for intensive therapy.This work revealed that patients with aneuploid DNA histograms and high SPF values had shorter survival time than those with diploid DNA histograms and low SPF values. Also short survival was associated with multiploid DNA histogram and with DNA aneuploid cells of ≥ 5. This finding is in agreement with the results of other studies [Tsutsui et al. 2001, Yildirim-Assaf et al. 2007].

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In a Cox multivariate analysis, DNA ploidy, age, and clinical stage were independent predictors of overall survival. DNA ploidy and clinical stage also proved to be independent predictors of disease-specific survival.

The decision cut points of 11% for SPF in the Libyan breast cancer tumor material was clearly significant ($p<0.0001$), and suggesting that this value might be used as quantitative criterion for separating patients into two groups with good and poor prognosis.

6. 3 Proliferation markers: Ki-67 and S-phase fraction (III)

The clinical course of breast carcinoma is partially unpredictable despite enhanced improvements in diagnoses and therapies. The patient outcome with neoplastic disease in terms of local recurrence, distant metastases and progression, is the major focus of several studies aimed to identify the most reliable prognostic factors. The proliferative rates of tumor have been investigated in an attempt to correlate them with prognosis.

These putative markers include immunohistochemical (IHC) analysis using antibodies directed against proliferation antigens such as Ki-67, cytometric S-phase fraction (SPF), proliferating-cell nuclear antigen, thymidine labeling index and mitotic index [Friedrich et al. 2000, Yildirim-Assaf et al. 2007].

The immunostaining analysis of Ki-67 and quantitatively measurable DNA content (which allows the evaluation of SPF) among women with breast cancer in Libya provide significant prognostic information as observed in this study. In this work it was found that median of the Ki-67 and SPF were higher than published data [Zhang et al. 1997, Jalava et al. 2006]. This difference could be due to differences of methodology, staining and sample preparation, and to variation in the interpretation between centers [Leivonen et al.1994]. It is also suggested that the patients in Libya have aggressive tumor type. Health care aspects are important but biological factors cannot be excluded.

This study confirmed many of prognostic associations between immunohistochemical Ki-67 expression, cytometric S-phase fraction value, traditional variables, and patient outcome [Collan et al. 1994, Urrticoechea et al. 2005, Jalava et al. 2006, Yerushalmi et al. 2010, Nishimura et al. 2010]. Comparisons with European studies are difficult, however, because our material had a greater fraction of advanced cases. Recent studies may suggest biological differences because Libyan cancers have the same fraction of hormone positive tumors although they have worse prognosis [Boder et al. 2011]. Number of patients, however, may be too small for final conclusions.

However, in this work it was found that low expression of Ki-67 was associated with early stages, hormone receptor positive tumors, good differentiation of tumors, negative axillary lymph nodes, low risk of metastasis, and a low SPF. On other hand high Ki-67 and SPF were more common with poor prognostic variables. These data suggest that patients with a lower Ki-67 expression and SPF value had a good prognosis, and patients with a higher Ki-67 expression and SPF value had a poor prognosis.

Some studies compared SPF with immunohistochemical indicators of cell proliferation, such as Ki-67 and proliferating cell nuclear antigen (PCNA) in breast cancer, and reported that SPF was superior in assessing prognosis [Gasparini et al. 1994]. In addition, clearly mitosis-associated evaluations (such as SPF and mitotic count) create stronger prognosticators than less directly mitosis-associated factors (e.g. K-67).

This is probably due to the fact that a fraction of cells which are proceeding in the cell cycle die through apoptosis before reaching mitosis [Jalava et al. 2006]. Anyhow, apoptosis and mitosis have a positive relationship with each other [Huovinen et al. 1993]. This study also observed that the SPF appeared more significantly associated with traditional prognosticators than Ki-67, suggesting that SPF is more useful in assessing prognosis.

One of important observation in this work is the linking of Ki-67 and SPF with the disease outcome, i.e., appearance of recurrence, disease free survival (DFS), and overall survival (OS).
Patients who had tumors with low Ki-67 and low SPF were associated with low rate of disease recurrence, and long survival time. Patients who had tumors with high Ki-67 and high SPF were associated with high rate of disease recurrence, and short survival time.

In this work it was found also that hormone receptor positive patients with high SPF values had poor survival similar to those of hormone receptor negative patients, suggesting that all patients with hormone receptor positive and negative tumors with high SPF value should be considered to intensive treatment. This was also suggested by Lipponen et al [1992], who reported that all women with steroid receptor-negative breast tumours and those receptor-positive tumours with high SPF value should be subjected to postoperative adjuvant chemotherapy immediately. In contrast, a particularly good prognosis was observed among hormone receptor positive and negative patients with low SPF values, suggesting that the need for additional therapy is questionable in hormone receptor positive patients with low SPF value.

Multivariate survival analysis in this study showed that Ki-67 was of borderline significance as an independent predictor for DFS. SPF was an independent prognostic factor for DSS with hormonal status and clinical stage. Dettmar et al [1997] also showed by multivariate analysis that SPF has the highest prognostic value.

In terms of prognostic significance, this is the first study allowing comparison between immunohistochemical Ki-67 expression and cytometric S-phase fraction in the Libyan breast cancer. The results suggest that SPF showed more significant association with prognostic traditional variables than Ki-67, and survival analysis (univariate and multivariate) also suggest that SPF was more useful in assessing prognosis.

6. 4 Immunohistochemical bcl-2 expression (IV)

Apoptosis (programmed cell death) is a physiological process in which cells die after various types of damage and it's an important mechanism for maintaining control over a population of cells under continuous renewal. Cancer cells may interfere with this mechanism by activating genes which inhibit apoptosis [Kumar et al. 2000].

The most important antiapoptotic protein is bcl-2 [Arun et al. 2003]. Bcl-2 is expressed in many types of normal tissue and belongs to a family of proteins which regulate apoptosis [Petros et al. 2004].
In human cancer, bcl-2 seems to acts as both an oncogene and a tumor suppressor gene in different tumor types. Bcl-2 expression in breast cancer is associated with favorable prognostic factors and the best survival [Divito et al. 2004, Callagy et al. 2006, Treré et al. 2007, Callagy et al. 2008, Nadler et al. 2008, Dawson et al. 2010].

This work investigates for the first time the association of the immunohistochemical bcl-2 expression in Libyan breast cancer with clinicopathological variables and patient outcome. Important observations were made and immunostaining analyses of bcl-2 in tumor cells provided significant prognostic information.
Anyhow, material may be too small for final conclusions. Further conclusions can only be drawn after more extensive studies.

Comparison to other published data [Zhang et al. 1997, Bottini et al. 2000], this study shows that patients with breast cancer in Libya have about the same fraction of bcl-2 positive tumors. However, as Libyan tumors have worse prognosis bcl-2 appears as a superior prognosticator, it may be that from biological point of view we have 2 types of Libyan breast cancers: bcl-2 positive ones with good prognosis and bcl-2 negative tumors with poor prognosis. Anyhow, expression of bcl-2 in breast cancer is widely variable [Zhang et al. 1997]. Population variations can be explained by different ways of quantification: intensity and percentage of positive cells [Kröger et al. 2006]; positive immunostaining reaction of more than 10% of tumor cells, or over 25% of staining tumor cells [Zhang et al. 1997, Tang et al. 2004].

This work shows that a lower grade of malignancy as in hormone receptor positive tumors, early stages, negative lymph nodes, small tumor size, and well differentiation of tumor were associated with positive expression of bcl-2, and bcl-2 negativity was more common with worse prognostic variables. Bcl-2 expression has been consistently associated with better prognosis for breast cancer in previous studies [Divito et al. 2004, Kumar et al. 2000, Callagy et al. 2008, Dawson et al. 2010]. These data also suggest that patients with breast cancer in Libya who had bcl-2 positive tumor had a favorable prognosis, and with those negative bcl-2 expression had a worse prognosis.

Bcl-2 expression in breast carcinoma may be an estrogen receptor regulated phenomenon. PR is under estrogen regulation via ER [Malamou-Mitsi et al. 2006, Zinkel et al. 2006]. The bcl-2 oncoprotein has been shown to be expressed in 45-93% of breast cancer, whereas 70% of breast cancer tumors are ER-positive, and most ER-positive cancers are also PR-positive [Bottini et al. 2000, Freedman et al. 2010].

Binding of estrogen with ER induces phosphorylation and dimerization followed by transcription of a variety of genes, including growth and angiogenic factors as well as PR and bcl-2 [Linke et. 2006]. Linke et al [2006] found that the disease-free survival and overall survival for patients with ER-positive/PR-negative tumor, and who had low bcl-2 expression were not significantly different from ER-negative tumor. However, if either PR was present or the bcl-2 expression was high, the ER-positive patients had a better prognosis [Linke et. 2006]. On the other hand, high expression of bcl-2 may be indicative of an intact ER pathway that is driving tumor growth and might be more responsive to hormonal therapy [Linke et. 2006]. The benefit of tamoxifen is better in the patients with ER-positive/bcl-2-positive tumors than in those who had ER-positive/ bcl-2-negative [Elledge et al. 1997, Linke etal. 2006]. The intense association between bcl-2, ER and PR may suggest co-targeting these molecules in hormone receptor-positive breast cancer. Understanding of this may play a role in beneficial planning for response to these tumors to chemotherapy [Nadler et al. 2008].

Furthermore, apoptosis is controlled by the ratio of various bcl-2 family members. When levels of apoptosis promoters (bax and bcl-X_S) increase, apoptosis is accelerated, whereas when the inhibitors of apoptosis (bcl-2 and bcl-X_L) increase, the cells are inclined to be resistant to apoptosis in response to external stimuli. Bcl-2 plays an important role in the inhibition of apoptosis stimulated by different factors, such as irradiation, chemotherapeutic agents, and withdrawal of growth factors [Jäättelä et al. 1995]. In cell culture, high concentrations of estrogen could induce apoptosis directly through a FAS/FASL pathway [Song et al. 2001]. However, physiological concentrations of estradiol could induce apoptosis in both cell culture and animal models [Jordan et al. 2002]. Furthermore, in MCF-7 cells, estrogen has been shown to inhibit cytotoxic drug-induced apoptosis through up-regulating bcl-2 levels [Teixeira et al. 1995].

In breast carcinoma, bcl-2 expression has been shown to be as a favorable prognostic factor, strong associated with ER status, and a predictor of response to endocrine therapy [Zhang et al. 1997]. The association with ER may explain the response to endocrine therapy in patients with bcl-2-positive tumors.This study found that the association between expression of bcl-2, and the expressions of ER, and PR were highly significant.

Bcl-2 expression may create a restrictive environment for the expansion of genetically unstable and potentially malignant cells, causing a delay in tumor progression [Hun et al. 2007]. The bcl-2 positivity in this work is high in small tumor is (T1) and decrease with the increasing size of tumors (T4).

In this study the expression of bcl-2 was also associated with tumors without lymph node metastasis. Bukholm et al [2002], and others [Alis et al. 2008] found a significant inverse correlation between bcl-2 expression and nodal invasion.

One of important findings was appears that patients who had tumors with positive bcl-2 were associated with lower incidence of disease recurrence, lower rate of death and longer survival time. This study also observed that patients who had tumors with high bcl-2 staining intensity (from moderate to strong) were associated with the best survival. The role of bcl-2 as an independent predictor was also assessed in this study. Cox regression analysis showed that bcl-2 and clinical stage were independent predicators of disease-specific survival. For disease-free survival, only bcl-2 proved to be an independent predictor.

Immunohistochemical analysis of the bcl-2 expression in Libyan patients shows that the bcl-2 is powerful prognosticator in both univariate and multivariate statistical analysis, and it has value that is equivalent to lymph node status, tumor size and histological grade.

Comparison between this study and others from Europe (Jalava et al, 2000) is interesting in light of the differences in African and European breast cancer. These differences may be associated with the differences in the health care, or be explained by differences in biology, genetics, or etiology. The results seem to suggest that even through bcl-2 expression is a favorable sign in both studies; the prognostic value is of different degree. The difference between 5-year survival rates was about 70% between bcl-2 positive and bcl-2 negative breast cancer among the Libyan patients. Among European patients this difference was 20% at the highest, but was only seen in postmenopausal lymph node positive (N+) patients. In Europe (Finland) bcl-2 can only be used as a prognosticator in the latter patient group. The question arises whether the difference is caused by stages of development in medical care between Europe and Africa (Libya), or whether there are other causes (including genetic differences). The differences found between Afro-Americans and Caucasian Americans with breast cancer seem to suggest the latter [Olopade et al. 2003, Loo et al. 2011]. However, final conclusions on the issue can only be drawn after more intensive studies in various parts or Africa, Europe and America.

7. SUMMARY AND CONCLUSION

Diagnosis delay (I)

This study shows that diagnosis delay is very serious problems in Libya and it's associated with complex interactions between social, medical and other patient-associated factors leading to advanced tumor stages, potentially resulting in high mortality. There is a need for improving breast cancer awareness and training of general practitioners to reduce breast cancer mortality by promoting early detection. The treatment guidelines should pay more attention to the early phases of breast cancer. Especially, guidelines for good practices in managing detectable tumors are necessary.

DNA image cytometry (II)

This study shows that static DNA cytometry with careful analysis of the histograms may provide valuable prognostic information in Libyan breast cancer, with potential clinical implications in patient management, particularly in predicting the patients at high risk for recurrence and metastasis, who should be considered as candidates for adjuvant therapy. Breast cancers with diploid DNA image histograms are more associated with good prognostic factors, especially with low grade and stage. Furthermore, in Libyan patients the SPF (11%) could be used as criterion for separation of patients into two groups with good and poor prognosis.

Proliferation markers: Ki-67 and S-phase fraction (III)

This work suggests that the SPF is very useful as proliferation cell marker to assess tumor prognosis. Ki-67 and SPF may reflect the aggressive behavior of Libyan breast cancer and predict the recurrence. It is therefore important to take these markers into consideration in selecting patients for the high risk subgroup for intensive treatment.

Bcl-2 expression (IV)

This study has found that bcl-2 is an independent predictor of breast cancer outcome and it is useful in providing prognostic information in Libyan breast cancer. Libyan breast cancers can be classified into two groups: bcl-2 positive ones with good prognosis and bcl-2 negative tumors with very poor prognosis. Thus, it could be used with classical clinicopathological factors to improve the selection of patients for different treatment groups.

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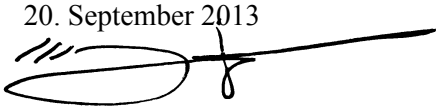

Eramah Ermiah

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