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**NUCLEAR MORPHOMETRY,  
APOPTOTIC AND MITOTIC INDICES,  
AND TUBULAR DIFFERENTIATION  
IN LIBYAN BREAST CANCER**

by

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**Nuclear morphometry, apoptotic and mitotic indices, and tubular differentiation in Libyan breast cancer.**

Institute of Biomedicine, Department of Pathology, University of Turku, Finland  
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**ABSTRACT**

Aims of this study were to evaluate the relations of nuclear morphometry, mitotic and apoptotic indices, and tubular differentiation with clinicopathological features and survival rate in Libyan women. The data were compared with corresponding results on Finnish, and Nigerian female breast cancer patients.

Histological samples of breast cancer (BC) from 131 patients were retrospectively studied. Mitotic activity indices (MAI and SMI), apoptotic index (AI), and fraction of fields with tubular differentiation (FTD) were estimated. Samples were also studied by computerized nuclear morphometry, such as mean nuclear area (MNA). Demographic and clinicopathological features were analyzed from 234 patients.

The Libyan BC was dominantly premenopausal, and aggressive in behavior. There were statistically significant correlations between the mean nuclear area, fraction of fields with tubular differentiation, apoptotic index and proliferative indices, and most clinicopathological features. The highest significances were shown between lymph node status and the proliferative and apoptotic indices ( $p=0.003$  with SMI, and  $p=0.005$  with AI). There were significant associations between clinical stage and SMI and AI ( $p=0.002$  and  $0.009$ , respectively). The most significant associations with grade were observed with MNA and FTD ( $p<0.0001$  and  $0.001$ , respectively).

The proliferative differences between Libyan, Nigerian and Finnish populations were prominent. These indices in Libyan were lower than in Nigerian, but higher than in Finnish patients. The Libyan patients' AI is slightly higher than in Nigeria, but much higher than in Finland.

The differences between countries may be associated with the known variation in the distribution of genetic markers in these populations. The results also indicated that morphometric factors can be reliable prognostic indicators in Libyan BC patients.

**Keywords:** Libyan breast cancer, nuclear morphometry, mitotic indices, apoptotic index, tubular differentiation, demographic features, clinicopathological features

Jamela Mostafa El. Boder

## **Tumamorfometria, apoptoottinen indeksi ja mitoottiset indeksit, ja tubulus-differentaatio libyalaisessa rintasyövässä.**

Biolääketieteen laitos, Patologia, Turun yliopisto, Suomi

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## **TIIVISTELMÄ**

Tämän työn tarkoituksena on arvioida tumamorfometrian, mitoosi- ja apoptoosi-indeksien ja tubulaarisen differentaation suhdetta libyalaiden rintasyöpäpotilaiden kliinispatologisiin piirteisiin ja eloonjäämiseen. Tietoja verrattiin suomalaisiin ja nigerialaisiin rintasyöpää sairastavien naisten tietoihin. Työssä tutkittiin 131 potilaan histologisia rintasyöpänäytteitä retrospektiivisesti. Mitoosiaktiiviteetti-indeksit (MAI ja SMI), apoptoottinen indeksi (AI) ja niiden mikroskooppikenttien osuus, joissa todettiin tubulaarista differentaatiota (FTD) arvioitiin. Myös kasvainsolujen keskimääräinen ala (MAI) arvioidiin tietokoneistettua morfometriaa käyttäen. Libyan rintasyöpäpotilaiden demografisia ja kliinispatologisia piirteitä analysoitiin 234 potilaasta.

Libyan rintasyöpä (BC) on etupäässä premenopausaalista ja käyttäytymiseltään aggressiivista. MNA, FTD, AI, MAI ja SMI olivat selvästi korrelaatioissa useimpiin kliinispatologisiin tietoihin. Merkittävin suhde todettiin imusolmukestatuksen ja proliferaatioindeksien ja apoptoottisen indeksin välillä (SMI  $p=0.003$ , AI  $p=0.009$ ). Histologinen gradus korreloi parhaiten MNA:n ( $p=0.001$ ) ja FTD:n ( $p=0.001$ ) kanssa.

Kasvainten proliferaatioindeksit Libyassa, Nigeriassa ja Suomessa olivat selvästi erilaisia. Libyan indeksit olivat matalampia kuin Nigerian indeksit, mutta korkeampia kuin Suomen indeksit. AI oli hieman matalampi kuin Nigeriassa, mutta selvästi korkeampi kuin Suomessa.

Erot maiden välillä voivat liittyä populaatioiden geneettisiin eroihin. Tulokset myös osoittavat, että morfometrisia tekijöitä voidaan käyttää libyalaiden rintasyöpäpotilaiden ennustetekijöinä.

**Avainsanat:** libyalainen rintasyöpä, tumamorfometria, mitoosiaktiiviteetti-indeksi, apoptoottinen indeksi, tubulusdifferentaatio, demografiset piirteet, kliinispatologiset piirteet

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**ABBREVIATIONS**

AI	Apoptotic index
AJCC	American Joint Committee on Cancer
AKT	alpha serine/threonine-protein kinase
AOI	African Oncology Institute
ATM gene	Ataxia teleangiectasia molecule gene
BC	Breast cancer
Bcl-2	B- cell lymphoma-2
BRCA 1	Breast cancer gene 1
BRCA 2	Breast cancer gene 2
CD	Cathepsin D
CA	Carbohydrate antigen
CEA	Carcinoembryonic antigen
CHEK2	Checkpoint kinase 2
CIS	Carcinoma insitu
CISH	Chromogenic in situ hybridization
CK	Cytokeratine
CNB	Core needle biopsy
CNS	Central nervous system
DCI	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
DSS	Disease specific survival
EGFR	Epidermal growth factor receptor
EGF	Epidermal growth factor
EM	Electron microscopy
EMA	Epithelial membrane antigen
ER	Estrogen hormone receptor
FCM	Flow cytometry
Fd	Field diameter
FISH	Fluorescence in situ hybridization
FNAB	Fine needle aspiration biopsy
FTD	Fraction of fields with tubular differentiation
G0	Gap 0, rest phase of the cell cycle (temporary or permanent)
G1	Gap 1, the interval in the cell cycle between mitosis and S-phase
G2	Gap 2 the interval in the cell cycle between S-phase and mitosis
GDP	Gross domestic product
HBOC	Hereditary breast-ovarian cancer syndrome
HE	Hematoxyline and eosin stain
HER2	Human epidermal receptor



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HMB45	Human melanoma black 45 monoclonal anti body against melanocyte
HPF	High power field
IDC	Invasive ductal carcinoma
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
ILC	Infiltrative lobular carcinoma
LCA	Leucocyte common antigen
LCI	Lobular carcinoma in situ
LN	Lymph node
MAI	Mitotic activity index
MIB	Monoclonal immunoglobolin G anti-body for detection of Ki-67
MRI	Magnetic resonance image
mTOR	Mammalian target of rapamycin
MVD	Microvessel density
MNA	Mean nuclear area
N-CAM	Neural cell adhesion molecule also known as CD56
Nm23	Nucleoside diphosphate kinase metastasis-associated gene 23
NOS	Not otherwise specified
NPI	Nottingham prognostic index
PAS stain	Periodic acid Schiff
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI	Prognostic index
PI3K	Phosphatidyl inositol-3 kinase
PR	Progesterone hormone receptor
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma gene
S	Synthesis phase of the cell cycle (DNA synthesis)
S100	Soluble protein present in cells derived from the neural crest
SMI	Standardized mitotic index, volume fraction corrected mitotic index
SPF	S phase fraction, fraction of cells in the synthesis phase of cell cycle
STK11	Serine/Threonine protein kinase 11
TDLU	Terminal duct lobular unit
TGF	Transforming growth factor
TK1	Thymidine kinase
TNM	Tumor size (T), Lymph node stage (N), Metastasis stage (M)
TP53gene	Gene that encoded tumor protein 53
VEGF	Vascular endothelial growth factor

## **LIST OF ORIGINAL PUBLICATIONS**

The thesis is based on the following publications

- I. Abdalla F, Boder J, Markus R, Hashmi H, Buhmeida A, Collan Y. Correlation of nuclear morphometry of breast cancer in histological sections with clinicopathological features and prognosis. *Anticancer Res* 2009; 29: 1771- 1776.
- II. Boder JME, Abdalla FBE, Elfageih MA, Abusaa A, Buhmeida A, Collan Y. Breast cancer patients in Libya: Comparison with European and central African patients. *Oncol Lett* 2011; 2: 323-330.
- III. Boder J, Abdalla F, Elfageih M, Buhmeida A, Collan Y. Apoptotic activity in Libyan breast cancer. *World J Surg Oncol* 2012; 10:102, 8 pages.
- IV. Boder J, Abdalla F, Elfageih M, Buhmeida A, Collan Y. Proliferative activity in Libyan breast cancer. *BioMed Research International* 2013; 2013:831714, 10 pages.
- V. Boder M Jamela, Abdalla B Fathi, Elfageih A Mohamed, Collan U Yrjö. Estimation of tubular differentiation in female Libyan breast cancer. *JCTR* 2013; 2:34, 5 pages.

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## 1. INTRODUCTION

Breast cancer is a major female public health problem for clinicians, radiologists and pathologists. With skin malignancies excluded, breast cancer ranks as the most common form of malignancy affecting women worldwide, accounting for nearly one of every three cancers diagnosed in Libyan women (Elmistiri 2006, Robert 2006, Sabratha Cancer Registry 2008). In developed countries one in seven to one in ten women who lives to the age of 90 will develop breast cancer. Breast cancer incidence rates in the United States and North European countries are more than five times higher than those in Africa. However, the rates are rising worldwide, and by 2020 it is estimated that 70% of cases will be in developing countries (American Cancer Society 2008, James 2000, Nadia et al. 2007).

Despite advances in diagnosis and treatment, breast cancer contributes to about 15% of cancer deaths in women, second only to lung cancer, and about one of every three affected women will die of the disease (Parkin et al. 2005). About half a million women will die every year from breast cancer worldwide (Coughlin and Ekwueme 2009). Even though screening programs and other advances in diagnosis and improvement in modern neoplastic therapy have improved survival rates for breast cancer during the last thirty years, mortality is still high, especially after extended follow-up.

Breast cancer is common among the Libyan female population, but relatively little is known about the biological behavior and mechanisms involved in the development and progression of the disease in Libya. The high number of premenopausal patients and the shortage of knowledge about the biology of breast cancer among Libyan and African patients may have resulted in difficulties when evaluating population by mammographic screening programs (Bassett et al. 1995, Elmistiri 2006, Sabratha Cancer Registry 2008). After the histological diagnosis is established, the clinician will determine whether the lesion has spread to the side breast. At a localized stage the cancer is potentially curable and the risk of recurrence is low. When cancer has spread beyond breast tissue it is often incurable. At the present time, the cure of breast cancer is limited to patients with localized disease. However, in part of the localized diseases the outcome cannot be predicted reliably. Biological differences between different countries may also cause variation when predicting the outcome.

In other words, it is important to find factors that could pick up patients with aggressive invading potential and recurring tendency. Various types of clinical and pathological prognostic factors may also have different significances in different patient populations.

The intention of this study is to evaluate the morphometric prognostic characteristics of Libyan female breast cancer patients. The findings are compared with those of Nigerian and Finnish breast cancer patients. At the moment we do not know a lot about

basic data on pathological and biological features of breast cancer cases in the Libyan population. However, these features should be well understood for the most appropriate development of breast cancer management in Libyan patients. In addition, such data could help produce a quantitative grading model for Libyan breast cancer patients, which may help in predicting the prognosis.

## **2. REVIEW OF THE LITERATURE**

Mammary gland (breast) may be considered to be composed of complex modified sweat glands. It is liable to modulation as a result of various physiological stimuli affecting all breast tissues elements. The basic structure is composed of a branching ductal system extending from the nipple inward to the breast stroma, which is composed of loose connective tissue and fat tissue lobules.

The ducts have an inner epithelial lining and an outer contractile myoepithelial layer resting on a basement membrane, surrounded by loose fibrous tissue stroma. The terminal duct lobular unit (TDLU) represents the functioning unit of the breast, and usually measures less than 1mm in an adult female breast. Thousands of lobules progress on full differentiation to form the collection of milk producing acini that empty into the duct system; this leads to the nipple through 20 or more distended ductal sinuses ending in orifices opening at the surface. The development of the breast is regulated by a complex mixture of stimulants and inhibitors including steroid hormones, somatomedins, insulin, thyroxin, epidermal growth factor (EGF), insulin-like growth factors (ILGFs), transforming growth factor (TGF $\alpha$  and  $\beta$ ) and prolactin, all of which are needed for mature development (Hunt et al. 2008, Rosen 2009). Most pathological alterations arise from the TDLU and its surroundings, whereas only a few conditions, notably papillomas and ductectasia, are produced in the major ducts (Rosen 2009).

### **2.1 Epidemiology of breast cancer**

#### **2.1.1 Incidence of breast cancer**

Globally, there are more than one million new breast cancer patients each year. After a period of constancy, the incidence of breast cancer began to increase in older women, partially due to mammographic screening in the 1980s (Bray et al. 2004). In Finland, the incidence of female breast cancer is 88/100 000 (Finnish cancer registry 2010). In USA, almost 240,000 women were diagnosed with breast cancer in 2007, of whom 62,000 were carcinoma in situ, and over 40,000 died of the disease (Parkin and Fernandez 2006) (American Cancer Society 2008). The incidence of breast cancer is almost non-existent in females before the age of 25 and uncommon before the age of 30. The incidence increases rapidly after 35 years of age, with significant geographical variation in the mean and median age of breast cancer. In populations of lower risk for breast cancer, such as African and Asian countries, the incidence reaches a plateau prior to menopause and then does not increase further (Chaouki and El Gueddari 1991, Hayanga and Newman 2007, Ikpat et al. 2002, Morris et al. 2001, McBride 2007, Nagata et al. 1997, Parkin and Fernandez 2006, Statistical Information Team 2009, Williams et al. 2006). Breast cancer

in young women under 40 is more often associated with inherited genetic defects, and it is obvious that the fraction of premenopausal cases is related to the studied populations (Hopper et al. 1999). Breast cancer rarely develops in men, but may be more aggressive than in women (Wernberg et al. 2009).

### 2.1.2 Risk factors for breast cancer

The most important step in the primary prevention of breast cancer is to understand and recognize the risk factors and how to avoid the risks. Irrespective of the identification of genetic risk factors (Madigan et al. 1995), about 35% of breast cancer can be explained by risk factors; such as age, family history, modifiable lifestyle (hormonal use, fatty diet, overweight, lack of exercise, and alcohol use) (Madigan et al. 1995, Polednak 1999).

**Aging:** The risk for developing breast cancer increases with age. The risk increases rapidly after 35 years (McPherson et al. 2000, Singletary 2003, American Cancer Society 2012). However, there are significant geographical differences in age-related risks. In European and American populations the mean age of breast cancer patients in years is about 58, but in amongst the African population the mean age of breast cancer is about 43 years (Williams et al. 2006, Ikpat 2002).

**Hereditary Predisposition:** Family history shows the strongest association with an increased risk for breast cancer. A first degree relative with breast cancer may increase the risk by three times (Casadei et al. 2011, Dumitrescu and Cotarla 2005). Familial breast carcinoma is linked to inherited mutations of BRCA1, BRCA2, and P53 genes, observed in 5-12% or more of breast cancers (Ponder 1994, Claus et al. 1996, Syrjakoski 2004, Dumitrescu and Cotarla 2005, Smith et al. 2006). BRCA1 (17q21) and BRCA2 (13q12-13), have gene products that play a role in the repair of DNA double strand breaks. Inheritance of cancer causing mutation predisposes 40-80% of these patients to breast cancer at a young age. Patients also have a greater risk of ovarian cancer and other cancers. Adolescents who carry BRCA1 or BRCA2 gene mutations should begin routine medical checkups at 20 years old (Smith et al. 2006). This is recommended to young females with Li-Fraumeni syndrome, and those with Bannayan-Riley-Ruvalcaba syndromes or Cowden syndrome. The medical checkup should also include females with first-degree relatives of previous syndromes (Saslow et al. 2007). Li-Fraumeni syndrome is associated with germline mutations of TP53 gene and characterized by tumors of brain and adrenals in young age and it accounts for 1% of breast cancer in young women. On the other hand, almost all women with Li-Fraumeni syndrome, who survive childhood cancers, will develop breast cancer (Li et al. 1969). Unlike BRCA genes the somatic TP53 gene mutations are common in sporadic breast cancers.

**Hormonal Status:** Increased exposure to estrogen over progesterone (e.g., early menarche, late menopause, nulliparity, first pregnancy late in life, exogenous intake) plays an important role in breast carcinogenesis (Yager and Davidson 2006). Nulliparous women, or those who become pregnant for the first time after the age of 35, have a two-

to threefold higher risk of breast cancer than women whose first pregnancy occurred before age 25. Metabolites of estrogen can cause mutations or generate DNA-damaging free radicals, or estrogen itself can drive proliferation of premalignant lesions and carcinomas directly or via TGF- $\alpha$  promotion effect (Cavalieri et al 2006, Lieher 1997). Oophorectomy before 35 years old reduces the risk of breast cancer by 30% (Yager and Davidson 2006).

**Previous Cancer of the Breast:** Women with earlier breast cancer have at least a fivefold increased risk of developing a second primary breast cancer, in the same or in the contralateral breast. Hormonal treatment by anti-estrogenic agents decreases the risk of a second primary cancer of the breast. Similarly, ladies with family history of atypical hyperplasia have a higher risk of developing breast cancer (Kelsey and Gammon 1991).

**Radiation:** Females who have been exposed to significant radiation have a high risk of breast cancer. The risk of breast cancer was increased in atomic bomb survivors, and in women irradiated for Hodgkin's disease (Hortobagyi et al. 2005). The increased risk of breast cancer is highest when exposure occurs during the developmental period of children and adolescents. Therapeutic radiation after the age of 40 is not known to increase the incidence of breast cancer. Modern mammographic techniques use extremely low doses of radiation that do not add a practically significant hazard (Hortobagyi et al. 2005).

**Lifestyle factors:** Many lifestyle factors are implicated in breast cancer risk; this includes diet factors, such as fatty diet and alcohol consumption. A diet that is rich in saturated fat raises the risk of breast cancer. Thiébaud et al. (2007) suggest that a diet rich in saturated fat is associated with a high risk of postmenopausal invasive breast cancer. At least moderate alcohol consumption also increases the risk of breast cancer (Chen et al. 2011, Dumitrescu and Shields 2005, Hortobagyi et al. 2005). Smith-Warner et al. (1998) state that each additional 10 grams of alcohol per day will increase the risk of breast cancer by about 10%. Several factors have an uncertain effect on breast cancer risk, e.g., tobacco smoking, viruses, obesity, night work and mothers avoiding breast-feeding. **Table 1** shows a summary of risk factors for breast cancer associated with patient history.

**Table 1** Risk factors for breast cancer which are associated with patient history

<b>Risk factor</b>	<b>Description</b>
<b>Sex</b>	Female to male 50:1
<b>Age</b>	Rare in females younger than 25 years; in Europe most common at the age of 58 years, in Africa most common in premenopause
<b>Family history</b>	Mother or sister with breast cancer (five fold), especially if bilateral or premenopausal
<b>Past history</b>	Previous breast cancer or atypical epithelial hyperplasia
<b>Menopausal status</b>	Late menopause (>50 years)
<b>Menstrual history</b>	Early menarche (<12 years)
<b>Pregnancy history</b>	Nulliparous and late age at first pregnancy (>35 years)
<b>Radiation</b>	History of exposure to ionizing irradiation
<b>Lifestyle-related factors</b>	Fatty diet, alcohol intake
<b>Uncertain risk factors</b>	Lack of breast-feeding after delivery, tobacco smoking, viruses, obesity, night work.

## 2.2 Morphological and pathological types of breast cancer

Even though genomic medicine has been able to classify breast cancers into different genomic classes with different prognostic implications, morphological typing remains the best standard for classification of breast cancer, and provides useful predictive and prognostic data. Breast cancer includes a heterogeneous group of malignant tumors of variable morphology. It is classified into non-invasive and invasive categories

### 2.2.1 Morphology of non-invasive carcinomas of the breast

Non-invasive carcinoma in situ (CIS) type means there is no invasion of malignant cells through the basement membrane of the gland. Non-invasive cancers are further classified into ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS), with incidence ratio of 4:1, respectively.

#### *Ductal carcinoma in situ (DCIS)*

DCIS has increased in incidence (from 5% to 15%) due to screening and early detection programs (Ernster and Barclay 1997). About 25% of DCIS progress to invasive cancer. Also DCIS is a highly heterogeneous group of lesions that can be distinguished on the basis of histomorphological criteria, clinical presentation, biological markers, and risk of progression to invasive cancer (Allred et al. 2008, Quinn and Ostrowski 1997). About 40% of DCIS can be classified as high grade lesions with marked anaplasia, necrosis, high proliferation index, multiple mutations, aneuploidy, high potency for recurrence and tendency to multifocality (Gandhi et al. 1998, O'Malley et al. 1994, Wen and Marsh 2005).

About 5% of comedocarcinomas can have LN metastases (Silverstein et al. 1990, Solin et al. 1992). Low grade non-comedo DCIS has mild anaplasia, low proliferation index,



and shows a much more favorable biology. Low grade DCIS includes cribriform, solid, clinging DCIS, micropapillary and intraductal or intracystic papillary carcinoma variants (Azzopardi 1979, Collins et al. 2006, Griffin and Frazee 1993, Hunter and Sawyers 1980, Pathology Reporting of Breast Disease 2005, Silverstein et al. 1995). Low grade DCIS should be differentiated from papillomas that have areas of atypical hyperplasia particularly after needle biopsy deformed architectures (Harvey and Fechner 1978). Double staining with P63 and high molecular weight cytokeratin might be used to differentiate invasive cancers from in situ lesions (Stefanou et al. 2004). However, intraductal carcinoma in a given patient can have more than a single microscopic structural appearance, cytological grade, or immunocytochemical phenotype (Patchefsky et al. 1989). Several studies concluded that surgical margin status and the biologic features of intraductal carcinoma such as tumor size, nuclear grade and the presence or absence of necrosis are the most important indicators for local recurrence in the breast after breast conservation with or without radiotherapy (Boland et al. 2003, Cheng et al. 1997, Solin et al. 2005).

### ***Lobular carcinoma in situ (LCIS)***

LCIS is an uncommon histopathologic finding; its incidence (1% to 6% of all breast cancers) has not been affected by the introduction of mammographic screening because it is not associated with calcifications or stromal reaction densities (Carter and Smith 1977). LCIS has a low risk for the development of further invasive tumors. In general, LCIS is a disease of premenopausal women, carrying a relatively high risk for multicentricity and bilaterality (Carter and Smith 1977). Morphologically, it is palpable distention of lobules by uniform, round, and loosely cohesive small cells with low nuclear grade (round normochromatic nuclei, minimal mitoses, minimal necrosis and minimal atypia). Myoepithelial cells are generally still present. LCIS carries low risk of invasive carcinoma, only 20% for more than 20 years follow-up. LCIS is a fundamentally different disease than DCIS: it is a marker for overall increased risk. This is why some pathologists prefer the term lobular neoplasia (Hanby and Hughes 2008, Lakhani et al. 2006, Li et al. 2006). Cases of carcinoma in situ that lack E-cadherin and ER and have high-grade nuclei should not be diagnosed as typical LCIS (Hanby and Hughes 2008).

### **2.2.2 Morphology of invasive carcinomas of the breast**

They are classified further into invasive ductal carcinoma NOS, invasive lobular carcinoma, and minor forms of carcinoma with special histological type.

#### ***The classic, not otherwise specified (NOS) invasive ductal carcinoma (IDC)***

They form about 75% to 80% of invasive carcinomas of the breast (Rosen 1979). Approximately 20% expressed combined features (Fisher et al. 1975), including tubular carcinoma component and invasive lobular carcinoma. Their morphology ranges from

neoplastic glands to sheets of highly pleomorphic neoplastic epithelial cells infiltrating fibrous stroma, with variation in mitotic activity and a tendency to show tubular differentiation. Invasive ductal carcinoma can be graded into three groups (I-III) based on the score for each percent tubule formation, nuclear pleomorphism, and mitotic count. Invasion in lymphatic system is about 33%, into perineural space (28%), and into blood vessels (5%).

### ***Invasive lobular carcinomas (ILC)***

This entity represents 10-12% of invasive carcinomas. They are similar to infiltrating ductal carcinomas except they are composed of noncohesive, small, uniform neoplastic cells commonly arranged in a linear pattern of stromal invasion (Indian files), often concentric to form targetoid patterns around benign glands (Cserni 1999, Kiaer et al. 1988). There is a slightly higher incidence of bilaterality (about 20%) (Broët et al. 1996). Nuclear ER positivity is more common than in ductal invasive carcinomas (Jalava et al. 2005). Invasive lobular carcinoma is graded using the same criteria as in other breast carcinomas (Ellis and Elston 2006, Yoder et al. 2007).

Rarely classic ILC shows more than 20% signet ring differentiation, it is named as ***signet ring variant*** (Frost et al. 1995, Steinbrecher and Silverberg 1976), and if most of the cells form sharply outlined groups then it is termed ***alveolar type*** (Shousha et al. 1986). ILC should be distinguished from lymphoma (CK+, LCA-) when the groups fused to form solid pattern (Stenman and Vaheri 1981). When the tumor has areas of invasive lobular and tubular carcinoma, it is named as ***tubulolobular carcinoma***, which more frequently shows multifocality (Esposito et al. 2007, Green et al. 1997, Marchio et al. 2006, Wheeler et al. 2004). Moreover, when the lobular carcinoma is composed of cells showing variability in shape and size with more mitotic activity, it is then classified as the pleomorphic variant of ILC (Eusebi et al. 1992, Reis-Filho et al. 2005). More than 90% of the tumor should exhibit one or more of the above patterns to be classified as lobular carcinoma. The E-cadherin stain can distinguish all variants of lobular carcinoma from ductal carcinomas in most instances (Brandt et al. 2008).

### **2.2.3 Morphologic variants of minor forms of breast carcinoma**

Even though the minor forms are rare types of infiltrative breast carcinoma, they are important to recognize because they have a more favorable outcome than the more usual invasive lobular or invasive ductal carcinomas.

***Medullary carcinoma:*** It contributes for less than 5% of breast cancers with a little higher percentage in Japanese and black American patients (Mitra et al. 1980, Rosen et al. 1989). Medullary carcinoma tends to be large, soft, and well-circumscribed with pushing borders, consisting of syncytial sheets of large polygonal cells associated with prominent lymphoplasmacytic infiltrate (which may contribute to good prognosis)

(Yoder et al. 2007). Medullary carcinomas have a basal-like gene expression profile (Bertucci et al. 2006, Rodriguez-Pinilla et al. 2007).

**Tubular carcinoma:** It tends to be a small tumor (McDivitt et al. 1982, Oberman and Fidler 1979, Rosen et al. 1989) composed of small, irregular angulated infiltrative tubules that are lined by a single cell layer. They show low mitotic activity and mild pleomorphism. Tubular carcinoma should be differentiated from radial scar, and microglandular adenosis; by haphazard infiltration to the stroma and surrounding fat, open lumina, basophilic secretion, apocrine-type apical cytoplasm (Abdel-Fatah et al. 2007, Shen and Sahin 2004).

**Cribriform carcinoma:** It is often seen with tubular carcinoma representing well-differentiated grade and favorable outcome (Venable et al. 1990). Invasive cribriform carcinoma is sometimes mistaken for adenoid cystic carcinoma (Harris 1977). Cell islands are unlike adenoid cystic; they have small uniform epithelial cell type and lack the basaloid cells. Therefore it is S100/CK14 negative and is usually ER and PR positive (Wells and Ferguson 1988).

**Invasive papillary carcinomas and invasive micropapillary carcinomas:** Most papillary carcinomas are in situ carcinomas (Carter 1977, Fisher et al. 1980). Invasive cystic papillary carcinomas with a pushing growth pattern should be distinguished from less favourable invasive solid papillary and micropapillary carcinomas. The latter are also more likely to be ER negative and HER2 positive and may have lymph node involvement (Nassar et al. 2006).

**Mucinous (colloid) carcinomas:** These carcinomas are seen usually in postmenopausal women (Park et al. 2010, Rosen et al. 1985). Mucinous carcinomas form gelatinous lakes of mucoid material in which single or groups of cancer cells float. The margin of mucinous carcinoma is well-circumscribed and determined by the extent of the mucinous component, even if no epithelial cells are seen in a peripheral zone (Goodman et al. 1995). Unlike papillary carcinoma, the calcification of mucin rarely shows psammoma body type of calcifications (Pillai et al. 2007). Pure mucinous carcinomas carry excellent outcome, low risk of LN metastases and recurrence (Andre et al. 1995, Clayton 1986, Park et al. 2010, Toikkanen et al. 1988).

**Juvenile (secretory) carcinoma:** This tumor can be seen in children, but the majority of cases have been reported in young adults (Ashikari et al. 1977). Juvenile carcinoma tends to be small with pushing margins, characterized by prominent secretion and central hyalinization. Larger lesions are found mainly in older patients (Lae et al. 2008).

**Adenoid cystic carcinoma:** This carcinoma is composed of small basaloid cells (S100/CK14 positive; Mastropasqua et al. 2005) surrounding two types of cavity; true glandular space, and eosinophilic cylindrical space filled with a basement membrane material (collagen IV material, positive PAS stain). It may show foci of sebaceous differentiation (Azumi and Battifora 1987). The presence of axillary metastases often indicates pulmonary metastases (Ro et al. 1987, Wells et al. 1986).

**Apocrine carcinoma:** This is a very rare carcinoma. The epithelium has apocrine cytological features: The cells are large and acidophilic and there are PAS positive granules in the cytoplasm (Matsuo et al. 1998).

**Carcinoma with neuroendocrine features:** This tumor includes a carcinoid-like tumor but without the clinical features of a classic carcinoid syndrome (Capella et al. 1990). The neuroendocrine differentiation is confirmed by IHC and E.M. (Miremadi et al. 2002). There are argyrophilic cells arranged in small solid nests, ribbons, or rosettes, separated by delicate fibrovascular stroma (Cubilla and Woodruff 1977).

**Metaplastic carcinoma:** This tumor includes a variety of rare types of breast cancer. The metaplasia can vary in extent from one focus in an invasive breast carcinoma to complete replacement of the whole glandular tumor by the metaplastic phenotype. The latter, pure metaplastic type, accounts for less than 1% of all breast cancers (Carter et al. 2006, Kaufman et al. 1984, Wargotz and Norris 2004). The tumors tend to be large with well-circumscribed borders (Kaufman et al. 1984, Shin et al. 2007, Velasco et al. 2005) consisting predominantly of cells with either squamous cell appearance (squamous cell carcinoma) or with a prominent spindle cell component (sarcomatoid cell carcinoma). They usually show positivity for mesenchymal markers such as vimentin in addition to the variable breast epithelial markers. However, when breast cancer shows prominent expression of both mesenchymal and epithelial markers, the cancer is termed as biphasic sarcomatoid carcinoma (Kaufman et al. 1984). The diagnosis should be confirmed by demonstrating myoepithelial markers e.g. P63 expression in addition to pankeratin and basal cell keratins reactivity (Carter et al. 2006, Leibl et al. 2005). Metaplastic carcinoma of breast is classified as high grade and hormone-receptor negative carcinoma but with limited prognostic value, may be because their low number in the breast cancer studies (Beatty et al. 2006, Carter et al. 2006, Chhieng et al. 1998, Luini et al. 2007, Wargotz and Norris 2004).

#### 2.2.4 Distinctive clinical type of breast cancer

A small number of breast carcinomas have a distinctive clinical presentation such as Paget's disease of the nipple and inflammatory breast carcinoma.

**Paget's disease:** It presents itself clinically as an eczematous-crusted lesion of the nipple and surrounding skin (Chen et al. 2006). It is characterized by the presence of large cancer cells in the epidermis of breast skin, which are different from epidermoid cancer cells because of an abundant mucin positive cytoplasm and a positive reaction for epithelial membrane antigen (EMA), and for low but not high molecular-weight cytokeratin (Ordonez et al. 1987, Shah et al. 1987). They differ from melanoma cells by being negative for S-100 protein and HMB45 (Gillett et al. 1990, Shah et al. 1987). In most cases, there is underlying intraductal carcinoma that may show an invasive component in about one third of cases (Kollmorgen et al. 1998, Shousha 2007). Patients

with Paget's disease have the prognosis of the underlying breast carcinoma (Fourquet et al. 1987).

***Inflammatory breast carcinoma:*** It presents itself clinically as a warm, painful swelling with peau d'orange appearance that mimics an acute mastitis (Chang et al. 1998). Inflammatory carcinoma is the result of permeations of the dermal lymphatics by undifferentiated carcinoma. Some cases may need immunohistochemistry stain to correctly differentiate tumor cells within true vascular space. Inflammatory carcinoma shows higher levels of E-cadherin expression than non-inflammatory carcinoma (Charafe-Jauffret et al. 2004). Inflammatory carcinoma is always categorized as high grade with a very poor outcome (Cristofanilli et al. 2007, Piera et al. 1986). However, the prognosis can be improved by neoadjuvant therapy (Carlson and Favret 1999, Hennessy et al. 2006).

## **2.3 Molecular pathology of breast cancer**

Approximately 5-12% of breast cancers have a familiar pathogenesis often caused by a germ-line mutation of a single gene (Ponder 1994, Syrjakoski 2004, Dumitrescu and Cotarla 2005, Smith et al. 2006). The majority of breast cancers are sporadic and usually caused by somatic mutations caused by agents related to lifestyle and environmental factors that might partially be avoided. Although hereditary breast cancers are rare, it is important that they are diagnosed because patients and their families have an increased cancer risk and they may need or want genetic counselling and screening mammogram (Bradbury and Olopade 2007, Garcia et al. 2008).

### **2.3.1 The multi-step progression model of breast cancer**

Sporadic breast cancer is probably a multi-step process. Progression pathways exist where the normal breast epithelium grows from hyperplasia to carcinoma in situ and finally to invasive cancer, which eventually can disseminate via lymphatics and/or blood vessels. Early events in sporadic breast cancer are often detected as allelic imbalance in cyclin D gene on chromosomal 11q13 (Larson et al. 2002). This will produce an overexpression of cyclin D mRNA as an early event that distinguishes the DCIS from the hyperplastic lesions (Guo et al. 2007, Weinstat-Saslow et al. 1995) and is associated with high telomerase activity (Landberg et al. 1997). The poorly differentiated invasive ductal carcinomas develop from poorly differentiated DCIS. Both lesions are more often characterized by an early p53 mutation (Albonico et al. 1996, O'Malley et al. 1994) and HER2 gene amplification (Bartkova et al. 1990, Collins and Schnitt 2005, van de Vijver et al. 1988) but less expression of ER (Roka et al. 2004) and E-cadherin (Bankfalvi et al. 1999, Gupta et al. 1997). On contrast to well-differentiated invasive ductal carcinomas develop from well-differentiated DCIS, both being characterized by

abnormalities in the E-cadherin gene on chromosome 16 which lead to epithelial cell adhesion abnormality (Asgeirsson et al. 2000, Bürger et al. 1999, 2000, 2001, 2000, Karray-Chouayekh et al. 2012). Therefore it can be concluded that the most common genetic changes seen in invasive breast cancer already take place in the carcinoma in situ stage. Here, the concept of multi-step progression model in breast carcinoma in situ probably differs from the ordinary multi-step progression model that is seen in the colon cancer (Karakosta et al. 2005, Fearon and Vogelstein 1990). In sporadic breast tumorigenesis there are multiple and different mechanisms of tumor progression for different grades (Weinstat et al. 1995). Thus a different breast cancer type could emerge with different cell biological characteristics such as mitotic and apoptotic activity, tubular differentiation, ER expression, metastasizing capability, and different clinical outcomes.

Large-scale gene expression profiling using DNA microarray analysis techniques has resulted in classification into clinically relevant breast cancer subgroups (Sorlie et al. 2003, Van de Vijver et al. 2002, Van't Veer et al. 2002). In one study, based on patterns of expression of over 500 selected genes, a subdivision into five distinct subtypes was obtained (Sorlie et al. 2003). These five subtypes represent different biological characteristics and might be originated from different cell types. One of the five subtypes was characterized by over expression of HER2 and poor prognosis. A second tumor type, lacking expression of the estrogen receptor and also with a poor clinical prognosis, has been termed (basal), as it resembles the pattern found in basal epithelial cells of the normal mammary gland. It is also termed as triple negative subtype (because it is ER-, PR- and HER2-). This basal tumor type differs from two other types; namely: luminal A and luminal B subtypes, which resemble cells that line the duct and give rise to the majority of breast cancers (Sorlie et al. 2003).

### **2.3.2 Examples of genes involved in breast carcinogenesis**

The different steps that are involved in breast cancer progression are thought to correlate with mutation in one or more regulating genes. Mutational activation of protooncogenes to oncogenes accompanied with inactivation of tumor suppressor genes are probably the first gene abnormality in the multi-step progression model. Alteration in genes that are responsible for regulation of proliferation, apoptosis and DNA repair mechanisms could lead to the status of genetic instability, which may result from genetic errors in single nucleotides, in microsatellites, in whole genes or in large part of chromosome (O'Connell 2003). Microsatellite instability can result from germ line mutation or from somatic mutation (Cristi et al. 2001, Balogh et al. 2003).

#### **2.3.2.1 Tumor suppressor genes**

**BRCA1 and BRCA2 genes:** BRCA1 and BRCA2 genes are located in chromosomes 17q12-21 and 13q12-13, respectively (Honrado et al. 2005). Both genes code for proteins that are involved in DNA repair, cell cycle control and in many transcriptional processes; and they

may also play a role in apoptosis as well as in maintaining genomic stability (Jhanwar-Uniyal 2003, Venkitaraman 2002). Females and males with mutation of these genes have an increased risk of breast cancer. Germline mutation in BRCA genes may lead to hereditary breast-ovarian cancer (HBOC) syndrome (Gareth et al. 2008, Metcalfe et al. 2009, Verhoog et al. 2000). In sporadic breast cancer, mutational inactivation of either BRCA1 or BRCA2 gene is of course a rare event (Venkitaraman 2002, Lambie et al. 2003). Whereas, the non-mutational suppression of BRCA1 function (Fraser et al. 2003, Jhanwar-Uniyal 2003, Lambie et al. 2003, Rosen et al. 2003, Venkitaraman 2002), such as by hypermethylation might be equivalent to effect of BRCA genes abnormality in hereditary breast cancer (Hughes-Davies et al. 2003). Metcalfe et al. (2004) reported that the risks for bilateral breast carcinoma increased in females with BRCA1 and BRCA2 mutation carriers. On the other hand, Shahedi et al. (2006) recognized that risk for bilateral breast cancer has also increased in women with familial breast carcinoma not associated with BRCA genes mutation. In general, BRCA-associated breast carcinomas are more aggressive than non-BRCA-associated carcinomas (Ana et al 2011, Robson et al. 2005). BRCA1-associated carcinoma is classified as basal subtype tumor with expression of CK5/6 and is more aggressive than BRCA2-associated carcinoma which is classified as luminal A subtype tumor (Sorlie et al. 2003, Van de Vijver MJ et al. 2002, Van't Veer et al. 2002).

**TP53 gene:** This gene is located on chromosome 17p13.1. and it codes p53 (tumor suppressor protein). TP53 gene is one of the most frequently mutated genes in sporadic breast cancer. Most mutations are point mutations producing nonfunctional long acting p53 protein leading to defect in activation of cell cycle inhibiting genes and the apoptotic genes that resulted in uncontrolled cellular growth (Kastan et al. 1991, Levine et al. 1997). On the other hand, the accumulation of long acting p53 protein in cancer cell nuclei can be detected by IHC (Allred et al. 1993, Kim et al. 2010). Some studies found a significant correlation between p53 and unfavorable aggressive tumor features such as: large size, high proliferation rate, ploidy and dynamic parameters of cell cycle (Allred et al. 1993, Kuopio et al. 1998, Tsutsui et al. 2001, Kim et al. 2010, Oren and Rotter 2010).

Other gene involved in pathogenesis of breast cancer is the retinoblastoma (Rb1) gene located in chromosome 13q14.1, and regulating the expression of BRCA1 via transcriptional activation. Rb1 mutation is common in sporadic breast cancers particularly basal-like tumors, and may play an important role in dictating their aggressive behavior and therapeutic responses (Fung and T'Ang 1992, Herschkowitz et al. 2008). ATM gene, PTEN gene, CHEK 2 gene, CDH1 gene are also contributed in familial and non familial breast cancer (Ueda et al. 1998, Lester and Cotran 1999, Vahteristo et al. 2002, Zhang et al. 2003, Smith and Robson 2006).

### 2.3.2.2 *Oncogenes*

**HER2 oncogene:** Located on chromosome 17q2, and it encodes for a protein that has tyrosine kinase activity (Ali et al. 2002). HER2 protein is a member of the EGFR family

of tyrosine kinase receptors that include EGFR, HER2, HER3, HER4. HER2 could form heterodimers with other members' ligands and it can also spontaneously homodimerize without ligand interaction (Ali et al. 2002). The HER2 gene undergoes an amplification and/or over-expression in about 15-30% of invasive breast cancers. Several studies have concluded that HER2 amplification and/or over-expression is associated with adverse outcome particularly in node positive patients (Gschwind et al. 2004, Jalava et al. 2002, Yeh et al. 2011).

**Apoptosis genes:** The role of apoptosis in oncogenesis is currently being studied intensively in breast cancer (BC) (Liu et al. 2003, Makin and Dive 2003). Apoptosis is needed to destroy cells with DNA damage, or those cells that have become cancerous, several oncogenes, such as Bax and Bcl-2 (van Slooten et al. 1998), c-myc (Koskinen and Alitalo 1993) and P53 (Wyllie 1992) are involved in the regulation of proapoptosis and anti-apoptosis signals are under control of several genes (Liu et al. 2003, Makin and Dive 2003). The bcl-2 regulates the release of mitochondrial proteins such as cytochrome. Cytochrome c binds with other factors to form an activating complex, called apoptosome (Acehan et al. 2002). The active apoptosome activates the caspases, which finally lead to apoptosis (Pinkoski and Green 2005). Steroid hormones are also known to either up regulate or down regulate apoptosis by controlling p53-mediated cell death (Kenemans and Bosman 2003, Bailey et al. 2012).

**Steroid receptor gene:** The estrogen receptor (ER  $\alpha$ ) gene is located on chromosome 6q25.1. It is the most important growth factor receptor involved in hormone-dependant breast carcinogenesis. Estrogen can act as a tumor initiator by causing direct DNA damage or by stimulating cell growth and proliferation supporting the malignant transformation process (Yager and Davidson 2006). Over-expression of ER  $\alpha$  is frequently observed in early stage of breast cancer. The significance of ER  $\beta$  in breast cancer is less clear than that of ER  $\alpha$ . Progesterone receptor isoform (PR-A and PR-B) in breast tumorigenesis is also less clear (Blanco et al. 1984, Harvey et al. 1999, Moelans et al. 2010).

**Invasive and cellular adhesion genes:** Invasion and loss of cell adhesion are essential steps in the metastatic spread of cancer cells. Several genes are involved in this process e.g. N-CAM, E-cadherin, catenins, cathepsin D, collagenase I, CD44, and metalloproteases (Asgeirsson et al. 2000, Ghadimi et al. 1999, Isola et al. 1993, Morris et al. 2001).

**Angiogenesis gene:** Growth and progression of tumors is accompanied by neovascularization (angiogenesis) (Folkman 1990, Kato et al. 2003). Tumor cells in the stroma contribute to an increase of angiogenic factors such as vascular endothelial growth factor (VEGF). Once the breast cancer cells start to produce angiogenic factors, malignant cells will begin to invade the surrounding tissue (Boudreau and Myers 2003).

### 2.3.3 Signalling pathways and cancers

During the last decade molecular pathologist have generated lots of data to distinguish between driver and passenger gene mutations. The somatic driver mutations are



responsible for cancer development and progression. Studies have reported that there are about 140 genes that drive the tumorigenesis (called Mut-driver genes). A typical cancer is caused by two to eight of the driver genes mutations; the remaining mutations are passengers and rarely have selective growth effect. The known driver genes generally act through one or more of 12 signaling pathways that regulate three critical cellular events: cell fate determination, cell survival, and genome maintenance (Vogelstein et al 2013, Leiserson et al 2013, Vandin et al 2012). PI3K/AKT/mTOR pathway (PI3K activation activates AKT which activates mTOR) is an important signalling transduction pathway. In breast cancer and many other cancers, this pathway is overactive and contributes to tumor cell growth by several ways, including the promotion of proliferation, reducing apoptosis, and stimulate angiogenesis (Zhang et al 2013, Grunt et al 2013). Genetic heterogeneity among the cells of the breast cancer is common problem that can affect the response to treatment. Recently, some signal transducers in PI3K/AKT/mTOR pathway provide potential targets for the development of novel anti breast cancer therapies that could be an effective step in developing personalized cancer therapy (Yap et al 2008, Ghayad et al 2010, Grunt et al 2013).

## **2.4 Diagnosis and clinical staging**

Many patients with breast cancer experience no symptoms in the early stages of the disease. When symptoms appear, they vary depending on the size and location of the tumour. In about 80% of the cases breast cancer is presented by painless or painful mass. However, it takes many years for breast cancer to develop from insitu carcinoma and progress to form a mass. Of the breast cancer masses 60% affect the outer hemisphere of the breast (American Cancer Society 2010, Smith et al. 2006). For an early detection of breast cancer at curable stage breast self examination and a mammographic screening program for women older than 40 years has been recommended (Keen and Keen 2009).

### **2.4.1 Triple diagnostic approach**

This approach can provide diagnostic accuracy that may exceeds 99% (Chaiwun and Thorner 2007, Sun et al. 2001). In practice, this approach includes mammographic imaging examination, clinical examination, and fine needle aspiration biopsy (FNAB) and/ or core needle biopsy (CNAB) examination.

Mammography can detect about 40% of impalpable cancers. However, 20% of palpable cancers are not detected by mammography and only 20% of the suspicious mammographic lesions are true cancers. So, cytological or histological examination is mandatory for diagnosis. Traditional ultrasound and MRI (magnetic resonance image) cannot detect microcalcifications (Keen and Keen 2009). Therefore, when radiological

findings are strongly suspicious or considered a proof for malignancy, and the FNAB/CNAB is normal or benign; it should be decided either to repeat the FNAB/CNAB or to do an open biopsy. On other hand, the malignant result of core biopsy in the absence of radiological and/ or clinical evidences of malignancy should be taken as an permission for surgical treatment. When there is strong disagreement between investigations an open diagnostic biopsy is the best choice forward.

Blood tumor markers such as carcinoembryonic antigen (CEA), carbohydrate antigen 15.3 (CA15.3) and thymidine kinase (TK1) levels are recommended to be determined preoperatively. TK1 is raised in more than two thirds of BC patients preoperatively. For the follow-up, CEA is most useful when found to be elevated preoperatively and then normalized after resection of the tumor, CEA may later rise due to recurrence, but only in about 60% of patients ( Elfagieh et al. 2012, Eskelinen et al. 1997)

## **2.5 Clinical staging of breast cancer tumors**

Tumor stage (**Table 2**) at the time of the surgical treatment is the most important guide for decision of breast cancer treatment. Traditional clinical staging and TNM staging system support each other (AJCC Cancer Staging Manual 2006, Rosen and Groshen 1990). The axillary lymph nodes are commonly involved (40-50%), while supraclavicular or internal mammary nodes in only about 20% of cases are involved. Supraclavicular nodes are unlikely to be involved if there is no axillary nodes involvement. Enlarged axillary LN in an adult female with unknown primary will usually be an example of breast cancer or malignant melanoma (de Vries et al. 2009). It is reminded that also basal-type breast cancers can be positive for S-100 (McKiernan et al. 2011) and it is recommended to use Melan-A for diagnosis. Breast cancer spreads either by direct invasion, through lymphatic, and/ or hematogenous spread, and produces recurrence within months, years, or decades after initial therapy. The common sites of breast metastases are bone, lung, pleura, peritoneum, liver, adrenal, and CNS (Nikolić et al. 2012). The invasive lobular cancer also metastases to stomach and bowel (Arrangoiz et al. 2011, Nikolić et al. 2012).

At initial presentation, about 10% of patients have distant metastasis, 25-40% of which have lung deposits. Several authors support the idea that breast cancer is a systemic disease from the beginning, due to vascular permeation (Lee et al. 2006, Nakagawa et al. 2007) Therefore, systemic treatment is recommended rather than an intensified loco-regional therapy to achieve long-term survival.

**Table 2** TNM\* staging of breast cancer.

Stage	T	N	M	
Stage 0	Tis	N0	M0	<b>T= primary tumor:</b> TX=Primary tumor cannot be assessed, T0=No evidence of primary tumour, Tis = Carcinoma in situ: intraepithelial.
Stage I	T1	N0	M0	
Stage IIA	T0,1	N1	M0	T1 = Tumor size < 2cm, T2 = Tumor size 2-5cm, T3 = Tumor size >5cm, T4 = Tumor of any size with direct extention to the chest wall-and/ or to the skin (ulceration or skin nodules).
	T2	N0	M0	
Stage IIB	T2	N1	M0	<b>N=Regional Lymph Nodes:</b> NX=Regional lymph nodes cannot be assessed, N0=No regional lymph node metastasis, N1=Metastasis in 1 to 3 axillary lymph nodes, N2= Metastasis in 4 to 9 axillary lymph nodes, N3= Metastasis in $\geq$ 10 axillary lymph nodes, or metastasis in infra- or supraclavicular nodes.
	T3	N0	M0	
Stage IIIA	T0,1,2	N2	M0	<b>M=Distant Metastasis:</b> MX=Distant metastasis cannot be assessed, M0=No distant metastasis, M1=Distant metastasis
	T3	N1,2	M0	
Stage IIIB	T4	Any N	M0	
Stage IIIC	Any T	N3	M0	
Stage IV	Any T	Any N	M1	

\*TNM classification presented here is accordance to AJCC Cancer Staging Manual 2006

## 2.6 Prognosis

The five-year survival of breast cancer is variable from areas of relatively low survival rate (Asian, East Europ, and African countries) to areas of higher survival rate (the United States, Western Europe, Northern Europe and Australia) (Jemal et al 2006, Parkin et al. 2005). For example, in the USA and North Europe, the five-year survival from breast cancer is more than 85% and 70%, respectively. Survival rates in East Europe and developing countries are less than 50%. (Jemal et al 2006).

Many factors can predict the risk of recurrence, overall survival and outcome of treatment. During the past several years, it has been well established that several clinical and histomorphology features, such as histological type, tumor grade, TNM and clinical stage, lymph node status, tumor size, proliferative activity, apoptotic activity, associated CIS, steroid hormone status include the factors that provide an estimate of the prognosis and suitable treatment of patients with breast cancer.

Other markers have potential value in predicting the clinical outcome of cancer patients. These include vascular and lymphatic invasion, angiogenesis (MVD), immunohistochemical proliferative indices such as PCNA, and MIB-1 staining, S-phase fraction (FCM), and DNA ploidy (FCM), tumor suppressor gene mutation (BRCA, Rb, p53, E-cadherin, mn23), DNA repair genes, HER2 status, and microarray profile.

Several studies concluded that breast cancer prognosis can be evaluated by combining classical prognostic factors particularly (morphometric features, tumor size, and lymph node status) (Kronqvist 1999, McBride et al. 2007)

## 2.6.1 Classical prognostic markers

### 2.6.1.1 Histological type

Breast cancer can be invasive (extending into the surrounding stroma) or non-invasive (confined just to the ducts or lobules). **Table 3** shows the major histologic types of non-invasive and invasive breast carcinomas, along with an estimate of type-associated prognosis along. The data are modified from Rosen 2009 and Tavassoli, Devilee 2003. Histological type is not an efficient prognostic factor. Histological grade is much more significant.

**Table 3** The major histological types of breast carcinoma, along with an estimate of type-associated prognosis (modified from Rosen 2009, Tavassoli, Devilee 2003)

Prognosis	Histological type
Excellent prognosis	Ductal carcinoma in situ (DCIS)
	Lobular carcinoma in situ (LCIS)
	Paget's disease of the nipple
	Papillary carcinoma
	Tubular carcinoma
	Cribriiform carcinoma
	Adenoid cystic carcinoma
Relatively good prognosis	Mixed tubulolobular carcinoma
	Medullary carcinoma
	Mucinous (colloid) carcinoma
Poor prognosis	Invasive ductal carcinoma (NOS)
	Infiltrating lobular carcinoma
	Neuroendocrine differentiation carcinoma (carcinoid-like tumor)
	Metaplastic Carcinoma
	Inflammatory carcinoma

\*NOS = *Not otherwise specified*

### 2.6.1.2 Histological grade

Because breast cancer is a heterogeneous disease, the histological type of the diagnosis alone does not satisfy the clinician for the final treatment decisions. Therefore histological grading systems have been developed. The grading system is good enough for the diagnostic and therapeutic decision making, but the subjectivity of the microscopists produces a limited number of overlaps and misgraded cases (Collan 1989a, b, Collan and Pesonen 1989, Kronqvist 1999). The grading systems were historically the earliest predictive factors for the tumors to be described and investigated. The first documentation on a histological grading system for breast cancer was published by Greenough in 1925. Greenough classified breast cancers according to the cyto and histomorphologic features (Kronqvist 1999). Bloom and Richardson (1957) produced a numerical scoring system

to evaluate the mitotic rate, nuclear pleomorphism, and the presence or absence of tubule formation. Each criteria was scored from 1 to 3. The sum of scores gives a grade for the tumor (G1 scored 3-5, G2 scored 6-7 and G3 scored 8-9).

In the 1980s the grading system by bloom and Richardson was modified by The Nottingham researchers and others who added valuable modifications regarding the field size that varies from microscope to microscope (Elston and Ellis 1991, Haapasalo et al. 1989). The modified Bloom and Richardson grading system has prognostic significance in almost all histological types of breast cancer. Cancers that have low grade have better prognosis than high grade tumors. The prognostic value of the grading system was also confirmed in early stage cancer with size less than 1 cm and without lymphnode involvement (Pereira et al. 1995).

The histological grading system gives highly reproducible results and identifies well-differentiated tumors with good prognosis, and poorly differentiated tumors, which progressing rapidly and have unfavorable prognosis (Collan et al. 1992), still, it is subjective, and leaves a large group of patients with unclear prognosis. New concepts have emerged suggesting that the grading system could be more objective and reliable for prediction of tumors outcomes. Quantitative histopathology offers a wide range of methods for unbiased assessment, as was shown by nuclear morphometry (Boon 1982, Schondorf and Naujoks 1985). Quantitative histopathology also allows reliable measurement of mitotic and apoptotic indices as well as the exact fraction of tubular differentiation which are able to distinguish between favorable and non favorable tumors. It was suggested that the quantitative methods in combination with other objective prognostic criteria can also predict the response to therapy (Kronqvist et al. 2000, 1998a, 1998b, 1999b).

### ***2.6.1.3 Mitotic activity***

The mitotic count is the best prognosticator of survival in breast cancer in Caucasian human population, particularly in lymph node negative patients (Aaltomaa et al. 1992, Elzagheid et al. 2006, Haapasalo et al. 1989, Ladekarl and Jensen et al. 1995, Van Diest et al. 1992). The classic morphologic parameters of tumor growth are mitotic indices (MAI and SMI); both are independent prognostic factors (Collan et al. 1996, Jalava et al. 2006). There is clear evidence that this is also true for African breast cancer (Ikpatt et al. 2002). There are more premenopausal breast cancer patients in African population than in European population (Ikpatt et al. 2002).

### ***2.6.1.4 Apoptotic activity***

Apoptosis or programmed cell death is a carefully coordinated collapse and death of the cell, associated with nuclear DNA fragmentation and protein degradation, and usually followed by rapid engulfment of remaining nuclear material by neighboring cells. It is an essential part of life for every multicellular organism (Tavassoli and Devilee

2003). Apoptosis plays a major role throughout life, from embryonic development to senescence. Apoptosis is needed to destroy cells infected with viruses, cells with DNA damage, and cancer cells. Several oncogenes, such as Bcl-2 (van Slooten et al. 1998, Dawson et al. 2010, Hanahan et al. 2011), and p53 (van Slooten et al. 1998, Wyllie 1992, Vousden et al. 2009) are involved in the regulation of apoptosis. The role of apoptosis in oncogenesis is currently being intensively studied in breast cancer.

There are several methods to detect apoptosis. Immunohistochemistry can detect death receptors and ligands, but also morphologic identification through microscopy is possible. Several studies concluded that apoptosis is strongly correlated to the histological grade of ductal carcinoma, in that poorly differentiated tumors show higher AI than well differentiated tumors (van Slooten et al. 1998, Zhang et al. 1998, Zheng et al. 1998). An earlier study has shown significant differences in apoptotic activity among patients from different populations (European and Nigerian) (Ikpatt et al. 2002). The same study has shown that apoptotic count is predictive in prognostic survival of breast cancer in Europe and in Nigeria women.

#### **2.6.1.5 Tubular differentiation**

Many studies have suggested that tubular differentiation could have potential as a prognosticator in different adenocarcinomas (Dalton et al. 1994, Fisher 1986, Theissig et al. 1990). Other studies, however, do not agree (Baak et al. 1985, Clayton 1991, Lipponen et al. 1991, Parham et al. 1992, Schumacher et al. 1993). Kronqvist et al. (2000) found that the FTD was an independent prognosticator in Finnish breast cancer. The difference in significance was smaller than in proliferative indices. Also Ikpatt et al. (2003) showed that tubular differentiation has prognostic significance in Nigerian breast cancer. However, mitotic activity (SMI, standardized mitotic index) is a better general prognosticator than the FTD. But FTD can add some valuable prediction particularly in premenopausal patients, when their tumors are large.

#### **2.6.1.6 Nuclear morphometry**

One of the most important prognostic and predictive markers in various human malignancies is the nuclear morphometry, which has been proven valuable in many connections (Baak et al. 1985, van Diest and Baak 1991). Currently, computer-assisted image analysis (nuclear morphometry) provides a powerful tool for high-precision measurement of several variables characterizing the size and shape of cancer cell nuclei in conventional tissue sections from solid tumors (Deans et al. 1993, Dundas et al. 1988). Several of these nuclear profiles seem to be useful prognostic predictors in breast cancer and other solid tumors (Jalava et al. 2001, Zhang et al. 2000). As expected, the nuclear size is usually larger and nuclear shape more irregular in undifferentiated cancers than in well differentiated cancers (Buhmeida et al. 2000, Kazanowska et al. 2004). Baak and Oort (1983) observed that nuclear morphometry has important prognostic roles in

separating long survivors amongst local stage patients from those who develop systemic disease. Since then, many histological studies (Epstein et al. 1984, Partin et al. 1989) have used nuclear morphometry to predict prognosis in patients with other cancers. Besides the prognostic and predictive power of morphometry, Elzagheid et al. (2003), and Abdalla et al. (2008) showed that nuclear size features are useful in distinguishing between different atypical groups of the breast gland lesions in fine needle aspiration biopsies.

#### **2.6.1.7 Clinicopathologic stage**

In clinical decision-making, the practical approach is to summarize the basic characteristics of a tumor by staging. Staging of breast carcinoma is in practice based on (TNM) classification (Table 2). Together with the morphological grade, the stage summarizes the basic clinicopathological aspects of the tumor. This provides a practical method for making the tumor management decisions in line to expectable behavior and prognosis. Staging is the most important predictor of survival. More than 90% of patients with stage I disease survive 5 years but the figures markedly drop in higher stages (Rosen and Groshen 1990, AJCC Cancer Staging Manual 2006).

#### **2.6.1.8 Lymph nodes and sentinel lymph nodes**

Axillary lymph node status is the most reliable clinicopathological prognosticator for breast cancer. Lymph node involvement is associated with unfavorable outcomes and the prognosis becomes worse as the number of lymph node metastases rises and/ or the capsule of lymph node is involved (Fisher et al. 1984, Hartveit and Lilleng 1996, Mambo et al. 1977, Nemoto et al. 1980, Rosen 2009). Recently sentinel lymph node biopsy is replacing axillary lymph node dissection in the management of early stage breast cancer (Jakub et al. 2003). Because sentinel nodes are more likely to contain metastatic disease than non sentinel nodes, and because their status determines whether or not there is need for radical axillary clearance, sentinel node status is important. Furthermore detection of micrometastasis with immunostains and molecular diagnostics of PCR can be applied (Jakub et al. 2003, Schoenfrid et al. 1994, Weaver 2003).

#### **2.6.1.9 Angiogenesis (neovascularisation)**

The neoplastic tissues induce vascular proliferation supporting cancer cell survival and growth through angiogenic factors, such as vascular endothelial growth factor (VEGF) (Folkman et al. 1989). Several authors have found that angiogenesis associated with breast carcinoma has significant prognostic influence (Gasparini 1996, Gasparini et al. 1992, Harris et al. 1993, Heimann et al. 1996), particularly in lymph node-positive (Harris et al. 1993, Simpson et al. 1996) breast carcinoma. Tumors rich with neovascularisation (high microvessel density with over-expression of endothelial surface receptor for VEGF) are associated with poorly differentiated duct carcinomas (Gasparini et al. 1992, Harris et al. 1993, Hilmi et al. 2012) and with axillary nodal metastases (Harris et al. 1993).

However, studies have failed to detect this significant relationship to prognosis (Goulding et al. 1995, Siitonen et al. 1995). One cause for the above discrepancies may be associated with methodological variability in the use of different markers to highlight blood vessels (CD31, CD34, and factor VIII), different methods for micro-vascular counting (manual, or image analysis), or differences in vascular distribution in different parts of the tumor.

The angiogenic ability of cancers is the basis for new therapy of the cancers by using the antiangiogenic agents (Hicklin et al. 2005, Teicher et al. 1994). Several new anti-angiogenic drugs (including endostatin, angiostatin, and Bevacizumab) have been proven to inhibit the neovascularization of tumors and to improve the efficacy of chemotherapy (Fan et al. 2012, Miller et al. 2005).

#### **2.6.1.10 Prognostic index**

The prognostic significance of the clinicopathological features can be separately evaluated for each factor. Several authors concluded that combining the most important clinicopathological factors in multivariate models can provide better prognosticator than a single clinicopathological factor. Nottingham researcher group produced a reliable and practical prognostic index including tumor size, lymph node status, and Nottingham histological grade in the following combining formula:

$PI = [0.2 \times \text{size}] + [\text{lymph node stage (N)}] + [\text{Nottingham histological grade}]$ . (N) is the number of lymph nodes involved; 0 = 1, 1-3 = 2, >3 = 3. The index values give a prognostic behavior of the tumor ( good index < 3.4, moderate index between 3.4 to 5.4 and poor >5.4) (Elston and Ellis 1991, Galea et al 1992). This index gave better separation of the best, moderate and worst prognostic groups than methods using tumor size, grade or lymph node metastases alone.

### **2.6.2 Molecular prognostic markers**

Mammographic screening programs detect more tumors in the earlier stages. However, the prognostic value of clinicopathological features in these early stage tumors is limited. New markers that can be used in these groups of tumors should be found. The improvements in molecular biology promises new prognostic factors such as oncogene and anti-oncogenes mutation or amplification, and expression of hormonal receptors, and growth factor receptors, and cell proliferation markers. They are not only for prognosis, but also for selecting the best individual targeted therapy. These molecular factors are especially important in the early stage of breast cancer patients. However, most molecular markers are still controversial, and are not routinely used. The traditional histological factors are still considered the most valuable prognostic factors (Fitzgibbons et al. 2000).

#### **2.6.2.1 Oncogenes and anti-oncogenes**

Cancer is a disease caused by alterations or mutations of specific genes. These mutations may be acquired or inherited in the germ line. HER2 and TP53 are currently the most



common genes examined in breast carcinomas to provide prognostic information. The overexpression of the oncogenes and/or mutation of the antioncogenes often indicate poor prognosis.

**HER2:** It is a gene that codes for a transmembrane glycoprotein with major homology to the epidermal growth factor receptor (EGFR). Slamon et al. (1987) first reported that breast carcinomas with amplified HER2 are associated with high rate of recurrence and short survival, but only in positive lymph node patients. It is well known that tumors that show over-expression of HER2 are associated with high grade of malignancy and high proliferative rates and have earlier recurrences and they metastasize faster (Thomas et al 2012, Yeh et al. 2011, Kaufmann et al. 2011). Excess HER2 may lead to tamoxifen resistance and increase in adriamycin chemotherapy sensitivity (Collins and Schnitt 2005).

A high proportion of DCIS is known to be positive for HER2 (predominantly high grade DCIS) suggesting that amplification of this protein is one of the early events in the process of tumorigenesis. Determination of HER2 protein over-expression by immunohistochemistry (IHC) has become an important method to investigate the HER2 status in breast cancer specimens. However, IHC results can be affected by technical factors and variation in sensitivity and specificity of commercially available antibodies, as well as inter observer variation due to subjectivity. Fluorescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH) methods are now considered to be the most sensitive techniques for exact evaluation of the HER2 gene amplification in breast cancer cells. Her2 determination has now become necessary in the selection of patients who should be targeted with trastuzumab therapy (Herceptin®) (Hurley et al 2006).

**Bcl-2 family:** These are intracellular mitochondrial proteins which have an apoptotic regulatory function in normal cells. The Bcl-2 family proteins consist of apoptotic inducers and inhibitors proteins. Bcl-2 is one of the most important antiapoptotic proteins that have been expressed in many types of human tumors (Petros et al. 2004). Bcl-2 expression in breast carcinoma has been usually associated with well differentiated tumors and longer overall survival (Ali et al. 2012, Dawson et al. 2010, Nadler et al. 2008, Ermiah et al 2013).

**p53:** Is an anti-oncogene whose alterations appear to be the most common genetic changes recognized in cancers. Mutations or deletions result in uninhibited cellular proliferation and they have been involved in many solid malignancies including prostate, colon, lung and breast cancer (Kim et al. 2010, Levine et al.1997, Stricker et al. 1996). Deletions in this gene have been identified in more than 50% of invasive breast cancers. The half-life of the wild type (normal) protein is short (6-30 min), without reaching high enough levels to be immunohistochemically detected. Mutant protein, by contrast, has an extended half-life, and its accumulation can be detected by immunohisto-chemistry stains (Allred et al. 1993, Kim et al. 2010). Expression of p53 has been reported to

correlate positively with ER negative tumors, high proliferative index, high histological grades, shorter disease-free interval and poorer overall survival (Oren and Rotter 2010, Kim et al. 2010, Kuopio et al. 1998, Tsutsui et al. 2001). No correlation has been shown with lymph node status. The role of p53 as a prognostic marker needs to be defined further (Allred et al. 1993, Kim et al. 2010, Rolland et al. 2007).

#### **2.6.2.2 *Hormone receptors***

The determination of estrogen receptors (ER) and progesterone receptors (PR) in breast cancer are important in treatment selection. More than two thirds of breast cancers are expressing ER and PR (Abdalla et al 2012, Freedman and Winer 2010). However, the expression of hormonal receptors is controlled by several factors such as menopausal status, histological type, HER-2 overexpression and BRCA genes mutations (Montemurro et al. 2012). Several studies shows an excellent correlation between ER positivity and response to hormonal treatment. The expression of both ER and PR increases the probability of response to the hormonal therapy. On the other hand, the positive ER and PR tumors are usually well differentiated; and many studies have confirmed that patients with those tumors have longer disease free survival and overall survival than ER and PR negative tumors (Arpino et al. 2004, Blanco et al. 1984, Collins et al. 2005, Ellis et al. 2001, Hilf et al. 1980, Jalava et al. 2005, Nadji et al. 2005, Goldhirsch et al. 2011, Prat et al. 2012). The traditional biochemical binding assay method was replaced by more accurate immunohistochemical staining (IHC) method and results were more accurate and sensitive (Allred et al. 1998, Bartlett et al. 2011). However, fixative choices and fixation times may affect the IHC detection of the receptors. Therefore, it is recommended to perform the staining en block containing normal breast tissue as internal positive control (Hammond et al. 2010). Some studies suggest that the presence of even a very small number of positive cells is enough to predict a good response to hormonal therapy (Goldhirsch et al. 2003, Hammond et al. 2010).

#### **2.6.2.3 *Insulin-like growth factor (IGF)***

The IGF system includes ligands IGF-1 and IGF-2, receptors IGF-1R and IGF-2R, and six known IGF-binding proteins. Many observers suggested that the IGF system is an important player in breast cancer growth, progression and metastasis (Toru et al 2012, Ellis et al. 1998, Yu et al. 1996). An overexpression of IGF-1R is usually associated with well differentiated tumors and favourable prognosis particularly in LN+ breast cancer patients (Eppler et al. 2002, Toropainen et al. 1995) IGF-1R targeting therapies may have some benefits in treatment of patients with breast cancer and other solid tumors (Aleksic et al. 2010).

#### **2.6.2.4 *Immunohistochemical cell proliferation markers and ploidy analysis***

In addition to classic method for measuring the tumor cell proliferation (mitosis counting) there are other tools that can reflect the status of cell proliferation such as:

**Estimation of S-phase fraction (SPF) and DNA ploidy** by flow or static DNA cytometry. Several studies have concluded that aneuploid DNA content and high S-phase fraction are significant prognosticators and associated with unfavorable prognosis (Bocking et al. 1989, Buhmeida 2006, Collan et al. 1992, Karra et al. 2012). Recently this was also shown in Libyan breast cancer (Ermiah et al. 2012a, Ermiah et al. 2012b). In addition to the prognostic role, the DNA content can also be helpful in distinguishing between benign and malignant breast lesions (Abdalla et al. 2010, Elzagheid et al. 2004).

**Immunohistochemistry:** Antibodies against proteins that are expressed during the cell proliferation have been applied to the quantitative analysis of the proliferation activity in the tumor tissues. For example, **Ki67** is a nuclear antigen that is expressed during the active phase of the cell cycle (G1, S, G2, M), and absent in resting cells (G0) (Gerdes et al. 1991, Isola et al. 1990). The MIB (MIB1, MIB3) antibodies recognize Ki67 in paraffin embedded tissue (Romero et al. 2011). The **PCNA** is a protein that also associated with the proliferation by reacting with DNA polymerase, and thus it is important role in the DNA repairing system. It has highest levels of expression during the cell replication (M) phases (Hall and Coates 1995, McCormick and Hall 1992, Strzalka and Ziemienowicz 2011, Stoimenov and Helleday 2009). There is high correlation between the expression of PCNA and S-phase fraction (SPF) determination by flow cytometry, as well as with mitotic index and nuclear grade. Some authors showed that high expression of the proliferative markers Ki67 or PCNA indicates a poor prognosis, high grade tumors, lymph node metastasis and shows a correlation with hormone receptor negative tumors such as luminal B breast cancer type (Aaltomaa et al. 1993, Ermiah et al. 2012b, Jalava et al. 2006, Jeziorski et al. 2000, Santamaria et al. 2005 Cheang et al. 2009, Yerushalmi et al. 2010, Nishimura et al. 2010).

### 2.6.2.5 Adhesion molecules

Several glycoproteins act as glues between the cells.

**E-cadherin** is the most important member of the glycoproteins family functioning as a cell adhesion molecule between the epithelial cells (Lipponen et al. 1994, Harris et al. 2010). E-cadherin expression is completely lost in invasive lobular carcinoma and LCIS and that feature can be useful in distinguishing diagnosis of lobular carcinoma from ductal carcinoma (Bratthauer et al. 2008, Charpin et al. 1997). Some authors have stated that lack or reduction of E-cadherin expression have been associated with high grade carcinoma and with unfavourable prognosis in LN+ but not in LN- cancers, particularly when combined with Her2 status (Elzagheid et al. 2006, Karray-Chouayekh et al. 2012).

**Catenins** are intracellular proteins that have an effect as glue between the epithelial cells (Hirohashi 1998). Catenins (alpha, beta and gamma) are important for the stabilisation of adhesive effect of E-cadherin and for the maintenance of a contact inhibition that has role in preventing cells to proliferate and migrate (Tripathi et al. 2012).

Some studies have recognized that reduction of catenins expression may be associated with poor prognosis (Uchino et al. 2010).

#### **2.6.2.6 Microarray analysis**

Recent techniques such as microarray analysis (gene expression profiling) has allowed for the measurement of thousands of genes in a single RNA sample. The RNA extracted from the tumor, is labelled with fluorescent dyes and hybridized with gene specific probes. This new technique has provided a new molecular classification of breast cancers (Benjamin et al 2012, Alan et al 2011, Perou et al 2000). Cancers are grouped (**Table 4**) into five major groups according the patterns of gene expression in each individual group: (1) Luminal A: Cancers that are ER positive and HER2 negative. (2) Luminal B: Cancers expresses ER but they are more aggressive (intermediate or high grade, high level of proliferation) and they may over-express of HER2. (3) Normal breast like: Usually well-differentiated tumors. This group was classified according the similarity to fibroadenomas and normal breast tissues; it was characterized by high expression of basal cell genes and low expression of luminal epithelial genes and enriched for genes usually expressed in adipose tissue (Peppercorn et al. 2008). However, some authors denied the existence of this group and suggested that the entity is just a results of contmination (Jones et al. 2012). (4) Basal-like: Expression of basal markers such as p63 and P-cadherin, CK5, 5/6, 17, 14, and EGFR is associated with these tumors. The tumors' lack of hormone receptors and HER2 expression i.e. they are so called triple negative. Triple negative and basal-like tumors share many characteristics but the entites are not synonymes. Most breast cancers with germline BRCA1 mutations belong to this group (Gazinska et al 2013, Cancer Genome Atlas Network 2012). (5) HER2 positive: These tumors are ER-negative and they over-express HER2. The cancers are usually poorly differentiated, they have a high proliferation rate, and they are associated with distant metastasis (Weigelt et al 2010, Sorlie et al. 2003, Van de Vijver et al. 2002, Van't Veer et al. 2002).

Commercial arrays are now available from several companies but some may not be purified especially if the RNA is not well-prepared. Recently, it has been reported that the data from the expression of 70 genes is quite enough to define the prognostic signature of breast carcinomas in premenopausal women (Linsley et al. 2002, Van't veer et al. 2002). However, Paik et al. (2006) have demonstrated that prognostic signatures developed from one study may differ from other studies.

The c DNA microarray technology is very expensive and generates a huge amount of data that requires complex analysis. Other major problems encountered include non-uniform methods of RNA extraction; different types of probe preparation probe labeling; and hybridization. All these obstacles are in front of this technique before it is ready and accepted for routine use (Ahmed and Brenton 2005, Reid et al. 2005).

**Table 4** Molecular and histopathological features of the breast cancer subtypes based on gene expression profiling (modified from Jones et al. 2012 and Weigelt et al 2010 ).

Molecular Subtypes	ER	PR	HER2	*MIB1	** Basal Markers	Histological Grade	Mitotic activity	TP53 Mutation	Prognosis
Luminal A	+	+	-	Low	-	Low	Low	Low	Most Favorable Prognosis
Luminal B	±	±	-/+	Intermediate / High	-	Intermediate / High	High	Intermediate	Intermediate
Basal Like	-	-	-	High	+	High	High	High	Poor
HER2 +ve / ER-	-	-/+	-/+	High	±	High	High	High	Poor
Claudin Low	-	-	-	Intermediate	±	High	High	High	Intermediate
Normal Breast- Like	-/+	NA	-	Low	+	Low	Low	Low	Intermediate

\* Considering the majority of tumors.

\*\* Basal markers: CK5,CK6,and epidermal growth factor receptor.

- negative, + positive, ± predominantly positive, -/+ predominantly negative.

NA not available

### 2.6.2.7 Next generation sequencing (NGS) methods

It was not long ago that the cloning and sequencing of a target gene could take months or years. Today the low-cost sequencing through the NGS or second-generation sequencing has revolutionized the genomic studies. This new technology may analyse thousands of DNA sequences in a short time and at low cost (Desmedt et al 2012, Hall 2007). Use of this technique has discovered many new driver genes that may have an important role in targeted therapy of breast and other cancers. NGS can also be useful in detection of minimal breast cancer-specific DNA rearrangements in a patient's plasma, suggesting that NGS may be used in the development of personalized treatment options for breast cancer patients. However, there are many unanswered issues, for example the responsibility for structural genomic changes in cancer progression and anticancer drug resistance (Desmedt et al 2012).

### **3. AIMS OF THE STUDY**

- I. Demographic and clinicopathological features of breast cancer patients in Libya, with a comparison of corresponding factors in breast cancer patients in Nigeria and Finland. (II)
- II. The relationship of nuclear size parameters, mitotic and apoptotic indices and the fraction of fields with tubular differentiation with clinicopathological features for Libyan female breast cancer patients. The data are compared with the corresponding factors in breast cancer patients in Finland and Nigeria. (I, III, IV, V)
- III. The prognostic significance of nuclear size parameters, mitotic and apoptotic indices and the fraction of fields with tubular differentiation in Libyan breast cancer material. The data are then compared with the corresponding findings for Nigerian and Finnish female breast cancer patients. (I, III, IV, V)

## 4. MATERIALS AND METHODS

### 4.1 Patient material

There are two groups of patients included in this study:

#### 4.1.1 Histopathological part of the study (I, III, IV, V)

##### 4.1.1.1 Clinico-pathological features

The current study was performed on paraffin-embedded Libyan female breast cancer (BC) samples. All cases were diagnosed at the Department of Pathology, African Oncology Institute, Sabratha, Libya, and Tripoli Medical Centre, Tripoli, Libya during the years 2000-2006. Patients were excluded from this study on the basis of the following exclusion criteria: histopathology was done elsewhere than in the mentioned study centers; the lack of patient history and medical files, or specimens; the follow-up was less than 3 months; and paraffin blocks were not available for re-cutting. After exclusions, 131 patients remained in the study. 116 patients were treated with modified radical mastectomy with axillary clearance. 15 patients were unfit for surgery due to distant metastases. Diagnostic biopsies were used for this study. A detailed history on clinico-pathological features (age, menopausal status, tumor size, clinical stage, histological types and grade, and lymph node status) was collected from patient files. For patients unfit for surgery, the stage was obtained from clinical examination or radiological assessment. In operable cases the stage was recorded according to the pathological report when available (**Table 5**). The mean age at the time of diagnosis was 46.5 (SD±13.4) years. Of the patients, 4.6%, 33.6%, 49.6% and 12.2% were at stages 1, 2, 3, and 4, respectively. The histological typing was based on the WHO Classification of Tumors (Tavassoli and Devilee 2003), and the grading was done according to the modified Bloom-Richardson histopathological grading system (Elston and Ellis 1991). There were 96 invasive ductal carcinomas (73.3%), 13 invasive lobular carcinomas (9.9%), 7 mixed ductal and lobular carcinomas (5.3%), 6 medullary carcinomas (4.6%), 3 papillary carcinomas (2.3%), 5 mucinous carcinomas (3.8%), and 1 metaplastic carcinoma (0.8%).

##### 4.1.1.2 Treatment and follow-up

103 (79.2%) patients were treated by modified radical mastectomy and axillary dissection, 9 (6.9%) patients received neoadjuvant chemotherapy with modified radical mastectomy and axillary lymph node dissection. Diagnostic lumpectomy was done in 2 (1.5%) patients, and simple mastectomy in 3 (2.3%) patients. No therapeutic surgical intervention was done for 13 (10.0%) patients with metastasis at time of diagnosis (diagnosis with core biopsy). Adjuvant and neoadjuvant chemotherapy with anthracycline was given to

96 (74.4%) patients, while combined chemotherapy of anthracycline and taxans were given to 17 (13.2%) patients. Three patients received chemotherapy of CMF regime (cyclophosphamide, methotrexate and 5- FU). No chemotherapy was given to 6 (4.7%) patients with early stage, and 7 (5.4%) patients were unfit to receive chemotherapy. Hormonal treatment (tamoxifen) was given to 69 (53.1%) patients who were hormone receptor positive. Axillary radiotherapy was given to node-positive patients (n = 103). One patient was in the first term of pregnancy and she was treated by modified radical mastectomy and radiotherapy with adjuvant therapy after therapeutic abortion was done.

The follow-up data were collected from patient files. The follow-up time ranged from 4 to 78 months. The average follow-up was 32.9 months. Some patients were lost from follow-up. Breast cancer was recorded as the underlying cause of death for 34 patients. Three patients died of causes unrelated to breast cancer and were not included as events in survival analysis. No autopsies were performed. The survival period was defined as the time from diagnosis either to the time of death or to the date on which the patient was last known to be alive.

#### **4.1.2 Epidemiological part of the study (II)**

A retrospective pathology study was conducted on 234 patients with breast carcinomas admitted to the African Oncology Institute (AOI) during the years 2002-2006. The data of clinical and pathological features were collected from pathology reports, hospital files of patients and from the Sabratha Cancer Registry (**Table 5**). Evaluation of incidence was based on data from 2006, from the Sabratha Cancer Registry. The incidence data are consequently based on the histologically verified cases of the year 2006, when the Sabratha Registry started to function.

## **4.2 Histological methods (I,III,IV,V)**

The tumor diameter was measured after surgical removal in 3 dimensions, then biopsy specimens were fixed in buffered formalin (pH 7.3), processed and embedded in paraffin. Sections of 5 $\mu$ m thickness were stained with the hematoxylin and eosin (HE) stain.

### **4.2.1 Estimation of nuclear parameters by morphometry method (I)**

The most representative nuclei from selected histological sections were analyzed by using an interactive digitizing image overlay drawing system run by Prodit morphometry program (Prodit 3.1, Promis Inc, Almere, and Buro medische Automatisering, De Meern, Holland).



**Table 5** The clinical characteristics of Libyan patients with breast cancer, in studies I, II, III, IV, and V.

Clinical characteristics	Descriptive data		
	Study I	Study II	Study III, IV, V
<b>Number of patients</b>	131	234	130
<b>Age at diagnosis (years)</b>			
Mean (SD*)	46.5 (13.4)	46.0 (12.3)	46.5 (13.4)
<b>Menopausal status</b>			
Premenopausal	81 (61.8%)	160 (68.4%)	80 (61.5%)
Postmenopausal	50 (38.2%)	74 (31.6%)	50 (38.5%)
<b>Nodal status</b>			
Positive	104 (79.4%)	173 (73.9%)	103 (79.2%)
Negative	27 (20.6%)	61 (26.1%)	27 (20.8%)
<b>Tumor size(cm)</b>			
Mean (SD)	5.6 (2.1)	4.8 (2.1)	5.6 (2.1)
<b>Clinical stage</b>			
Stage1	6 (4.6%)	12 (5.1%)	6 (4.6%)
Stage 2	44(33.6%)	103(44.1%)	44(33.8%)
Stage 3	65(49.6%)	86 (36.6%)	64(49.2%)
Stage 4	16(12.2%)	33 (14.1%)	16(12.3%)
<b>Histological grade</b>			
1	11 (8.4%)	11 (6.6%)	10 (7.7%)
2	70 (53.4%)	104(62.3%)	70 (53.8%)
3	50 (38.2%)	52 (31.1%)	50 (38.5%)
<b>Histological type</b>			
Ductal	96 (73.3%)	191(81.6%)	95 (73.1%)
Lobular	13 (9.9%)	17 (7.3%)	13 (13.0%)
Others	22 (16.8%)	26 (11.1%)	22 (16.9%)
<b>Duration of follow-up (ms)</b>			
Mean (Range)	32.9 (4-78)	22 (1-74)	32.9 (4-78)

\*SD= standard deviation

The system consisted of a light microscope, a personal computer (Compaq Deskpro 386/20e; Compaq Computer Corporation, Houston, TX, USA), a video camera attached to the microscope (JVC TK-870U; JVC, Japan) and a digitizer board (PIP-512B video digitizer board; Matrox Electronic Systems, Dorval; Quebec, Canada). Analog images of the nuclear profile were outlined on the monitor screen using a computer mouse, and consequently a digital database was created of the nuclear features in computer memory. The instrument was calibrated in 2 perpendicular directions with a micrometer scale before each session of measurement. Measurement was carried out at x2600 magnification on the monitor screen (x40 objective lens magnification, x10 video ocular and x1.25 internal magnification). The computer automatically created the following nuclear morphometric features: (i) area; (ii) perimeter, which is the length around the nuclear border; (iii) diameter; (iv) the longest axis of the best fitting ellipse; and (v) the shortest axis which is measured perpendicular to the longest axis. Furthermore the following parameters were measured: (i) AR form factor; (ii) PE form factor; (iii) NCI

form factor; (iv) the longest/shortest axis ratio (LS ratio); (v) nuclear roundness. In a circular nucleus, the values of the roundness and the LS ratio (ellipticity rate) correspond to 1. If the nucleus is elliptic, the roundness becomes less than 1; in contrast, the LS ratio is higher than 1; (vi) contour ratio, the shape factor calculated by using the formula  $(\text{perimeter})^2 / (4 \pi \text{ area})$  (Prodit manual).

At the end of each case measurement, the system automatically calculated 18 basic statistical parameters (mean, median, mode, range of values, minimum and maximum values, standard deviation and standard error, variation, skewness, kurtosis, and the percentiles 5%, 10%, 25%, 50%, 75%, 90% and 95%) for each nuclear feature, resulting in a total of 198 features. The total number of nuclei measured in each case was approximately 50 nuclei of cells presenting sharp nuclear borders that did not overlap. Cell nuclei were contoured by tracing nuclear margins with the aid of a mouse and a cursor on the screen.

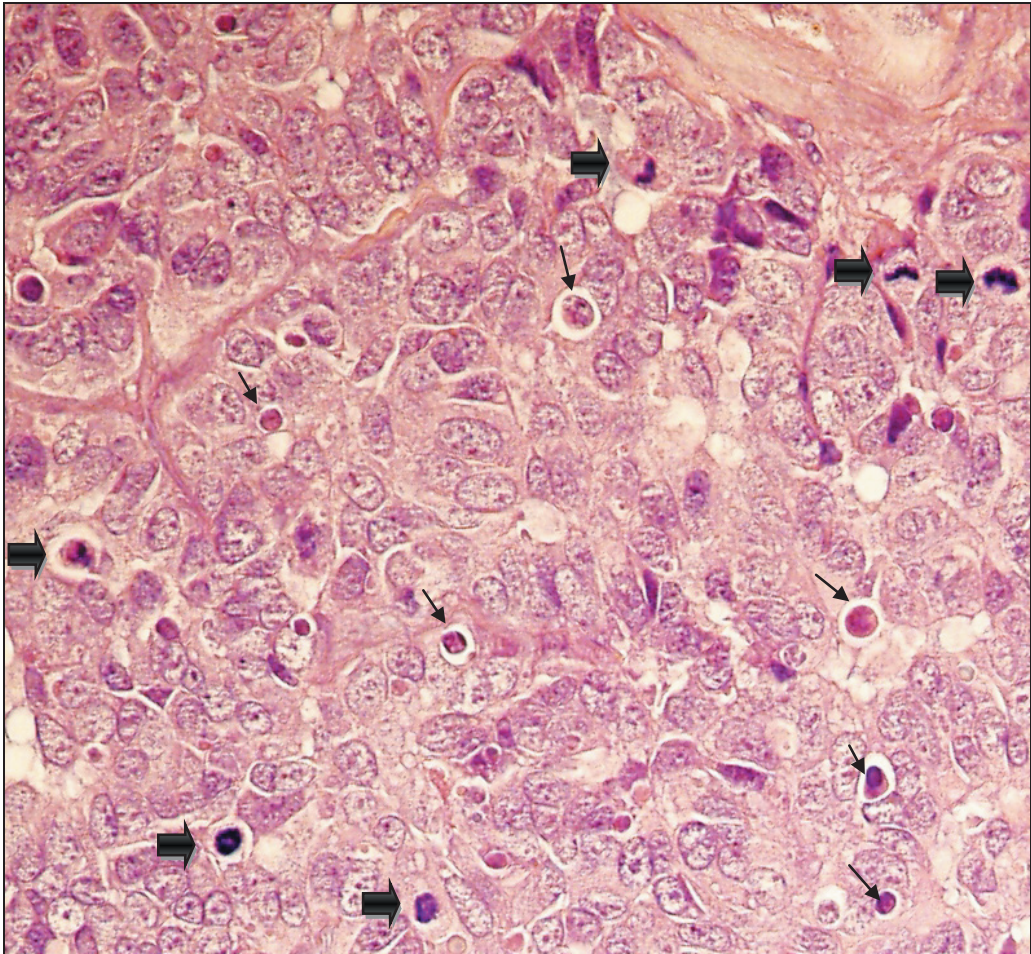
#### **4.2.2 Histological identification and counting of the mitotic figures and the apoptotic bodies (III, IV)**

In identifying the mitotic figures and the apoptotic bodies we applied criteria described by Baak and Oort (1983) and van de Schepop et al. (1996), respectively.

Mitotic figures were characterized by an absent nuclear membrane with clear, hairy extension of nuclear material (condensed chromosomes) either clumped (beginning metaphase), in a plane (metaphase/ anaphase), or in separate chromosomal aggregates (anaphase/ telophase). The basic idea was that at least one chromosomal end was seen in a mitosis (Baak and Oort 1983). Two parallel, clearly separate chromosome clumps were counted as one mitotic figure. The cytoplasm of mitotic cells in mitosis was often larger than in the resting cells.

Shrunken acidophilic bodies with fragmented nuclei and condensed nuclear chromatin characterized apoptotic cells. The apoptotic cells were separated from their neighbors and lacked associated inflammatory reaction (**Figure 1**).

Dr. Jamela Boder carried out counting after a five-week training program on counting of mitotic and apoptotic figures from a set of 10 Libyan female BC samples. During that period counts were repeated on 10 separate occasions, 2-4 days apart. Areas of necrosis, inflammation, in situ carcinoma, and calcification were avoided. We used an Olympus laboratory microscope (x40 objective lens magnification, numerical aperture 0.75, field diameter 490 $\mu$ m). The number of mitotic figures, as well as the apoptotic bodies counted in the same 10 consecutive fields from the most cellular area of the sample, were termed as mitotic activity (MAI) and apoptotic activity (AA), respectively.



**Figure 1.** Invasive ductal carcinoma shows high mitotic and apoptotic indices. This field shows multiple mitotic figures (thick arrows) and apoptotic bodies (thin arrows). The apoptotic bodies are characterized by shrunken acidophilic cell cytoplasm, occasionally with fragmented nuclei and condensed nuclear chromatin. The apoptotic cells are often separated from their neighbors and lack associated inflammatory reaction.

The estimation of volume fraction corrected mitotic index (SMI) as well apoptotic index (AI) expressed per  $\text{mm}^2$ , provide the mitotic and apoptotic count as the number of mitotic figures and apoptotic bodies by the area of the neoplastic tissue in the microscopic fields. In this method, the area fraction (as an estimate of volume fraction) of neoplastic tissue in the microscopic field is evaluated in parallel with the apoptotic count (Haapasalo et al. 1989, Lipponen et al. 1994).

$\text{SMI} = k \frac{(\sum \text{MI})}{(\sum V_v)}$  and  $\text{AI} = k \frac{(\sum \text{AA})}{(\sum V_v)}$ , where  $k = 100 \sqrt{\pi} r^2$ ;  $r$  is the radius of the microscopic field;  $\text{MI}$  = the number of mitotic figures in the studied field;  $\text{AA}$  = the number of apoptotic bodies in the same studied field.  $V_v$  is the volume fraction (estimated by the area fraction, in percent) of neoplastic tissue in the studied field.

The ratio of SMI and AI (SMI/AI) was also calculated to evaluate the balance between cell proliferation and cell death.

#### **4.2.3 Estimation of fraction of fields with tubular differentiation (FTD) (V)**

Tubular differentiation was evaluated in each sample as the fraction of fields showing tubular differentiation (FTD) (Kronqvist et al. 1999, Kronqvist et al. 2000). According to this method tubular differentiation was assessed in the whole tumor area. The samples were screened at x10 magnification and the presence or absence of malignant tubular structures in each microscopic field was registered. By this method the field was registered positive if a single clearly malignant tubular structure was identified. The final result was the fraction of fields presenting tubular differentiation. This assessment method is especially recommended because it has proved to be the most efficient and fastest way to quantitatively evaluate the tubular differentiation in invasive breast cancer. In a previous study (Kronqvist et al. 1999) on Finnish material comparing several evaluation methods for tubular differentiation (Kronqvist et al. 2000), FTD turned out to be the most practical, accurate and reproducible way to determine tubular differentiation in invasive breast cancer. In the evaluations, special emphasis was placed on histological identification of the malignant tubule. The required features for registering a tubule was a clear lumen within a tubular or alveolar pattern created by surrounding malignant epithelial cells. Special consideration was taken to avoid counting adipocytes, benign ducts, and central necrosis or artifact clefts as malignant tubules. Luminal structures in cribriform malignant epithelium were not counted either.

### **4.3 Data from Finland and Nigeria**

The comparison with Nigerian material is based on data presented by Ikpat et al. (2002;4 publications, 2003). In addition, updated data were used when available. The comparison with the Finnish material is based on the database developed in the study group "Prognostication and cancer" and is best presented as part of the papers by Kronqvist et al. (1995, 1998a, 1998b, 1999, 2000).

### **4.4 Statistical analyses (I-V)**

Statistical analyses were performed using SPSS software packages for Windows, versions 16.0/19.0 (SPSS, Inc., Chicago, USA). The variables of the material were grouped into logical classes and descriptive statistics calculated for the continuous variables. For survival analysis, Kaplan-Meier curves were plotted, and differences between the curves analyzed using the log-rank test. The MNA, FTD, AI, MAI and SMI thresholds were chosen in such a way that the patients were divided into two or three groups. Differences

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between the curves were analyzed also using the log-rank test. Pearson and Spearman's correlation tests were used for comparison between two variables. P-values below 0.05 were regarded as significant. Comparison of numerical data was done by the chi-square test. Student t-tests and ANOVA were also used to test differences between the groups. Univariate and multivariate analyses were performed for all studied prognostic features to estimate their effect on disease outcome, together or separately. Microsoft Excel 2007 was used to draw graphs and to evaluate relationships between variables.

## 5. RESULTS

### 5.1 Demographic and clinicopathological features of the Libyan female breast cancer population (II)

The Libyan breast cancer incidence was 18.8 per 100,000 females. The occurrence of breast cancer in the female Libyan population is strongly related to young age (mean age is 46.0 years). About 70.9% of cases arise in women who are 50 years or younger. The average age at first pregnancy was 22.1 year. Libyan breast cancer is dominantly premenopausal (68.4%) and displays unfavourable features such as high histological grade and stage, large size and frequent lymph node metastases (**Table 6**).

### 5.2 Correlation of measured morphometric factors with the clinicopathological features (I, III, IV, V)

A statistically significant correlation was observed between the mean nuclear area, fraction of fields with tubular differentiation, apoptotic index, proliferative indices and the most clinicopathological features, with the strongest association observed for lymphnode status and histological grade. **Table 6** shows most of these correlations.

#### 5.2.1 Correlation of MNA with the clinicopathological features (I)

There was a statistically significant correlation between the mean nuclear area (MNA) and most clinicopathological features, with the strongest correlation observed for the nuclear grade (Spearman's test  $r = 0.52$ ,  $P < 0.0001$ ). Correlation was also found between the nuclear area and tumor stage (Spearman's  $r = 0.16$ ,  $P = 0.05$ ), tumor size (Pearson's test  $r = 0.24$ ,  $P = 0.005$ ) and lymph node (LN) status (Spearman's  $r = 0.32$ ,  $P = 0.001$ ). The difference in the nuclear area between invasive ductal carcinoma and lobular carcinoma was statistically significant ( $p < 0.0001$ ). There was also a significant difference between special types of carcinomas and lobular carcinomas ( $p < 0.0001$ ). On the other hand, the difference in the nuclear area among infiltrating ductal carcinomas (IDC) and special types of breast carcinoma was not statistically significant. Menopausal status does not have a significant impact on the MNA or any of the nuclear features.

#### 5.2.2 Correlation of proliferative indices with the clinicopathological features (IV)

A statistically significant correlation was observed between the proliferative indices particularly SMI and most clinicopathological features, with the strongest correlation observed in tumor stage (Spearman's test  $r = 0.35$ ). There was correlation also between SMI and lymphnode status (Spearman's test  $r = 0.31$ ), histological grade (Spearman's

$r = 0.22$ ,  $P = 0.001$ ), and tumor size (Pearson's test  $r = 0.24$ ). The correlations between proliferative indices and most clinicopathological features were more significant with SMI than MAI. Age and menopausal status showed a higher significant relationship with MAI. The histological types of the neoplasm had no a significant relationship with the mitotic counts.

**Table 6** Morphometric features in 131 Libyan histological breast cancer samples, and in subgroups defined by menopausal status, clinical stage, histological grade, histological type and nodal status. The values are presented as means + SD. The p-values refer to significance of difference between the subgroups (based on analysis of variance, ANOVA)

Clinico-pathological features	MNA(SD)	SMI(SD)	MAI(SD)	AI(SD)	FTD(SD)
<b>Whole material</b>	74.3 (23.7)	32.1(20.9)	27.3 (18.5)	12.8 (9.6)	23.4 (21.6)
<b>Menopausal status</b>	<b>p = 0.62</b>	<b>p = 0.01</b>	<b>p = 0.008</b>	<b>p = 0.26</b>	<b>p = 0.22</b>
Premenopausal	75.1(24.1)	28.5 (16.6)	23.9 (17.0)	12.07 (9.7)	25.3 (22.8)
Postmenopausal	72.9 (23.3)	37.7 (25.7)	32.7 (19.8)	14.05 (9.5)	20.5 (19.4)
<b>Clinical stage</b>	<b>p = 0.05</b>	<b>p = 0.002</b>	<b>p = 0.07</b>	<b>p = 0.009</b>	<b>p = 0.07</b>
Stage 1	57.9 (11.2)	15.7(9.3)	10.8( 8.1)	5.0 (5.1)	27.7 (21.9)
Stage 2	70.6 (22.0)	25.0(14.5)	20.9(3.1)	10.2(6.7)	
Stage 3	78.0 (24.1)	36.1(22.2)	16.7(2.1)	14.5(10.5)	
Stage 4	75.0 (27.0)	41.3(21.0)	18.7(4.7)	16.4(11.4)	20.8 (21.1)
<b>Histological grade</b>	<b>p &lt; 0.0001</b>	<b>p = 0.01</b>	<b>p = 0.003</b>	<b>p = 0.035</b>	<b>p = 0.001</b>
Grade 1	58.9 (8.1)	14.9 (9.2)	12.8 (9.6)	5.9 (5.7)	40.0 (17.5)
Grade 2	66.0 (20.2)	32.1 (19.3)	25.4 (17.1)	12.7 (7.4)	26.1 (23.5)
Grade 3	98.2 (23.0)	35.4 (23.3)	32.7 (20.0)	14.5(9.0)	16.1 (16.5)
<b>Histological type</b>	<b>p &lt; 0.0001</b>	<b>p = 0.7</b>	<b>p = 0.8</b>	<b>p = 0.35</b>	<b>p = 0.46</b>
Invasive ductal	76.8 (23.3)	32.7(21.2)	26.9(18.6)	13.3(10.6)	22.6 (20.8)
Invasive lobular	52.8 (16.5)	32.9(16.4)	30.1(17.1)	10.8(5.8)	
Others	75.9 (23.9)	28.7(27.7)	27.7(19.7)	11.5(6.3)	25.7 (23.8)
<b>Nodal status</b>	<b>p = 0.001</b>	<b>p = 0.003</b>	<b>p = 0.035</b>	<b>p = 0.005</b>	<b>p = 0.007</b>
Negative	60.6 (16.3)	21.5 (13.0)	20.6 (19.7)	8.3(7.0)	33.3 (24.6)
Positive	77.9 (24.1)	34.8 (21.8)	29.0 (17.9)	14.0(9.9)	20.8 (20.1)

### 5.2.3 Correlation of apoptotic index with the clinicopathological features (III)

There were statistically significant correlations between the apoptotic index and most clinicopathological features. The strongest correlations were observed in clinical lymph node status and tumor size (Spearman's test  $r = 0.3$  and Pearson's test  $r = 0.3$ , respectively). Significant differences were also seen among different histological grades and clinical stages ( $p = 0.035$  and  $0.009$ , respectively) (**Table 6**). Although the differences in the apoptotic index between different histological types of invasive breast cancer was statistically insignificant, the mean AI values observed in the invasive ductal, medullary and mixed ductal-lobular carcinomas (13.3, 13.7 and 13.5 apoptotic cells per

mm<sup>2</sup>, respectively) were higher than that observed in invasive lobular, mucinous and papillary carcinomas (10.8, 11.2 and 9.1 apoptotic cells per mm<sup>2</sup>, respectively).

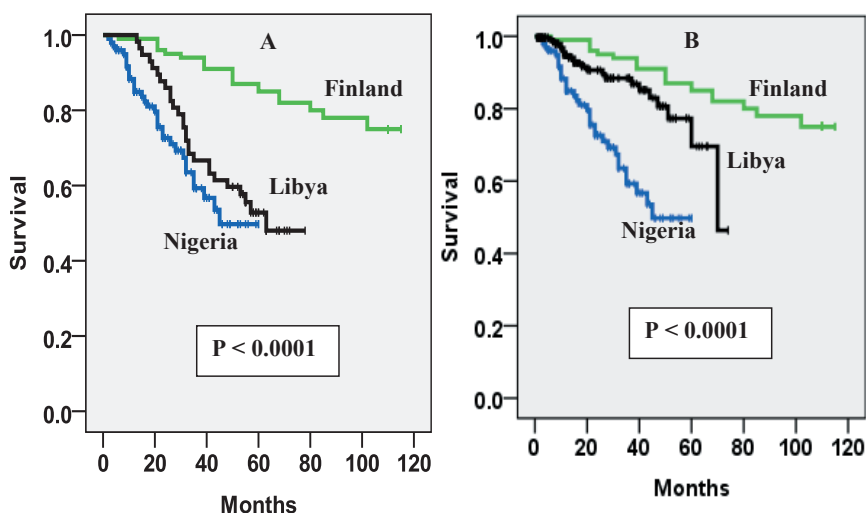
### 5.2.4 Correlation of FTD with the clinicopathological features (V)

There were negative correlations between the FTD and some clinicopathological features, with the strongest correlations observed in histological grade and tumor size (Spearman's test  $r = -0.3$  and Pearson's test  $r = -0.3$ , respectively). Statistically significant differences in the FTD were also observed between patients with positive and negative lymph nodes ( $P = 0.007$ ). The difference in the FTD between invasive ductal carcinoma and other types of breast carcinomas was statistically insignificant ( $P = 0.46$ ) (Table 6).

## 5.3 Survival analysis (I, II, III, IV, V)

### 5.3.1 Clinicopathological features and survival (II)

The survival curves of the 3 countries show statistically significant differences: the Libyan patients have better survival than the Nigerian patients, but worse than the Finnish patients ( $p < 0.0001$ ) (Figure 2). This result was obtained when survival curves of breast cancer patients in 3 countries; estimated in both whole Libyan material, and Libyan material with only stage 1,2 and 3 to be justified the comparison of our results with the results of Ikpat et al. and Kronqvist et al.

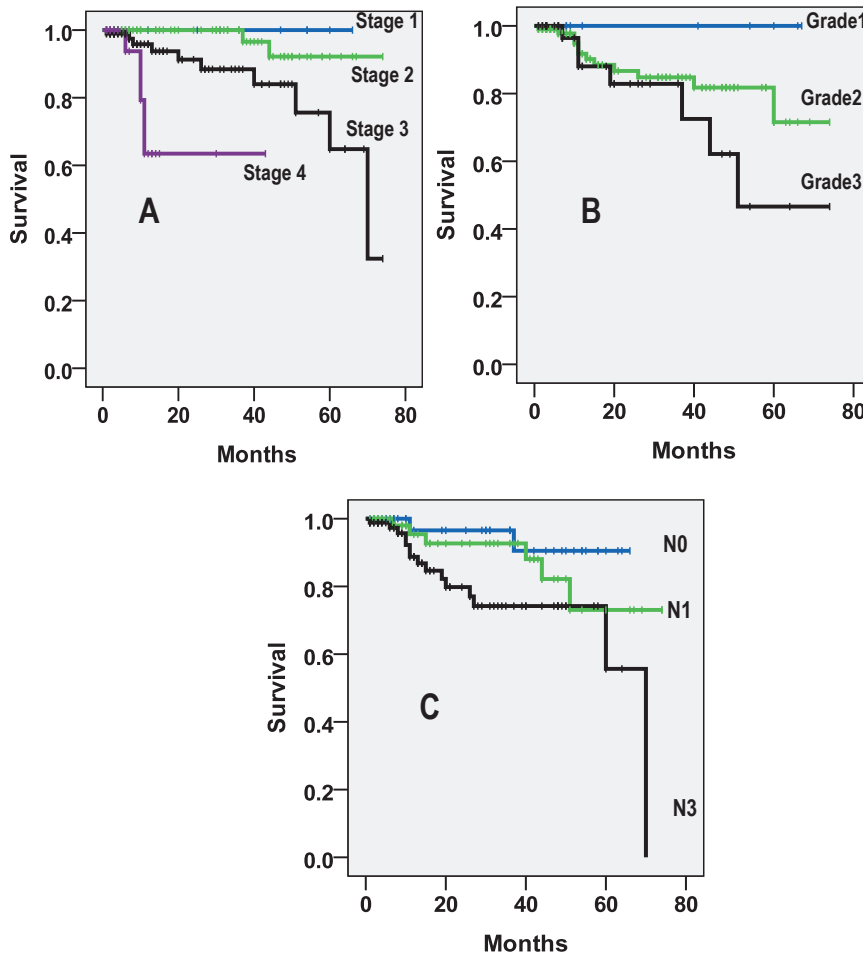


**Figure 2.** Survival curves of breast cancer patients in 3 countries: A The curves in whole Libyan material; B The curves in Libyan patients with stages 1-3 to justify the comparison with other studies. In both curves the Libyan patients have better survival than the Nigerian patients, but worse than the Finnish patients ( $p < 0.0001$ ).



The survival rates in Libyan breast cancer behaves similarly to the European breast cancer in respect of the grade, stage, and LN status at diagnosis. This also indicates that long survival time is associated with well-differentiated tumors, tumors detected at an early stage, and patients without lymph node involvement (**Figure 3**).

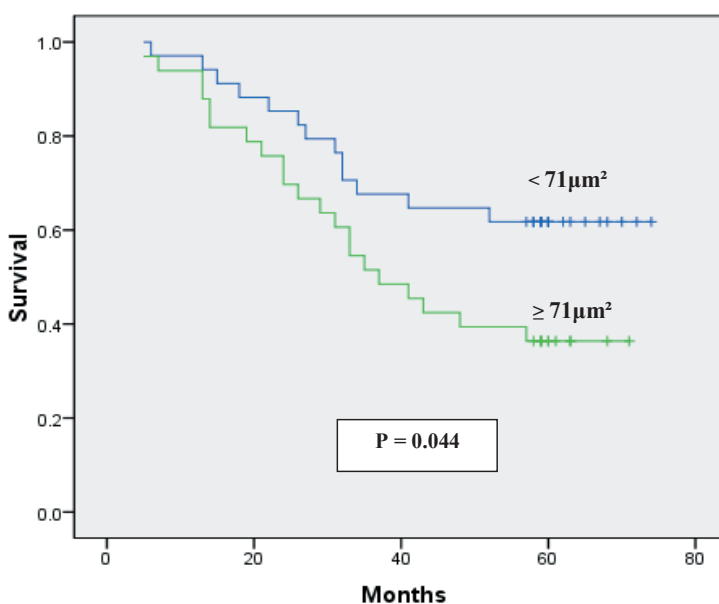
Among Libyan patients the menopausal status, histological type of tumor, and age of patient did not seem to influence survival rates.



**Figure 3.** A. Survival curves of Libyan breast cancer patients in different clinical stages. The group of patients with stage 1 had the best 5-year survival ( $p < 0.0001$ ). B. Survival and histological grade. G1 (N=11) patients have better survival than G2 and G 3 patients ( $p = 0.09$  “almost significant”, 167/234 grades available). C. Survival curves and lymph node status (N0, N1, N2). The group of patients without involvement had better 5-year survival than the group with N1 and N2 status ( $p = 0.002$ ).

### 5.3.2 MNA and survival (I)

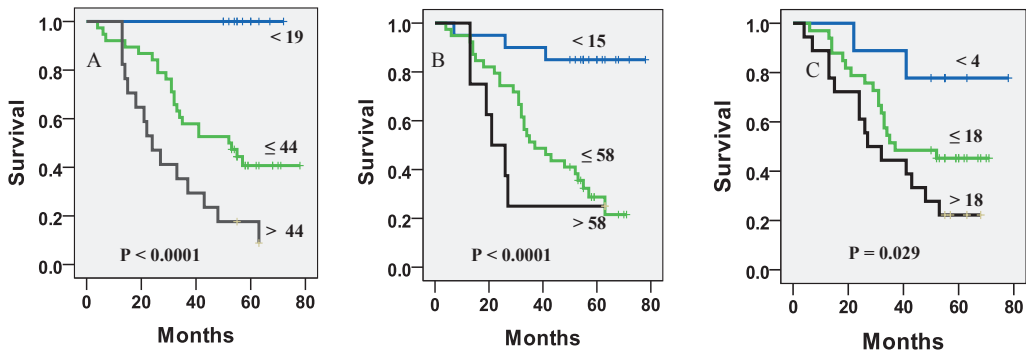
The univariate analysis and survival Kaplan-Meier analysis indicates that short survival time was associated with high nuclear morphometric size values. Determination of decision cut-points for MNA in the Libyan material resulted in an obvious cut-point at  $71\mu\text{m}^2$  (**Figure 4**). At  $71\mu\text{m}^2$  the groups with higher or lower means were prognostically most significantly different. The analysis detected only one cut-point surrounded by less significant cut-points. MNA was more significant than other morphometric features in respect of significant potential cut-points. On the other hand, the current study also recognizes that all the studied morphometric size parameters were correlated significantly with survival. Shape parameters were not significantly associated with clinical features or survival.



**Figure 4.** Survival curves associated with the mean nuclear area measured in 131 Libyan breast cancers. The cut-point at  $71\mu\text{m}^2$  was the most significant cut-point and the corresponding survival curves are shown here. The survival curves are significantly different at 5 and after 5 years (Log Rank test,  $P = 0.044$ ). The upper curve started with 65 patients, the lower curve with 66 patients. At 5 years the upper curve had 21 survivors, the lower curve had 12 survivors.

### 5.3.3 Proliferative indices and survival (IV)

The survival analysis indicated that short survival time was associated with high mitotic indices values. The proliferative indices can identify aggressive tumors and provide significant prognostic support. The cut-point (19 and 44 mitosis/ $\text{mm}^2$ ) of SMI might be applied as quantitative criterium for Libyan breast cancer to separate the patients into good, moderate and bad prognosis groups. **Figure 5A & 5B.**



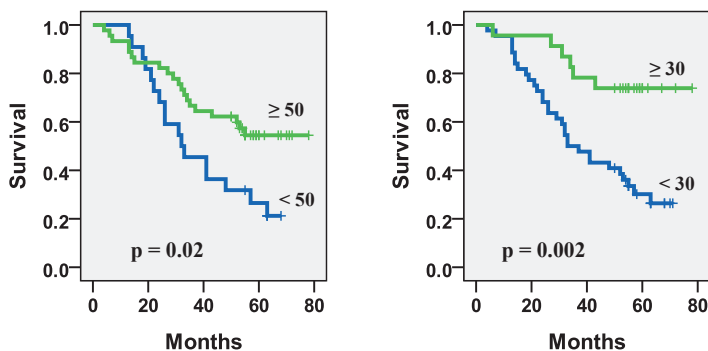
**Figure 5.** Survival curves for 130 Libyan female patients with breast cancer divided by A: SMI cut-points of 19 and 44 mitoses/mm<sup>2</sup>. B: MAI cut-points of 15 and 58 mitotic figures/10 hpf. The differences between the curves are highly significant. C: AI cut-points of 18 and 4 apoptosis/mm<sup>2</sup>.

### 5.3.4 Apoptotic index and survival (III)

The apoptotic index (AI) was a significant predictor of survival in the overall material. A high apoptotic index was associated with short survival time. The analysis of cut-points resulted in two AI values (4 and 18 apoptotic cells per mm<sup>2</sup>), which divided the patients into significantly different groups with good and bad prognoses. The difference was more significant when the cut-point was at 18 apoptotic cells per mm<sup>2</sup> (Figure 5C).

### 5.3.5 FTD and survival (V)

Survival analysis indicated that high FTD was associated with prognosis (Figure 6). The cut-points at 30% and 50% divided the patients into significantly different groups with good and bad prognoses. The difference was more significant when the cut-point was at 30% (Figure 4). In multivariate analysis (all patients, with SMI, MAI, nuclear area and apoptotic count) FTD was not a significant prognosticator.



**Figure 6.** Survival curves for 130 female Libyan patients with breast cancer divided by FTD cut-points of 30% and 50%. The differences between the curves are significant.

## **6. DISCUSSION**

### **6.1 Quantitative analysis of morphometric features: applicability and limitations (I, III, IV, V).**

Cancer develops after a sequence of cellular events associated with various degrees of atypia. Several studies have concluded that such events are associated with alteration in nuclear morphometric size and shape features as a result of excessive DNA synthesis followed by proliferative and apoptotic activity (Barker and Sanford 1970, Brawer 1992, Merkel and McGuire 1990). Estimation of nuclear size, proliferative indices and tubular differentiation has been used to improve classic grading systems of breast cancer (Kronqvist et al. 1998). However, all of these methods have some limitations.

#### **6.1.1 Application of quantitative analysis methods**

The histopathology grading system and the TNM staging system are still at present the gold standard as prognostic indicators of breast cancer (AJCC Cancer Staging Manual 2006, Tavassoli and Devilee 2003, Elston and Ellis 1991). However, the TNM staging of breast cancer is not always accessible by histological examination of the primary tumor, particularly when the tissue is obtained through breast fine needle or tru-cut biopsies, where the tumor size and tumor progression data cannot be obtained. The applied histological grading system can be improved by using the quantitative measurements making the grading system more accurate and reproducible (Kronqvist et al. 1998). The improved grading system could provide useful prognostic information (Haapasalo et al. 1989, Aaltomaa et al 1992, Collan et al. 1994, Ikpatt et al. 2002). The new powerful tool is formed of high accuracy measurement of several histopathological features including the nuclear size and shape, mitotic and apoptotic activity, and fraction of fields with tubular differentiation of breast cancer.

The present study is the first study to estimate the quantitative features of different morphometric characteristics in Libyan breast cancer. Several interesting and important observations were made. However, the reasons for the increase in the author's interest in applying quantitative pathology methods to breast cancer diagnosis and prognosis rather than molecular analysis are mainly down to the fact that quantitative analysis methods are fairly simple and inexpensive. The cost is much less than that of molecular analysis and IHC stain (Baak et al. 1985, Buhmeida 2006, Abdalla et al. 2010). Therefore, it is quite evident that quantitative analysis methods can be used to support the classic prognostic indicators in predicting BC outcome in developing countries. Some quantitative methods such as SMI and AI do not seem challenging to use. But it should be remembered that both molecular analysis and IHC stain also have

false-positive and false-negative results. The paraffin blocks of the current study were obtained from old archival material. The conditions of fixation of archival material are not always known and therefore the results of IHC must be carefully interpreted and with caution. Moreover, while quantitative analysis is potentially fast, molecular analysis methods may be more time-consuming and in some cases may lead to delays in the final decision on prognosis and treatment.

Automated quantitative computer-assisted analysis of nuclear morphometric features showed very high sensitivity and reproducibility (Kronqvist et al. 1997). These methods also allow for exact measurement of cell and nuclear size, shape and quantity. Quantitative analysis methods provided detailed information on the morphological criteria of breast cancer (Baak et al. 1985).

Finally, the quantitative pathological analysis is more reproducible than the classic histopathology examination (Baak 1991, Mariuzzi and Collan 1995, Abdalla et al. 2010).

### **6.1.2 Limitation of quantitative analysis methods**

There are some limitations to the application of different quantitative analysis methods. This is true even though quantitative estimations of different morphometric characteristics are sensitive methods. Selection of examined areas of study and the number of selected cells (studies I, III, IV) or of selected tubules (study V) will affect the results to a considerable extent. Selections of examined areas of study as well as the number of examined cancer cells still require some subjectivity. In this study, with the exception of a couple of cases, because of a little amount of tumor tissue, the selection of examined areas and malignant cells were large enough to at least partly avoid this problem. Sampling methods affect comparison between different studies, and to a less extent within the same study. As a part of the efforts to introduce suitable sampling rules for BC, Kronqvist and her colleagues tested a number of sampling rules in nuclear morphometry (Kronqvist et al. 1995). Furthermore, the sources of inter-observer and intra-observer variation were also examined (Kronqvist et al. 1997). Analyses of some cells with broken nuclei or with nuclear overlapping may be one source of errors.

A significant source of nuclear morphometric size variation has been found as a result of delay in tissue fixation. It is, however, greatly dependent on the standardization of laboratory methodology, including: fixation delay, type of fixation, formalin pH and formalin dilution. This has been especially studied in respect to pH. The pH of the different fixatives varied from 5 to 9 (Baak et al. 1989). Different fixative pH levels can cause significant morphometric differences (Baak et al. 1989, Fleege et al. 1991). Fixation delay is a common problem in the Nigerian material (Ikpat et al. 2002b). For this reason, the results of the current study should be confirmed by further studies with more standardized preparation of tissue samples.

On the other hand, mitotic and apoptotic indices can be affected by work methodology (Woosley 1991). To minimize this possibility, the present study used similar equipments and methodology as used in earlier studies of Ikpatt (2002) and Kronqvist (1999).

In addition, quantitative estimations of different morphometric characteristics (SMI, MAI, AI, MNA and FTD) cannot be undertaken perfectly when only a small number of tumor cells are examined. This is due to the heterogeneity of breast cancer, and the interpretation of the cancer grading system is still hampered by the lack of a large body of examined tumor tissue in tru-cut biopsy. An adequate amount of tissue could make the interpretation of grading system more objective for diagnosis, prognosis and treatment decision-making (Buhmeida 2006).

Finally, the presence of necrotic cellular debris, cell aggregates, broken nuclei, unwanted cellular artefacts, and stromal and inflammatory cells have to be taken into account when enumerating apoptotic and mitotic figures.

## **6.2 Differences between Libyan, Finnish and Nigerian breast cancers (I, II, III, IV, V) (Table 7)**

Recently, discussion on geographical variation and variation between populations in breast cancer has emerged (American Cancer Society 2008, Bray et al. 2004, Hall et al. 2000, Ikpatt 2002, James 2000, McBride et al. 2007, Simon and Severson 1997). If blacks and whites show differences, what are the characteristics of breast cancer in the Saharan (African) human population? Libyan breast cancers are optimal for evaluating this. In the current work, it was our intention to study Libyan breast cancer in respect to demographic and clinicopathological features and connect these with morphometric characteristics (nuclear size, proliferative and apoptotic activity and tubular differentiation). Although there were no differences in the methodology between the current study and earlier studies of Ikpatt (2002) and Kronqvist (1999) in estimating the morphometric features of nuclear size, proliferative indices and tubular differentiation, and apoptotic activity (Ikpatt 2002); the differences between breast cancers in Africa (Libya, Nigeria) and Finland were pronounced.

### **6.2.1 Differences in epidemiology (II)**

The incidence of breast cancer in the Libyan female population was much lower than in Finland and Nigeria (0.19‰, 0.88‰, 0.34‰, respectively) (Williams et al. 2006, Ikpatt et al. 2002).

**Table 7** The most important demographic and clinicopathological features related to population and to breast cancer among the populations of Libya, Nigeria, and Finland.

Characteristic	Libya	Nigeria	Finland
Age structure			
0-14	30% <sup>1</sup>	44.4% <sup>4</sup>	17.1% <sup>5</sup>
15-64	66.2% <sup>1</sup>	52.6% <sup>4</sup>	66.4% <sup>5</sup>
64+	3.8% <sup>1</sup>	3.0% <sup>4</sup>	16.5% <sup>5</sup>
Population growth rate	2.3 <sup>2</sup>	2.67 <sup>4</sup>	0.46 <sup>5</sup>
Total fertility rate	3.34 <sup>2</sup>	5.9 <sup>1</sup>	1.8 <sup>5</sup>
Life expectancy			
Total	76.6 <sup>2,1</sup>	52.0 <sup>3</sup>	79.3 <sup>5</sup>
Female	78.8 <sup>2,1</sup>	52.0 <sup>3</sup>	82.8 <sup>5</sup>
Infant mortality	24.6 <sup>2</sup>	74.18 <sup>4</sup>	3.82 <sup>4</sup>
Literacy	84.1 <sup>2</sup>	76.3% <sup>1</sup>	100% <sup>1,3,4,5</sup>
GDP (in US\$)	8298 <sup>2</sup>	970 <sup>4</sup>	21000 <sup>4</sup>
Mean age at diagnosis (SD)	46.0 (12.3) <sup>6</sup>	42.7(12.1) <sup>4</sup>	58.8(12.5) <sup>4</sup>
Mean age at 1 <sup>st</sup> pregnancy	22.1 <sup>6</sup>	20.8 <sup>4</sup>	25.6 <sup>4</sup>
Menopausal status			
premenopausal patients(%)	68.4 <sup>6</sup>	74.3 <sup>4</sup>	32.6 <sup>4</sup>
postmenopausal patients (%)	31.6 <sup>6</sup>	21.3 <sup>4</sup>	67.4 <sup>4</sup>
Tumor size (diameter in cm (SD)	4.8 (2.1) <sup>6</sup>	4.8 (2.4) <sup>4</sup>	2.6 (1.9) <sup>4</sup>
Positive LN (%)	73.9 <sup>6</sup>	79.1 <sup>4</sup>	34.0 <sup>4</sup>
Grade 3 tumors (%)	52 (31.1) <sup>6</sup>	137 (45.1) <sup>4</sup>	45 (15.8) <sup>4</sup>
Low stage (1&2, %)	49 <sup>6</sup>	47 <sup>4</sup>	87.5 <sup>4</sup>
High stage (3&4,%)	51 <sup>6</sup>	53 <sup>4</sup>	12.5 <sup>4</sup>

<sup>1</sup> King et al. 2012, <sup>2</sup> Libyan National Statistics Figures: 2003, <sup>3</sup> Brazier and Hamed 2003, <sup>4</sup> Ikpatt. 2002, <sup>5</sup> Statistics Finland: Finland in Figures 2010, <sup>6</sup>Original publication II.

The incidence in Libya is in line with results published from other low BC incidence countries from North Africa (Tunisia 19.6%, Egypt 24.2%, Algeria 23.4%) (Sabratha Cancer Registry 2008, Williams et al. 2006). The apparent slow increase in incidence may be related to improved diagnostic practice (mammography, immunostaining) during the last few years in Libya. Studies have observed that women who migrated from low BC incidence countries (in Africa and Asia) to high risk areas (US and Europe) typically experienced a rapid increase in breast cancer incidence suggesting that the difference could be explained by factors other than genetics. These include general standard of living factors such as lifestyle, education, life expectancy and health care, environmental factors such as geographic location, and different types of pollution, and reproduction related risk factors such as age of first pregnancy and oral contraceptive use (Dumitrescu and Cotarla 2005, MacMahon 2006, American Cancer Society 2008). The current study recognizes that the demographic differences between the populations of Libya, Nigeria and Finland are prominent. The Finnish population is older than the populations of Libya and also Nigeria. The populations aged over 65 years were 16.5%, 3.8% and 3.0% in

Finland, Libya and Nigeria, respectively. However, the age group of 15 to 64 years was smaller in Nigeria than in Libya and Finland (52.6%, 66.2% and 66.4%, respectively). This can be partially explained by the difference in life expectancy which is about 52 years in the population of Nigeria, and more than 75 years in the population of Libya and Finland. Clearly Libyan and Nigerian women had higher parity; about threefold in respect to the Finnish female population. The age of first pregnancy in Libya among breast cancer patients was about the same as in Nigeria (22.0 years and 21.0 years, respectively), but Finnish women had a higher mean age of first pregnancy (25.6 years). This might be an additional factor that has resulted in an increasing of parity in Libyan and Nigerian women. The increase in parity could be responsible for the low incidence of breast cancer in postmenopausal patients in Libya and Nigeria.

On the other hand, this may have resulted in a higher fraction of premenopausal breast cancer, which is more likely to be an aggressive type with unfavorable survival in the Libyan and Nigerian population (Largent et al. 2005, Shantakumar et al. 2007).

Differences in socioeconomic and educational facilities are also remarkable among the three countries studied. Most Libyan and Finnish women have an economic status above the poverty line, whereas about a third of the Nigerian population live below the poverty line (less than 1000US\$ per year). The low GDP contributes to low economic status, low education, less awareness of the disease, and poor healthcare. Healthcare indicators such as life expectancy and infant mortality rate are clearly different among the three countries (**Table 7**). On the other hand, the economic improvement in Libya may will link to an improvement in early detecting of breast cancer. However, in view of the fact that a national breast-screening program not is yet established in Libya, affecting the delay-time of diagnosis (Ermiah et al 2012) which could be explaining the increase risk of advanced tumors.

### **6.2.2 Differences in clinicopathological features of the disease progression (II)**

On average, Libyan and Nigerian breast cancer is a premenopausal and progressive disease with aggressive features like high histological grade and stage, large size and frequent lymph node metastases.

**Age:** The occurrence of breast cancer in female Libyan population is strongly related to youth with nearly 70.9% of cancers occurring in women 50 years or younger. The mean age of breast cancer patients in Libya was 46.0 years which is about the same as in Nigeria (42.7 years), but much lower than in Finland (58.8 year, p-value 0.001). Libyan and Nigerian breast cancer is predominantly premenopausal. African breast cancer was clearly different from European breast cancer in this respect. In Europe and among US whites, most patients are postmenopausal (**Table 7**). The lack of older patients in Libya could not be explained by the low average life expectancy, because female mean life expectancy in Libya is close to the figure in Finland (78.8 years, and 82.8 years, respectively). The variation in genetic marker distribution between Central,



North African, and European populations may be associated with the distribution of the premenopausal and postmenopausal type of breast cancer (Alero and Lisa 2005, Jobling et al. 2004).

**Stage and disease progression:** In the present study and in the study of Ikpatt et al. 2002 the authors observe that African patients were usually diagnosed in advanced stages and with aggressive behavior. For example, 74% and 79% (in Libya and Nigeria respectively) were presented with positive lymph nodes (LN+). In contrast, only 34.0% were LN+ in Finland. These results are in line with various studies in the US that report that breast cancer among females of African descent is predictive of higher mortality than among Caucasian women. Cancer is more likely to be diagnosed at an early age and with advanced stage of disease (American Cancer Society 2008, Bray et al. 2004, James 2000, McBride et al. 2007). In Egypt, researchers recognized that the majority of breast cancer patients are premenopausal and cancers are diagnosed at an advanced stage (Nadia et al. 2007).

**Histological grading:** The current study and the study of Ikpatt (2002) found that the BC in Libyan and Nigerian patients had higher histological grade than the BC in Finnish patients. BC in Libya and Nigeria showed higher mitotic indices, larger nuclear size and had poorly tubular differentiation, which reflected the aggressiveness of the disease in Libyan and Nigerian patients. This result is in line with the results on Afro-American patients (American Cancer Society 2008, McBride et al. 2007). This may be associated with premenopausal breast cancer, which is more common in African countries.

### **6.2.3 Differences in morphometric features in breast cancer patients in Libya, Nigeria, and Finland (I, II, III, IV, V)**

The variation was prominent in the size of breast cancer cell nuclei, proliferative and apoptotic activities and tubular differentiation between Libyan, Nigerian and Finnish populations.

Current findings of morphometric features in breast cancer patients in Libya fall between the findings in Central African and European patients (Ikpatt 2002, Kronqvist 1999) (**Table 8**). For example, There are differences in the fixation and preparation of the tissue samples. Fixation delay is a common problem in Nigerian material (Ikpatt et al. 2002b) and some Finnish materials were fixed after frozen sections analysis (Kronqvist et al. 2000). However, Kronqvist et al. concluded that at least in their material there was no significant variation in the counting of mitosis between both types of specimens. It is well known that the effect of variation in the fixation time and fixation methods on counting of the mitosis and apoptosis and on estimation of tubular fraction is neither significant nor explains the morphometric features differences between various populations (Kronqvist et al. 2000, Bergers et al. 1997, Ikpatt 2002b).

**Table 8** Differences in morphometric values in female breast cancer patients in Libya, Nigeria, and Finland.

	Libya	Nigeria	Finland
MNA $\mu\text{m}^2$	74.25	89.2*	38.6°
SMI mitoses/mm <sup>2</sup>	32.1	42.6*	13.8°
MAI mitoses/10HPF	27.3	30.53*	10.7°
AI apoptosis/mm <sup>2</sup>	12.8	9.6*	5.2*
FTD (%)	23.4	16.7*	30°

\*From Ikpatt 2002, °From Kronqvist 1999.

Another important factor is that the screening programs for BC are well established in Finland and other European countries compared to African countries, which might indicate that European breast cancers are detected at earlier localized stages and with less aggressiveness behaviour. On the other hand, the differences may be partly related to biological difference and variation in genetic marker distribution between Central and North African, and European populations (Williams et al. 2006). The variation in haplotype marker distribution has taken place under selective environmental stresses (Jobling et al. 2004). However, there is no need to believe that the environmental influences are not operating at the moment. So far the viral association with African breast cancer is far from proven, but if found, can potentially be an explanation for differences (Ikpatt and Olopade 2005). The new classification of breast cancer according to gene expression analysis has recognized that basal-like breast tumours occur at a significantly higher rate for premenopausal African-American patients than postmenopausal African-American and non-African-American patients (Carey et al. 2006). The present study has also shown difference between Libyan and Nigerian subgroups (**Table 9**) further strengthening the idea of biological breast cancer differences between North and sub-Saharan African populations. Studies of ethnic differences in pathological features are limited except for those between Afro-American women, and Caucasian American women (American Cancer Society 2008, Bray et al. 2004, James 2000, McBride et al. 2007, Simon and Severson 1997). Afro-American women are known to have an aggressive BC compared with Caucasian American women. For instance, the proliferative differences particularly in terms of SMI between Libyan and Nigerian tumors and between Libyan and Finnish tumors were significant in the whole material ( $p < 0.0001$ ). Differences are also present in subgroups and thus it seems to indicate the reliability and consistency of this study's results. The proliferative (SMI) difference in the lymph node positive subgroup is clearly significant between Libyan and Finnish women, and on the other hand, between Libyan and Nigerian cancers ( $p < 0.0001$  and  $p = 0.001$ , respectively). In lymph node negative subgroups the proliferation measured with SMI was highest among Nigerians and lowest among Finnish patients.

In premenopausal patients, SMI shows statistical significance between Libyan and Nigerian and between Libyan and Finnish tumors. Interestingly, the breast cancers

of postmenopausal Libyan patients have higher mitotic activity than the cancers in premenopausal patients (age group 40-49 years). But there were no significant differences in mitotic activity between cancers in patients below 40 years and postmenopausal patients ( $p=0.13$ ,  $p=0.15$  in MAI and SMI respectively). The reason for this difference remains unclear. Several studies have demonstrated that younger breast cancer patients are estrogen hormone receptor negative (Jalava et al. 2005, Fowble et al. 1994, Gnerlich et al. 2009, Klauber 2006, Hartley et al. 2006). A recent study on genomic analysis of breast cancer shows that cancers in young women have distinct biological characteristics and distinct deregulated signaling pathways (Colak et al. 2013). On the other hand, the high mitotic activity of Libyan patients older than 49 years can be explained by diagnosis delay, which is a serious problem in Libya (Ermiah et al. 2012). Diagnosis delay was significantly associated with old age and advanced stages (Ermiah et al. 2012). Age and advanced stages are indicators of advanced disease. Ikpatt et al. (2002) also reported higher mean values of proliferative indices in postmenopausal than in premenopausal cancers Nigerian patients.

The SMI differences in high grade cancers are significantly larger in Nigerian breast cancer patients than in Libyan patients. This may reflect the fact that Nigerian cancers, especially, have high proliferative activity. This may be related to the reported obesity (which is associated with higher blood estrogen levels) of Nigerians (Ikpatt et al. 2002). Proliferation difference was obviously significant between Libyan and Nigerian cancers in stages 2 and 3. Lower number of stage 1 cases in Libya may be related to the diagnosis delay, and the absence of screening programs for early detection of breast cancer in Libya (Ermiah et al. 2012).

Basically, all pairwise differences among different subgroups were statistically significant. The differences between the Libyan and Nigerian populations are less obvious. The proliferative difference between Central African, North African and European subgroups of patients support the idea of true biological differences as also suggested by genetic marker distributions between Central and North African and European populations. We cannot exclude “African” genomic pattern (as contrasted to “European” genomic pattern) in breast cancer. Healthcare and the screening programs for BC are well organized in Finland and other European countries when compared to African countries, which might explain the fact that the European breast cancers are detected at earlier localized stages and with low proliferative activity. Improvement of the African health care system and health education are important in order to increase women’s awareness and knowledge about breast cancer.

On the other hand there were significant AI differences between Libyan, Nigerian and Finnish populations. The Libyan AI is slightly higher than in Nigeria and much higher than in Finland. The differences between countries are seen throughout the samples as well as in certain subgroups, further strengthening our previous suggestion of biological differences between North and Central African, and European populations. Studies of

ethnic differences in BC pathological features are still limited, except for those between African-American women, and Caucasian American women. African-American women are known to have aggressive BC compared with Caucasian American women (McBride et al. 2007). Finally, current results on FTD reflect the same differences between countries in proliferative and apoptotic indices, although, the significant differences between Libyan and Finnish populations are less obvious.

### **6.3 Prognostic significances (I, III, IV,V)**

#### **6.3.1 Mean nuclear area (MNA) (I)**

In the multivariate analysis of the present study, the mean nuclear area was the strongest prognostic factor among the other nuclear parameters. Several authors have also recognized the prognostic values of nuclear area in breast cancer (Ikpatt et al. 2002, Kronqvist et al. 1995). These findings were confirmed in the univariate analysis of the current study. But the multivariate analysis of the present study shows that the MNA is a weak prognostic factor in relation to the classical and proliferative and apoptotic indices.

Kronqvist et al. (1998) suggest two thresholds for the MNA ( $32\mu\text{m}^2$  and  $47\mu\text{m}^2$ ) that could separate the Finnish patients into three subgroups with favorable, intermediate and unfavorable prognosis. In the present study, survival among patients with  $\text{MNA} < 71\mu\text{m}^2$  was significantly better than among patients with  $\text{MNA} \geq 71\mu\text{m}^2$ . So, the current study suggests that this value might be used as quantitative criteria for separating patients into two groups with good and poor prognosis in Libyan female patients. Ikpatt et al. (2002) also found only one decision cut-point, far higher than our cut-point. These differences, clearly, are related to the patient populations studied.

#### **6.3.2 Proliferative indices (SMI and MAI) (IV)**

Proliferation activity can be evaluated by several methods including immunohistochemistry (IHC) with antibodies directed against different proliferation antigens, such as Ki-67 (Ermiah et al. 2012b, Karanikas et al. 2010), and (PCNA) (Stuart-Harris et al. 2008), and estimation of the S-phase fraction method using DNA cytometry (Collan et al. 1992, Buhmeida 2006, Karra et al. 2012, Ermiah et al. 2012a). However, the S-phase fraction is a weak method due to potential intra-tumoral heterogeneity (Abdalla et al 2010, Bergers et al. 1996). Also, Ki-67 has been reported to be a less independent prognosticator than the proliferative indices by classic microscopic methods (Collan et al. 1996, Baak et al. 2007, Jalava et al. 2006). Baak et al. (1983) found that mitotic count was the best prognosticator in European breast cancer, and later it was shown that of the two mitotic counting indices (SMI and MAI), the SMI was prognostically stronger than the MAI (Aaltomaa et al. 1992, Laroye et al. 1991). The present study is in line with studies of Baak et al. (1983), Collan et al. (1996), Ikpatt et al. (2002), Jalava et al. (2006) and

Kronqvist et al. (1997). All show that low proliferative indices and particularly SMI are associated with good prognosis.

LN+ and LN- patients had different prognosticators. The data in Elzagheid et al. (2006) was on Caucasians, and proved that mitotic count is the best prognosticator of survival in breast cancer particularly in lymph node negative patients. However, in our study the proliferative indices did not show statistically significant differences of survival in LN- patients. The reason for that was purely technical, as the algorithm did not calculate the prognostic influence if there were no deaths in the studied group (**Table 9**).

**Table 9** Proliferative indices of Libya, Nigeria and Finland in whole patient materials and in subgroups. Data on Nigeria\* and Finland<sup>o</sup> are basically the same as those published in the study of Ikpat et al. (2002c) and Kronqvist 1998b, respectively. Significance estimated by one-way ANOVA test

Mitotic index	Subgroups	Libya n (130)	Finland <sup>o</sup> n (364)	Nigeria* n (300)	p-value	
					Libya vs. Finland	Libya vs. Nigeria
<b>SMI</b>	Whole material	32.1(21.0)	13.8(17.8)	42.6(27.5)	< 0.0001	< 0.0001
	<b>MAI</b>	27.3(18.5)	10.7(16.5)	30.5(25.1)	< 0.0001	0.192
<b>SMI</b>	LN+	34.8(21.8)n=103	17.8(19.3)n=131	45.4(27.6)n=235	< 0.0001	0.001
	LN-	21.5(13.0)n=27	11.6(16.5)n=232	32.6(25.1)n=65	0.003	0.032
	Postmeno-pausal	37.7(25.7)n=50	11.2(13.4)n=249	44.9(26.6)n=77	< 0.0001	0.134
	Premeno-pausal	28.5(16.6)n=80	19.6(29.0)n=114	41.9(27.8)n=223	0.014	< 0.0001
	Grade1	14.9 (9.2)n=10	NA	11.8 (11.4)n=44	NA	0.427
	Grade2	32.1 (19.3)n=70	NA	31.7(16.5)n=119	NA	0.880
	Grade 3	35.4 (23.3)n=50	NA	61.9(24.5)n=137	NA	< 0.0001
	Stage 1	15.7(9.3)n=6	NA	32.6(25.1)n=65	NA	0.108
	Stage 2	25.0(14.5)n=44	NA	41.9(28.3)n=75	NA	< 0.0001
	Stage 3	36.1(22.2)n=64	NA	48.9(28.8)n=98	NA	0.003
	Stage 4	41.3(21.0)n=16	NA	44.3(24.4)n=72	NA	0.650
	LN+	29.0(17.9)n=103	13.4(17.4)n=131	32.7(25.6)n=235	< 0.0001	0.184
	LN-	20.6 (19.7)n=27	9.2(15.8)n=232	22.6(21.2)n=65	0.001	0.675
	Postmeno-pausal	32.7(19.8)n=50	8.8(11.9)n=249	33.8(24.9)n=77	< 0.0001	0.793
Premeno-pausal	23.9(17.0)n=80	14.9(23.2)n=114	29.4(22.1)n=223	0.004	0.044	
<b>MAI</b>	Grade1	12.8 (9.6)n=10	NA	4.2(3.8)n=44	NA	< 0.0001
	Grade 2	25.4(17.1)n=70	NA	16.8(10.1)n=119	NA	< 0.0001
	Grade 3	32.7(20.0)n=50	NA	50.9(21.9)n=137	NA	< 0.0001
	Stage 1	15.7(9.3)n=6	NA	22.6(21.2) n=65	NA	0.434
	Stage 2	25.0(14.5)n=44	NA	28.3(24.4) n=75	NA	0.416
	Stage 3	36.1(22.2)n=64	NA	36.6(26.8) n=98	NA	0.901
	Stage 4	41.3(21.0)n=16	NA	31.9(24.7) n=72	NA	0.162

The current study suggests two significant cut-points for the proliferative indices to separate the patients into three subgroups with favorable, intermediate and unfavorable prognosis (Figure 5). These cut-points may be more suitable for the Libyan material than the cut-points used by Ikpat et al. (2002) in the Nigerian material (17 and 92 mitoses/mm<sup>2</sup> for SMI and 10 and 92 mitoses/10HPF for MAI), or by Kronqvist et al. (1998) in the Finnish material (17 and 32 mitoses/mm<sup>2</sup> for SMI and 13 and 35 mitoses/10 HPF for MAI) (**Table 10**) or by Buhmeida et al. (2011) who suggest one threshold for each index in the Saudi Arabian material (4 mitoses/mm<sup>2</sup> for SMI and 13 mitoses/10HPF for MAI). These data clearly indicate that breast cancer with high SMI and MAI is at high risk of local or distant recurrence. Because of the high adverse prognostic impact of disease recurrence, patients with high indices of proliferation are also more likely to die of their disease. To avoid this, patients are appropriate candidates for intensive follow-up and active therapeutic strategy.

In the present study, univariate and multivariate Cox regression analysis showed that SMI, MAI at cut-point 58, and clinical stage were independent predictors for overall survival in the whole material. Whereas, in IDC with stage 1-3, the SMI, MAI at cut-point 58, age, and tumor size proved to be the independent predictors. This result accorded with the work of several authors (Collan et al. 1996, Ikpat et al. 2002b, Jalava et al. 2006), who confirmed that the mitotic indices are independent prognostic markers in breast cancer. Studies of the SMI and MAI in Nigerian (Ikpat et al. 2002b) and Finnish patients (Kronqvist et al. 1998) showed that the grouping variable of SMI is a powerful prognosticator in both univariate and multivariate analysis. In breast cancer patients in Saudi Arabia, neither MAI nor SMI proved to have any value as independent predictors in a study in which several investigators performed mitotic counts (Buhmeida et al. 2011). Furthermore, the multivariate analysis in Libyan material suggested that SMI is more useful in assessing prognosis than MAI. This seems to be in line with earlier studies (Collan et al. 1996, Ikpat et al. 2002b, Jalava et al. 2006).

### 6.3.3 Apoptotic index (AI) (III)

The present results show that AI was associated with long-term disease specific survival (DSS) and separated the patients into subgroups with significantly different prognosis in the Libyan breast cancer material. This finding is in line with earlier studies showing that increase in apoptosis was related to poorer prognosis and a shorter lifespan (de Jong et al. 2000, Berardo et al. 1998, Ellis et al. 1998, Lipponen et al. 1994). However, there is no evidence that AI has independent value in multivariate analysis especially when analyzed together with proliferative indices and classic prognosticators (Ikpat et al. 2002a).

Some studies observed that the AI was an independent prognosticator (de Jong et al. 2000). However, the present study and study of Ikpat et al. (2002a) on Nigerian and Finnish material show that the AI can be used only as a general prognosticator.

The current study also suggests two thresholds for the AI (4 and 18 apoptotic cells per mm<sup>2</sup>) (**Table 10**) that could separate patients into significantly different groups with good, moderate and bad prognoses. It was found that survival among patients with AI < 4 apoptotic cells per mm<sup>2</sup> was significantly better than among patients with AI ≥ 18 apoptotic cells per mm<sup>2</sup> ( $p < 0.0001$ ). The apoptotic index in addition to proliferative indices could be very useful in grouping patients with good or poor prognosis. Some authors suggested that AI might be reliable in predicting unfavorable outcomes (de Jong et al. 2000).

#### 6.3.4 Fraction of fields with tubular differentiation (FTD) (V)

Several authors have reported on the prognostic value of the FTD in breast carcinoma (Dalton et al. 1994, Davis et al. 1986, Fisher 1986, Theissig et al. 1990). On the other hand, there are a lot of papers concluding that tubular differentiation lacks prognostic significances and is inferior to the proliferative activity and nuclear size features (Baak et al. 1985, Clayton. 1991, Le Doussal et al. 1989, Lipponen et al. 1991, Parham et al. 1992, Rank et al. 1987, Schumacher et al. 1993, Theissig et al. 1990, van der Linden et al. 1989). The multivariate analysis of the present study shows that the FTD is a weak prognostic factor in relation to the proliferative index SMI. The study of Ikpatt et al. (2003) also shows that the FTD could not be accepted as an independent prognosticator due to the fact that SMI and FTD are factors that have corresponding changes. Kronqvist et al. (2000) did their work with the same methodology and found that the FTD was an independent prognosticator in Finnish breast cancer, but below the significance of the proliferative indices.

The current study shows that FTD can be used as a general prognosticator. However, mitotic activity (SMI, standardized mitotic index) is a better general prognosticator than FTD. However, FTD can add some valuable prediction particularly in premenopausal patients.

The present study suggests two significant cut-points for the FTD in Libyan material (30% and 50%) (**Table 10**), which could separate patients into three subgroups with favorable, intermediate and unfavorable prognosis (Figure 6). These cut-points may be more suitable for the Libyan material than the cut-points suggested by Kronqvist et al. (2000) on Finnish material (23% and 59%).

**Table 10** Comparison of the most significant quantitative threshold values for proliferative indices of Libyan, Nigerian, and Finnish female breast cancer patients.

Country	MNA µm <sup>2</sup>	AI apoptosis/ mm <sup>2</sup>	SMI mitoses/mm <sup>2</sup>	MAI mitoses/10hpf	FTD %
Libya	71	4,8	19 and 44	15 and 58	30 and 50
Nigeria	111*	NS*	17 and 92*	10 and 92*	15*
Finland	32 and 47°	NS*	17 and 32°	13 and 35°	23 and 59°

\*From Ikpatt et al 2002, °From Kronqvist 1999.

NS = there is no statistically significant cut-point

## 7. CONCLUSION

1. Compared with western countries Libyan breast cancer is characterized by low incidence, and it is dominantly premenopausal. In this sense it reminds the breast cancer in European patients have higher incidence and is dominantly postmenopausal in character. The Libyan finding also supports the idea that breast cancer is not one disease but should be classified in to premenopausal and postmenopausal types.
2. At present Libyan breast cancer displays unfavorable features such as high histological grade and stage, large size and frequent lymph node metastases. These features are associated with poor survival in Libyan premenopausal breast cancer patients.
3. A positive correlation of nuclear size parameters, and mitotic and apoptotic indices with clinicopathological characteristics were observed in Libyan breast cancer. Of the morphometric parameters the mean nuclear area (MNA), and of the proliferation- associated features the standardized mitotic index (SMI) were most clearly associated with the grade and the clinical stage of the tumor.
4. Histomorphometric parameters can be used as prognostic tools. Especially high values of MNA, high mitotic indices (SMI, MAI) and high apoptotic index (AI), showed highly significant association with aggressive tumor nature and poor survival.
5. Grading cut-points in Libyan breast cancers in respect to survival were evaluated for proliferation (mitotic indices), apoptotic index (AI), tubular differentiation (FTD = fraction of fields with tubular differentiation) and mean nuclear area (MNA). Cut-points for standardized mitotic index (SMI) and mitotic activity index (MAI) were 19 and 44 mitotic figures/mm<sup>2</sup>, and 15 and 58 mitotic figures /10HPF, respectively. Cut-points for apoptotic index (AI) were 4 and 18 apoptosis /mm<sup>2</sup>. For the fraction of fields with tubular differentiation (FTD) the cut-points were 30% and 50%, and they could be used as criteria for separation of patients into three groups with good, moderate and poor prognosis. Survival analysis revealed one significant cut-point (71µm<sup>2</sup>) for mean nuclear area. This cut-point allowed separation of patients into groups of good and poor prognosis.
6. The results suggest, but do not prove that the differences in morphometric features in breast cancer in Libya, Nigeria, and Finland may be associated with variation in the distribution of genetic markers in these populations. Improvement in health



care and introduction of screening programs, however, could be very helpful clarifying the situation in the Libyan population.

7. In the hospital setting, the MAI, SMI, AI, FTD and MNA in breast carcinoma may be prognostically useful markers in guiding decision on future treatment.

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