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APPLICATION OF MORPHOMETRY, STATIC DNA PLOIDY ANALYSIS, AND STEROID RECEPTOR EXPRESSION IN DIAGNOSIS AND PROGNOSIS OF LIBYAN BREAST CANCER

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Fathi B. Elmabrouk Abdalla: Application of morphometry, static DNA ploidy analysis, and steroid receptor expression in diagnosis and prognosis of Libyan breast cancer

ABSTRACT

The aim of this study was to describe the demographic, clinicopathological, biological and morphometric features of Libyan breast cancer patients. The supporting value of nuclear morphometry and static image cytometry in the sensitivity for detecting breast cancer in conventional fine-needle aspiration biopsies were estimated. The findings were compared with findings in breast cancer in Finland and Nigeria. In addation, the value of ER and PR were evaluated. There were 131 histological samples, 41 cytological samples, and demographic and clinicopathological data from 234 Libyan patients.

The Libyan breast cancer is dominantly premenopausal and in this feature it is similar to breast cancer in sub-Saharan Africans, but clearly different from breast cancer in Europeans, whose cancers are dominantly postmenopausal in character. At presention most Libyan patients have locally advanced disease, which is associated with poor survival rates.

Nuclear morphometry and image DNA cytometry agree with earlier published data in the Finnish population and indicate that nuclear size and DNA analysis of nuclear content can be used to increase the cytological sensitivity and specificity in doubtful breast lesions, particularly when free cell sampling method is used. Combination of the morphometric data with earlier free cell data gave the following diagnostic guidelines: Range of overlap in free cell samples: 55 μm^2 -71 μm^2. Cut-off values for diagnostic purposes: Mean nuclear area (MNA) >54 μm^2 for 100% detection of malignant cases (specificity 84 %), MNA < 72 μm^2 for 100% detection of benign cases (sensitivity 91%).

Histomorphometry showed a significant correlation between the MNA and most clinicopathological features, with the strongest association observed for histological grade ($p < 0.0001$). MNA seems to be a prognosticator in Libyan breast cancer (Pearson's test $r = -0.29$, $p = 0.019$), but at lower level of significance than in the European material. A corresponding relationship was not found in shape-related morphometric features.

ER and PR staining scores were in correlation with the clinical stage ($p = 0.017$, and 0.015, respectively), and also associated with lymph node negative patients ($p=0.03$, $p=0.05$, respectively). Receptor-positive (HR+) patients had a better survival. The fraction of HR+ cases among Libyan breast cancers is about the same as the fraction of positive cases in European breast cancer. The study suggests that also weak staining (corresponding to as few as 1% positive cells) has prognostic value. The prognostic significance may be associated with the practice to use antihormonal therapy in HR+ cases.

The low survival and advanced presentation is associated with active cell proliferation, atypical nuclear morphology and aneuploid nuclear DNA content in Libyan breast cancer patients. The findings support the idea that breast cancer is not one type of disease, but should probably be classified into premenopausal and post menopausal types.

Fathi B. Elmabrouk Abdalla: Morfometria, staattinen DNA sytometria, ja steroidireseptorit libyalaisen rintasyövän diagnostiikassa ja ennusteen arvioinnissa.

YHTEENVETO

Väitöskirja kuvaa rintasyöpää sairastavien libyalaisten naisten elinoloja, ja heidän rintasyöpänsä kliinispatologisia, biologisia ja morfometrisia piirteitä. Tutkimuksessa arvioitiin, miten tumamorfometria ja staattinen DNA sytometriä lisäävät ohutneulabiopsian herkkyyttä löytää syöpäkasvain. Löydöksiä verrattiin suomalaisten ja nigerialaisten rintasyöpäpotilaiden tietoihin. Työssä arvioitiin myös steroidireseptorin merkitystä rintasyöpämateriaalissa. 131 histologista ja 41 sytologista näytettä analysoitiin, ja 234 libyalaisen potilaan kliinispatologiset ja väestötiedot tutkittiin.

Libyalainen rintasyöpä on etupäässä premenopausaalista, ja eroaa siksi eurooppalaisesta rintasyövästä, joka on pääosin postmenopausaalista. Saharan eteläpuolinen rintasyöpä Afrikassa on myös selvästi premenopausaalista. Taudin toteamisvaiheessa useimmilla libyalaisilla naisilla oli paikallisesti levinnyt rintasyöpä johon liittyy huonompi ennuste kuin vain maitorauhasen sisäiseen syöpään.

Tumamorfometrian ja DNA sytometrian tulokset ovat yhteneväisiä suomalaisesta rintasyöpämateriaalista julkaistujen tulosten kanssa. Menetelmiä voidaan käyttää lisäämään ohutneulabiopsiatutkimuksen herkkyyttä ja spesifisyyttä. Kun aikaisempien tutkimusten tulokset yhdistetään tässä tutkimuksessa havaittuihin, saadaan diagnostiikassa käytettäviksi tuloksiksi: hyvänlaatuisten ja rintasyöpäsolujen yhteinen kokoalue oli 55-71 neliömikrometriä. Kaikki syöpätapaukset löydettiin niiden näytteiden joukosta, joissa tumien alojen keskiarvo oli yli 54 neliömikrometriä. Tämä vastaa 100%:n herkkyyttä. Vastaava spesifisyys oli 84%. Kaikki hyvänlaatuiset näytteet sisältyivät tapauksiin, joissa tuman keskimääräinen ala oli alle 72 neliömikrometriä. Tällä alueella rintasyövän toteamisen herkkyys oli 91%.

Rintasyöpäsolun keskimääräinen tuman pinta-ala oli suhteessa useimpiin ennusteellisiin kliinispatologisiin tietoihin. Vahvin korrelaatio oli suhteessa histologiseen erilaistumisasteeseen (gradus). Tilastollinen merkitsevyys ei kuitenkaan ollut libyalaisessa materiaalissa samaa luokkaa kuin aikaisemmin julkaistussa suomalaisessa materiaalissa. Kasvainsolun tuman muotoon liittyvillä tekijöillä ei ollut ennusteellista merkitystä.

Steroidireseptorien värjäytyvyyttä arvioitiin histologisesti. Värjäytyvyydellä oli selvä yhteys kliiniseen levinneisyysasteeseen ja imusolmuke-etäpesäkkeiden esiintymiseen. Steroidireseptoripositiiviset syövät liittyivät pitempään keskimääräiseen eloonjäämisaikaan. Steroidireseptoripositiivisten potilaiden osuus libyalaisessa materiaalissa oli sama kuin suomalaisessa materiaalissa. Tutkimus osoitti, että myös heikko värjäytyminen oli ennusteellisesti merkitsevä. Reseptorien ennusteellinen merkitys voi liittyä siihen, että positiivisia potilaita hoidetaan antiestrogeenihoidolla.

Rintasyövän heikompi ennuste Libyassa ja taudin levinneisyys diagnoosivaiheessa näyttää liittyvän lisääntyneeseen proliferaatioaktiivisuuteen, poikkeavaan tumarakenteeseen ja aneuploidiseen DNA pitoisuuteen. Löydökset tukevat ajatusta, että rintasyöpää ei välttämättä ole pidettävä yhtenäisenä biologisena tautina, vaan se voitaisiin ehkä jakaa premenopausaaliseen ja postmenopausaaliseen rintasyöpään.

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ABBREVIATIONS

AOI	African oncology institute
AR	Area axies ratio
BRCA	Breast cancer gene
CA	Carcinoma
CNB	Core needle biopsy
CSC	Cancer stem cell
DNA	Deoxy ribonucleic acid
ER	Estrogen receptor
FA	Fibroadenoma
FCD	Fibrocystic disease
Fd	Field diameter
FNAB	Fine needle aspiration biopsy
FU	Follow-up
GDP	Gross domestic product
HBOC	Hereditary breast-ovarian cancer syndrome
HE	Eosin and Hematoxiline stain
HR	Hormonal receptors
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
ILC	Infiltrative lobular carcinoma
LN	Lymph node
LS	Long and short axies
MNA	Mean Nuclear Area
Mi RNA	Micro RNA
N	Number of patients
NCI	Nuclear countour index
NOS	Not otherwise specified
NS	Not statistically significant
PR	Progesterone receptor
SD	Standard deviation
TNM	Tumor size, Lymph node stage, Metastasis stage
TT	Triple Test Method

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications

I. Abdalla F, Boder J, Buhmeida A, Hashmi H, Elzagheid A, Collan Y: Nuclear morphometry in FNABs of breast disease in Libyans. Anticancer Res 2008; 28: 3985-3990.

II. 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.

III. Abdalla FBE, Boder JME, Buhmeida A, Elzagheid AI, Collan Y: Image DNA cytometry in FNABs of Libyan breast disease. Anticancer Res 2010; 30: 175 – 182.

IV. Abdalla FBE, Markus R, Buhmeida A, Boder J, Syrjänen K, Collan Y: Estrogens receptors, progesterone receptors, and nuclear size features in Libyan female breast cancer: Correlation with clinical features and survival. Anticancer Res 2012, 32: 3485-3494.

V. 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.

1. INTRODUCTION

Female breast carcinoma is one of the most common malignant diseases in the world, contributing to 23-31% of all cancers, with over one million new cases diagnosed annually (Parkin et al. 2005, Parkin and Fernandez 2006, Sabratha Cancer Registry 2008, American Cancer Society 2010). There are about 4.4 million women are living with the disease and over 400,000 annual deaths. Breast cancer accounts for 10-18% of all cancer deaths. It is the first most common cause of female death in industrialized countries and the third most common in developing countries (Lester 2007, Draper 2006, Parkin et al. 2005, Wiliams et al. 2006, Bray et al. 2004).

In Libya and in developing countries, in general, breast cancer management constitutes a big medical, social and economic issue. The hallmarks of the detection and treatment level of breast cancer in most developing countries are presentation at advanced stage, lack of adequate mammography screening programs, dominant presence in young age; premenopausal status, and high death rate (Ikpatt 2002, Elmistiri 2006, Adesunkanmi et al. 2006, Wiliams et al. 2006, Sabratha Cancer Registry 2008, Misurata Cancer Registry 2010). Despite major advances in treatment regimes, there has been only a little improvement in mortality rates. Therefore, it is important to achieve better cancer control by two different strategies, first by improvements in early detection, and second by better selection of prognostic factors which should be applied to predict the outcome of the individual patient, and to select appropriate therapy (Ikpatt 2002).

As to the first, we could increase diagnostic sensitivity of fine needle aspiration biopsy by supportive methods such as DNA cytometry, cDNA array, chromatin texture analysis, and morphometric measurements.

As to the second, because breast cancer is one of the hormone dependent tumors many studies have been dealing with the relationship between steroid hormone receptors, such as oestrogen receptors (ER), and progesterone receptors (PR) and breast cancer outcome. It is now well established that determination of progesterone (PR) and estrogen receptors (ER) in breast cancers can be used as prognostic and predictive factors (Barbareschi et al. 2002), especially when associated with antisteroidal receptor therapy. The presence of ER and PR are related to a favorable response to endocrine therapy and improve overall survival. The general practice is that the choice among endocrine treatments is made on the basis of hormone receptor status. Some studies show a significant endocrine therapy benefit in women with tumors containing only 1% of positive cells (Goldhirsch et al. 2003). Many molecular markers are available for the better evaluation of breast cancer tumorigenesis, disease progression and as guide to treatment (Casadei et al. 2011, Statistical information UK team 2009, Fillmore and Kuperwasser 2008). However, few studies have been performed on the Libyan breast disease; and the tumor phenotypic alterations in Libyan population are not well

known. Another reason for the limited understanding of the clinical and pathologic prognostic factors of breast cancer in female Libyan patients can be due to the low incidence and low prevalence of this disease in Libya. Also the follow up of patients is very variable due to the fact that patients are often partly or fully treated outside the hospital which made the histopathological diagnosis, in other Libyan or north African hospitals, or abroad.

2. REVIEW OF THE LITERATURE

The female breast is a collection modified sweat glands composed of lobes and lobules interspersed with adipose tissue and connective tissue. Ducts drain from each lobule. These converge to form a lactiferous duct that drains from each lobe. The mature female adult breast is composed of 15 to 25 grossly defined lobes corresponding to parenchyma associated with each of the major lactiferous ducts, each emerging independently at the nipple. The functional secretory unit in lactation is the terminal duct lobular unit. Here, each duct has a lining epithelium surrounded by a thin myoepithelial cell layer responsive to oxytocin, the hormone that stimulates lactation. Neoplasia may arise in the ductular epithelium, lobules, or the stroma. However, the majority of cancers arise in the ducts (Keith & Arthur 2006, Rosen 2009).

2.1 History of struggle against breast cancer

Breast tumors were historically the earliest tumors to be described and treated. The first ancient documentation on breast tumor was written in Egyptian surgical papyri (between 1500 and 3000 BC). Eight patients (one male and seven females) were described with breast mass lesions, and all were treated by cauterization. During the middle of the second century, Claudius Galenus in Rome confirmed the Hippocratian theory that described the tumor as a crab, which has legs on both sides of his body. In this disease the blood vessels extending out from the tumor take the shape of the crab's legs (Ismail et al. 2006). During early 19th century the new concept emerged suggesting that the disease could be cured in its early stages, but not after it had reached a large size. In the early stages of the history of medicine, surgical operation was considered the only treatment. Halsted and Meyer were in 1894 established radical mastectomy as the standard for breast cancer treatment. At the end of 19th century Beaston was the first to recognize the effect of hormonal status on breast cancer, when he treated patients with advanced breast cancer by bilateral oopherectomy. During the latter part of the 20th century, there was a change from Halsted radical mastectomy to modified radical mastectomy (MRM) (Stone et al. 2003, Ismail et al. 2006). In the 1950s breast cancer was often diagnosed as a systemic disease at presentation. Later the treatment of primary breast cancer improved from surgical therapy only to multidisciplinary management that used chemotherapy, surgery, radiation and hormonal therapy. Today, pathologists generate lots of data for therapeutic decisions. Surgery of breast cancer is now more conservative and sparing than earlier and includes lumpectomy or quadrantectomy. The recent four decades have shown development in the knowledge and understanding of the basic science of the disease. Particularly the genetic and molecular basis of the disease has been evaluated and more attention has also been given to hormonal and targeted therapy (Stone et al. 2003, Rosen 2009).

2.2 Epidemiology of breast cancer

2.2.1 Breast cancer incidence

Today breast cancer constitutes a major public health problem worldwide with over 1 million new cases diagnosed annually, 99% of them in female patients, (Ahmedin and Melissa 2010, American Cancer Society 2010, Parkin et al. 2006). The disease is rarely diagnosed in women younger than 25 years. Past that age the incidence rises steadily to reach a peak at the age of menopause. The increase in incidence falls after menopause, but older women are still at increasing risk (Singletary 2003).

The breast cancer incidence is increasing worldwide, but it varies from areas of low incidence (Japan and other Asian, Latin American, and African countries) to areas of high incidence (the United States, Western Europe, Northern Europe and Australia). For example, in the USA there were less than 0.9 new cases per 1000 women in 1990s, and more than 1.4 new cases per 1000 in 2006 (American Cancer Society 2010, McCracken et al. 2007, Parkin et al. 2005, Wiliams et al. 2006, Bray et al. 2004). Also in the Nordic countries, the incidence of breast cancer has been increasing steadily during the last 30 years. The incidence in Finland rose from 0.63 per 1000 female population in 1987 to 0.94 in 2010 (Finnish cancer registry 2010). In UK the incidence of breast cancer has increased from 0.75 per 1000 in1977 to 1.2 per 1000 in 2006 (Statistical information UK team 2009). Even the low incidences of breast cancer in Eastern Europe and Japan have started to rise (Pompe-Kirn et al. 2000, Nagata et al. 1997). However, breast cancer is much less common in Asia and Africa. Many studies in the USA show that black women have a lower breast cancer incidence but higher breast cancer mortality rates than white women (Bray et al. 2004, American Cancer Society 2008, McBride et al. 2007, Rowan et al. 2005, James 2000). In Africa, breast cancer has overtaken cervical cancer as the commonest malignancy affecting women and the incidence rates appear to be rising. In Nigeria for example, incidence rate has increased from 13.8-15.3 per 100,000 in the 1970s, to 33.6 per 100,000 in 1990s (Adebamowo and Ajayi 2000, Ikpatt 2002, Wiliams et al. 2006, Adesunkanmi et al. 2006).

In sub-Saharan African population there are low rates of breast cancer, and majority of the patients presented in advanced stage (Fregene and Newman 2005, Wiliams et al. 2006). In the Arabic countries the studies are not fully covering. In Morocco the most frequent cancer in the female is cervical uterine neoplasia (35%) followed by breast cancer (22.3%), which is also presented at advanced stages (Chaouki and El Gueddari 1991). In Egypt about 35% of all female cancer is breast cancer (Nadia et al. 2007).

Table 1 Population data of Libya, Nigeria and Finland. Data on Nigeria and Finland are basically the same as those published in the study of Ikpatt et al (2002). However, new data, when available, were used for updating.

	Libya	**Nigeria**	**Finland**
Total population	6,546,000(10)[1]	158,259,000(10)[1]	5, 364,000 (10) [1]
Age structure			
0-14	30.1% [1]	43.1%[1]	16.7%[1]
15-59	63.4%[1]	52.1%[1]	59.5%[1]
60+	6.5%[1]	4.8%[1]	23.8%[1]
Population growth rate	2.3[2]	2.67[4]	0.44[5]
Birth rate	2.68%[1]	3.99%[1]	1.12%[1]
Death rate	0.35%[1]	1.68%[1]	0.92%[1]
Sex ratio (m/f)	1.08[1]	1.03[1]	0.96[1]
Infant mortality rate	2.14%[1]	10.9%[1]	0.26%[1]
Life expectancy			
Total	76.55[1]	46.85[1]	79.65[5]
Female	78.8[1]	47.3[1]	83.0[5]
Male	74.3[1]	46.4[1]	76.3[5]
Total fertility rate	3.34[1]	5.3 [1]	1.85[1,5]
Literacy	88.1 [1]	73.1%[1]	100%[1,3,4,5]
GDP(in US$/capita)	11.590[1]	1.160[1]	48.120[1]

[1, 2, 3, 4, 5] See references:
[1] King et al. 2012, [2] Libyan National Statistics Figures: 2003, [3] Brazier and Hamed 2003, [4] Ikpatt et al. 2002, [5] Statistics Finland: Finland in Figures 2010

The background of Arabic patients may be more related to other African breast cancer patients than to European breast cancer patients, although also demographic and environmental differences e.g. between Arabic Libyan, African (Nigerian) and European (Finnish) populations are prominent (Table 1, identical with **Paper V**: Table I).

These increases in incidence are due to changes in the demography, socio-economic parameters, epidemiologic risk factors, better reporting and awareness of the disease. On the other hand, the mortality rates are declining in the developed world (Americas, Australia and Western Europe) as a result of early diagnosis, screening, and improved cancer treatment programs.The converse is true in the developing world as well as in eastern and eastern central Europe (Parkin et al. 2005, Adesunkanmi et al. 2006).

2.2.2 Risk factors

Although a single specific cause for breast cancer has not been identified, there are many established risk factors that increase the likelihood that a woman will develop a breast cancer (McPherson et al. 2000). The most potential risk factors are increased estrogen exposure, proliferative breast disease, increased age of patient and family and/or personal history of breast cancer.

Age

Like in many other carcinomas, age is also a risk factor in breast carcinoma (McPherson et al. 2000). The risk is low before age 25 and increases with age, leveling off at the age of 80 (Singletary 2003). Most breast cancers in white women occur after the age of 50 with incidence decreasing after the age of 60. In Arabian, South American, African and African-American women, the average age is approximately 10-15 years younger than in European and American white women (Ikpatt 2002, Fregene and Newman 2005, Wiliams et al. 2006, Parkin et al. 2005, American Cancer Society 2008). Some authors suggested that risk of age could partly be associated with the age distribution of populations in respective countries, and partly due to indirect influence of other factors such as females with early age of menarche, and/or late age of menopause. However, biological difference may also be involved, including the variation in genetic marker distribution between countries, and underlying genetic difference such as BRCA1 or BRCA2 mutations (Ford et al. 1998, Ikpatt and Olopade 2006).

Gender

It is well known that the female gender has breast carcinoma incidence 100 times greater than the male. In Europe and United States, breast cancer the men accounts less than 1% of all breast cancers, and is responsible of 0.1% of breast cancer mortality. However, in some African countries, the male breast cancer forms more than 5% of all breast cancers. The latter is true especially in Kinia, Ethiopia and Uganda (Ersumo 2006, Fentiman et al. 2006, Alterman et al. 2008). Male breast cancer patients usually have higher age and more aggressive disease than female patients (Andre et al. 2001).

Previous breast disease

Females who have a past history of breast cancer will have an added 1% per yearly risk of developing a new invasive breast cancer. The risk among women with proliferative disease in form of atypical hyperplasia is 3.0 to 5.0 times that of women with non-proliferative benign breast disease. Women, who have atypia-free proliferative disease including moderate to florid epithelial hyperplasia and sclerosing adenosis, are associated with a little increased risk (1.5 fold to 2.0 fold) (London et al. 1992). An increased risk of breast cancer has also been demonstrated for women who have increased density of breast tissue as assessed by mammograms. Compared to women with no visible breast density, a breast density of 75% or greater is associated with an approximately 5-fold increase in risk (95% confidence interval 3.6-7.1) (Byrne et al. 1995). Women with a previous primary breast cancer have a 3 to 4 fold increase in risk of a second breast cancer in the contralateral breast (Kelsey and Gammon 1991). Risks are higher for women diagnosed at a younger age, with a lobular histology in the original cancer and/or with a family history of breast cancer (Habel et al. 1997).

Geographic differences

There is a big variation in age adjusted incidence rate for breast cancer seen among countries. More than fivefold differences are possible, when the lowest incidence countries in South-East Asia and Africa are compared with highest rate countries including USA, North-West Europe, Australia and New Zealand. The risk for women who migrate from low to high risk areas typically increases suggesting that difference could be explained by other than genetic factors (lifestyle, environmental and reproduction related risk factors). For example, Asian female migrants to the USA experience rapid increase in breast cancer incidence rates (Dumitrescu and Cotarla 2005, MacMahon 2006, American Cancer Society 2008).

Family history and genetics

Although the aetiology of breast cancer is not fully understood, many studies have been done on the effects of family history and inherited mutation. Up to 10% of breast cancers have been found to have genetic predisposition. Women, whose first-degree family member had breast cancer at a young age, have 2- to 4- fold risks for development of breast cancer than females who have not got an affected family member (Casadei et al. 2011, Dumitrescu et al. 2005). Those females should be studied with breast cancer screening at an early age. The suitable age for starting screening is at least one decade younger than the age at which the affected relative got breast cancer. Of patients with family history many have mutations in BRCA1 and 2 genes, and other genes such as PALB2 gene which is partner and localizer of BRCA2, mutations at PALB2 among male and female increases risk of breast cancer 2- to 6-fold (Cao et al. 2009, Casadei et al. 2011). Mutations are inherited, usually in an autosomal dominant pattern with different penetrance, and are located on the long arms of chromosomes 17q21 and 13q12, respectively (Gareth et al. 2008). These are tumor suppressor genes which are important in the repair process after DNA damage and preservation of genomic integrity (Jhanwar Uniyal 2003). Women with these mutated genes have a 50 to 80% chance of getting breast cancer in their lifetime. Because also ovaries can be involved the condition is called hereditary breast-ovarian cancer (HBOC) syndrome (Lester and Cotran 1999, Gareth et al. 2008, Metcalfe et al. 2009). Risks for colon and prostatic cancers are also increased. BRCA2 mutations are associated with further cancers, such as non-Hodgkin lymphoma, Fallopian tube, pancreatic, bladder, and male breast cancers (Lakhani 1999, McPherson et al. 2000). On other hand, little data is available about genetic mutations as a cause of breast cancer in non-Caucasian population, where the hereditary cancer may be the cause for only 1% of all breast cancers. Studies that have been done in African patients, a considerable number of BRCA-1 and BRCA-2 mutations have been recognized, but so far we do not have evidence that breast cancer genes (BRCA1 and BRCA2) are more often involved in Africa than other population (Fregene and Newman 2005, Ikpatt and Olopade 2006). In addition, there are other gene mutations that may also be accompanied with breast carcinoma such as: mutation in P53 tumor suppressor gene e.g. in Li-Fraumeni syndrome, mutation PTEN (phosphate and tension) tumor suppressor gene in Cowden syndrome (multiple hamartoma syndrome), STK11gene mutation in Peutz-Jeghers syndrome (Lester and Cotran 1999, Smith and

Robson 2006) and mutation ATM gene in Ataxia telangiectasia patients is associated with non-hodgkin lymphoma, ovarian and breast carcinoma (Statistical information UK team 2009, Zhang et al. 2003)

Environmental factors

It is thought by many that environment does not much affect breast cancer risk. The most well-defined environmental risk is exposure to ionizing radiation. Presence breast cancer has been demonstrated in patients who received prolonged therapeutic radiation for thymus tumor, Hodgkin's disease, and thyroid malignancy. This also applied to Hiroshima/Nagasaki survivors of the atomic bomb, and X-ray medical technicians and workers (Hortobagyi et al. 2005). Some reports show that there is association between radiation sensitivity and increased rate of breast cancer and multiple primary malignancies in persons with familial disorders like ataxia-telangiectasia (Kastan 1995). So breast cancers can potentially have resulted from a genetic susceptibility to the mutagenic effect of radiation exposure (Lakhani et al. 1999), best described by a multistep progression model (Beckmann et al. 1997). Low dose of diagnostic radiation exposure, including mammography and therapeutic radiation may have carcinogenic risk in patients with radio-sensitivity. On other hand, radiation sensitivity could make tumors in women with genetic susceptibility to breast cancer more responsive to radiation treatment. But experimental confirmation is essential to establishing these suggestions.

Lifestyle risk factors

Several lifestyle factors are associated with breast cancer risk. These include obesity, lack of exercise and physical activity, dietary fat intake, alcohol use and smoking. Obesity, high body mass index and lack of exercise are risk factors especially in post-menopausal women. Obesity is associated with 2-fold increase in the risk of breast cancer in postmenopausal women, and associated with a reduced incidence of breast cancer in premenopausal women as a result of its association with anovulatory cycle (McPherson et al. 2000). However, there are inconsistent results on the association of postmenopausal obesity with breast cancer risk according to ER and/or PR status (Yoo et al. 2001). Kumar et al. in 1995 suggested that women, who gained weight from puberty to adulthood, and specifically after the third decade of life, should be considered a higher risk group. Alcohol-rich and saturated fat rich diets raise the risk, while smoking does not appear to affect the risk (Dumitrescu et al. 2005). However, the epidemiologic relation between fat intake and breast cancer does not appear to be particularly strong (Kuller et al. 1997).

Hormone and reproductive related risk factors

Reproductive hormones are thought to increase risk of breast cancer through effects on cell proliferation and promotion of cancer growth (American Cancer Society 2010). Many risk factors for breast cancer are associated with long-term exposure to estrogen hormone. These include; long duration of reproductive life (early age at menarche, late

age at menopause), intake of estrogen hormone replacement therapy, nulliparity, and delay the age of first pregnancy especially post thirties (Pathak et al. 2000). However, young ladies who get full term childbirth have a low risk of breast cancer. Because some anti-estrogenic factors are synthesized by well developing fetal liver, like alpha-fetoprotein, mother can be protected from the risk of estrogen effect. But because those anti-estrogenic factors will often be at a maximum at the full term, any gestation that is not ended by full term cannot yield the same protective effect (Lambe et al. 2003). Some reports suggested that the risk of breast cancer has been also slightly raised among females who have prolonged intake of contraceptive pills (Butler et al. 2000). We feel that, as the living standards in Africa improve, the age at menarche will decrease and the age of menopause will increase. The level of education and health care may increase the number of young ladies who use contraceptive methods to avoid or delay pregnancy, and avoid breast feeding. These factors, with the absence of screening programs are likely to have an effect on the incidence of breast cancer in African countries. However there is reason to believe that the differences between African type breast cancer and the type seen in more developed countries will not disappear. The studies on African American breast cancer support this view (Fregene and Newman 2005, Alford et al. 2009)

2.2.3 Screening for breast cancer

Screening means the use of investigations on asymptomatic persons, to detect the disease at an early stage in order to lower the risk of death, or complications of therapy (Jerzy et al. 2002, Smith et al. 2006). Annual screening program by using mammography method usually begins at an age of about 50 years. This is a useful method for detection of impalpable tumor with a size less than 1 cm in diameter. The increased use of mammography screening program among women between the ages of 50 and 64 (may extend to 70) resulted in more than 20% relative risk reduction in breast cancers mortality (Keen and Keen 2009). For example the American breast cancer mortality was 20.8% lower in 2001 than in 1991. The decline was predominantly due to a shift towards the use of annual screening program, which can reveal small size cancers at an early stage. The early stage allows more conservative and sparing treatment, rather than mastectomies (Smith 2006). However, mammography may fail in young women. Therefore the effectiveness of screening mammography in younger females (i.e. 40–49 years) is not well established. Such uncertainty has yielded conflicting answers about its use for this age group. For individuals who have high risk factors a yearly mammography screening program from the age of 40 years is recommended (Keen and Keen 2009). The occurrence of breast cancer in female African population is strongly related to the younger age, which may be problem because of low diagnostic value of mammography in young patients.

Other simple and important methods for early detection of breast cancer are self breast examination, accompanied with clinical breast examination. A few studies have suggested, such methods have not significantly decreased breast cancer mortality (Gaskie and Nashelsky 2005).

The screening of large populations is associated with anxiety and negative psychological effect. The life style and cultural attitudes have essential roles in compliancy of screening programs in some countries. Introduction of the breast health education at schools, in the media and in the primary health care centers of such countries may well be a useful option to changing the attitudes towards acceptance of breast screening programs (Smith et al. 2006).

2.3 Diagnosis of breast cancer

One of the best methods for detection of breast abnormalities is a screening program with self and routine physical examination. However, a breast cancer may have been present for 5 to 10 years before reaching a size (about 1 cm) that is detectable by palpation. In a developed practice and at many centers (including the AOI in Libya) all patients presenting with a symptomatic breast lump are assessed by means of triple assessment: clinical examination, radiology image in the form of mammography and/ or ultrasonography, and fine needle aspiration/ core needle biopsy (Clarke et al. 2001, Smith et al. 2006, American Cancer Society 2010).

2.3.1 Clinical examination

The usual clinical picture of an early breast cancer is a painless mass in the breast particularly in upper outer quadrant. Other less common clinical presenting features of breast cancer are; breast size and shape asymmetry, nipple or skin retraction, blood-stained discharge from the nipple, areolar eczema (e.g. in Paget's disease), ulceration, erythematous rash of the nipple or surrounding skin area and palpable regional lymph node. Cancer in the axillary tail can be mistaken clinically for an involved lymph node. There are also systemic complaints like fatigue, cough, anemia, ascites, or musculoskeletal discomfort, especially in advanced disease. During palpation oddly shaped, hard lump that feels firmly fixed within the breast is likely to be cancer (American Cancer Society 2010).

2.3.2 Radiological imaging techniques

The radiological imaging tools including mammography, ultrasonography and MRI are very useful in annual screening programs and clinical diagnosis of breast lesions.

Mammography

The mammography is the commonest diagnostic X-ray image of breast that can be used to detect impalpable cancers in their pre-invasive or early invasive stage and aid to clinically distinguish between benign and malignant diseases with high sensitivity rate. Therefore it is commonly recommended for breast cancer screening (Keen and Keen 2009). Specific mammography abnormalities that suggest a diagnosis of breast carcinoma include heterogeneous high density mass in breast or/and axilla, with or without micro-calcifications related to the ducts. The suspicious calcifications are

usually clustered, angular and irregular patterns and often branching. Mammography can also be used in guiding needle localization, fine-needle aspiration biopsy, and core-needle biopsy. However, we know that some tumors detected by palpation are not easy to find in mammography (Rubio et al. 2003). If the mammographic films show some abnormalities, a range of following techniques may be utilized to further aid in diagnostic investigations such as ultrasound, magnetic resonance imaging and breast biopsies.

Ultrasonography (US)

Although mammography is usually superior in sensitivity to US in detecting breast tumors, (particularly those which are 1cm or less in diameter), US is fast and easy to apply and may be used along with a mammogram. US is poor as a screening test, but on the other hand, about 10-15% of clinically palpable breast tumors are not detected by mammography (Rubio et al. 2003 and Bassett et al. 1990). Ultrasonography is an important technique in helping to resolve an equivocal mammography finding, define cystic lesions, and demonstrating the echoes qualities of specific solid abnormalities. Moreover, ultrasonography may also be used in guiding needle biopsy and pre-operative needle localization of selected breast lesions. US is highly reproducible and has a high patient acceptance rate. However, young women with high-risk for breast cancer, e.g. women with a strong family history of breast cancer or who carry known genetic mutations require screening with US at an early age when the mammography evaluation is of limited value because of the increased breast density in younger women. In these cases ***magnetic resonance imaging (MRI)*** is superior in detecting breast lesions (Pavic et al. 2004, American Cancer Society 2010).

2.3.3 Breast biopsies (FNAB, CNB and open biopsy)

Following detection of an abnormality by palpation and/or by mammography, a tissue sample can be obtained. For pathologic diagnosis of small breast lumps that in imaging are not clearly cancers, a number of biopsy techniques are used. The needle biopsies may be performed with using imaging procedures to guide the needle. Needle biopsy methods are divided into two types.

Fine needle aspiration biopsy (FNAB)

FNAB has been routinely applied as a part of triple assessment of breast mass in combination with mammography and clinical examination. Aspiration cytology allows an early diagnosis of breast diseases (Chaiwun et al. 2002), with good specificity and moderate sensitivity. However, it cannot differentiate between in situ or invasive disease, and usually cannot give the histological type. Also, in many cases the diagnosis can only be done with reasonable uncertainty (Zuk et al. 1989, Teague et al. 1997, Chaiwun et al. 2002).

The cells are aspirated into the needle with several passes through the abnormal area, and production of suction in the needle. Ultrasound or other imaging techniques are used to

guide the needle precisely into the suspicious lesion. Imaging is especially important for obtaining enough cells from non-palpable lesions (Paredes et al. 1998).

Cells are smeared on glass slides, and stained, to be examined by a cytopathologist. The response after investigation according to the national breast screening program guidelines for categorisation of FNAB (National Breast Cancer Centre 2004), findings will generally be one of these five categoris:

C1 = Unsatisfactory. A definite microscopic diagnosis cannot be presented. The sample does not contain enough cells, or sample is not satisfactory because distortions due to fixation, defects in processing or laboratory performance.

C2 = Benign. The mass is not of serious concern in respect to cancer.

C3 = Atypical but indeterminate. Other tests are needed to determine the nature of the lesion.

C4 = Suspicious/ probably malignant. Also this type of diagnosis requires additional investigations. The lesion should be re-biopsied, with lumpectomy or core needle biopsy.

C5 = Malignant. The diagnosis can be considered certain for cancer.

Exact tumor type, histological grade and stage will be determined after Surgery (Sun et al. 2001). In general, the FNAB is useful, simple, quick, highly reproducible, minimally invasive, and with rare false positive diagnoses. However, with this technique, false negative diagnoses (when a cancer cells may be missed) are possible in a few cases due to sampling error or too small number of cells examined. When experienced radiologist used modern equipment, the false negative rate of fine needle aspiration biopsy (FNAB) was 2-10% (Zuk et al. 1989, Teague et al. 1997, Chaiwun et al. 2002).

Core biopsy / Tru-cut biopsy

The tissue cores are usually with size about of 10x1 mm, and the biopsy can be performed under local anesthesia. The core biopsy allows the diagnosis as well as the distinction between invasive and in situ cancer. However, both false positives and negatives can occur. It is also very helpful in the differential diagnosis of an abscess, and sclerosing adenosis from cancer, but needs experience from the side of the examiner. Both fine-needle aspiration cytology and core biopsy are useful in the diagnosis of breast cancer (Sun et al. 2001). The development and increased utilization of FNAB and core needle biopsies for obtaining tissue samples have been major advances in both detection and diagnosis.

Open biopsy

The proper diagnostic procedure for patients with suspected breast cancer is the open excisional biopsy of the mass. Generally this often applied to benign lesions that the patient wants be removed, and also to removal of doubtful lesions, if a malignancy has

not been demonstrated by FNAB or core biopsy. When the lesion is still suspected to be cancer, or if a lump is likely to be malignant, and the biological make up of the tumor is looked for open biopsy can be done. Specific characteristics of cancer cells make them more or less sensitive to different cancer treatments. The biopsy can be examined as a frozen section by the pathologist for a quick, but preliminary diagnosis. More commonly, the biopsy is processed routinely, and a diagnosis is made. If a malignancy is found, the cancer cases are studied routinely further with immunohistochemistry staining for the estrogen and progesterone receptor status (American Cancer Society 2010).

2.3.4 Triple Test (TT) Method

The triple test comprises correlating the results of physical examination, radiological imaging (mammography, MRI, etc.) findings and cytological results (Clarke et al. 2001). When all these suggested cancer, the diagnosis is very reliable with extremely high sensitivity, specificity and diagnostic accuracy. The false positive and false negative rates are similar to biopsies obtained by more invasive surgeries. The TT method should always be used in diagnosis of a breast mass when using FNAB (Sun et al. 2001, Chaiwun and Thorner 2007).

2.3.5 Classification of breast cancer

After diagnosis of breast cancers they should be classified histologically. Carcinomas can be invasive (extending into the surrounding stroma) or non-invasive (confined just to the ducts or lobules). Table 2 identifies the major histologic types of invasive cancers, along with their frequency, and overall relative survival rate. The data are modified from Rosen 2009. The "NOS" categories contain carcinomas not classified into more specific histologic types, the specific histologic types should be limited only to those tumors composed entirely or in very large part (90%) of the designated pattern (Tavassoli and Devilee 2003, Rosen 2009).

Table 2 The major histological types of invasive breast carcinoma, along with their frequency, and overall relative 5 and 10 year survival (modified from Rosen 2009, and Tavassoli and Devilee 2003).

Histological Type	Frequency (%)	5-year Survival (%)	10-year Survival (%)
Invasive ductal carcinoma (NOS)*	75-80	79	35-60
Infiltrating lobular carcinoma	5-14	86	35-70
Mixed tubulolobular carcinoma	6	>85	50-80
Medullary carcinoma	3-10	>80	50-80
Mucinous (colloid) carcinoma	2	90-100	80-90
Papillary carcinoma	1-2	90-100	80-90
Tubular carcinoma	2 -7	90-100	90-100
Cribriform carcinoma	3-4	90-100	90-100

**NOS = Not otherwise specified*

2.4 Classical clinicopathological prognostic markers

2.4.1 Tumor size

It is important to make macroscopic measurement and the greatest diameter is taken as the tumor size, but in case of any doubt about the tumor measurements, then the exact size should be confirmed microscopically from histological sections by using the stage micrometer, or a micro scale. In general the survival deteriorates with increasing tumor size, and the best prognosis will accompany cancers less than 10 mm in diameter. Many studies concluded that tumor size is an independent prognostic factor particularly in axillary node-free patients (Elston et al. 1982, Neville et al. 1992). On the other hand very small tumors in axillary node-postive patients may predict for higher breast cancer–specific mortality compared with larger tumors (Wo et al. 2011). Tumor size correlates well with lymph node involvements. The percentage of axillary lymph node metastasis in tumors less than 10 mm is 15-20%, compared with over 40% in tumors measuring 15 mm or more (Rosen and Groshen 1990).

2.4.2 Lymph node status

The clinical status of the axillary nodal is the single most important prognosticator for breast cancer. A better prognosis will accompany cancers without axillary lymph node involvement. The average 10-year survival rate is decreased from 65-75% for nodal free patients, to 20-30% in nodal metastasis patients (Elston et al. 1982, Galea et al. 1992, Hartveit and Lilleng 1996, AJCC Cancer Staging Manual 2006). Evaluation of prognosis can be more valuable by using the number of regional lymph nodes involves than does anatomic staging. The greater the number of nodes involved the worse is the prognosis (Nemoto et al. 1980, Fisher et al. 1984). A single lymph node obtained for pathologic examination is likely to provide inaccurate information; therefore, it has been advised by many authors that at least 10 nodes should be obtained before calling the patient node negative (AJCC Cancer Staging Manual 2006, Rosen 2009). Some retrospective studies suggested that presence of extra-nodal spread conveyed poor prognosis (Mambo et al. 1977). Fisher et al. 1976 and Donegan et al. 1993 demonstrated that the greater number of nodes involved gives more frequently extra-nodal spread and concluded the extra-nodal spread alone had no significant influence on prognosis and tend to be a function of the total number of involved nodes. The authors also suggested that the number of lymph nodes is more important indicator than the extra-nodal spread for radiotherapy after complete axillary clearance. The higher metastatic lymph node levels of the axilla carry an unfavorable outcome. However, when the number of nodes with metastases is constant, the level of lymphnode involvement has no additional predictive value (Barr et al. 1992).

Status of sentinel node

The sentinel node biopsy is an alternative for accurate prognostication, with minimal lymph node sampling (Boer et al. 2009). The sentinel node is the first lymph node to which lymph drainage and metastasis from tissues infiltrated by a malignant tumor and biopsy of the sentinel node will reflect the true regional lymph node status and is an accurate determinant of stage. Cabanas is the pioneer in introducing the sentinel node to the lymphatic drainage of penile cancer (Cabanas 1977). In breast cancer the detection rate of carcinoma in axillary sentinel lymph node improved from 65% in the first report (Giuliano et al. 1994) to 93% at the second series of the same group (Giuliano et al. 1996). An accurate staging was provided in all cases (100%) of those in whom sentinel LN was found. The sentinel node detection rate was improved by the introduction of the lympho-scintigraphic techniques (Albertini et al. 1996). The sentinel LN sampled can be used to stage the axilla in primary breast carcinoma. However it can also be used in DCIS with extensive involvement of the breast, and/ or when the triple test findings suggested invasiveness (Lester et al. 2008). The using of IHC and polymerase chain reaction improves the detection of micrometastasis in axillary lymph nodes (Rutgers et al. 2009. Schoenfrid et al. 1994). Some studies show that micrometastases in regional lymph nodes were associated with a reduced 5-year rate of disease-free survival among women with favorable early-stage breast cancer who did not receive adjuvant therapy (Boer et al 2009). However, the new cohort study on early stage breast cancer concluded that micrometastasis did not have any clinical significance (Wu et al. 2012)

2.4.3 Clinical staging

The practical clinical decision is dependent on summing the above basic features of a tumor by means of a staging system. American Joint Committee (AJC) on cancer staging has modified the TNM staging system that was proposed by the International Union Against Control of Cancer (IUAC) and based upon the size of tumor (T), degree of spread to lymph nodes (N) and systemic metastasis (M) at the time of diagnosis. The staging system goes from stage I to stage IV. Staging is regarded as the most important prognostic factor. As stage increases the prognosis deteriorates. For example; the 5 years survival in stage I breast cancer is more than 90% while patients with stage IV disease have very poor prognosis and a 5 years survival are less than 30% (Rosen and Groshen 1990, AJCC Cancer Staging Manual 2006).

2.5 Classical prognostic markers in histopathology

2.5.1 Histological grade

Breast cancers can be graded according the degree of differentiation to well differentiated (grade I), moderately-differentiated (grade II) and poorly differentiated (grade III)

carcinomas. Higher rates of systemic metastasis and decreased survival are associated with decrease of tumor differentiation (Contesso et al. 1987, Elston and Ellis 1991, Henson et al. 1991, Tavassoli, Devilee 2003). The grading of invasive breast cancers is a useful prognosticator even in a tumor with size less than 10 mm. Evaluation of grade may has powerful prognostic value similar to the staging system. Cancers that are well differentiated (have low grade) have usually better prognosis than high grade tumors. Bloom and Richardson in 1957 had added numerical scoring to the previous grading system described by Patey and Scarff in 1928. The resulting grading was then called modified Scarff-Bloom-Richardson grading system (Bloom and Richardson 1957). The Nottingham researchers have added further modification with the idea of making the grading system more objective and reproducible, and they have suggested that grading could be applied for practically all types of breast carcinoma (Elston and Ellis 1991). These grading systems (i.e. Scarff-Bloom-Richardson and Nottingham systems) are based on evaluation of three histological characteristics of breast carcinoma: (1) Formation of tubules; only structures exhibiting clear central lumina are counted. (2) Nuclear pleomorphism; the regularity of nuclear size and shape is comparison with the nuclei of normal breast epithelial cells. (3) Number of mitotic figures per 10 fields; count only clearly defined mitotic figures; hyperchromatic and pyknotic nuclei are ignored since they are more likely to represent apoptosis than mitosis. The Nottingham researchers' modification allows consideration of the field size which varies between different microscopes, and affects mitosis counts as already shown by Haapasalo et al. (1989) (Table 3).

The above histological characteristics are scored from 1 to 3. For example, a tumor with many tubules would score 1 whereas a tumor with no tubules would score 3.

These score values are summed and converted into three groups: grade I (score 3-5), grade II (scores 6 and 7), and grade III (scores 8 and 9) (Table 3, Elston and Ellis 1991). This modified Scarff-Bloom-Richardson grading system with Notingham modification is an important predictor of both disease free and overall survival. The Nottingham histological grading has prognostic significance in almost all histological types of breast cancer, particular in LN- patient (Pereira et al. 1995). Moreover the relationship between biological behavior of cancer and response to chemotherapy is established since several years. Poorly differentiated (high grade) cancers with high mitotic acivity can produce a better responsiveness to adjuvant chemotherapy than well-differentiated cancers with low mitotic acivity among both node-positive and node-negative patients. This may decrease the difference in survival between well-differentiated and poorly differentiated patients treated with chemo-therapy (Singletary et al. 2004).

Table 3 Summary of semi-quantitative method for assessing histological grade in breast carcinoma (modified from Elston and Ellis 1991, and Tavassoli and Devilee 2003).

Feature				Score
Tubule formation				
Majority of tumour (>75%)				1
Moderate degree (10-75%)				2
Little or none (<10%)				3
Nuclear pleomorphism				
Small, regular uniform cells				1
Moderate increase in size and variability				2
Marked variation				3
Mitotic counts per 10 fields				
Dependent on microscope field area				
Fd= 0.4 mm	Fd=0.5 mm	Fd=0.6 mm	Fd=0.7 mm	
0-4	0-6	0-9	0-13	1
5- <10	7- <14	10- <20	14- <28	2
10 or more	14 or more	20 or more	28 or more	3

Fd is field diameter

2.5.2 Mitotic activity

Several authors suggested that evaluation of mitotic activity alone can be as prognostic as the grading system or even more powerful (Baak et al. 1982, Contesso et al. 1987, Collan et al. 1997, Kronqvist et al. 1998). There are many available ways to measure cell proliferative activity e.g. by counting the number of mitoses from ten high power fields from the most cellular area of the sample, using a standard light microscope (objective, x40; numeric aperture, 0.75; field diameter, 420μm) (Baak et al. 1982, Baak et al. 1985, Kuopio and Collan 1996), or by expressing the count by square millimeter, which produces the standardized mitotic index (SMI), also called volume fraction corrected mitotic index (M/Vv index) (Haapasalo et al. 1989, Collan et al. 1997, Kronqvist et al. 1998). Many studies demonstrated that the mitotic activity index (MAI) is an independent prognostic factor for recurrence free survival. SMI is a bit more efficient than MAI as a prognosticator (Collan et al. 1996, Kronqvist et al. 1998).

2.5.3 Histological type

Carcinoma in-situ (CIS) is composed of ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). They are classified as pre-invasive carcinomas. In theory, CIS is curable and with no threat on life when completely surgically removed. However, 16% of DCIS patients with such local excision develop recurrence as invasive ductal carcinoma usually of high grade (Silverstein 1998, The University of Southern California 2003). Similarly, 18% of patients develop invasive recurrence after the local excision of LCIS (Andersen 1974). Of breast cancer types, tubular, papillary, invasive cribriform

and mucinous carcinomas have most favorable prognosis. Tubulolobular and medullary carcinomas have intermediate prognosis. The classic infiltrating lobular carcinoma has a slightly better prognosis than invasive ductal carcinoma (NOS). This may be due to the fact that classic infiltrating lobular carcinomas show estrogen receptor (ER) expression more frequently than invasive ductal carcinomas. However, in general, invasive ductal NOS and solid type infiltrative lobular carcinomas both have a 10 year survival not better than 60% (Ellis et al. 1992, Green et al. 1997, Tan et al. 2008, Rosen 2009).

2.5.4 Vascular invasion

Because it is difficult to determine whether vascular spaces are lymphatics, capillaries or venules, the vascular permeation should be left unspecified and 'vascular invasion' used as broad term. The prognostic value of the estimation of vascular invasion is still debated. A few studies have concluded that there is no significant correlation between vascular invasion and clinical outcome (Ejlertsen et al. 2009, Sears et al. 1982). Others have recognized that the presence of vascular invasion has significant prognostic value e.g. on short term survival, particularly in lymph node negative patients (Mohammed et al. 2011, Ragage et al. 2010, Lee et al. 2006, Rosen 1983, Pinder et al. 1994). lee et al. 2006 recognized that the risk of death after adjuvant hormonal and chemotherapy was high in patients with vascular permeation. One cause for the above discrepancies may be that there is low reproducibility in distinction between true vessels and tumor cell groups within artefactual tissue spaces caused by tissue shrinkage and poor fixation (Pinder et al. 1994). Immunostaining may be helpful in excluding shrinkage artifact (Marinho et al. 2008, Martin 1987, Kahn and Marks 2002). The problem could also be reduced by good fixation. To be counted the vascular spaces should not be within the tumor itself, and tumor emboli should clearly be present within an endothelium-lined vessel. Several studies have found that vascular invasion has significance correlation with axillary lymph node involvement (Marinho et al. 2008, Pinder et al. 1994, Bettelheim et al. 1984). However, 18% of breast cancers show vascular invasion with no obvious lymph node metastasis (Pinder et al. 1994). It has been suggested that vascular invasion can provide powerful prognostic information, independent of lymph node status (Rakha et al. 2012, Mohammed et al. 2011, Bettelheim et al. 1984). Several studies have shown that vascular invasion predicted local recurrence after mastectomy (Bettelheim et al. 1984, O'Rourke et al. 1994) and conservation surgery (Pinder et al. 1994).

2.5.5 Angiogenesis (Microvessel density)

Neo-vascularisation is the formation of new micro vessels in stroma. There is general agreement that angiogenesis has an important role in growth and metastasis of malignant tumors (Folkman 1990, Kato et al. 2003). For quantitative estimation of microvessel density (MVD) in breast carcinomas, one should use immunostaining for expression of endothelial markers such as Factor VIII, CD 34 and CD 31. It has been found that breast carcinomas showing a lot of neo-vascularisation are rapidly metastasizing. These tumors also are of

larger size, higher stage, and with lymph node metastasis, and have poorer survival than those with relatively little angiogenesis (Toi et al. 1993, Bhatavdekar et al. 2000, Kato et al. 2003). On the other hand, a few studies have failed to detect such a correlation (Sightler et al. 1994). Anti-angiogenic factors in cancer therapy may slow down cancer growth. Microvessel-targeted antibody therapy may be a successful future treatment of malignant tumors (Gordon et al. 2001). Novel antiangiogenic factors have positive effect on breast cancer therapy. Bevacizumab, one of the angiogenesis inhibiting antibodies is suitable for the first-line treatment of metastatic breast cancer (Fan et al. 2012). Although their role in primary tumors needs further research, the anti-angiogenesis agents in general are promising in breast cancer treatment (Bossung and Harbeck 2010).

2.5.6 Tumor necrosis

Tumor necrosis is morphologically characterized by nuclear karyorrhexis, pyknosis and karyolysis, often accompanied by granular eosinophilic cytoplasm. When tumor necrosis has been present for a prolonged time it may be associated with fibrosis. Necrosis is usually related to invasive ductal carcinoma of non specific type, especially when of has high grade (Page and Anderson 1987, Fisher et al. 1978). A few studies suggested that tumor necrosis is accompanied with lowered survival and early treatment failure (Fisher et al. 1978). However, low reproducibility in evaluating necrosis may limit its use as a prognostic factor.

2.5.7 Stromal fibrosis

The presence of stromal fibrosis in invasive carcinoma of the breast still has uncertain prognostic significance. Some studies confirmed that stromal fibrosis was accompanied with good prognosis (Fisher et al. 1983). Others showed no significant effect on survival and concluded that localized stromal fibrosis is a benign condition and reflects the ductal and lobular atrophy secondary to stromal proliferation that may radiologically mimic malignant lesions (Page and Anderson 1987, Dawson et al. 1982, Taskin et al. 2011).

2.5.8 Stromal elastosis

There is no full agreement on the prognostic value of elastosis either. A few studies have shown that the presence of stromal elastosis is correlated with a favorable prognosis (Masters et al. 1979). Giri et al. in 1987 reported that central elastosis had significant prognostic value; but this result was based on a patients group with short follow-up time. It may be that elastosis is not an independent prognostic factor, but related to histological type, because tubular and cribriform carcinomas, with relatively good prognosis, often show general stromal elastosis.

2.5.9 Ductal carcinoma with in situ component

Some studies have observed that breast cancer with predominant DCIS has a favorable prognosis and lower rate of lymph node involvement (Matsukuma et al. 1991). However,

the risk of local recurrence after conservative surgery is associated with abundant intraductal component (Van Dongen et al. 1989, Holland et al. 1990 and Jacquemier et al. 1990). On the other hand, the presence of cancer in the surgical margins is probably a more important factor affecting local recurrence than in-situ component (Gage et al. 1996, Tadashi et al. 1999). About 5-30% of DCIS treated with breast-conserving surgery, with or without radiotherapy, develops a local recurrence, and half of these recurrences are invasive. However, the clinicians are unable to predict the risk of local recurrence or progression to invasive breast cancer. A few studies have concluded that size, margin status, nuclear grade, architectural pattern, and the presence of necrosis are predictors of recurrence in DCIS lesion (Kuerer 2010). As yet, we do not know how the most recent histological markers behave in this picture (Lari et al. 2011).

2.5.10 Multivariate clinicopathological prognostic indices

In last few years many authors combined different independent prognostic factors in multivariate indices. They found that these models are better prognostic indicators than the traditional grading system alone and can be expected to be more reproducible (Ellis 1981, Haapasalo et al. 1989, Aaltomaa et al. 1992, Collan et al. 1994, Ikpatt et al. 2002). Typically, mitotic count has an important position in these models. It is important to notice that most multivariate models include lymph node status as a contributing feature and for that reason multivariate models or indices are applicable to both LN+ and LN- patients. One of the most common prognostic indices is Nottingham prognostic index which combines tumor size, lymph node status, and Nottingham histological grade (Elston et al. 1991). The applied histological features can be measured morphometrically (Quantitive pathology) making the grading more robust and reproducible by using the morphometric grading of breast cancer (Kronqvist et al. 1998).

2.6 Quantitive pathology associated prognostic factors

2.6.1 DNA ploidy and S- phase fraction

DNA analysis in a breast tumor by flow or static image cytometry produces useful data on the DNA content of single cells, and the fraction of cells in active DNA synthesis (S-phase fraction, SPF). Normally, diploid cells are in the resting phase (G0) or in the first gap phase of the cell cycle (G1), cells with twice the normal DNA content are in either the G2 or early mitotic phase (M), and cells with intermediate amounts of DNA are in the synthesis phase (S), which reflects the proliferative activity.

DNA content

Static image DNA cytometry analysis allows determination of ploidy in both cytological smears and tissue sections even with relatively small amounts of tumor (Buhmeida

2006). The cytometric quantitation of nuclear DNA content can assist in the diagnosis and grading of malignant tumors. It is known that of malignant tumors about 20% are diploid but have small chromosomal variations, not detectable by cytometry. The rest of the neoplasms are aneuploid to various degrees (Ruiz-Sauri et al. 1995, Bocking et al. 1995, Elzagheid et al. 2004).

Aneuploid DNA content has been shown to be associated with a worse prognosis (Auer et al. 1980 and Bocking et al. 1989), whereas patients with diploid cancer have more favorable prognosis (Tsutsui et al. 2001). However, Chassevent et al. 2001 showed that ploidy status in general is a weak prognostic indicator and in combination with axillary status fails to add independent information of prognostic value. In additional to prognostic importance, the DNA content is strongly correlated to differentiation of ductal carcinoma, Grade 3 tumors were more likely to be aneuploid than lower grades (Bracko et al. 2001). The strong correlation with tumor grade may explain the lack of independent statistical significance of DNA content as a prognostic factor.

S-phase fraction (SPF)

Flow and static cytometry can be used to estimate the S phase fraction (SPF), a rough estimate of neoplastic growth rate (Montironi et al. 1992). Some authors say that low SPF is associated with an excellent prognosis particularly in LN- breast patients (Michels et al. 2000, Jones et al. 2001). Moureau-Zabotto et al. (2005) suggested that combination of DNA ploidy and SPF improve the prediction of patient's outcome, especially in LN- breast cancer patients. SPF and DNA ploidy can be combined with other features in efficient evaluation of prognosis (Stenkvist et al. 1982, Buhmeida 2003). SPF estimation may reach efficiencies comparable to that of mitotic counts (Collan et al. 1992).

2.6.2 Nuclear and nucleolar morphometry

Morphometry of nuclei and nucleoli may helpful in diagnostic and prognostic evaluations, and improve the sensitivity and specificity of cytological diagnosis (Davaris et al. 2000, Elzagheid and Collan 2003, Karslioglu et al. 2005). On the other hand, Baak et al. (1985), and Tosi et al. (1986) introduced nuclear morphometry for prognostication of breast cancer. They found that nuclear variables were very useful in identifying an aggressive tumor and separated early stage from late stage malignancies. Since then, many histological studies have used morphometry in infiltrating breast cancer. The most useful prognostic factor was the mean nuclear area and the nuclear diameter (Aaltomaa et al. 1992). As the nuclear area increased the patient's survival decreased (Tosi et al. 1986). Many studies confirmed the strong relation between high mean nuclear area and high histological grade (Kronqvist et al. 1998, Sarker et al. 2002, Ikpatt et al. 2002).

2.7 Molecular prognostic markers in breast cancer

Some women have carcinomas for several years and at presentation in the oncology clinic may have a large tumor of high grade, and with active cell proliferation. However, despite the advanced clinic presentation, many of such patients survive for years after treatment. Other women may receive medical help promptly after faintly palpable small mass but die within a short time. Thus clearly there are differences between breast carcinomas and these are probably related to prognosis-affecting biological factors (Elzagheid 2006). Several factors have been identified which may help us to predict how an individual carcinoma will behave, and may help in planning therapy. However, despite the great advances in this area, the only major clinical situation is increased recurrence-free time rather than improved survival (Geyer et al. 2009)

2.7.1 Onco-suppressor genes products

P53

P53 gene is located on chromosome 17p13.1, and encodes for p53 which is a protein thought to be a gatekeeper in cell cycle, and also called the guardian of the genome. When active, the main function of p53 is suppression of cell proliferation and activation of apoptosis. When DNA is damaged, p53 inhibits the progression of cell cycle from G1 to the S-phase and activates DNA repair genes. Cell with un-repairable DNA is directed to apoptosis through activation of the apoptotic genes (Levine et al. 1991, Kastan et al. 1991). The point mutations in one allele of the p53 gene accompanied with congenital or acquired loss of the other allele results in continuous cellular growth, which can promote carcinogenesis in many organs including colon, lung and breast (Levine et al. 1991). Mutation of p53 leads to an increased half-life of non-functional p53 protein, accumulating in cancer cell nuclei. The accumulated p53 protein can be recognized with IHC (Allred et al. 1993, Kim et al. 2010). Overexpressed p53 protein has been detected in many human cancers including breast cancer (Kim et al. 2010, Levine et al. 1991, Temmim et al. 2001), and is usually associated with poor prognosis (Temmim et al. 2001, Rolland et al. 2007, Kim et al. 2010). P53 mutations, detectable by DNA sequencing appear to be independent prognostic indicators (Tsutsui et al. 2001). Allred and his group reported in 1993 that p53 predicted disease free survival in patients with LN- breast cancer. Kuopio et al. 1998 added that expression of mutant p53 protein was also associated with more aggressive tumors, and included early disease recurrence and early death in LN- breast cancer.

P21 (WAF)

P21 is a cyclin-dependent kinase inhibitor (CDKI), which binds to CDK4 complexes and cause cell cycle arrest at the G1 phase as result of inhibiting DNA replication. P21 is transcriptionally activated partially by p53 (Gohring et al. 2001, Skildum et al. 2002) and partially by Sp1/Sp3 (Mottet et al. 2009) as well as by FOXP3 (Liu et al. 2009). No

correlation between p53 mutation and down-regulation of p21 would suggest that p53 mutation is perhaps not the major underlying cause for p21 loss in breast cancer. On other hand, Mottet et al (2009) found that histone deacetylases have an important role in the repression of p21 through Sp1/Sp3, but not through p53-binding sites. The prognostic value of P21 in breast carcinomas is still ambiguous (Liu et al. 2009). Although Gohring et al. (2001) could not find a significant prognostic indicator, Thor and co-workers (2000) show that P21 expression in the LN+ breast cancer patients show a weak correlation with their survival.

P27

P27 is also an inhibiting factor of cyclin-dependent kinase that down regulates cell prolifration, with potential tumor suppressor gene function (Chiarle et al. 2001). Loss or mutated P27 continuously activates cyclin-cdks during G1 phase, and leads to uncontrolled cell proliferation and neoplasia formation (Chiarle et al. 2001). Reductions of expression have been correlated with bad prognosis in some cancer patients including breast cancer patients (Tsuchiya et al. 1999) and have potential therapeutic implications in various types of human cancers (Wander et al. 2011). Moreover, Foulkes and his group (2004) demonstrated that the p27 was an independent predictor in LN- patients but not in LN+ patients. On the other hand, Pillay et al. (2011) concluded that reduced p27 immunoreactivity has little prognostic value in patients with early-stage breast carcinoma in addition to the influence of grade, lymph node status and vascular invasion.

c-Myc

c-Myc gene is proto-oncogen, located on chromosome 8q24, encodes for a protein that binds to the DNA of other genes. It is suggested that gene is involved in apoptosis. When c-Myc gene is mutated, or overexpressed, the c-Myc protein doesn't bind correctly, and often result in malignant transformation progression and angiogensis (Chen and Olopade 2008). c-Myc is mutated in about 20% of non invasive ductal carcinoma (Aulmann et al. 2002) and in 30% of primary breast cancers, particularly in patients with high proliferation and poor differentiation (Naidu et al. 2002). Expression of c-Myc can also be correlated with larger tumors and/or with lymph node involvement (Nass and Dickson 1997). Some studies demonstrated that over-expression c-myc may be related to breast cancers that have worse prognosis Naidu et al. (2002) particularly in basal-like tumor types (Xu et al. 2010). In addition, Myc amplification is an important predictor of response to HER2-targeted therapies. In BRCA1-associated breast cancer c-Myc is an important in targeting therapy particularly in basal-like/triple-negative breast cancers (Chen and Olopade 2008, Xu et al. 2010).

Bcl-2

Bcl-2 is an intracellular mitochondrial protein which has an anti apoptotic function in normal cells; the Bcl-2 gene is commonly over-expressed in follicular B-cell type non

Hodgkin's lymphoma, but it has also been over-expressed in epithelial tumors (Tsujimoto et al. 1985, Olopade et al. 1997). Lack of Bcl-2 expression due to abnormal regulation of Bcl-2 gene can be associated with increase tumor aggressiveness and presence of chemotherapy resistance (Olopade et al. 1997, Jalava et al. 2000). An over expressed of Bcl-2 protein has been detected by IHC stain in well differentiated breast cancer and/or with positives ER and PR hormonal receptors (ER and PR) (Jalava et al. 2000, Park et al. 2002). Several authors concluded that Bcl2 overexpression has independent prognostic value in all type of early stage breast cancer (Callagy et al. 2006, Dawson et al. 2010) but others confirmed this only among LN+ patients (Bhatavdekar et al. 2000, Jalava et al. 2000). Lack of Bcl-2 expression, when accompanied with negative ER phenotype, is associated with poor prognosis (Xu et al. 2010). Ali et al. (2012) have found that Ki67/ BCL2 stain index had significant correlation with favorable out come in ER positive breast cancer.

2.7.2 Cell proliferation markers

MIB-1 (Ki-67)

Ki-67 is a cell proliferation marker. It is a non- histone DNA binding protein that can be detected by immunohistochemistry (Cooper et al. 1998, Romero et al. 2011). It is expressed in all phases of cell cycle but the resting phase (Gerdes et al. 1991). The Ki-67 positive cells are in preparing for the S phase. Ki-67 labeling index (fraction of Ki-67 positive nuclei of all cells nuclei) indicates the fraction of cells in proliferation or near S-phase in the cell cycle. Several authors detected that high Ki-67 labeling index correlates with high histological grade (Cooper et al. 1998, Isola et al. 1990) and large tumor size of breast cancer (Isola et al. 1990, Railo et al. 1993). Ki-67 labeling index (determined e.g. by the MIB1; IgG monoclonal antibody used for detection Ki-67 in paraffin embedded material) shows a significant adverse correlation with survival in breast cancer, particularly among patients without LN involvement (Pietilainen et al. 1996, Santamaria et al. 2005, Jalava et al. 2006). However, the independent prognostic significance of MIB-1 is much less than that of mitotic count (as determined with either MAI or SMI) (Collan et al. 1996, Jalava et al. 2006).

AgNOR

Special silver stain has been applied to the study of another marker of cell proliferation, the argyrophilic nucleolar organizer region (AgNOR). NORs are loops of deoxyribonucleic acid (DNA) that encode ribosomal ribonucleic acid (RNA) and are located in the nuclei. NORs proteins are argyrophilic and can be detected by using modified silver staining method (AgNOR technique). The number of silver binding dots is a valuable marker of proliferative activity as it reflects the extent of ribosomal biogenesis (Smith and Crocker 1988, Vijaya et al. 2008). AgNOR score is useful for estimating the cell proliferative activity of neoplasms, and increased AgNOR counts are consistent with increased growth activity of the cells (Dasgupta et al. 1997). Prognostic significance of AgNOR counts

in breast carcinoma is controversial. Some researchers have reported that AgNOR has a prognostic value especially among LN+ patients (Aubele et al. 1994, Derenzini et al. 2004). Others were not able to find any prognostic significance in AgNOR counts for breast cancer (Toikkanen and Joensuu 1993, Simha et al. 1996). However, the best results may be achieved if AgNOR measurements are used in combination with MIB-1 positive nuclei (Jeziorski et al. 2000, Biesterfeld et al. 2001).

PCNA

The cell proliferation markers include PCNA that stands for "Proliferating cell nuclear antigen" which is forming a trimeric ring structure around DNA to facilitate and control DNA replication and repair (Stoimenov and Helleday 2009, Strzalka and Ziemienowicz 2011). PCNA is a DNA polymerase-cofactor that has ability to stimulate the activity of DNA polymerase and to interact with p21in regulation of the cell cycle. PCNA was expressed in proliferating cells, particularly at the transition from the G1 to the S phase (Linden et al. 1992), and it is important for cell replication (Strzalka and Ziemienowicz 2011, Stoimenov and Helleday 2009, McCormick and Hall 1992, Hall and Coates 1995). Stoimenov and Helleday (2009) reported that specific DNA lesions will signal for different PCNA modifications which may be of importance for the outcome of treatment. Some authors detected that high PCNA expression correlates with high histological grade and high ploidy of breast cancer (Aaltomaa et al. 1993). Some authors showed that PCNA has significant prognostic value in breast cancer (Jeziorski et al. 2000), but Aaltomaa and his co-worker (1993) confirmed this only among LN- patients.

Cathepsin D (CD)

Cathepsin D is an acidic lysosomal protease that can be found within the epithelial cells of breast cancer in various forms. They have proteolytic activity that can degrade basement membranes and extracellular matrix. Cathepsin D also has a mitogenic activity, thus Cathepsin D is suggested of facilitating cell proliferation, invasion and metastasis of breast cancer (Greco et al. 2000). Several researches have concluded that over-expression of Cathepsin D is a useful prognostic indicator in breast carcinoma particularly in lymph node negative cases (Isola et al. 1993). Cancers with positive Cathepsin D immunohistochemistry stain often show high rates of recurrence, nodal and systemic involvements and an unfavorable prognosis (Isola et al. 1993, Rochefort 1998, Greco et al. 2000). However, there were some studies that did not detect any prognostic value of Cathepsin D in breast cancer (Aaltonen et al. 1995, Ramirez-Ortega et al. 1997).

2.7.3 Growth factors and their receptors

The oncogenic production of either growth factors or their cell surface receptors (either by over-expression or by point mutation) can activate the cell cycle, and cause abnormal cell growth or cell transformation. The most important of these growth factors and receptors are epidermal growth factor (EGF), transforming growth factor alpha and beta

isoforms (TGFα, TGFβs), insulin-like growth factor and vascular endothelial growth factor (VEGF) (Reynolds et al. 1996).

Epidermal growth factor receptor (EGFR)

EGFR is a transmembrane glycoprotein receptor which one of tyrosine kinase growth factor receptor family that plays an important role in breast carcinogenesis (Abd El-Rehim et al. 2004). EGFR is a helpful prognostic indicator in patients with solid tumors (Lippman et al. 1987). EGFR over-expression and/ or amplification have been recognized in breast carcinoma patients with high grade and poor survival (Jeziorski et al. 2000, Kumar et al. 2001, Aziz et al. 2002 Abd El-Rehim et al. 2004). Rampaul et al. (2004) demonstrated that the EGFR is a significant prognostic indicator mainly in carcinomas with lymph node involvement. Tsutsui and coworkers in 2002 observed that EGFR when combined with ER was a significant prognostic factor for both disease free survival (DFR) and overall survival (OS) both in patients with LN- and LN+ breast cancer. Patients with EGFR (+) and ER (-) had worse DFS and OS. Anti-EGFR monotherapy alone or combined with other chemotherapy may slow down the cancer progression (Normanno et al. 2009, Lu et al. 2009, Guise 2009).

Transforming growth factor receptor (TGFR).

Transforming growth factors (TGFs) are multifunctional growth factors that show variable expression, and include TGF-α and TGFβs, and have produced different roles during cell proliferation (Ghellal et al. 2001, Baumeister et al. 2009). IHC expressions of both TGF-α and EGFR have important independent prognostic influnce in patients with solid tumors, and also can be useful in detecting the aggressive category of breast cancer (Lippman et al. 1987, Umekita et al. 2000, Baumeister et al. 2009). All TGFβ isoforms bind to the same cellular receptor of normal cells (Massague 1990). During embryogenesis TGFβs are involved in cell migration and cell differentiation, they have also a role in cell proliferation (Kehrl et al. 1986, Hsu et al. 2009). TGFβ isoforms act can both stimulate and inhibit cell proliferation, depending upon cell type. They usually have a suppressive effect on epithelial cell proliferation in breast tissue, through inhibiting the cell cycle at the G1-phase by causing increase in the CDK inhibitors P15 and P27 (Hsu et al. 2009). Thus cells proliferation continues when cells are no longer exposed to TGFβ (Massague 1990). When TGFβ is mutated and loses its ability to inhibit the cell cycle, cells then may proliferate in an uncontrolled manner and may lead to benign or malignant tumors (Massague 1990). TGFβ2 and TGFβ3 may have favorable prognostic value in breast cancer (Ghellal et al. 2001 Laverty et al. 2009).

Vascular endothelial growth factor and receptor (VEGF-R)

Several growth factors have been identified that regulate angiogenesis in breast cancer; the most important of these is thought to be VEGF. Most of the reported studies have suggested that endothelial cell surface receptors for VEGF may be over-expressed in

association with cancers with adverse prognosis (Yoshiji et al. 1996, Fukumura et al. 1998, Hilmi et al. 2012). VEGF targeting has been used for the first-line treatment of metastatic breast cancer (Hilmi et al. 2012, Fan et al. 2012). However, resistance to the therapy due to VEGF splicing may affect the outcome of treatment (Hilmi et al. 2012)

Insulin-like growth factor (IGF) system

This system has 2 ligands (IGF-1 and IGF2) 2 receptors (IGFRI and IGFRII) and 6 binding proteins (IGFBP1to 6). The system plays an important role in the biological activity of the cell. The IGF-1 and IGF-2 are circulating peptide hormones and have mitogenic and apoptotic effect on breast cancer possibly are through autocrine mechanisms (Ellis et al. 1998). The role of the IGF system in the progression of breast tumors is still a controversial issue. Some studies suggested that IGFs have important role in the progression of breast cancer (Yu et al. 1996 and Surmacz et al. 1998, Maor et al. 2007). Others recognized that breast cancer with positive IHC stain for IGF-1 has lower grade than breast cancer with negative staining (Eppler et al. 2002). Toropainen et al. (1995) reported that, in a univariate analysis, IGF-1 was related to good survival particularly in LN+ breast cancer patients. Recent studies have focused on the IGF-1R as a target for cancer treatment and they suggest that this type of therapy may have an effect on treatment of breast cancer and other solid tumors (Aleksic et al. 2010)

HER2/neu or c-erbB-2 (HER2)

HER2 is proto-oncogene located on chromosome 17; the gene encodes HER2, which is a protein of a family of four transmembrane receptor tyrosine kinases that mediate the growth, differentiation, and survival of cells (Gschwind et al. 2004), basically this is an epidermal growth factor receptor with extracellular domain and intracellular tyrosine kinase activity (Ali et al. 2002). Abnormalities of HER2 occur in about 15-20% of breast cancers. There is amplification of the gene with resultant over-expression of the membrane-related protein. Carcinomas which have these abnormalities are often associated with aggressive character, and are poorly differentiated, lymph node positive, hormone receptor negative, DNA aneuploid and show high proliferation rates (Jeziorski et al. 2000, Bhatavdekar et al. 2000, Jalava et al. 2002, Gschwind et al. 2004, Yeh et al. 2011). HER2 protein over-expression can be identified at cell membrane by immunohistochemistry (IHC), while the HER2 amplification is usually detected by fluorescence in situ hybridization (FISH) or chromogenic in situ hybridization (CISH). These methods can be performed on paraffin-embedded tissue and are used as an eligibility criterion for anti-HER2 therapy, such as Herceptin (trastuzumab), used to treat women even with advanced disease (Jalava et al. 2002). HER2 status is important in predicting prognosis in breast cancer patients (Jalava et al. 2002, Winters et al. 2003, Gu et al. 2005). Several studies revealed that elevated HER2 protein is associated with poor prognosis (Jalava et al. 2002, Abd El-Rehim et al. 2004, Gschwind et al. 2004). In addition, the expression of HER2 is also thought to have positive predictive value as regards the response of breast cancer to anthracycline-based chemotherapy and negative predictive value to tamoxifan

(Arpino et al. 2004) and to taxan and/or methotroxate-based regimes (Winters et al. 2003, Gu et al. 2005). Seidman et al. (2001) and Joensuu et al. (2006) demonstrated that the overall therapy response rate was better among patients showing HER2 gene amplifiction or intensive membrane staining by immunohistochemistry than patients with tumors having normal HER2 expression, particularly when used with combined chemotherapy and anti-HER2 therapy regimes. The effect was better on disease-free survival and when there was no lymph node involvement or distant metastasis. About 20% of metastatic breast cancers may have overexpression of HER2 receptors. Anti-HER2 therapy combined with other chemotherapy may also suggest slows down or even stops the growth of such cancers (Salminen 2001, Bilous 2003).

2.7.4 Adhesion molecules

E-cadherin

E- cadherin is an epithelial cell adhesion molecule which plays a significant role in the maintenance of tissue architecture (Gonzalez et al. 1999). E-cadherin is a transmembrane glycoprotein that mediates calcium dependent cell-to-cell adhesion in epithelial tissues (Lipponen et al. 1994). Loss in cancer can promote invasion and metastasis (Asgeirsson et al. 2000). Many studies have recognized that loss or reduction in E-cadherin expression is associated with increase in breast cancer infiltration and metastasis (Asgeirsson et al. 1998, Karray-Chouayekh et al. 2012). The expression of E-cadherin correlates with histological type and grade. The expression of E-cadherin in infiltrative lobular carcinoma and LCIS is mostly lost (Bratthauer et al. 2008). However, infiltrating ductal carcinoma shows higher positivity in low grade than in high grade carcinomas. Thus E-cadherin may have a role in histological diagnosis of ambiguous CIS and infiltrative carcinomas (Charpin et al. 1997, Bankfalvi et al. 1999, Berx et al. 2001). Bankfalvi et al. (1999) also reported that decrease in E-cadherin expression was significantly associated with regional lymph nodes metastasis. Some authors stated that the exepression of E-cadherin was associated with favorable outcome and its prognostic value was stronger among patients with LN+ breast cancer (Gamallo et al. 1993, Elzagheid et al. 2006, ElMoneim and Zaghloul 2011, Karray-Chouayekh et al. 2012) than among other patients. Other publications observed that there was no significant association with prognosis among LN- patients (Lipponen et al. 1994, Rakha et al. 2005), as well as on long term outcome of women with breast cancer (Goyal et al. 2008).

Catenins

These are intracellular proteins (α-β-γ-catenins), and have a link to actin microfilaments, and react with E-cadherin. This reaction is important for the stabilisation of adhesive effect of E-cadherin (Hirohashi et al. 1998). Reduction of intracellular catenins may lead to loss of cellular adhesion, and may be associated with cancer cell infiltration and distant metastasis (Ghadimi et al. 1999). The lack of β-catenins expression was detected in in-situ and invasive lobular carcinoma and poorly differentiated invasive

ductal carcinoma (Bankfalvi et al. 1999). Several observers have stated that lack or reduction of catenin expression may be correlated with poor survival in breast cancer patients (Yoshida et al. 2001, Uchino et al. 2010). The results suggest that loss of catenin expression could be one of the mechanisms responsible for the loss of E-cadherin mediated cell-cell adhesion (Bankfalvi et al. 1999, Yoshida et al. 2001, Uchino et al. 2010).

CD 44

CD44 is a family of cell surface transmembrane glycoproteins with different isoforms. CD44 isoforms are expressed in most human tissues and linked to metastatic spread in a variety of tumors (Foekens et al. 1999 and Morris et al. 2001). CD44 was detected in normal breast myoepithelial cells (Bankfalvi et al. 1999). It has been observed in 40% of invasive ductal carcinoma and correlated with aggressive features including large size, high stage, high histological grade, and lymph node metastasis (Bhatavdekar et al. 2000, Uchino et al. 2010). However, some authers suggested that CD44 is not considered as an independent prognosticator in breast cancer (Joensuu et al. 1993, Tempfer et al. 1996). Horiguchi and his co-worker (2010) found that CD44 expression had an independent prognostic value in breast cancer. This discrpancy may be explaned by that other prognostic features carry the same information in respect to prognosis. Foekens and his group (1999) reported that the expression of CD44 may be a marker for identifying patients with relatively favorable prognosis only among LN- patients.

2.7.5 Estrogen and progesterone receptors

The presence of estrogen receptors within a carcinoma indicates that the tumor cells have a higher degree of functional differentiation. It is thus not surprising that women whose tumors are estrogen-receptor-positive have better survival figures than those whose carcinomas are estrogen-receptor-negative. More importantly, they are more likely to benefit from tamoxifen, an estrogen receptor antagonist. Breast cancer is often hormone dependent, and determination of estrogen receptor (ER) and to a lesser extent, progesterone receptor (PR) status in breast cancer is important in the proper treatment selection. The American Society of Clinical Oncology (ASCO) in 2007 concluded that ER and PR should be measured on every primary breast cancer as well as in metastatic lesions, because if primary or metastatic disease is ER or/ and PR positive that will identify patients likely to benefit from adjuvant hormonal therapy. Almost three-fourths of breast cancers expressing ER will respond to this type of therapy, whereas about 5% not expressing ER will also respond especially in postmenopausal women (Silvestrini et al. 1995, Barbareschi et al. 2002, Hayashi et al. 2003, American Cancer Society 2007). However, in the adjuvant setting, about 50% of those (ER) positive patients undergoing breast cancers recurrence (Ma et al. 2009). Several different semi-quantitative and quantitative scoring systems were

available for evaluating hormone receptor status (HR+ or HR-), based on the intensity of nuclear stain and/or percentage of positive cell nuclei, such as Allred's score (Allred et al. 1998) and J-score (Kurosumi 2007). Most labs report just the actual percentage of positive cells in every examined case (Talley et al. 2002). In addition to the strong predictive value, ER and PR have a weak prognostic value. Cancers which express ER and/or PR in their nuclei have a better prognosis than those with negative expression, especially those cells with overexpression of ERα was expected to be associated with a best significantly longer survival and have maximum respond to hormonal manipulation. Tamoxifen and other anti-estrogens (including toremifene) are used for this purpose (Barbareschi et al. 2002, Harvey et al. 1999, Blanco et al. 1984, Moelans et al. 2010). On other hand, the prognostic value seems to be greater among axillary LN+ than among LN- patients (Nomura et al. 1992, Abd El-Rehim et al. 2004), and the optimal cut-points for defining patient groups with good or worse prognosis may differ between LN- and LN+ patients (Jalava et al. 2005). The importance of progesterone receptor positivity in breast cancer is less well understood than the ER positivity. Although Allred et al (1998) stated that ER positive cancers will also be PR positive; Wood et al. 2007 observed that PR expression is lower than ER, because PR is more sensitive to the fixation process than ER. Although breast cancers that are PR positive, but not ER positive, may have a worse prognosis, the double positive cancers indicate even better prognosis and survival. On other hand, expression of ERs and/or PRs within tumors correlates well with low histological grade and low mean nuclear area (MNA) value (Giardina et al. 1990, Larsimont et al. 1989).

2.7.6 Cancer stem cells (CSCs)

Cancer stem cells (CSCs) have been found in different human cancers, and recent studies recognized that many breast cancers originate from CSCs (Vargo-Gogola and Rosen 2007, Nakshatr et al. 2009). CSCs may be responsible of the variability of hormone receptor staining in breast cancer (Allred et al. 2004). Stem cells are CD44+ and CD24- and may express high levels of aldehyde dehydrogenase. Numerous stem cells are associated with aggressive type of breast cancer (Nakshatr et al. 2009).

2.7.7 MicroRNAs (miRNAs, miRs)

MicroRNAs (miRNAs, miRs) are a class of small, non-coding RNA molecules which can be important regulators of gene expression. Micro RNAs seem to have an important role in the development of tumors, and in other diseases (Ha 2011). MicroRNAs either act like oncogenes or like tumor suppressor genes (Voorhoeve et al. 2006, Zhang et al. 2007, Finoux and Chartrand 2008, Shenouda and Alahari 2009). Studies have suggested that micro RNAs potentially represent new prognostic and predictive markers (Kuo et al. 2012, Hummel et al. 2010, Mattie et al. 2006; Yanaihara et al. 2006). Today, hundreds of specified micro RNAs are known. Abnormal expression of specific micro RNAs is found in many cancers (Kuo et al.

2012, Ha 2011, Vasilatou et al. 2010; Dacic et al. 2010, Yanaihara et al. 2006). Iorio et al. (2005) found that micro RNA hsa-miR-21 can be up-regulated in breast cancer. Several studies reported that high miR-21 expression was associated with features of aggressive breast cancer (Qian et al. 2008, Yan et al. 2008, Lee et al. 2011) particularly in early stage patients (Qian et al. 2008). On other hand, studies also suggested that MiRNAs can be biomarkers for diagnosis of early stage breast cancer (Alshatwi et al. 2012, Schrauder et al. 2012).

2.8 The molecular classification of breast cancer based on gene expression profiling

Breast cancer in practice is sub-classified on the basis of microscopic cellular and histopathologic characteristics and the existence of hormonal and HER2 receptors identified by IHC staining (Tavassoli 2010). Although such classifications have proved helpful in terms of predicting prognosis and guiding the treatment, they might not be fully suitable to all clinically progressive elements of the disease. Gene expression profiling technologies have further assessed breast cancer by determining gene activation through mRNA expression patterns (called expression signature). This may provide further accurate prognostic assessment that could lead to a decrease in overtreatment of low-risk individuals and could improve overall survival by correctly identifying high-risk individuals who might need aggressive systemic therapy (Weigelt et al. 2010). In addition, it may help to reveal novel biological targets which could help in efforts to find new anti-cancer drugs (Colombo et al. 2011).

Perou and co-workers in 2000 were pioneers in the comprehensive gene expression patterns. They did RNA expression arrays on a series of breast cancers, and recognized that there are at least four major molecular classes of breast cancer (Table 4). These are: (1) The luminal class; with expression of luminal epithelial cytokeratins 8 and 18, and is usually ER-positive. Later this group was subtyped (type A has negative HER2 and type B has positive HER2). (2) Basal like class; with IHC similar to basal cell in expressing basal-cell cytokeratins 5/6 and 17, usually negative for ER, PR and HER2 receptors. This group is today called triple negativity breast cancer. (3) HER-2 positive class (more than 90% of this class is HER-2-amplified cancers). (4) normal-like class (IHC cancer cells reminds normal breast tissue) this group is characterized by high expression of basal cell genes and low expression of luminal epithelial genes. The gene expression differences between above 4 types may suggest they originate from different cell type within the breast. The different molecular classes also differ with respect to prognosis and chemotherapy sensitivity. For instance, luminal cancers tend to be associated with the most favorable prognosis.

Table 4 The Classification of breast cancer according the gene and immuno profile (modified from Perou et al. 2000, Paik et al. 2006, and Ishihara et al. 2009).

Immuno and gene features	**Luminal A**	**Luminal B**	**Basal like Triple -ve**	**Her 2 +ve**
ER, PR	+, +	+, +	-, -	-, -
HER2	-	+	-	+
Ck5/6&17	-	Variable	+	Variable
Cell prolifration markers	Low expression	High expression	High expression	High expression
Gene profile pathway	CKs, ER responsive genes (e.g. TFF1, GATA3)	CKs, ER responsive genes (e.g. TFF1, GATA3) TP53 mutations	Basel CKs, TP53 mutations, BRCA1 pathway	HER2 amplicon genes (e.g. GRB7, GATA4) TP53 mutations
Treatment senstivity	Respond to HT but not to standard CT	Less respond to HT but may senstive to CT	More sensitive to CT	More sensitive to CT
Prognosis	Most favorable prognosis	Favorable prognosis	Unfavorable prognosis	Less favorable prognosis than luminal type

On other hand, Basal-like and HER-2-positive cancers are more sensitive to chemotherapy but with less favorable prognosis (Sorlie et al. 2001, Paik et al. 2006, Ishihara et al. 2009). Although the gene signature seems to add independent prognostic information to clinico-pathologic risk assessment for patients with breast cancer, there are some major limitations. Some authers e.g. have noitced that gene singnature failed to detect a group of cancers which expresses stem cell genes (Tavassoli 2010), and the most useful application is more specific for early stage of breast cancer (Reis-Filh and Pusztai 2011), but not necessarily for progressed disease. Also difficulties in choosing prediction rules could be the major limitation for gene expression profiling applications (Paik et al. 2006). At the moment expression profiling seems to have only limited applications in diagnostic and therapeutic decision making in practice (Colombo et al. 2011).

3. AIMS OF THE STUDY

The idea of this study concentrates on the following issues

1. The study describes clinicopathological and demographic features in Libyan breast cancer. These features are then compared with corresponding data from sub-Saharan Africa (Nigeria) and Europe (Finland). (V)

2. The study evaluates the supporting value of various nuclear morphometric measurements in breast FNAB diagnosis, after different sampling methods. The Libyan results are compaired with results from Finnish patients. (I)

3. The study estimates the supporting value of image DNA cytometry in breast FNAB diagnosis, after using different sampling methods. (III)

4. The study evaluates the relationship of nuclear morphometry measurements with clinicopathological features, and prognosis in invasive Libyan female breast carcinoma. The data are then compared with corresponding results on Finnish, and Nigerian female breast cancer patients. (II)

5. The study evaluates the status and prognostic value of estrogen receptor and progesterone receptor expression in Libyan breast cancer. Special attention is given to the relationship between receptor positivity and mean nuclear area (MNA) and clinical and histopathological features. (IV)

4. MATERIALS AND METHODS

4.1 Patient material (I-V)

The paraffin embedded histological samples of 171 patients with breast cancer, diagnosed between 2000 and 2006 in the African Institute of Oncology and Tripoli Medical Centre were collected for this retrospective study. 40 paraffin blocks were excluded because the sections did not show viable malignant tissue. This left 131 samples for the study. A detailed history and clinicopathological data included: age, menopausal status, tumor size, stage, grade, lymph node status, and the follow-up and survival data, all collected from patients files. Age of the patients ranged from 25 to 85 years with a medium at 45 years. 4.6%, 33.6%, 49.6%, and 12.2% of patients were at stages 1, 2, 3, and 4, respectively.

Patients were followed-up until death or to the end of the observation period at the mid of July 2007. Some patients were lost from the follow-up. The follow up period ranged from 4 to 78 months, the average being 32.9 months. In most instances, the causes of death were obvious on clinical grounds alone, and autopsy was not performed for any case. Breast cancer was recorded as the underlying cause of death for 34 patients, and unrelated to breast cancer for 3 patients. At least one section of 5 μm thickness was stained with hematoxylin and eosin stain for re-grading according to the modified Bloom and Richardson grading system (Bloom and Richardson 1957, Elston and Ellis 1991), and for the morphometric measurements. The tumor diameters were measured after surgical removal in 3 dimensions in the pathology laboratory and the largest diameter was entered in the database of the study. 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%). The survival period was defined as the time from diagnosis of the tumor either to the time of death, or the latest date on which the patient was known to be alive.

Sixty two histological samples out of the above 131 female breast cancer patients were available for ER and PR immunohistochemistry staining (Table 4).

In the cytology part of the study (I, III), 41 fine-needle aspiration samples were biopsied between August 2004 and April 2007, from Libyan patients with breast masses later diagnosed histopathologically as benign lesions (n = 18), or invasive ductal carcinoma (n = 23). None of the patients had a previous history of malignant disease. The provisional diagnosis was made by fine-needle aspiration biopsy (FNAB) and classified in cytological groups (C2, C3, C4, C5), and confirmed by histopathological analysis at the African Oncology Institute. The smears collected were submitted to the cytomorphometry study and then to DNA image analysis following a three-step protocol. Smears stained with

the HE method were destained, and then restained with Feulgen staining, and finally analyzed using image analysis cytometry.

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

Clinical characteristics	**Descriptive data**		
	Study II	**Study IV**	**Study V**
Number of patients	131	62	234
Age at diagnosis (years)			
Mean (SD*)	46.5 (13.4)	46.7 (13.9)	46.0 (12.3)
Median	45	45	44
Range	25 - 85	25 - 80	20 - 85
Menopausal status (n**)			
Premenopausal	81 (61.8%)	37 (59.7%)	160 (68.4%)
Postmenopausal	50 (38.2%)	25 (40.3 %)	74 (31.6%)
Nodal status (n**)			
Positive	104 (79.4%)	47 (75.8%)	173 (73.9%)
Negative	27 (20.6%)	15 (24.2%)	61 (26.1%)
Tumor size(cm)			
Mean(SD)	5.6 (2.1)	5.6 (2.1)	4.8 (2.1)
Range	1.5-12.5	1.5-12.5	1.5-12.5
Duration of follow-up (months)			
Mean	32.9	36.7	22
Range	4 - 78	9 - 72	1 - 74

**SD= standard deviation, ** n= number of patients*

In epidemiology part of study (V): A retrospective pathology study was conducted on 234 patients with breast carcinomas, admitted at 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 the data of 2006, from Sabratha Cancer Registry. The incidence data are consequently based on the histologically verified cases of year 2006, when the Sabratha Registry started to function.

4.2 Methods

4.2.1 Nuclear morphometry methods (I&II)

The most representative nuclei from selected FNAB samples and 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 Automatiserving, De Meern, Holland). 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 following nuclear morphometric features: (i) area; (ii) perimeter; the length around the nuclear border; (iii) diameter; (iv) the longest axis of the best fitting ellipse; and (v) the shortest axis which 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 (perimeter) 2 / (4 π 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.

In FNAB study (I, III): two types of sampling strategy were tested for malignant and benign cases: cell group sampling and free cell sampling. The total number of nuclei measured in cell groups or in the free cell file was approximately 60 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 moving on the screen. Because of the quality of slides or number of cells available (or both), 4 cancer and 2 benign samples had a nuclear count of less than 60; in addition one free cell sample and one cell group sample had no nuclei available. From fibroadenoma samples (92 cell groups in all), a range of 3 - 32 cell groups were analyzed; from fibrocystic disease samples (74 cell groups in all), a range of 7 - 13 cell groups were analyzed; and from malignant samples (266 cell groups in all), a range of 4 -19 cell groups were analyzed. That only a limited number

of cell groups were analyzed in certain samples depended solely on the fact that these samples did not contain many cell groups. During measurement, most abnormal nuclei with obvious boundaries were measured; compressed or apparently deformed nuclei were avoided. The diagnostic value of all different nuclear features was studied: eleven size and shape features for each of the total of 6,288 nuclei from different breast lesions were measured, which equals 69,168 individual values, in addition to 15,444 statistical nuclear features resulting in 18 statistical measurements for all 11 nuclear features of the total 78 free and cell group samples (18x11x78). Thus, the total dataset was 84,612 different variables. A total of 60 nuclei were measured in each sample category which can be considered adequate for morphometry, when an experienced pathologist selects the most atypical nuclei (enlarged and more deeply stained than other nuclei), and the adequacy in free and cell group categories was tested with the cumulative mean plot method (Romppanen and Collan1983).

4.2.2 DNA imaging cytometry method (III)

Feulgen staining

The FNAB samples were stained with Feulgen stain according to method of Gaub et al. 1975. Before staining the samples were washed in xylene for 3-7 days to remove the cover glass and the embedding medium. Xylene was removed with sequential immersion in 100%, 95%, 70%, and 50% ethanol series. The samples were washed in distilled water, followed by acid hydrolysis in 5M hydrochloric acid at room temperature (20°C) for one hour. After washing in distilled water, samples were treated in darkness with Schiff's reagent (stain: pararosaniline) for 2 hours 45 minutes at room temperature (20°C), rinsed in distilled water, treated for 3 × 10 minutes in fresh aqueous sodium tiosulphate (180 ml distilled water, 10 ml 1 M HCl, 10 ml 10% $Na_2S_2O_5$), and rinsed for 5 minutes with distilled water. After dehydration the smears were treated with xylene and mounted, then stored in shade. During the staining process, especially during the hydrolysis in 5M hydrochloric acid, destaining also took place, because the original stain was washed away.

Image analysis cytometry

The intensity of Feulgen staining was measured using a computer-assisted image analysis cytometry system AHRENS ICM with a Nikon microscope (Eclipse E 400; Japan) (designed and produced by Olaf Ahrens; Meßtechnische Beratung, Bargteheide/ Hamburg, Germany). The field of view from the camera (JAI DSP surveillance (color, American English) CCD camera, CV-S 3200/3300) was stored in image memory with resolution of 736 by 560 pixels. The image was produced by a plan objective (Nikon; X 40, numerical aperture 0.065) and the measurements were made from that image. Prior to each measurement session, the illumination of the microscope was adjusted according to the method of Köhler (Bohnhoff, 1979). Several histograms were produced twice, and they turned out to be very similar.

Sampling rules

200 nuclei were sampled, if available, from each case for each type of sampling. Artificially smeared nuclei were excluded. Thirty small lymphocytes, in a few cases also granulocytes, served as internal controls. The DNA values of the lymphocytes were set at 2c, and showed a thin diploid peak. Different sampling strategies were applied. Two methods were used: (i) cell group sampling, and (ii) atypical free cell sampling.

Sampling method 1: cell group sampling. Cells from the cell groups in the sample (cell group defined as more than 2 cells in contact with each other) were selected and the DNA histograms from cell nuclei produced. Free cells were excluded from the analysis at this stage. There was a risk of nuclear overlap within cell groups, but overlapping nuclei were not measured.

Sampling method 2: atypical free cell sampling. Only free cells were measured. Cells were called free when present as single cells, or when two cells were in contact with each other. If there were 3 or more cells in contact, cells were said to form cell groups and not measured for atypical free cell sampling. The aim was our aim to measure the most atypical free cells.

Interpretation of the histogram

The diploid region was viewed to be situated within the gate of 1.7-2.3c. A small number (<10%) of all cells in the tetraploid region (3.4-4.6c) were not considered to represent abnormality. When the mode of the peak and the peak were completely within the gate of 1.7-2.3c, the peak was defined as diploid. When some of the cells represented by the peak were outside 1.7-2.3c, but within gate 1.5-2.5c, they were called peridiploid. Aneuploid peaks were those with modes outside these defined gates (1.7-2.3c, 1.5-2.5c, 3.4-4.6). Individual cells between 2.3-3.4c (without peak) were classified as proliferative cells and individual cells >5c were classified as aneuploid cells. Non-identical peaks had the mode of the peaks was located within different gates. For clinical application the decision rules given by Elzagheid et al. (2004) were tested.

4.2.3 Immunohistochemistry method (IV)

Formalin-fixed, paraffin-embedded primary breast tumor tissue was obtained from 62 patients. Sections were cut serially at 5µm for routine haematoxylin and eosin staining, and for immunohistochemical (IHC) analysis. An experienced pathologist confirmed all histological diagnoses. IHC analysis was done using the automatic system (BenchMark XT, Ventana Medical Systems, Inc. Tucson, Arizona, USA). This fully automated processing of bar code labeled slides included baking of the slides, solvent free deparaffinization, antigen retrieval in a cell conditioning buffer CC2 (Mild: 30 minutes conditioning, and standard: 60 minutes conditioning, at 95°C), incubation both anti-estrogen (ERA) rabbit monoclonal antibody (clone: SP1, isotype: IgG, Zymed Laboratories, San Franscisco), and anti-progesterone (PGR) also rabbit monoclonal antibody (clone 1E2, isotype: IgG Zymed Laboratories, San Franscisco), at 32 min,

37˚C, the application of ultraView™ Universal Diamynobenzidine (DAB) (a biotin-free, Multimer-based detection system for the specific and sensitive detection of mouse IgG, mouse IgM, and rabbit IgG primary antibodies). UltraView DAB Detection Kit includes: ultraView Universal DAB Inhibitor, Streptavidin HRPO, DAB Chromogen substrate, DAB H_2O_2, and DAB Copper enhancer. Counterstaining with blueing reagent (2037) took 4 minutes, and post-counterstaining also with blueing reagent (2037) took 4 minutes as well. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips. The anti-ER and anti-PR react directly with human ER and PR proteins located in the nuclei.

Scoring system and assessment for ER, PR status

Immunostained slides were scored after the entire slide was evaluated by light microscopy. ER and PR expressions were determined according to J-Score method (Kurosumi 2007) (Table 6).

Table 6 J-Scoring system for ER and PR immunostaining.

J - Score	Assessment of staining
0	no neoplastic cells stained
1+	≤ 1% neoplastic cells stained
2+	>1% to <10% neoplastic cells stained
3+	≥ 10% neoplastic cells stained

Negative stain receptor; when score of 0, weak stain receptor; when score of 1+ or 2+, Positive: when score of 3+ (Kurosumi 2007).

When there were discrepancies of the nuclear grade, histological type, or ER and PR receptor status from the original pathological report, the re-evaluation results were recorded for our data analysis.

4.3 Statistical analyses (I-V)

Statistical analyses were performed by using SPSS for Windows, version 15 / 16.0. (SPSS, Inc., Chicago, USA), software packages. Frequency tables were analyzed using the Chi-square test, with likelihood ratio (LR), or Fischer's exact test to assess the significance of association between the variables. Differences in the means of continuous variables were analysed using non-parametric tests (Mann-Whitney or Kruskal-Wallis) for 2 and multiple independent samples, respectively. Comparison of numerical data was done by the chi-square test and Student's t test. Analysis of variance (ANOVA) was used for deriving the mean values (and their 95%CI) of each individual stratum. For survival analysis, Kaplan-Meier curves were plotted, and differences between the curves analysed using the log-rank test (KM-LR). And P values < 0.05 were considered statistically significant. Several statistical analyses and graphs were also performed by Excel (Microsoft office).

5. RESULTS

5.1 Epidemiology and clinicopathological features (V)

5.1.1 Breast cancer incidence in Libya (V)

Based on the data from the Sabratha registry region (most north western part of Libya), the incidence estimate was 18.9 new cases per 100.000 Libyan females, and also there was some evidence that breast cancer in this region has been increasing (Figure 1). This can be attributed to several factors, including the development in health care, and associated improved diagnostic facilities.

5.1.2 Clinicopathological characteristics (V)

The occurrence of breast cancer in female Libyan population is strongly related to the young age with nearly 70.9% of cases arising in women who are 50 years or younger. The median age was 44.0 years, and mean age 46.04 (12.28) years (Figure 2, Table 10; Page 71 (**Paper V**: Figure 2, Table IV, VI, and VII)). The age at first pregnancy was available only from 44 Libyan breast cancer patients with an average was 22.1 years. A large fraction of the patients were premenopausal (68.4%).

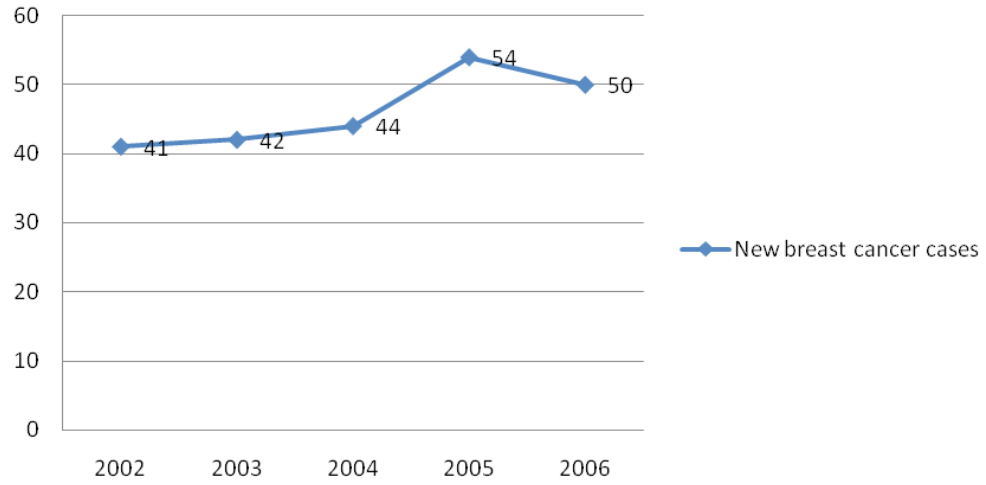

Figure 1. New infiltrating breast carcinoma cases in Sabratha Cancer Registry over the 5 year period 2002-2006.

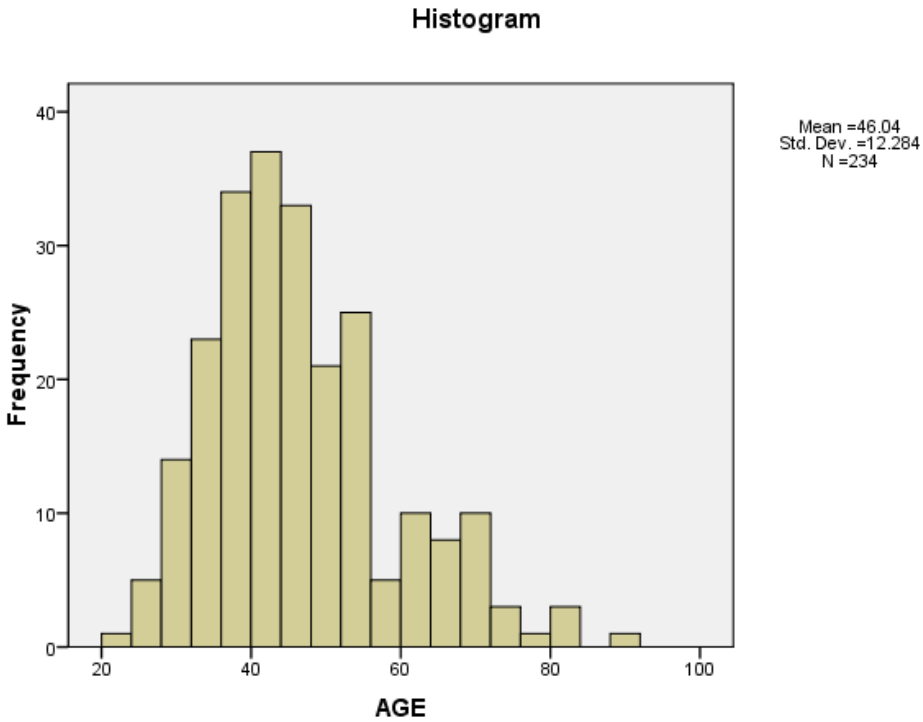

Figure 2. Age distribution at diagnosis of histologically verified breast cancer patients in Western-Libya (Sabratha region) in 2002-2006. The graph is based on 234 patients.

Many of Libyan patients were presented with lymph node involvement (73.9%), a large tumor size (mean 4.8 cm, SD 2.1 cm) and more than 50.0% were classified as belonging to stages 3 and 4.

5.2 Diagnostic support of nuclear morphometry and nuclear DNA content to FNAB examination (I, III)

Size variables and DNA content were significantly different between malignant and benign lesion of breast. The ductal carcinoma cells had higher values for mean nuclear area, perimeter, diameter, and long and short axis than the benign cells (Table 6).

5.2.1 Variations between sampling methods (I)

The mean nuclear area (MNA) of the free cell sample was larger than the mean nuclear area in cell group samples (Table 7 and Figure, identical with **Paper I**: Table I and Figure 2).

Table 7 Ranges of the nuclear size means in malignant and in benign breast lesions of the breast after 2 types of sampling (free cells, cell groups). P values reflect the significance of difference between benign and malignant cases.

Nuclear size features	ranges of the means in free cells		P Free cells	ranges of the means in cells groups		P Cell groups
	Breast cancer	Benign Samples		Breast cancer	Benign Samples	
Area	55.30 -169.70	30.00 - 62.70	<0.001	59.20 -137.80	27.80 - 61.80	<0.001
Perimeter	27.60 - 47.30	20.20 - 29.50	<0.001	28.47 - 42 .40	19.33 – 29.55	<0.001
Diameter	08.26 - 14.39	06.11 - 08.79	<0.001	08.64 - 12.80	05.88 - 08.99	<0.001
Short axis	06.91 - 12.95	05.33 - 07.57	<0.001	07.33 - 11.33	05.16 - 07.54	<0.001
Long axis	10.18 -16 .60	07.22 - 10.98	<0.001	10.19 - 15.70	06.90 - 10.99	<0.001
Axis ratio	01.24 -01.60	01.23 - 01.61	>0.05	01.27 - 01.60	01.22 - 01.53	>0.25

The mean nuclear area of free cell samples was between 30.0 – 169.7 μm^2, and in cell group samples the mean nuclear area ranged from 27.8 to 137.8 μm^2.

5.2.2 Value of nuclear size in distinguishing between benign and malignant lesions (I)

The mean nuclear area was significantly higher in carcinomas than among benign cases ($p < 0.001$). The MNA in cell groups of carcinomas ranged between 59.2 and 137.8 μm^2 and in benign cases between 27.8 and 61.8 μm^2. The MNA of free cells in carcinomas ranged from 55.3 to 169.7 μm^2 and in benign cases from 30.0 to 62.7 μm^2 (Table 7; **Paper I**: Table I).

Figure 3 shows the overlap of mean nuclear areas between the fibrocystic change, fibroadenomas, and breast carcinomas. Fibrocystic change clearly overlaps with carcinoma. On the other hand, the overlap with fibroadenoma is not to the same degree significant. In these samples mean nuclear areas above 62.7 μm^2 always represented carcinomas. 21/23 of carcinomas showed mean nuclear area higher than 62.7 μm^2.

Combination of the present data with earlier published free cell data in the Finnish population gave the following diagnostic guidelines: Range of overlap in free cell samples: MNA 55 μm^2 - 71 μm^2. Cut-off values for diagnostic purposes: 100% detection of malignant cases: MNA >54 μm^2 (specificity 84 %), 100% detection of benign cases: MNA < 72 μm^2 (sensitivity 91%).

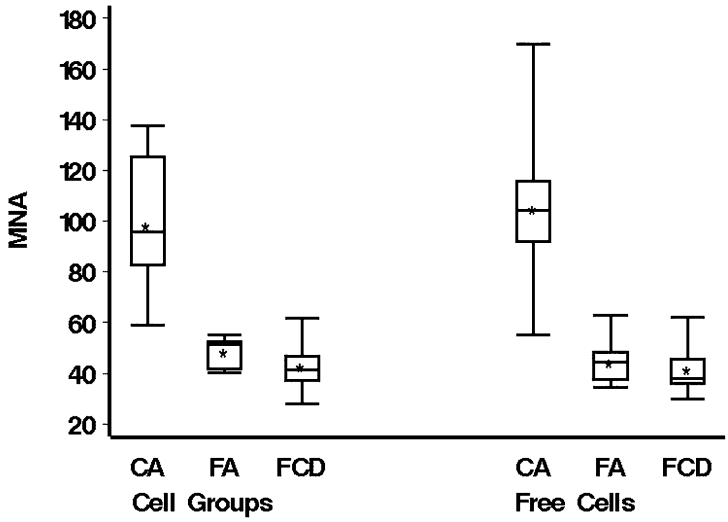

Figure 3. The mean nuclear area of the same 40 FNABs of the breast, studied by 2 sampling methods (cell group sampling and free cell sampling). There were 23 malignant samples (CA), 9 fibroadenomas (FA) and 8 fibrocystic disease (FCD) samples. Asterisks indicate the mean MNA. The upper and lower ends of the box represent the 75th and 25 th percentiles, respectively. The uppermost and lowermost bars represent the highest and lowest mean nuclear areas, respectively. Clearly the mean nuclear areas of free cells and cell groups have value in distinguishing between malignant and benign lesions.

5.2.3 Value of image DNA cytometry in distinguishing between benign and malignant lesions (III)

All benign cases had diploid histogram (modal peak between 1.7 c - 2.3 c limits), and the histograms of benign lesions were not affected by the different methods of sampling. However, a few had one or two cells of > 5 c category. There was not enough follow-up for these patients for more detailed analysis, but there was no history of carcinoma. It can be suggested suggest that with this type of proliferative histograms (a few > 5 nuclei) patients should be placed in a follow-up category which includes at least yearly mammograms. Most histologically malignant cases were aneuploid. Only three invasive ductal carcinomas showed diploid histograms and the histograms could not be distinguished from benign histograms (Figure 4). All samples with aneuploid histograms were malignant. The results also showed that DNA cytometry was able to support a diagnosis of carcinoma and to improve sensitivity, especially in moderately atypical cases where only one of three had peridiploid histogram. However, the method was less powerful in improving sensitivity for detecting carcinoma among highly suspect cases.

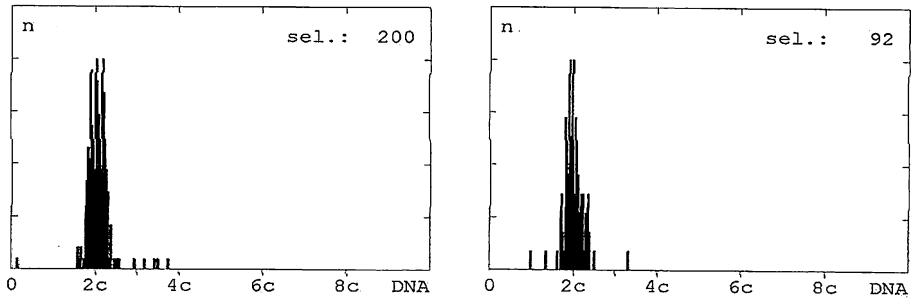

Figure 4. The cell group sampling methods in fine needle aspiration biopsy shows diploid histogram (A) in a benign case – and (B) in a malignant case. The histograms are very similar.

5.2.4 Influence of sampling methods in malignant lesions (III)

DNA cytometry confirmed the cytological diagnosis in most of definitely benign (C2) and malignant cases (C5). Among highly suspicious and definitely malignant samples, DNA cytometry supported the presence of carcinoma in 75% of samples, when the interpretation was based on cell groups. From histograms of free cells, the diagnosis of carcinoma was supported in 85% of samples. Cell group sampling had a sensitivity of detecting carcinoma of 73.9%. Free-cell analysis increased sensitivity to 82.6.0%. These results show clearly that sampling methods can influence the ability of DNA cytometry to detect malignant lesions (Figure 5, identical with **Paper V**: Figure 2). In some cases, nuclear overlapping in cell groups made it impossible to produce adequate histograms, *i.e.* a sufficient number of non overlapping nuclei were not available.

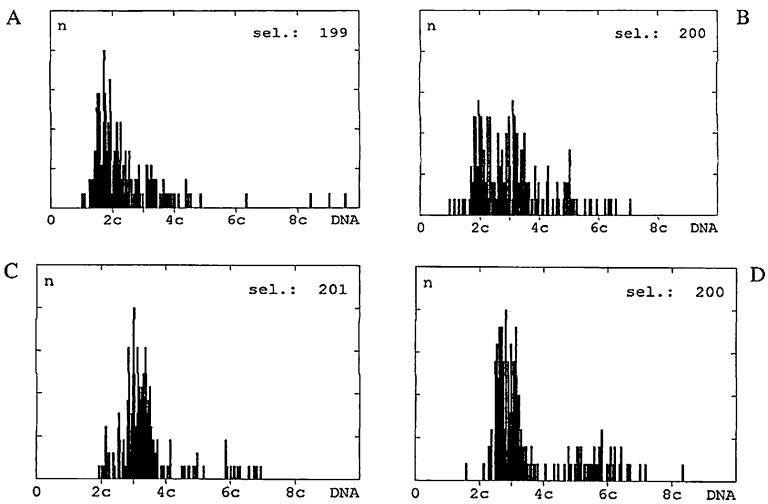

Figure 5. Examples of the use of different sampling methods in image DNA cytometry of fine needle aspiration biopsy. A and B: same cancer after cell group (A) and free cell sampling (B). C and D: another neoplasm after cell group (C) and free cell sampling (D). Note that free cell sampling can give wider separation of DNA value, and more clearly suggest anaploidy.

5.3 Nuclear morphometry and the clinicopathological features (II, IV)

Nuclear morphometric features were analyzed in the whole material, and in groups defined by the nodal status, tumor size, histological type, stage, and grade, as shown in (Table 8, identical with **Paper II**: Table II and III). A statistically significant correlation between the mean nuclear area (MNA) and most clinicopathological features were observed. The strongest association was observed for nuclear grade ($p < 0.0001$).

Table 8 Means of morphometric nuclear variables in the whole Libyan material (n=131), and in subgroups defined by menopausal status, tumor size, clinical stage, histological grade, histological type and nodal status. The feature values are presented as means with the SD shown under the mean. The p-values refer to significance of difference between the subgroups.

Clinico-pathological features	Area (SD)	Perimeter (SD)	Diameter (SD)	Long axis- (SD)	Short axis- (SD)
Whole material	74.3 (23.7)	31.6 (5.2)	9.5 (1.5)	11.7 (2.00)	8.1 (1.3)
Menopausal status	***p* = 0.62**	***p* = 0.55**	***p* = 0.64**	***p* = 0.27**	***p* = 0.88**
Premenopausal	75.1(24.1)	31.8 (5.2)	9.6 (1.5)	11.9 (2.0)	8.1 (1.3)
Postmenopausal	72.9 (23.3)	31.2 (5.2)	9.5 (1.5)	11.5 (1.9)	8.2 (1.3)
Tumor size	***p* = 0.03**	***p* = 0.04**	***p* = 0.03**	***p* = 0.08**	***p* = 0.01**
<3cm	64.0 (19.6)	29.4 (4.9)	8.9 (1.4)	11.0 (2.0)	7.5 (2.0)
>3cm	76.2 (24.0)	32.0 (5.2)	9.7 (1.5)	11.9 (2.0)	8.3 (1.3)
Clinical stage	***p* = 0.05**	***p* = 0.05**	***p* = 0.04**	***p* = 0.09**	***p* = 0.02**
Stage 1	57.9 (11.2)	27.9 (3.6)	8.5 (1.1)	10.5 (1.3)	7.1 (1.0)
Stage 2	70.6 (22.0)	30.9 (5.0)	9.3 (1.5)	11.5 (1.9)	7.9 (1.3)
Stage 3	78.0 (24.1)	32.5 (5.0)	9.7 (1.5)	11.9 (1.9)	8.4 (1.2)
Stage 4	75.0 (27.0)	31.5 (6.4)	9.5 (1.8)	11.6 (2.5)	8.2 (1.4)
Histological grade	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**
Grade 1	58.9 (8.1)	12.0 (4.1)	28.2 (2.3)	8.6 (0.6)	7.4 (0.5)
Grade 2	66.0 (20.2)	15.3 (5.9)	29.8 (4.7)	9.0 (1.4)	7.7 (1.2)
Grade 3	98.2 (23.0)	18.6 (7.0)	34.7 (4.8)	10.5 (1.4)	8.9 (1.1)
Histological type	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**	***p* < 0.0001**
Invasive ductal	76.8 (23.3)	32.1 (5.1)	9.7 (1.5)	12.0 (1.9)	8.3 (1.2)
Invasive lobular	52.8 (16.5)	26.3 (4.2)	8.1 (1.3)	9.7 (1.6)	7.0 (1.1)
Others	75.9 (23.9)	32.1 (4.7)	9.7 (1.5)	11.8 (1.7)	8.3 (1.3)
Nodal status	***p* = 0.001**	***p* = 0.001**	***p* = 0.001**	***p* = 0.005**	***p* = 0.0001**
Negative	60.6 (16.3)	28.7 (3.8)	8.7 (1.2)	10.8 (1.5)	7.3 (1.0)
Positive	77.9 (24.1)	32.3 (5.3)	9.8 (1.5)	12.0 (2.0)	8.4 (1.3)

There was also correlation between nuclear area and node status ($p = 0.006$), seen in figure 6 **(Paper II**: figure II and III). The difference in the mean nuclear area between invasive ductal carcinoma and lobular carcinoma was statistically significant ($p = 0.02$). Mean nuclear area was larger in receptor positive tumors, but the statistical significance was less than in the relationship between lymphnode status and receptor positivity. The MNA was higher in the premenopausal patients than in the postmenopausal patients, but the difference was not statistically significant. The MNA was also higher in larger

tumor and in advanced stages (stages 3, 4). However, the difference was not statistically significant. A corresponding relationship was found with the other nuclear size related features but not with the shape related features.

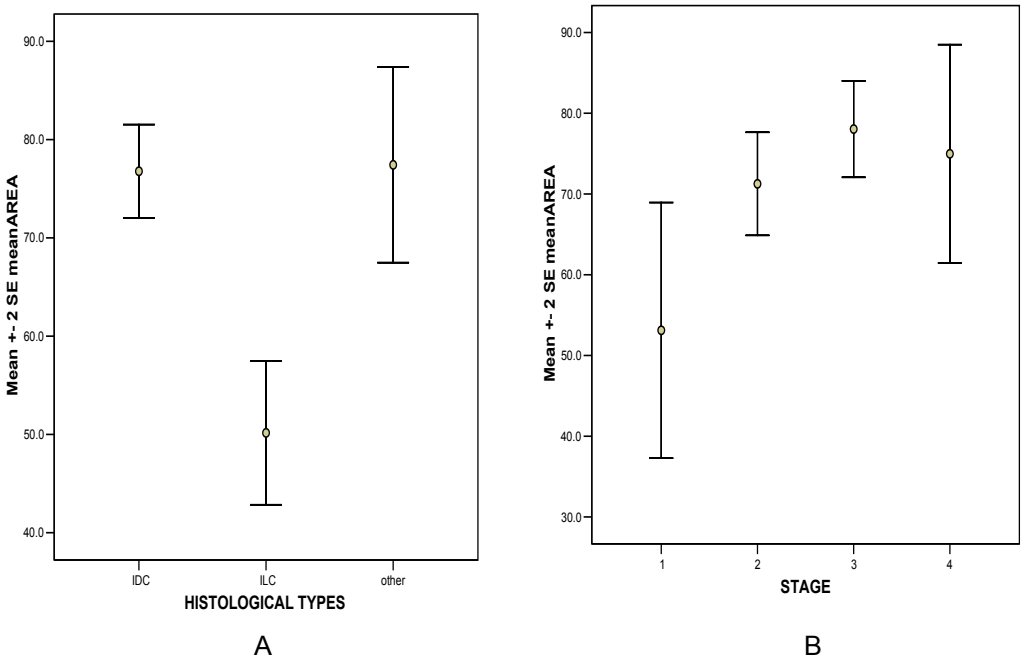

Figure 6. Mean nuclear area (mean Area) ± 2SD: A. the mean nuclear area of different histological types of breast cancer in Libyan female patients (IDC = infiltrating ductal carcinoma ILC = Infiltrative lobular carcinoma, other = other types of infiltrating carcinoma; not IDC, B. nuclear area in histological sections of breast cancer of Libyan female patients in different clinical stages (stages1-4). Clearly the mean nuclear area is in correlation with the stage (Pearson's r = 0.173, P=0.04).

5.4 ER and PR expression and the clinicopathological features (IV)

ER or PR expression did not show any significant relation with age, menopausal status, or histological type. The time between onset of symptoms and diagnosis by histopathology exceeded six months, in 36 of 62 (58.1%) cases. This did not appear to influence the staining positivity. The size of the tumor mass was variable. 53(85.5%) were > 3 cm, 14.5% < 3 cm (p=*0.27*). The size difference was significant (p=*0.05 in PR*) at the cutpoint of 5 cm (Table 8). High stage tumors (stages 3-4) on average were negative in hormonal staining and low stage tumors (stages 1-2) were positive (ER; p = 0.017, PR; p = 0.015). 47 of 62 patients (75.8%) had lymph nodes involvement. 32 of 62 patients (51.6%) had N1 status. 14 cases (22.6%) were of N2, and one case was N3. The prevalence of lymph node involvement was significantly lower in patients

with positive hormonal receptors than in patients with negative hormonal receptors (p = 0.03 in ER and p = 0.05 in PR). The significant relationships are shown in Table 9 **(Paper IV**: Table 3 and 4).

Table 9 Summary of immunohistochemical staining versus histological grade, lymph node positivity, early (Stage I/II)/late stages (Stage III/IV), (size of tumor smaller or larger than 5cm), Nuclear area (MNA) and cumulative number of death.

Feature	PR-	PR +	P value	ER-	ER +	P value
Histological grade						
1	2	6		2	6	
2	14	20		12	22	
3	14	6	0.041	13	7	0.05
Nodal status						
negative	4	11		3	12	
positive	26	21	0.05	24	23	0.03
Clinical stage						
I–II	7	17		6	18	
III–IV	23	15	0.015	21	17	0.017
Tumor size						
≤ 5 cm	10	18		9	19	
> 5cm	20	14	0.05	18	16	0.08
Nuclear area						
<71μm²	13	23		11	25	
≥71μm²	17	9	0.022	16	10	0.015
Cumulative No. of death	9	3	<0.0001	8	4	0.001

5.5 Distribution of hormonal receptors (IV)

The nuclear staining of benign proliferative lesions and normal breast tissue were used as internal controls for ER and PR staining. The number of completely negative cases was 27 and 30 in ER and PR staining, respectively, and the positive staining was usually of weak to moderate intensity. About 57% and 52% of all tumors showed positive epithelial nuclear staining for ER and PR, respectively.

Three patients (4.8%) were positive for only ER and negative for PR. Thirty two patients were recognized positive for both PR and ER. Therefore, altogether 27 (43.5%) patients were negative for both ER and PR. The results of the immunohistochemical analysis of both ER and PR in the whole material are summarized in Table 10 **(Paper IV**: Table 2).

5.6 Prognostication (II, IV, V)

5.6.1 Morphometric features (II, IV)

After 5 years, 34 patients were known to have died, and 33 patients were known to be alive. Median survival for the whole series of 131 patients was 33 months (mean 33.9 months, range 4 - 78 months). The nuclear morphometric parameters can identify the aggressive tumor phenotype and provide significant prognostic information in predicting survival and tumors at risk of progression. Determination of decision cut points for MNA in the Libyan material resulted in an obvious cut point at 71 μm². At 71μm² 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 to significant potential cut-points. The size parameters were significantly correlated with survival. The shape parameters were not significantly associated with survival. The analysis using Kaplan Meier curves of MNA indicated that short survival time was correlated with high mean nuclear area (Figure 7; **Paper II**: Figure I). Moreover, the Pearson's correlation analysis showed that MNA had the highest negative correlation with survival ($r = 0.29$, $p = 0.019$). The same survival analysis was also applied on data from 62 patients in hormonal receptor study. Also here, the short survival time was associated with high nuclear morphometric values (p value = 0.04).

Table 10 Distribution of ER and PR expression as determined by immunohistochemistry (IHC) in 62 Libyan breast cancers.

IHC	Patients ER positive		Patients PR positive	
J- score	Number	Percent	Number	Percent
0 (negative)	27	43.5	30	48.4
1 (weak positive)	4	6.5	5	8.1
2 (mild to moderate positive)	14	22.6	14	22.6
3 (strong positive)	17	27.4	13	21.0

Also in this group of patients the morphometric shape features did not show any statistical significant association with clinical features or survival.

5.6.2 Clinicopathological features: (IV, V)

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

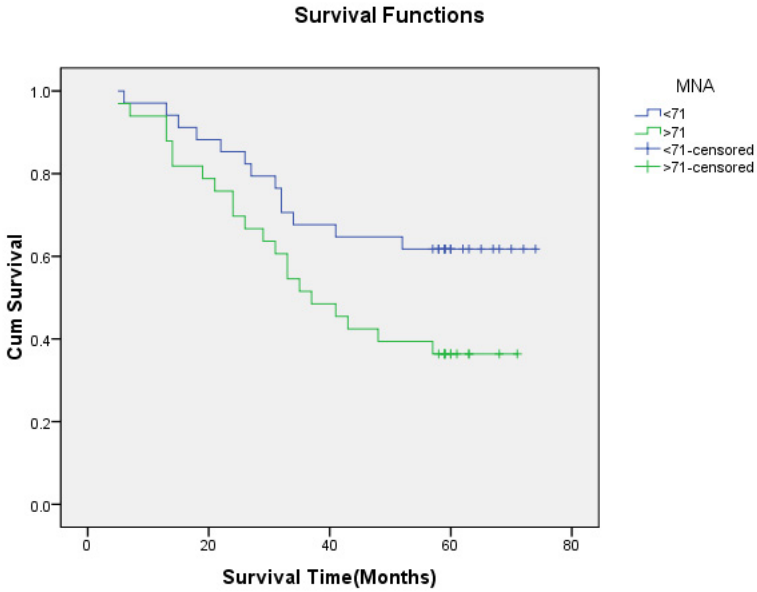

Figure 7. Survival curves associated with the mean nuclear area as measured from 131 Libyan breast cancers. The cutpoint at 71μm² was the most significant cutpoint, and the corresponding survival curves are shown here. The survival curves are significantly different at 5 and after 5 years (Log Rank test, P = 0.044). The upper curve started with 65 patients, the lower curve with 66 patients. At 5years the upper curve had 21 survivors; the lower curve had 12 survivors.

However, advanced tumor stage, LN involvement and large size tumors were strongly associated with shortened survival rate (Kaplan-Meier and log rank ($p < 0.0001$, 0.002 and 0.003 respectively), while high histological grade also associated with shortened survival rate but with only marginal significance (Kaplan-Meier and log rank ($p = 0.05$), Figures (8: A-D) (**Paper IV**).

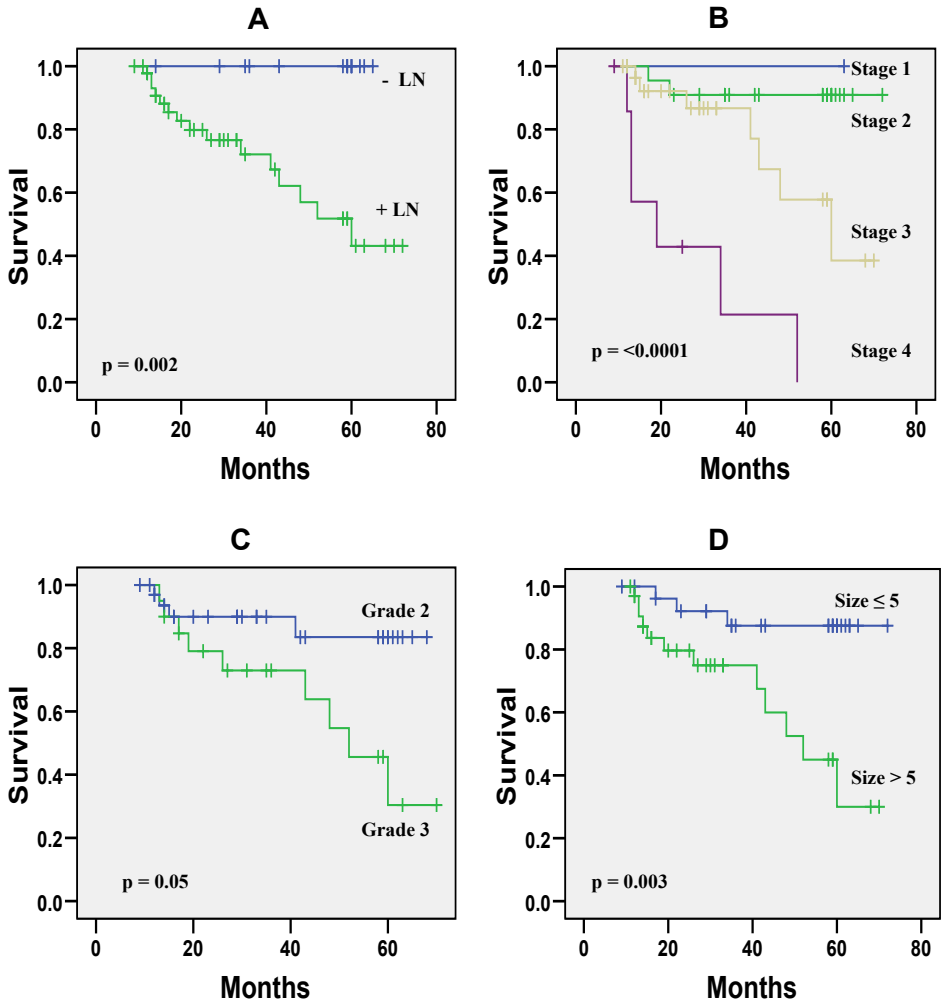

Figure 8. A. Survival curves based on LN involvement (p value = 0.002) B. Survival curves based on the clinical staging. The group of patients with stage 1 had good 5 -year survival ($p < 0.0001$). C. Kaplan Meier curves for patients with histological grade 2 and grade 3 ($p = 0.05$ "just significant difference"). D. Survival curves associated with tumor size. The cut-point at 5cm was the most significant cut-point (Log Rank test, $p = 0.003$).

5.6.3 Hormonal status (IV)

Of the 32 PR-positive patients 28.6% showed metastases, and of the 35 ER-positive patients 32.1% showed metastases after an average follow-up of about 41.0 months.

Furthermore, the present results; which are shown in Figures (9A-9D; **Paper IV** Figure I), demonstrated that patients with high ER or PR expression had better survival than those with low or no exepression ($p = 0.002$ for ER, and PR), while the overall survival for cut point at score 2, had lesser significance ($p = 0.03$ for ER and 0.02 for PR, Kaplan-Meier and log rank).

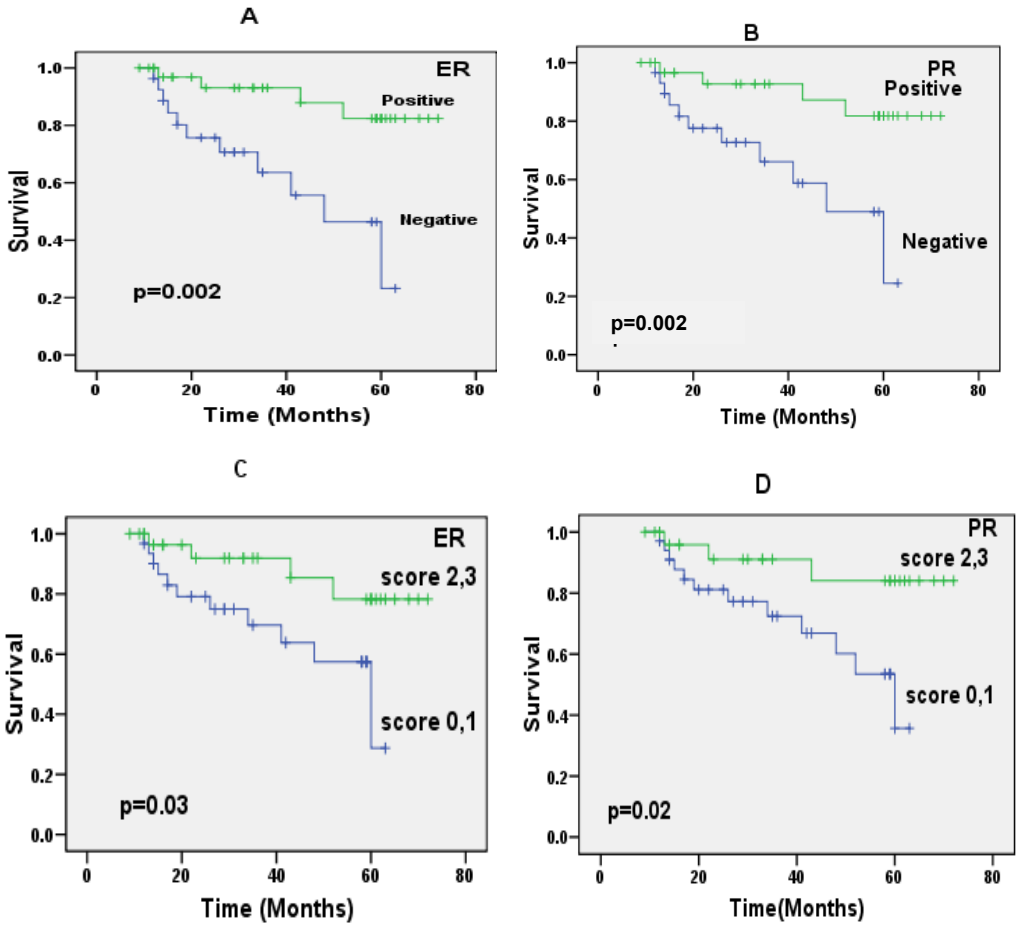

Figure 9. Overall survivals of patients according to expression of ER and PR in primary breast cancer. (**A**, **B**): Expression status was evaluated by J score (cutoff point 1 (between negative (0) and 1, 2, 3 (any positivity detected)); differences are highly significant, positive ER and PR were associated with better survival (Log Rank test, p = 0.002 for ER and 0.002 for PR). In this study on Libyan material, the receptors are much better prognosticators than in the previous studies on Finnish materia (**C**, **D**): Expression status was evaluated by J score (cutoff point of 2 (between scores 0 with 1 and scores 2 with 3). Higher expression levels of ER and PR were associated with better survival, the separation in these group of patients were not as striking as for cutoff point 1 between negative and positive (Log Rank test, p = 0.03 for ER and < 0.02 for PR).

6. DISCUSSION

6.1 Epidemiology and clinicopathological features of Libyan breast cancer as compared with cancer elsewhere

The clinicopathological differences between Libyan, Nigerian and Finnish population were prominent. The Libyan and Nigerian patients were younger than European at presentation with mean age of about 46 years, and they displayed unfavorable features such as high histological grade and stage, large size and frequent lymph node metastases. However, the histological types and histopathological risk features showed similar importance in respect to survival as European breast cancer (see Table 1, p 15 and Table 11).

Table 11 Comparison of distribution of age at diagnosis, menopausal status, tumor size, lymph node status, histological type and grade and stage at diagnosis in breast cancer patients in Libya, Nigeria, and Finland.

Variable		**Libya**	**Nigeria***	**Finland***
Mean age at diagnosis (SD)		46.0± 12.3	42.7 ±12.1	58.8±12.5
Menopausal status	No.of premenopausal patients (%)	160 (68.4)	223 (74.3)	93 (32.6)
	No. of postmenopausal patients (%)	74 (31.6)	77 (21.3)	192 (67.4)
Tumor size	Diameter in cm (sd)	4.8 (2.1)	4.8 (2.4)	2.6 (1.9)
	Range in cm	1.5-12.5	1.0-11.0	1.0-15.0
Nodal status	No. of patient with positive LN (%)	173 (73.9)	235(79.1)	97(34.0)
	No. of patient with negative LN (%)	61 (26.1)	62(20.9)	188(66.0)
Histological grade	Number of patient with grade1 (%)	11 (6.6)	44 (14.8)	67(23.5)
	Number of patient with grade2 (%)	104 (62.3)	119(40.1)	173(60.7)
	Number of patient with grade3 (%)	52 (31.1)	137(45.1)	45 (15.8)
Histological type	Ductal	191(81.6)	252(84.0)	244 (85.6)
	Medullary	11 (4.7)	8 (2.7)	2 (0.7)
Clinical stage at diagnosis	Stage 1	12 (5.1)	65 (21.7)	95 (31.25)
	Stage 2	103 (44.1)	75 (25.0)	171(56.25)
	Stage 3	86 (37.6)	97 (32.3)	19(6.25)
	Stage 4	33 (14.1)	63 (21.0)	19(6.25)

**From Ikpatt et al 2002*

6.1.1 Breast cancer incidence (V)

There was some evidence that breast cancer in western Libya has been increasing (Fig. 1, p 57). This can be attributed to development in health care, including improved diagnostic facilities (mammography, immunostaining) in the last few years in Libya. The breast cancer incidences in Libya, Nigeria, and Finland were 18.9, 33.6, and 87.6 per 100,000, respectively. The incidence in Libya is clearly lower than in Europe or USA, but also lower than in Nigeria. Life style differences may be involved, but biological differences as causes are not excluded (Ikpatt and Olopade 2006, Jobling et al. 2004). The results are in line with neighboring North African countries (Libya 18.9, Tunisia 19.6, Egypt 24.2, Algeria 23.4) (Sabratha Cancer Registry: First annual report, 2008).

6.1.2 Age and menopausal status at the onset of disease (V)

Libyan and other African breast cancer patients are clearly dominantly of premenopausal type. In Europe and US most patients are postmenopausal at diagnosis. The younger age presentation in African patients may partly be associated with the age distribution of populations in respective countries, but biological difference may also be involved. The variation in genetic marker distribution between central and north African, and European populations may also be involved (Jobling et al. 2004, Alero and Lisa 2005), suggesting that in the African population, characterized by ‘’African’’ genomic haplotypes the premenopausal type of breast cancer is more common than the postmenopausal type. In Europe, the population of which is characterized by ‘’European’’ genomic haplotypes, the contrary is true.

6.1.3 Clinicopathological description of Libyan patients (V)

At diagnosis, a large fraction of Libyan patients are in advanced stages, have large tumors and lymph node involvement. These may reflect the delayed presentation and late diagnosis which was also obvious in the study by Ikpatt et al. 2002 on Nigerian breast cancer. Mammography in Libya is not part of a screening program, but it can be speculated that the potential of mammography is limited because of the difficulties of making early mammographic diagnosis in premenopausal breast cancer (Keen and Keen 2009). Also biological aggressiveness of premenopausal type seems to limit the value of early screening (Alban et al. 1994 and Gao et al. 2000). These results are in line with other North African results. In Egypt, the carcinoma of the breast is responsible for a large fraction of all cancer deaths among women (8.2%) and the tumors are advanced at presentation (Nadia et al. 2007). In Tunisia breast cancer has poor survival due to late diagnosis (Ben Ahmed et al. 2002). Approximately 55% of the breast cancer patients presenting at the Tunisian Oncology Institute of Salah Aziiz are characterized by rapid disease progression, inflammation, and edema (Ben Ahmed et al. 2002). The present study and the study of Ikpatt et al. 2002 found that fraction of medullary carcinoma is a little higher than in European countries. It may be that genetic factors are involved, but so far we have a little evidence that breast cancer genes (BRCA1 and BRCA2) are

more often involved in African population (Alero and Lisa 2005). The Libyan patients had higher histological grade than Finnish patients. This result is in line with the results on Nigerian (Ikpatt et al. 2002) and on African American patients (McBride et al. 2007 and American Cancer Society, 2007). One explanation for grade differences may just be the more active cell proliferation in premenopausal type of breast cancer, which is more common in Africa.

6.2 Diagnostic tools (I, III)

The cytological analysis of FNAB actually is a successful application for diagnosis in malignant diseases. However, for some patients, the identification of malignant cells was difficult (specificity is high but sensitivity is variable 70–90% (Teague et al. 1997, Alatise et al. 2007). The factors contributing to this low sensitivity are the presence, in some cases, of only few malignant cells and the difficulty in differentiating low grade malignant cases from reactive and or benign epithelial lesions. Further techniques have been proposed by some authors to increase the sensitivity of the FNAB cytology diagnosis (Elzagheid and Collan 2003, Buhmeida 2002). Cytometric quantification of nuclear morphometry and nuclear DNA content by static cytometry can be expected to be more reproducible (Böcking et al. 1995).

6.2.1 Diagnostic value of nuclear morphometric parameters after different sampling methods (I)

Our cytomorphometric study showed that benign and malignant lesions can be distinguished with a considerable degree of accuracy with quantitative pathological methods The results support other previous works for lesions of the breast (Elzagheid 2003, Mapstone 1990, Pattari 2000, Schöndorf 1985, Stenback 1984, Wittekind and Schulte 1987) and other organs (Collan et al. 1987), such as thyroid (Karslioglu et al. 2005), prostate (Buhmeida 2000), and liver (Davaris 2000). The present results demonstrate that the nuclear size parameters are the most appropriate nuclear

morphometric parameters for differentiating between benign lesions and infiltrative ductal carcinoma of the breast. These parameters showed significant differences between benign breast lesions and infiltrative ductal carcinoma ($p<0.001$). Many studies suggested that the perimeter and not the mean area was the most powerful feature for differentiation between benign and malignant breast lesions (Stenback 1984, Elzagheid and Collan 2003, Buhmeida et al. 2000, Wittekind & Schulte1987). However, as a general rule the value of these features is at about the same level of significance.

Boon et al. 1982 preferred to use the nucleus/cytoplasm ratio for characterizing cells of different tumors. We feel that such design should be avoided because outlining of cellular margins is difficult due to more indistinct outline than nuclear outline, making the measurement less reproducible and more subjective. Some authors mentioned that

there is considerable variation in the cytomorphometric results in smears obtained from the same patients by different sampling methods (Elzagheid and Collan 2003, Collan et al., 1987, Cui et al. 2007). The use of all observed cells in morphometry may cause a dilution effect in recognizing diagnostically important features (Karslioglu et al. 2005). The current sampling tried to combine the proper selection of abnormal cells and careful morphometry in both free and cell group sampling categories. We can agree that careful selection of abnormal cells ensures retrieval of most of the diagnostically and prognostically important information (Stenkvist 1981, Elzagheid and Collan 2003). The study of Stenkvist (1981) detected that smaller size of isolated cell groups in FNAB was associated with poor differentiation and a poor prognostic outcome. In the current study, free cells of malignant cases had higher values in nuclear size features than nuclei of cell group categories. Clearly lowered cohesiveness was also associated with larger nuclear size.

Table 12. The ranges of mean nuclear area of different sampling categories based on pooled results from FNABs from Libyan and Finnish patients (see Elzagheid and Collan 2003).

Sampling method	Fibrocystic disease (n = 18)	Fibroadenoma (n = 19)	Ductal carcinoma (n = 46)
Free cell	33-70 μm^2	30-61 μm^2	55-181 μm^2
Cell group	30-55 μm^2	26-61 μm^2	42-137 μm^2

The present results were similar in respect to distinction between benign and malignant lesions of the breast with those by Elzagheid and Collan (2003) on Finnish cases. In their study, the sensitivity for discriminating benign lesions from infiltrative ductal carcinoma in free cell and cell group sampling categories were 90.9%, and 78.3%, respectively. The current results (Table 12; **Paper I**: Table III), were about the same in both categories, moreover the range of values in benign and malignant categories was similar. This clearly makes it possible to combine the results.

On the basis of this combination the value ranges for definitely malignant cases were 71-181 μm2 (free cells), and 63-137 μm2 (cell groups). All cases were benign within the range of 30-54 μm2 (free cells) and 26-41 μm2 (cell group). But within the range of 55-70 μm2 (free cells) there were 6 benign and 4 malignant cases. Within the range 42-62 μm2 (cell group) there were 18 benign and 9 malignant cases. In pooled results, at a cut-off of 62 μm2 in association with free cells the sensitivity of detecting carcinoma was 95.5 %, and specificity 90.3 %, at a cut-off of 52 μm2 in cell groups, the figures were 87% and 84%, respectively. Therefore the method of cell sampling significantly influenced the results, but without altering the general conclusions regarding evolution of the morphonuclear features (Salmon et al. 1991).

6.2.2 Diagnostic value of image DNA cytometry after different sampling methods (III)

The present results on the various ways of DNA cytometry sampling, however, show that different ways of sampling should be considered when DNA histograms from FNABs are produced. This is consistent with the finding of Elzagaheid et al. (2004). In both studies (in the present paper and in that of Elzagaheid et al.) the most atypical DNA histogram patterns are to be found among free cancer cells, and not among cells of the cell group sampling. The current study showed that all but one of the cytological definitely malignant cases had a non-diploid histogram, and truly Ludwig et al. (1973) and others have mentioned adenocarcinomas showing diploid cells. In the current study three malignant cases were diploid, and one peridiploid in free cell sampling. Some carcinomas may present slight chromosomal variations that are difficult to detect with the analysis of nuclear DNA content. Such cases can represent diploid carcinoma (Ludwig et al. 1973 and Ruiz-Sauri et al. 1995). Furthermore, some FNABs may not be representative of the lesion with few malignant cells mixed to a large amount of benign cells (Zuk et al. 1989 and Grosby 1996).

6.3 Survival and patient outcomes in Libyan breast cancer (II,V,IV).

6.3.1 Prognostic value of histomorphometry in Libyan female breast cancer (II)

Several authors have reported on the prognostic importance of estimates of nuclear area in breast carcinoma (Kronqvist et al. 1998). These finding were confirmed in the univariate analysis of the current study.

There was considerable difference in nuclear area and other parameters values reported by Kronqvist *et al.* 1998 and Ikpatt *et al*. 2002. The mean of Finnish's MNA value was 38.6 μm² (SD 15.0) which was within the range of previous European data [from 24.4 (SD 12.8) up to 67.8 μm² (SD 18.35)] (Baak et al. 1982, Aaltomaa et al. 1991, Ladekarl et al. 1993 and Kronqvist et al. 1995). This was lower than the Libyan mean MNA value 74.25 μm² (SD 23.74). The Nigerian mean MNA value was highest; 89.2 μm² (SD 34.0). These differences might be due to the fixation technique employed. Another explanation may be that screening programmes were operated in European countries for years for early detection of cancer. The African females came to the hospital in very advanced stage and grade.

In the future, after establishment of screening programs in Libya, the cut point difference will probably decrease. Biological factors as explanation of difference should not be excluded, either (Wiliams et al. 2006, Jobling et al. 2004).

There were no differences in the used morphometric methods between the 3 studies (Libyan, Finnish, Nigerian). The same equipment was used and the technique was standardized and uniform, with regular calibration of the computerized morphometric

equipment with a micrometer slide, which should ensure reproducible results (Kronqvist et al. 1997).

Nuclear size features were in correlation with histological grade, stage, and nodal status in all 3 studies, and these findings support the other corresponding studies by Van Diest et al. (1991), and Ladekarl (1995). That African and European breast cancers are different in many respects is one of the dominant discussion aspects today (Ikpatt et al. 2002 and Morris et al. 2007). The present findings on Libyan breast cancer fall between the findings from Nigerian and Finnish populations (Ikpatt et al. 2002). This is very much in line with the idea that the differences may have a genetic basis (Ikpatt et al. 2005). The variation in the distribution of different genetic marker haplotypes makes this easily understandable. There is a clear difference between the marker haplotype distribution in western central Africa and northern Africa (Jobling et al. 2004). A similar difference is to be found between North-Africa and Europe (Joblink et al. 2004). The variation in haplotype marker distribution has taken place under selective environmental stresses. However, the environmental influences could not be ruled out (Ikpatt et al. 2005). Kronqvist et al. (1998) suggested that the mean short axis was the strongest prognostic factor among the nuclear parameters. The present results show that the mean nuclear area was still better. Kronqvist et al. (1998) has suggested two thresholds for the MNA ($32\mu m^2$ and $47\mu m^2$) that could separate patients into three subgroups with favorable, intermediate and unfavorable prognosis. In this study, it was found that survival among patients with MNA $< 71\mu m^2$ was significantly better than among patients with MNA $\geq 71\mu m^2$. So, it can be suggested 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 the present cut-point.

According to Giardina et al. (1996), the nuclear shape parameters allowed good discrimination between cases with good and poor prognosis. However, the current results on FNAB and tissue paraffin embedded material showed that the nuclear shape parameters have no statistically significant correlation to clinicopathological feature and neither have prognostic nor diagnostic importance. The same results were obtained in other studies as well (Stenback 1984, Elzagheid and Collan 2003, Buhmeida et al. 2000, Baak et al. 1985).

6.3.2 Prognostic value of clinicohistopathological features in Libyan female breast cancer (V)

The Libyan and other African breast cancer patients clearly have worse prognosis than that in European breast cancer patients (Ellis et al. 1992, Helmrich et al. 1983). However, in respect to stage and lymph node involvement, large size African breast cancer behaves as the European breast cancer as shown by the Libyan material. Among Libyan patients the menopausal status, histological type of tumor, and age of patient did not seem to influence survival.

Comparative survival curves of breast cancer in Libya, Nigeria and Finland are shown in Figure 10 (**Paper V**: Figure 3).

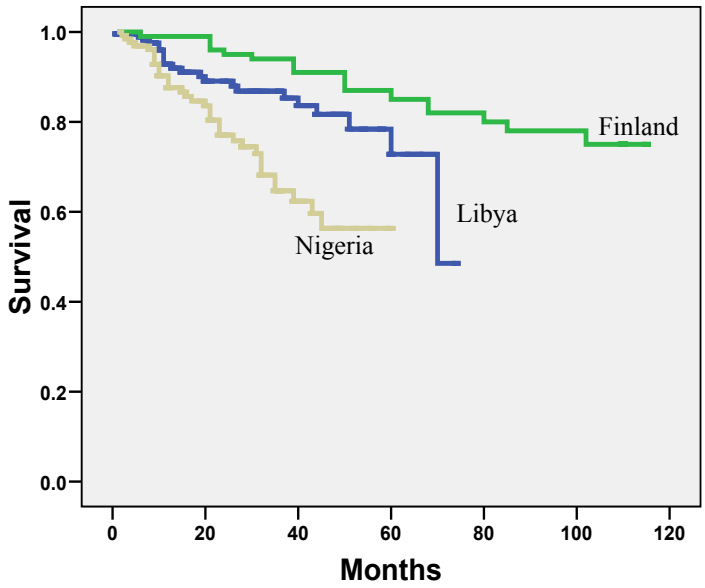

Figure 10. Survival curves of breast cancer patients in 3 countries. The Libyan patients have better survival than Nigerian, but worse than the Finnish patients ($p < 0.0001$).

There is a clear difference in survival between the 3 countries; the Libyan survival curve is located between the Nigerian survival curve and Finnish curve, which is the best of three survival curves.

6.3.3 ER and PR as useful biomarkers in determining the prognosis of female breast cancer (IV)

The current study is in line with the studies of Arpino et al. (2004), Ellis et al. (2001), and Blanco et al. (1984) and shows that positive expressions of ER and PR correlate with better survival and response to estrogen antagonists such as tamoxifen, regardless of tumor size, stage, and age. The study of Jalava et al. (2005) showed that immunohistochemical ER score is associated with prognosis. However, cutpoints for defining the groups with good or worse prognosis may differ between LN- and LN+ patients.

The present results indicated that the cutpoints for defining the groups with good or worse prognosis might be set low. Also a weak positivity is meaningful, and cancer with positive cells had better overall survival, as well as disease free survival. It was reported by Harvey et al. (1999) that tumors with low scores predict better survival, compared with those with higher scores. There may be several explanations as to why such weakly

positive cells predict better survival, including that weakly positive cells may correspond to an ER-positive stem-cell population (Allred et al. 2004, Nakshatr et al. 2009).

6.4 Immunohistochemical analysis of steroid receptors (IV)

6.4.1 Distribution of ER and PR immunohistochemistry stain in Libyan female breast cancer (IV)

Both steroid hormone receptors are present in the present population of Libyan breast cancer patients. However, it seems that in the current test systems ER positive expressions are clearer to detect than PR positive expressions. The ER-positive rate in the present series (57%) was higher than PR-positive rating (52%) %. These frequencies are in agreement with those reported by other authors on European female breast carcinoma patients (Blanco et al. 1984, Jalava et al. 2005, Helin et al. 1989 and Belkis et al. 1991). Fifty two % of the present patients had both receptors positive in their tumors. This is in good agreement with the 49% of patients who had both receptors positive in the study by Belkis et al. (1991). (The proportion of patients increased toward lower receptor values).

6.4.2 Hormonal receptor expression related to pathological parameters (IV)

There are conflicting data on the correlation of ER with age and menopausal status (Jalava et al. 2005, Thike et al. 2001, Pichon et al. 1980). This study showed that neither of the receptors correlated with age and this is in line with previous results of Thike et al. 2001. However, Jalava et al. (2005) noted that ER but not PR had positive correlation with age. Some authors have reported presence of correlation of ER with menopausal status. They found that a higher ER value was seen in postmenopausal women (Jalava et al. 2005). The current results lack such correlation which is consistent with other studies (Thike et al. 2001 and Pichon et al. 1980). In this current study, no correlation was found between PR and ER receptor status and tumor size. This lack of correlation has been previously described by Jalava et al. 2005, Aaltomaa et al. 1991, Thike et al. 2001 and Blanco et al. 1994. Although Blanco et al. (1984), Jalava et al. (2005) and Helin et al. (1989) reported a significant correlation between HR positive and invasive lobular type, Belkis et al. (1991) reported a lack of correlation between HR status and histological type of carcinoma. The present results are in line with latter findings. Several studies recognized that expression of ERs and PRs correlates well with low histological grade (Blanco et al.1984, and Helin et al. 1989, Jalava et al. 2005, Thike et al. 2001). Furthermore, positive hormonal status was more common in low stage patients, and in node-negative patients. These results are consistent with findings of Belkis et al. (1991), Blanco et al. (1984), and Helin et al. (1989), although not all investigators have obtained the same results (Jalava et al. 2005).

6.4.3 Hormonal receptor expression related to nuclear size parameters (IV)

The study of Giardina et al. (1990) showed that ER negative or weakly positive breast cancers possess cells with significantly bigger nuclei than ER highly positive tumors. However Belkis et al. (1991), Blanco et al. (1984), and Helin et al. (1989) showed that as the size of tumor nuclei increased, the hormonal receptor expression decreased, and this is in line with the current results

6.4.4 Quantitative relationship between ER and PR (IV)

The two type's steroid hormone receptor values show good correlations with each other. As receptor values increase for one receptor, there is a corresponding increase in values for the other receptor. This has been previously described by Allred et al. (1998) that ER positive cancers will also be PR positive. However, Wood et al. 2007 observed that PR expression is a little lower than ER and that is because PR is more sensitive to the fixation process than ER. Although breast cancers that are PR positive, but not ER positive, may have a worse prognosis, the double positive cancers are more common phenotype and also indicate a better prognosis.

7. CONCLUSION

I. In Libyan and other African countries premenopausal breast cancer is more common than postmenopausal breast cancer. The premenopausal type has lower incidence and unfavorable features such as high histological grade and stage, large size and lymph node metastases much more often than the postmenopausal type predominant in Finland and other European countries. Population differences between Libya and Finland may be involved in explanation of the above differences. However, the expected life span in Libya is about the same as in European countries. This may suggest that the mentioned differences are understandable in light of genetic instability and markers differences in these populations. Different environmental influences, however, cannot be excluded.

II. The morphometric FNAB seems to be efficient in distinguishing malignant from benign breast lesions. Nuclear morphometry improves reliability of interpretation, especially after free cells sampling. However, there are still some overlapping cases which will be classified into the uncertain category. The latter group can be further studied with supporting methods such as mammography, DNA cytometry, chromatin texture analysis, and cDNA array analysis. The results on Libyan cases were surprisingly similar to the earlier results on Finnish cases. However, further and larger studies will be necessary for producing universally more applicable guidelines.

III. The DNA cytometry results confirm data from literature and indicate that cytometric analyses of nuclear DNA content by different sampling methods are helpful for identification of malignant cells in FNAB. DNA cytometry can be used to improve cytological sensitivity in doubtful breast lesions.

IV. Nuclear size features seems to be reliable prognostic indicators in Libyan female breast carcinomas, as they were among Finnish and Nigerian females. The nuclear morphometric parameters can identify the aggressive tumor phenotype and provide significant prognostic information in predicting survival and tumors at risk of progression. The cut-off (71.0μm²) of MNA might be applied as quantitative criterium for Libyan breast cancer cell nuclear grading to separate patients into good and poor prognosis groups. A positive correlation between nuclear morphometric parameters and clinicopathological features was observed, and the mean nuclear area (MNA) showed the strongest correlation with histological grade and clinical stage.

V. In addition, potential prognostic markers of ER and PR were evaluated. The cut points for defining the groups with good or worse prognosis were between scores 0 and 1 (the latter corresponding to as few as 1% or less positive cells). Patients with ER and PR positive cancer had better overall survival than patients with negative cancer.

In the hospital setting, the ER and PR expressions and MNA in breast carcinoma may be prognostically useful markers in guiding decision on future treatment.

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