Molecular markers in breast carcinoma

A study with focus on molecular phenotypes, angiogenesis and stem cells in an African population

Hawa Nalwoga

Dissertation for the degree of philosophiae doctor (PhD)

The Gade Institute, Section for Pathology
University of Bergen
NORWAY

2010

This thesis is dedicated to my beloved daughters Sophia and Shamim
as well as little Michael Joel
I love you more than you can imagine

May God bless you


SAHARA
AFRICA
Kalahari Desert
Uganda


150 mi
150 km
Ethiopia
Mountain Nile
Sudan
Dem. Rep. of the Congo
Albert Nile
Kenya
Arura
UGANDA
Gulu
Lake Kwania
Moroto
Soroti
Lake Albert
Lake Kyoga
Kibale Forest Nat. Park
Victoria Nile
Mbale
Kampala
Jinja
Tororo
Ruwenzori Mtns.
Entebbe
EQUATOR
Lake George
Mbarara
Port Bell
Sese I.
Lake Edward
Lake Victoria
Rwanda
Tanzania


©GraphicMaps.com
Arctic
Atlantic
Pacific
Pacific
Indian
Southern
Capital City
Regional Capital City
Significant City
Important City - Town
Attraction - Landmark
River
Highest Point

CONTENTS

Acknowledgements

I acknowledge my main supervisor Professor Lars A Akslen for tirelessly guiding me through the process, for the professional advice and the ensuring that I have everything that I needed in a timely and friendly manner. I thank my co-supervisor Professor Henry Wabinga for coordinating the collaboration successively and for the scientific nurturing that he provided whenever I approached him. I acknowledge Professor Leif Bostad for giving me guidance when I most needed it.

I would like to extend my gratitude to all my colleges and the staff at the Department of Pathology MUCHS for accepting my long periods of absence and for the endless and warm welcomes that they offered whenever I returned home. They made me feel more determined to achieve my goal.

I acknowledge my colleges Grace Ndeezi, Henry Wamani, Sissy Mitike, Kamaludeen Baba, Wevica Gronberg, Sam Kalungi, Robert Byamugisha, Jolly Kaharuza, Karsten Gravdal and Charles Karamagi with whom I shared a lot. I would to give special thanks to Annet Birungi, Rebecca Babirye, Robina Kusiima, Syllivia Nampebwa, Ssendikadiwa Emmy, Yahya Baweemu, Joseph Kaaya and Martin Kizito for all the social support and encouragement that they provided to my family. I acknowledge my sisters Pastor Gift Murungi and Monica Mariríre as well as Betty Namuyiga and Siragi Ahebwa for the wonderful company they offered my family during my absence. I thank Sam Nkata and my brother Swaibu Muhumza Metha for the undying support they always offered whenever need arouse. I wholeheartedly thank my dear Mummy and Daddy for the wonderful blessings and wise encouragement they always offered me throughout the process. I am highly indebted to Lynnette K Tumwine for the unlimited social support which she offered my family, without her my studies would have been next to impossible. I thank her dearly for accepting to parent my daughters. I deeply appreciate the academic advice she offered whenever I felt like the

opposite. In addition, I acknowledge Professor James K Tumwine and Professor George B Kirya for the academic inspiration and advice through the process.

I extend my sincere gratitude to the Norwegian government, which funded this study through NUFU project # 64/2003. In addition, I acknowledge Makerere University for establishing the collaboration with the University of Bergen through which this study was funded. I thank the Department of Pathology, MUCHS and the Kampala Cancer Registry for allowing me to use the archival tissue and the data. I am indebted to Sarah Nambooze, Ruth Nakigudde, Gerd Lillian Hallseth, Bendik Nordanger, Randi Nygaard and Dorothy Lynn Nabbale for excellent technical assistance. I also extend my thanks to all the staff at Centre for International (CIH) and The Gade Institute, University of Bergen and Haukeland University Hospital for the friendly working environment that they offered. At CIH, special thanks go to Solfrid Hornell, Borgny Kvalnes Lavik and Unni Kvernhusvik. I wholly heartily thank Lillian for the motherly love and care that she always offered to me unreservedly. I would in particular like to acknowledge my colleague Jarle Arnes for accepting to help me at a time when I hardly knew anyone at the Institute. To Gyrild, you were a blessing, the speed and efficiency at which you made things happen is beyond what words can express; thank you so much, you made my study at the Institute enjoyable. In addition, I acknowledge the Centre for Disease Control and Prevention (CDC) for computerizing the records at the Department of Pathology, MUCHS which made data retrieval a lot easier.

Special acknowledgement goes to my lovely daughters Sophia and Shamim for honorably accepting my long period of absence and for seeing me through the process. Thank you for the beeping, it kept me going and alert.

Lastly but not least, I thank you all who have made the completion of this thesis possible. May God bless you all.

Bergen, March 2010 ***Hawa Nalwoga***

List of publications

This thesis is based on the following articles referenced to in the text by their respective roman numerals:

I. **Nalwoga H**, Arnes JB, Wabinga H, Akslen LA. Frequency of the basal-like phenotype in African breast cancer. *APMIS 2007; 115:1391-1399.*

II. **Nalwoga H**, Arnes JB, Wabinga H, Akslen LA. Expression of EGFR and c-kit is associated with the basal-like phenotype in breast carcinomas of African women. *APMIS 2008; 116:515-525.*

III. **Nalwoga H**, Arnes JB, Wabinga H, Akslen LA. Expression of aldehyde dehydrogenase 1 (ALDH1) is associated with basal-like markers and features of aggressive tumors in African breast cancer. *Br J Cancer 2010; 102:369-375.*

IV. **Nalwoga H**, Arnes JB, Stefansson IM, Wabinga H, Foulkes WD, Akslen LA. Tumor angiogenesis is increased in basal-like breast cancer. *Manuscript.*

List of abbreviations

AJCC	American Joint Committee on Cancer
ALDH	Aldehyde dehydrogenase
BLP	Basal-like phenotype
CBP	Core basal phenotype
CK	Cytokeratin
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
HIF-1α	Hypoxia inducible factor-1α
HIV	Human Immunodeficiency Virus
IHC	Immunohistochemical
mRNA	Messenger Ribonucleic acid
MUCHS	Makerere University College of Health Sciences
MVD	Microvessel density
PcG	Poly comb genes
pMVD	Proliferative microvessel density
PR	Progesterone receptor
SI	Staining index
TNP	Triple negative phenotype
UICC	International union against cancer
VEGF	Vascular endothelial growth factor
VPI	Vascular Proliferation Index

INTRODUCTION

INCIDENCE AND MORTALITY

Worldwide, breast cancer is the most prevalent cancer as well as the most common female neoplasm accounting for 23% of all female cancers.[1, 2] According to the world cancer report, more than one million cases occur worldwide each year, and 45% of these are in developing countries.[1, 3] The incidence of breast cancer is increasing in most countries[1, 4-7] but the outcome is now much better in the western world. The five-year survival rates are over 70% in most of them[1, 3] although racial differences still exist.[3, 8, 9] This reduction in the morbidity and mortality rates of breast cancer in the developed countries has been due to increasing early detection by way of mass screening as well as improved targeted therapy.[1, 3, 8, 10]

In spite of this, breast cancer still remains the leading cause of cancer mortality in women worldwide. In 2002, the estimated number of deaths was about 411,000 (14% of female cancer deaths).[1] Although the risk is still low in sub-Saharan Africa, the incidence of breast cancer is increasing rapidly in most African countries,[2, 11] where breast cancer is more common in the urban population compared to the rural population.[12] In Uganda it has doubled over three decades from 11/100,000 in 1965 to 22/100,000 in 1995 (Figure 1).[13] It is now the second most common non-HIV related cancer[14-16] affecting women in Uganda.[13] Unfortunately, the outcome is still very poor. Five-year survival rates have been found to be very low 29% and 34% for patient with grade 3 and 2 tumors, respectively, in one study,[17] and similarly the overall 5-year survival rate was 38%[18] and 56%[19] in previous reports.

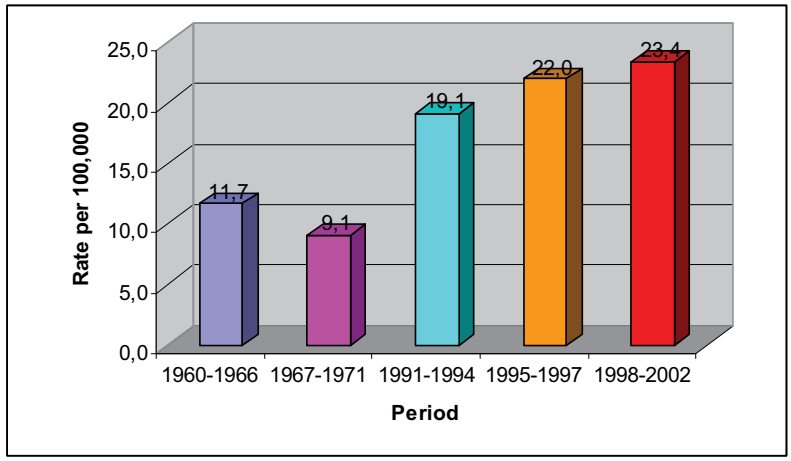

Figure 1: ***Trends in age-standardized incidence rate of breast cancer in Kampala, Uganda Adapted in part from Parkin et al. (2008)***[12]

ETIOLOGY AND RISK FACTORS

The etiology of breast cancer is multi-factorial involving both genetic and environmental influences. Well known factors include genetic, dietary and reproductive factors plus related hormonal imbalances. Numerous studies have shown that most etiological and risk factors for breast cancer are related to the cumulative exposure of the breast to estrogens both endogenous and exogenous and include early menarche, nulliparity, late age at first pregnancy, late menopause (after 55 years) and hormonal replacement therapy. In addition, the other major influences on the risk of breast cancer include genetic susceptibility, body size and obesity, alcohol, physical activity, and possibly diet plus the western lifestyle.[1-3, 20]

Regarding genetic susceptibility, these factors contributes about 5-10% of breast carcinoma risk.[21] The highest risk is due to germline mutations in the high penetrance breast cancer genes which include *BRCA1, BRCA2, TP53, CDH1, PTEN* and *LKB1/STK11*. Breast cancer susceptibility genes with low to moderate risk include *CHEK2*, *TGFβ1*, *CASP8* and *ATM*.[22] Of these, *BRCA1* and *BRCA2* germline mutations have been extensively studied.

Mutations of the other genes such as *TP53*, *PTEN, STK11 CHEK2* and *ATM* result in a small proportion of breast cancer syndromes.[23] Although family history has been reported to be a marker of risk of breast cancer in the African setting, the prevalence of *BRCA1* mutations in African populations is not clearly known.[24]

CLINICAL FEATURES

Breast cancer is a highly heterogeneous disease with regards to morphology, hormonal receptor expression, invasive behavior, metastatic potential, as well as clinical behavior including response to treatment. Nevertheless, most primary invasive breast cancers are characterized by a palpable mass or lump, most frequently located in the upper outer quadrant and most often discovered by the patient.[25] Other symptoms include nipple discharge, nipple lesions, skin edema plus redness and axillary lymphadenopathy. A small proportion will present with skin ulceration and skin retraction of the overlying skin or nipple.[25]

However, the spectrum of breast cancer clinical presentation has been considerably changed by the introduction of mass screening by use of mammography. As a result of mammography screening, breast cancer tumor size and stage at presentation or detection have decreased.[26-28] Whereas detection of non-invasive disease[29] as well as impalpable breast lesions[30] is more frequent. This has major implications on the management of breast cancer as well as on screening programs because of false positive cases and the lead time bias effects. Further, some racial differences in tumor size at presentation have been reported.[31]

Regarding clinical presentation in African populations, it has been noted that African and African-American patients with breast cancer present in the late stages of the disease.[12, 19, 32-34] In Uganda, a recent retrospective study of medical records of breast cancer presenting at the national referral hospital revealed that a majority (77%) of patients presented in the late

stage according to the AJCC staging.[35] Stage III was the peak stage at presentation with 51% of all patients, whereas 26% of patients had metastatic disease at presentation (Figure 2).[19]

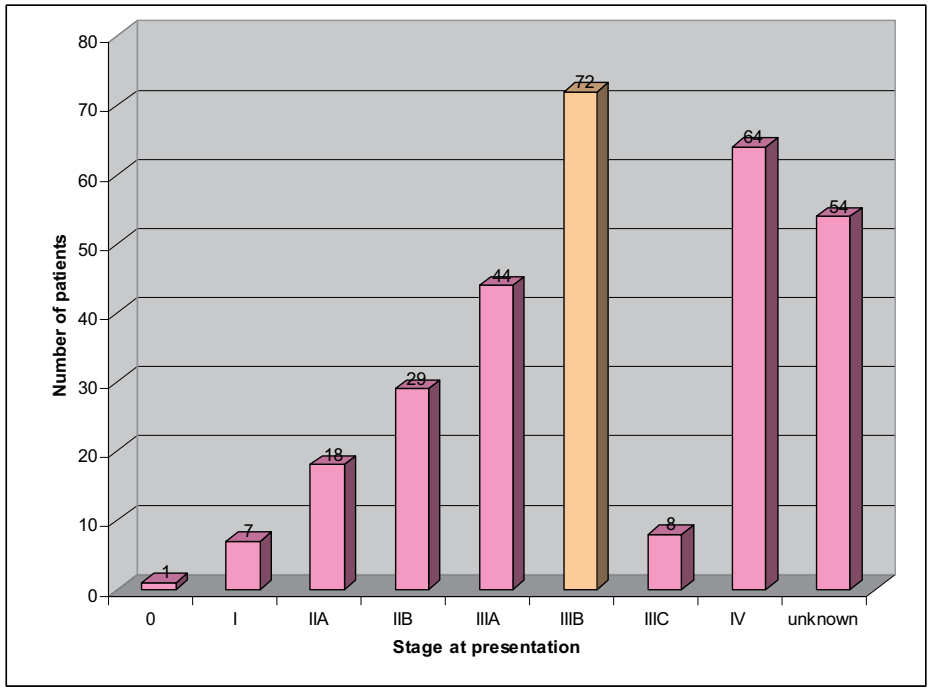

Figure 2: ***Stage at presentation of breast cancer patients presenting at the national referral hospital from 1996-2000. Adapted in part from Gakwaya et al. (2008)***[19]

TUMOR BIOLOGY

The complex processes that characterize the development and progression of malignant tumors, the *hallmarks of cancer*, have been well described. They include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis and tissue invasion and metastasis (Figure 3)[36] plus the ability of the cancer to escape the immune response through several complex processes and events.[37, 38]

Further, the identification of cancer stem cells in breast cancer has led to more elucidation about evolution and progression of breast cancer.[39] Also, research on morphologic and molecular features of hereditary breast cancer, especially in patients with germline mutations in *BRCA1*,[23] a candidate stem cell regulator,[40, 41] has increased our understanding of breast cancer biology. Gene expression profiling studies have extended our understanding of the molecular mechanisms involved in tumorigenesis and progression of breast cancer. Basic research on genes involved in signaling pathways modulating proliferation, apoptosis, survival, angiogenesis, invasion, metastasis and drug resistance have provided more answers to the heterogeneity of breast cancer. There is increasing evidence that this heterogeneity finds its source in genetic variability.[42-45]

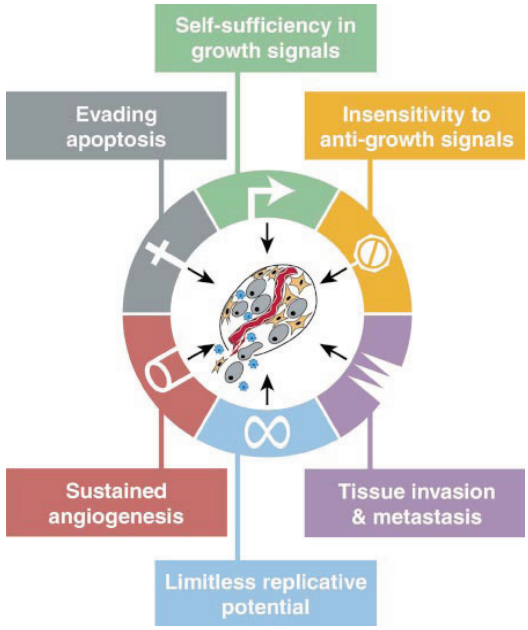

Figure 3: ***Hallmarks of cancer (Adapted from Hanahan and Weinberg, 2000).[36] Loss of normal growth control as a hallmark of cancer which encompasses four (self-sufficiency in growth signals, insensitive to antigrowth signals, sustained proliferation and evasion of apoptosis) of the six hallmarks of cancer as defined by Hanahan and Weinberg (2000)[36] involve control over the cell cycle.***

Cell cycle regulators and proliferation

The cell cycle is a highly organized and complex process comprised of a series of tightly controlled events that drive the replication of DNA and ensures correct cell division. Cells are normally in the resting phase G_0, and after appropriate stimuli they enter the proliferative phases of the cell cycle which is made of four phases; G_1, S, G_2 and M phase (Figure 4).

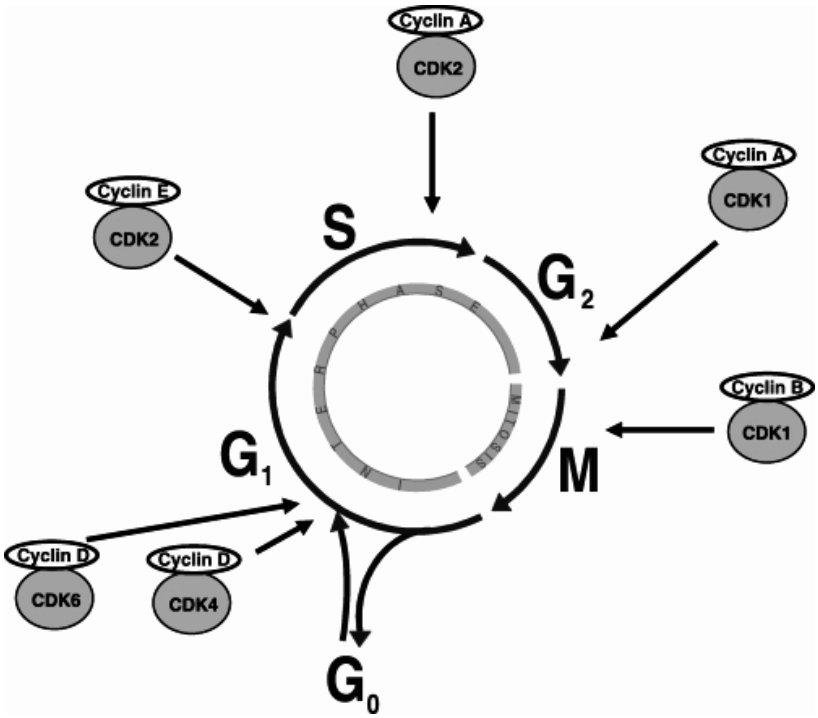

Figure 4: ***The stages of the cell cycle indicating site of activity of regulatory CDK/cyclin complexes.***[46]

In the G_1 phase, the cell is in a preparation for the S phase, in which DNA synthesis occurs followed by a second gap phase (G_2) in preparation for the phase M in which the cell undergoes mitosis to generate two diploid G_0 cells which may reenter the cell cycle or persist in the resting phase.[47, 48] Cells are stimulated to divide in response to numerous external signals, including growth factors, hormones and cellular adhesion.[47-49] During the G_1 phase of

the cell cycle, cells are responsive to the external stimuli and are dependent on them until they reach the restriction point (R). This is a point of no return beyond which the cell is committed to enter the cell cycle and thereafter the process becomes autonomous.[49]

The transition through the cell cycle phases is mediated by sequential assembly and activation of a family of serine/threonine proteins, the cyclin dependent kinases (CDK; CDK1, CDK2, CDK4, CDK6 and CDK7) and the CDK inhibitors (CKI; INK4 family: p15, p16, p18, p19; Cip/Kip family: p21, p27). The CKI are regulated by both internal and external signals such as the *TP53* tumor suppressor gene and Transforming Growth Factor β (TGF-β). The cell cycle has several check-points[48] (Figure 5) to ensure an orderly sequence of events in the cell cycle as well as complete and accurate replication of the cell before division.[48] Of these, the DNA damage check points (G_1/S and G_2/M) are well elucidated. Although it appears that oncogenic defects may target any major check-point, the most frequently

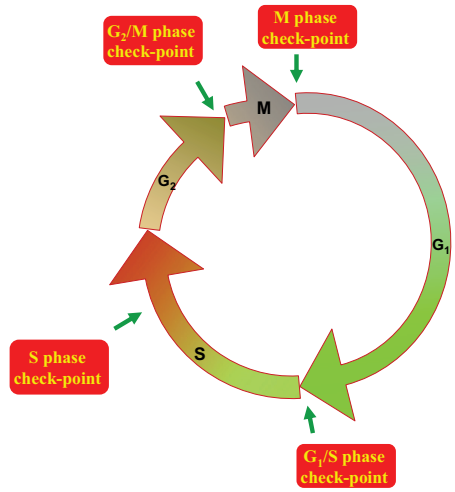

Figure 5: ***Check-points of the cell cycle. Adapted in part from Gillet et al. (1998).***[48]

involved is the G_1/S transition, and it encompasses many of the important cell cycle events that may be specifically altered in breast cancer including actions of the oncogenes (such as cyclin D1 and cyclin E) and tumor suppressors (such as p27).

Control of cell proliferation in the normal mammary gland is steroid hormone dependent, and it involves complex interactions with other hormones, growth factors and cytokines as well as three proto-oncogenes (c-myc, cyclin D1 and cyclin E1). Proliferation is essential for tissue turnover but it exposes the cell to the occurrence of DNA damage.[50] Cell proliferation plays an important role in the clinical behavior of breast carcinoma[51] and it is a significant prognostic factor in breast cancer.[52] Tissue homeostasis results from the balance between cell proliferation, differentiation and death in the form of apoptosis. An imbalance between cell proliferation and apoptosis contributes to tumorigenesis and tumor progression.

Genetic factors

Cancer is considered to be a genetic disease caused by genomic instability at the chromosomal or DNA level, and breast cancer has all the hallmarks of a multistep genetic disease. Studies have shown that the development of human breast cancer is based on the accumulation of various genetic alterations,[44] and almost every chromosome presents at least one site involved in cancer-related genetic alterations (chromosomal losses, DNA amplifications, mutations or altered DNA methylation patterns).[45] The multistep process in breast cancer is driven by both inherited and acquired genetic alterations which result in changed expression of mRNA and various proteins.[44] These abnormalities may be categorized into two; *loss-of-function* defects of tumor suppressor genes that have been inactivated by DNA mutation and unmasked by deletion or allelic loss, and *gain-of-function* genetic events that activate oncogenes.[44] Several genetic alterations have been identified;[36, 44, 53] somatic and

germline mutations have been described in tumor suppressor genes whereas oncogenes have been found to be activated.

Tumor suppressor genes

Several tumor suppressor genes have been implicated in breast carcinogenesis; mutations in genes such as *BRCA1*, *BRCA2*, *TP53*, *PTEN* or *ATM* or epigenetic functional inactivation of genes such as *SYK* and *NESI* play important early roles in formation of some breast cancers.[54] Of particular significance are the germline mutations in the *BRCA1/BRCA2* genes and somatic alterations in the *TP53* gene. Other genes of interest include the retinoblastoma gene (*pRb*), *p16*, *NM23* and *MASPIN*.[55]

BRCA1 and BRCA2 genes

Studies have indicated that *BRCA1* and *BRCA2* are tumor suppressor genes which are essential for cellular development and are involved in repair of double-stranded breaks (DSB) and the maintenance of genome integrity as well as cell cycle control.[56-59] *BRCA1* has also been suggested to represent a stem cell regulator.[40, 41] Mutations in these genes contribute to about 25-30% of hereditary breast cancer among young patients. In addition, it has been suggested that hypermethylation of *BRCA1* and *BRCA1* with inactivation may have a potential role in the carcinogenesis and aggressive phenotype of sporadic breast cancer.[60-64]

Further, breast carcinomas occurring in women with *BRCA1* mutations are more likely to occur at an earlier age and are frequently high grade, aneuploid, estrogen receptor (ER) and progesterone receptor (PR) negative, p53 positive, have abundant lymphocyte infiltration and pushing margins.[21, 22] They have also been shown to be associated with the basal-like phenotype,[65] and are enriched with CD44+/CD24− stem cells.[66] Decreased BRCA1 expression has been associated with acquisition of metastatic capacity, the solid-tubular

phenotype, poor tubular formation, high tumor grade and overexpression of HER2 in sporadic tumors.[61, 63, 64, 67]

TP53 gene

The *TP53* gene is the most frequently mutated gene in breast cancer and other human cancers.[68] About 25% of breast cancers have somatic *TP53* mutations,[2] and 30-50% of breast tumors have overexpression of p53 protein.[42] p53 is a nuclear transcription factor that is involved in control of gene transcription in the cell cycle (check-points) and promotes chromosomal stability maintaining the integrity of the genome. It regulates cell proliferation and apoptosis by preventing replication of damaged DNA and division of genetically altered cells.[69] p53 protein binds to damaged DNA and regulates transcription of a number of genes. Some of these genes, such as *GADD45*, *p21* and *MDM2*, are transcriptionally activated by p53 whereas genes such as *c-myc* and *c-fos* are repressed by p53.[70] The transcriptional activation of *p21* during the G_1 phase leads to cell cycle arrest and prevents cells with damaged DNA from entering the cell cycle phases of DNA synthesis and replication.[70] In addition, the *p53* gene transcriptionally activates *bax*, a pro-apoptotic gene and down regulates transcription of *bcl-2* which is a powerful antiapoptotic proto-oncogene.[70] Consequently, inducing apoptosis through the bcl-2/bax pathway in susceptible cells in which the damage is beyond repair thereby protects the tissue against transmission of DNA abnormalities.[50, 71-73]

Mutations in *p53* adversely affect its ability to bind regulatory DNA sequences of these genes and thus to inhibit their transcriptional regulation resulting into a cascade of downstream effects.[70, 73] Mutation of the *p53* gene increases the risk of developing breast cancer and affects the biology of cancer cells and their response to therapy.[70, 73] Mutations are more common in ductal carcinomas than in lobular carcinomas and are commonly associated

with *BRCA1* and *BRCA2* germline mutations.[55] Also, *TP53* mutations have been associated with more aggressive disease.[74, 75]

Oncogenes

Oncogenes refer to those genes whose alterations cause *gain-of-function* effects that lead to activation and can contribute to the development of cancer.[76] Activation of oncogenes can occur through various ways; gene amplification, point mutation and chromosomal translocation.[76] Oncogenes may also act cooperatively with other genetic or epigenetic changes.[76] Numerous oncogenes have been characterized in human cancers but only few oncogenes are crucial in the development of breast cancer.[44, 76] Amplification and overexpression of these oncogene and oncogene products are the major mechanisms through which they contribute to carcinogenesis.[76]

In breast cancer, oncogene amplification is a common mechanism,[44] and is an important mechanism for oncogene overexpression.[77] The *HER2*, *EGFR*, *c-myc, CCNDI, FGFR1, ESR1* and *MDM2* are among the frequently amplified oncogenes. Coamplifications (*HER2/c-myc* or *CCND1/FGFR1*) have also been reported.[45, 77]

HER2/neu gene

The *HER2/neu* proto-oncogene is amplified in 15-30% of breast cancer.[78] HER2 (also known as *neu*, c-*erb*B-2 or human epidermal growth factor 2) is a transmembrane protein with tyrosine kinase activity. *HER2* has been implicated in breast carcinogenesis and plays an important role in development and progression of cancer.[78] HER2 overexpression has been reported in 10-44% of human breast cancers.[79-81] Overexpression in breast carcinoma occurs through either amplification of the gene or mRNA overexpression. This results in increased

cell proliferation, inhibition of apoptosis, and angiogenesis leading to poor prognosis in breast cancer.[78, 82-85]

EGFR gene

EGFR is another member of the tyrosine kinase family of receptors which are transmembrane proteins regulating major cellular events such as cell proliferation, differentiation, apoptosis, adhesion and cell migration.[86-89] Several studies have established that *EGFR* gene acts as a cellular oncogene. *EGFR* gene amplification has been identified in 0.8-14% of breast cancers.[77, 90, 91] Epidermal growth factor influences the proliferation and differentiation of a wide variety of cancer cells, and plays a role in the pathogenesis of breast cancer.[92, 93] In addition, it influences cell proliferation and a number of other processes in tumor progression such as cell survival, cell adhesion, cell motility, angiogenesis and tissue invasion.[92] EGFR expression has been reported in about 45% (range 14-91%) of all breast cancers.[94-96]

Amplification of *EGFR* in breast cancer indicates a more aggressive tumor behavior and a poor patient outcome.[91, 97-100] Similarly, EGFR expression in breast cancers has been associated with features of poor prognosis including high tumor grade, elevated growth fraction, ER negativity and poor response to endocrine therapy and reduced survival.[96, 101-104]

C-myc gene

C-myc amplification is relatively common (8-37%) in breast cancer and may provide independent prognostic information.[105] It encodes for a helix-loop/leucine zipper protein and myc responsive genes include those whose protein products regulate cell proliferation and apoptosis.[105] The *HER2/c-myc* coamplified tumors have worse prognosis than tumors with only one of these amplified.[45, 77]

CCNDI gene

The *CCNDI* gene located on chromosome 11q3 and coding for the G_1-cyclin protein (cyclin D1) involved in regulation of the cell cycle[47] has been found amplified in 10-27% of breast cancers.[77] *CCNDI* amplifications are associated with ER and PR positivity, but studies on prognostic significance are still controversial.

MDM2 gene

The *MDM2* gene protein product down regulates the *TP53* tumor suppressor gene and is amplified in 4-7.7% of breast cancers and has been associated with poor prognosis in some studies.[45, 77]

DNA ploidy

DNA aneuploidy is a manifestation of chromosomal instability which is recognized as an early feature of malignant transformation and found to be an indicator of prognosis in breast cancer.[106] The mechanisms responsible for the frequent instability of genomes of breast cancer cells have been poorly understood although recent functional findings on oncogene and tumor suppressor genes have provided more information about this matter.[107] Studies have suggested that the DNA content of breast cancer cells reflects biologic properties associated with malignant behavior of the tumors.[106, 108, 109]

Steroid receptors

Ovarian steroid hormones estrogen and progesterone are necessary for normal mammary development and growth. The estrogen (ER) and progesterone receptors (PR) belong to the steroid hormone receptor family of inducible transcription factors that play a role in the development and progression of breast cancer.[110-112] Studies have shown that

estrogen directly increases the growth of breast cancer cells in culture by increasing the number of cells entering the cell cycle (Figure 4). ER directly regulates several key G_1 phase cell-cycle regulators (such as cyclin D1, Myc, cyclin E-CDK2 complex, CDK4 and CDK inhibitors) and those required for S phase entry.[113-115] In addition, studies have suggested that PR induce cell cycle progression via activation of mitogen activated protein kinases in breast cancer cell lines.[116] Steroid hormone receptors are directly involved in the development, progression and therapeutic responsiveness of breast cancer.

ER is expressed in about 50-95% of breast carcinomas, while PR is expressed in 60% to 70% of the cases.[117, 118] However, previous independent studies have shown a low prevalence of ER (23-33%) in women of African and African-American populations,[32, 119-122] although some studies reported higher frequencies (64-65%).[123, 124] ER/PR positive tumors are more common in postmenopausal women and are more likely to be diploid, well differentiated, to have lower proliferative rates, and to be less aggressive than the receptor negative tumors.[42]

Furthermore, estrogens in mammary epithelial cells and ER positive breast cancer cell lines have been shown to regulate,[125] the expression of *bcl-2,* a powerful antiapoptotic proto-oncogene.

Apoptosis

Apoptosis is a highly complex and tightly regulated process of cell death which deprives the proliferating cellular pool and allows the elimination of genetically damaged cells after their division. It is also a cellular protective mechanism against malignant transformation. Apoptosis regulation is ensured by various genes often associated with breast carcinogenesis, mostly pro-apoptotic (*c-myc*, *p53* and *ras*) and rarely antiapoptotic (*bcl-2)*. The *bcl-2* gene was the first antiapoptotic gene to be described and is able to antagonize

apoptosis induced by several stimuli. Bcl-2 is one of the important regulators of apoptosis,[126] and it delays the induction of apoptosis in mammary glands.[126, 127] The expression of the *bcl-2* gene is regulated by estrogens[125] as well as down regulated by p53 in breast cancer cell lines.[128] Several independent studies have shown that Bcl-2 overexpression in breast cancer correlates with biologic features of a differentiated phenotype (low proliferative rate, high levels of steroid receptor, weak or absent p53 expression and absence of HER2 expression).[129]

The ability of tumor cells to evade apoptosis, as a *hallmark of cancer*,[36] leads to continued proliferation of tumor cells and ultimate tumor expansion. Thus, dysregulation of apoptosis plays an important role in the pathogenesis and progression of breast cancer. The development and continued growth of cancers involve an interaction between cell proliferation and apoptosis.[130-133] It has been shown that apoptosis is increased in invasive breast cancer[134] and is positively correlated with Ki-67 expression.[135] Breast tumors with increased apoptosis are more likely to be of high histologic grade and to be ER negative. Further, studies have shown that the rate of tumor growth depends in part on the excess of proliferation over apoptosis,[130-133] and partly on angiogenesis.[136]

Angiogenesis

In 1971, Judah Folkman suggested that the growth and spread of malignant tumors were dependent on the process of angiogenesis, and that tumors could be treated by attacking their blood supply.[136-139] Tumor-associated angiogenesis is now considered one of the key elements which contribute to tumorigenesis.[140-143] Sustained angiogenesis, another *hallmark of cancer*,[36] is a tumor micro-environmental process that is necessary for tumor cell survival, tumor growth, invasiveness, progression and development of metastasis, and beyond a critical volume a tumor can not expand further in absence of neovascularization.[139, 142, 144-146] Angiogenesis is a complex multi-step process, consisting of coordinated, sequential and

interdependent steps leading to formation of new blood vessels from pre-existing vascular networks.[146, 147] It is a highly restricted process in normal human adult tissues, and in order to initiate it, a tumor must switch to the angiogenic phenotype. This occurs early in tumor development and limits or determines the rate of tumor progression.[139, 141, 142, 146-148] The *angiogenic switch* is induced by the secretion of specific endothelial cell growth factors like VEGF (vascular endothelial growth factor) produced by the tumor cells plus other non-malignant host cells recruited by the tumor.[142, 146]

Vascular Endothelial Growth Factor

The *angiogenic switch* of a tumor is related to a balance between positive and negative regulators (Figure 6). Several pro-angiogenic factors have been identified, and the vascular endothelial growth factor (VEGF) family plays a key role in this process as the major mediator of breast cancer angiogenesis.[139, 141, 146, 149] VEGF is the most active, specific and potent mitogen for vascular endothelium among the endothelial cell growth factors,[146, 150, 151] and is a potent inducer of angiogenesis.[139, 141, 149, 150, 152] It is secreted in response to environmental stimuli like hypoxia which is the main stimuli, certain cytokines and estradiol.[146, 149] It plays crucial roles in cancer biology including endothelial cell proliferation and migration, promotion of tumor angiogenesis and metastasis.[152, 153] Studies in breast cell lines showed that down regulation of the *VEGF* gene expression inhibited breast cancer cell-induced angiogenesis and suppressed breast tumor metastasis in mice.[154]

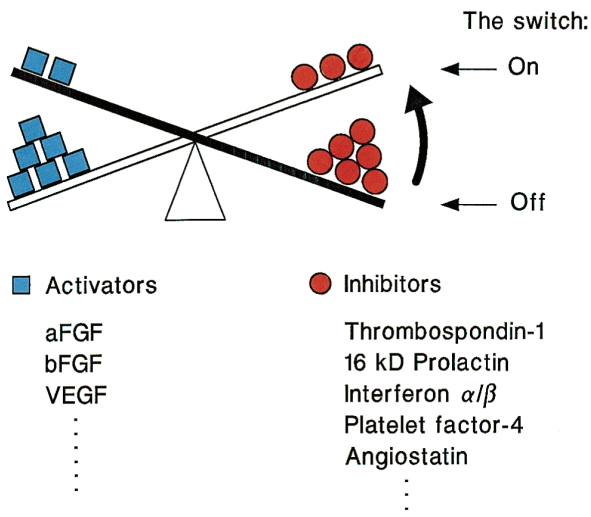

Figure 6: ***The levels of the angiogenic inhibitors and activators factors control the angiogenic switch as well as the angiogenic activity of a tumors including breast cancer. Adapted from Hanahan (1996).***[141]

Furthermore, *in vitro* studies have shown that angiogenesis is also related to other molecular mechanisms involved in tumor growth and metastasis. Certain oncogenes such as *HER2* signaling pathways promote angiogenesis by up-regulating cancer-cell production of angiogenic factor like VEGF.[155, 156] In contrast, the p53 transcription factor has been reported to have a role in suppressing angiogenesis through enhancing the expression of Thrombospondin-1, an angiogenic inhibitor,[157] as well as down regulating VEGF expression[158] (Figure 6). p53 contributes to the angiogenic switch during tumorigenesis. It inhibits Hypoxia-inducible factor-1 (HIF-1) activity by targeting the HIF-1α for ubiquitination and proteasomal degradation.[159] Thus, loss of p53 function leads to an amplification of normal HIF-1-dependent response to hypoxia,[159] which is a key signal for induction of angiogenesis.[160] Indeed, hypoxia is one of the most potent inducers of VEGF mRNA synthesis, a function achieved through inducing HIF-1α.

Studies have shown that tumor growth, invasion and metastasis of breast carcinoma depend partly on angiogenesis.[136, 154] Thus increased tumor angiogenesis has been associated with increased incidence of metastasis.[161, 162]

Invasion and metastasis

Most deaths from cancer result from progressive growth of metastases that are resistant to conventional therapies, and in a significant number of patients metastases occur before diagnosis of the primary tumor.[154] Tissue invasion and metastasis are exceedingly complex processes whose mechanisms are closely related but are poorly understood and are some of the acquired capabilities of cancer.[36] The existence of an invading cancer does not necessarily imply metastasis, but invasive growth is a prerequisite for metastasis. Cancer cell invasion involves the breaching of tissue barriers by the cancer cells, and subsequent infiltration of these cells throughout the surrounding tissue.[163] Several gene families are involved in this process. Acquired genetic alterations conferring growth advantage to the cells in addition to loss of cell-cell adhesion or cell-matrix adhesion and matrix remodeling all interplay to confer a migratory plasticity to the cancer cells.[163, 164] Studies have indicated that the motility machinery of the cells is extremely important; and acquisition of a motile phenotype is essential for the tumor cells to become invasive.[164, 165] Tumor cell motility is the hallmark of invasion and an essential step in metastasis, and evidence shows that tumor microenvironment might initiate the expression of genes that induce cell motility, invasion and metastasis.[165-168]

Single epithelial cells can migrate through two predominant mechanisms.[169] The mesenchymal migration which requires an epithelial-mesenchymal transition (EMT) is the predominant mechanism and requires matrix degrading enzymes. The second type, the amoeboid migration, enables cells to squeeze their way through the matrix without need for

the proteases and requires a mesenchymal-amoeboid transition (MAT). [164, 169, 170] This migratory method has implications for the treatment of breast cancer since it is used as a compensatory mechanism when the predominant one has been blocked.[164, 170] Further, factors from the tumor microenvironment such as cytokines, growth factors, proteases and angiogenic factors secreted from multiple cell types plays a major role in determining the potential invasion and later metastasis in cancer.[164]

Cell adhesion molecules play major roles in the invasion-metastasis cascade. Whereas activation of integrin αvβ3 initiates calcium-dependent signaling pathway leading to increased cell motility and proteolysis,[171] loss of E-cadherin expression facilitates tumor cell detachment enabling invasion and metastases.[172] During tumor progression *E-cadherin* can be functionally inactivated or silenced by different mechanisms and loss of E-cadherin expression and/transcriptional repression of its mRNA are hallmarks of epithelial-mesenchymal transition (EMT).[173]

Epithelial-mesenchymal transition (EMT)

By this process, polarized epithelial cells are converted into motile mesenchymal cells. The initial step of metastasis is epithelial-mesenchymal transition (EMT) which involves disruption of the adhesive interactions with surrounding cells and the acquisition of a motile phenotype. EMT is characterized by loss of polarity and down regulation of epithelial proteins, mostly E-cadherin, but also occludin, claudins, cytokeratins or catenin proteins in addition to inducing mesenchymal proteins like N-cadherin, vimentin and others.[174] Multiple signaling pathways and effectors induce or contribute to the EMT and the key players include Receptor Tyrosine Kinases, the Transforming Growth Factor β superfamily, NF-κB, WNT signaling, Notch signaling and Hedgehog signaling.[173, 174] In addition, the EMT transcriptome program is controlled by several transcription factors outlined in Table 1.

Transcription factor
Snail family
SNAI1 (Snail)
SNAI2 (Slug)
ZEB family
SIP1/ZEB-2
δEF-1/ZEB-1
TWIST1
TWIST2
E12/E47 (*E2A* gene product)
FOXC2
Goosecoid

Table 1: ***Transcription factors involved in the epithelial-mesenchymal transition.***[173-175]

The current model proposes that EMT is a two way process and EMT occurs at the invasion front of tumors whereas mesenchymal-epithelial transition (MET) occur at the secondary site (Figure 7).

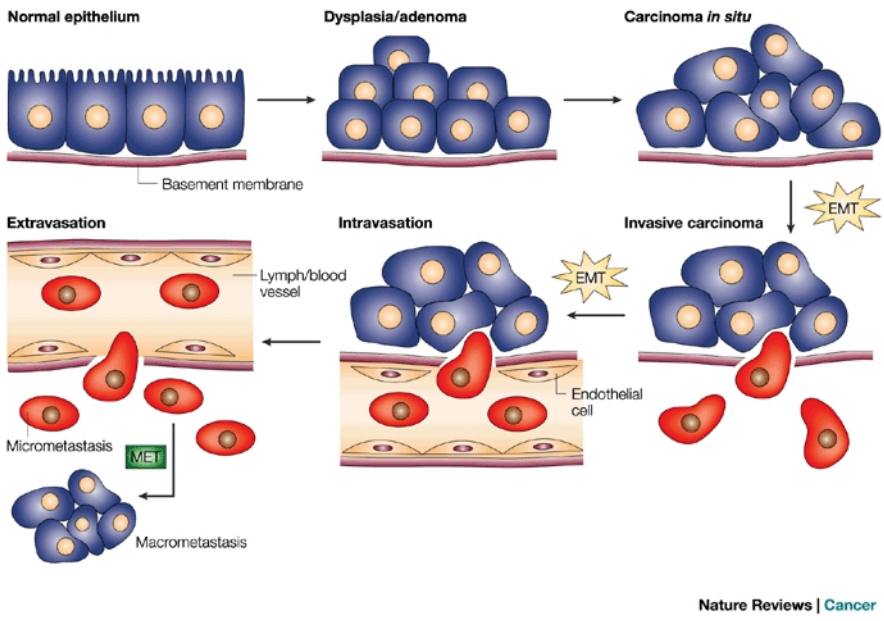

Figure 7: ***A reversible EMT model in tumor metastasis, with deregulation of cell proliferation and eventual acquisition of a motile phenotype; tumor cells breach the***

basement membrane and enter the blood or lymphatic vessels. At the distant organ, the cancer cells exit the vessels and undergo a reverse mesenchymal-epithelial transition (MET) and regain their ability to proliferate. Adapted from Thiery et al.(2002)[176]

EMT can promote metastasis in several ways and some of the EMT mediators also inhibit apoptosis (snail and twist families) which promotes tumor growth and expansion and mediate tumor immunosuppression (snail) potentially facilitating metastasis.[174]

Metastasis is a complex process including primary tumor growth, local invasion through basement membrane and extracellular matrix, angiogenesis and lymphangiogenesis, dissemination to lymphatic and/or blood circulation, transport to distant organs and colonization at the secondary site.[154] Recent evidence indicates that metastatic capacity is an early and inherent feature of breast tumors and not a late event. In breast cancer, metastases occur most commonly in the bone, lung and liver (Figure 8). Other relatively frequent sites include adrenal glands, pleura, gastrointestinal tract, brain and the peritoneum.[177] Studies have shown that gene expression signatures can predict the likelihood[178] of distant metastases with 90% accuracy as well as the site[179] of breast cancer metastases.

In addition, gene expression studies[178] identified a poor-prognosis signature which included genes involved in the cell cycle, signal transduction, angiogenesis, invasion and metastasis. These also included genes almost exclusively expressed by stromal cells such as *MMP1* and *MMP9* which are required for extracellular matrix (ECM) degradation and tumor invasion.[180, 181]

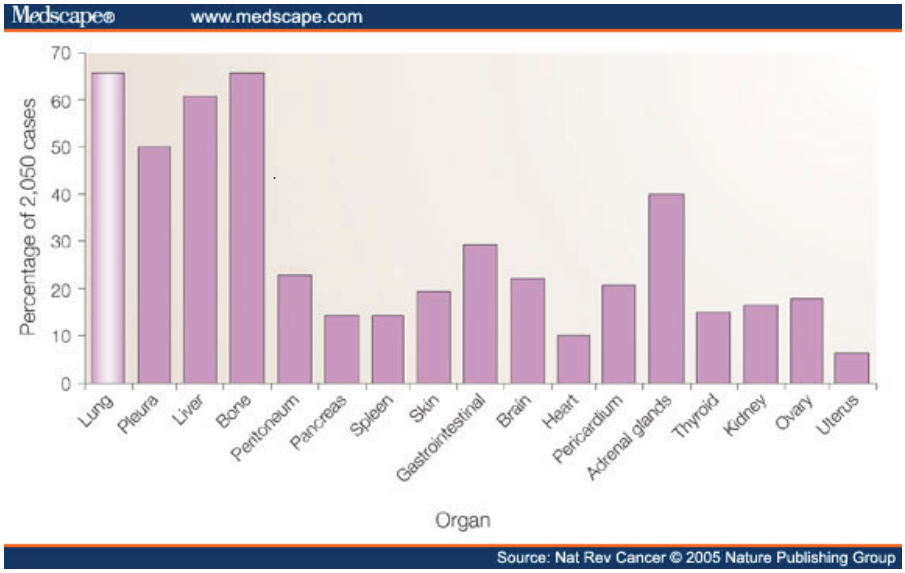

Figure 8: ***Common metastasis sites of breast cancer as seen at autopsy. Adapted from Weigelt, (2005).***[182]

The integrated model of breast cancer metastasis

Further, gene expression profiles have demonstrated that the tumor microenvironment plays a significant role in tumorigenesis.[166] Current evidence shows that tumor microenvironment initiates the expression of genes that induce cell motility, invasion and metastasis. Many of the EMT-inducing pathways play prominent roles in development and stem cell self-renewal.[174] There is rapidly accumulating evidence which suggest that a link exists between stem cells and EMT.[174] Mani et al. (2008) demonstrated that EMT induced by twist or snail endows breast epithelial cells with stem cell-like properties.[183] Conversely, normal and neoplastic stem cells isolated from breast tissues show several features of EMT, and several signaling pathways that mediate stem cell self-renewal also induce EMT.[174] Further, it has been proposed that the biological and molecular heterogeneity[184] as well as the risk of distant metastasis[185, 186] corresponds with the amount of breast cancer stem cells (see

next section) in the tumor. Consequently, a new integrated model of breast cancer metastasis which is illustrated in Figure 9 has been proposed by Weigelt et al. (2005).[182]

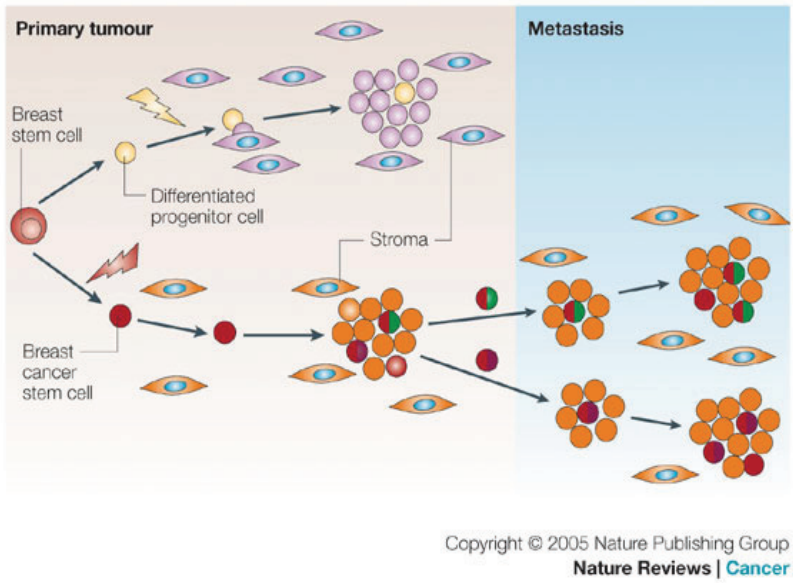

Figure 9: ***The integrated model of breast cancer metastasis, adapted from Weigelt et al. (2005).[182] Oncogenic mutations occurring in the breast stem cells (red) and the differentiated progenitor cells (yellow) generate metastatic 'poor prognosis' (orange) and non-metastatic 'good prognosis' breast cancers (pink), respectively. In the metastatic tumors, under the influence of stromal fibroblasts, a small population of breast cancer stem cells has the ability to metastasize. There might be variants of cancer stem cells that differ in their tissue selectivity for metastasis, expressing additional tissue-specific profile (such as green; bone, purple; lung).***

Interestingly, studies in brain tumor cell lines have shown that cancer stem cells (CSC) contributes to the angiogenic drive in tumors by generating VEGF and other factors to induce angiogenesis.[187, 188] The CSC-mediated VEGF production led to amplified endothelial cell migration and tube formation *in vitro* suggesting that cancer stem cells may be a crucial source of key proangiogenic factors in cancers.[189] At the same time, tumor vasculature aids in maintaining CSC self-renewal and maintenance. Cancer stem cells depend on CSC

maintenance signals created by the vasculature similar to what has been observed in normal stem cells (Figure 10).[187, 188]

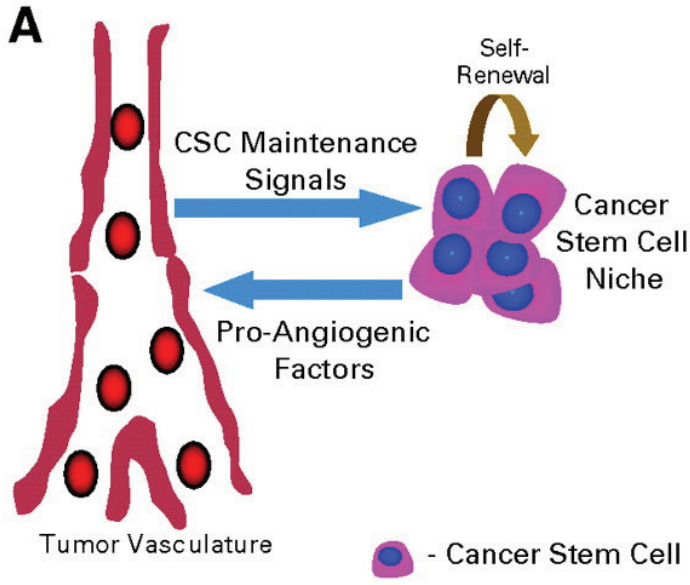

Figure 10: ***CSC generate pro-angiogenic factors to stimulate angiogenesis while the tumor vasculature aids in maintaining CSC self renewal and maintenance. Adapted in part from Eyler and Rich (2008).***[188]

Cancer stem cells

The term 'cancer stem cell' is an operational term defined as a cancer cell that has the ability to self-renew giving rise to another cancer stem cell as well as undergo differentiation to give rise to phenotypically diverse mixed populations of non-tumorigenic tumor cell populations in the tumor.[190-192] The cancer stem cell hypothesis has fundamental implications for cancer biology and clinical management of patients.[193, 194] It implies that breast cancers arise in mammary stem or progenitor cells through dysregulation of the normally tightly regulated process of self renewal.[195] These "cancer stem cells" are thought to drive the growth and spread of tumors.[190, 191] Therefore, failure to target them would set the stage for

recurrences and treatment failures.[190, 191, 194] Studies in mouse models and established breast cancers have suggested that breast cancer behavior may be programmed in the precancer stem cells,[196] and the amount of cancer stem cells within breast tumors may correspond to the biologic and molecular heterogeneity of the tumors[184] as well as to risk of distant metastases.[185, 186] Pece et al. (2010),[184] characterized the transcription signature of human normal mammary stem cells (hNMSC signature) and by using markers of this signature isolated stem cells from both the normal gland and breast tumors. In xenografts, the hNMSC signature was able to predict the biologic and molecular features of breast cancers.[184] The ability to identify these tumorigenic cancer cells has facilitated the elucidation of pathways that regulate their growth and survival,[190, 191] and might lead to development of novel CSC-targeted therapies[197-199] which will eliminate breast CSCs.[198, 199]

Importantly, exploration of cellular and molecular mechanism involved in the relationship between CSC and tumor angiogenesis that has been established in brain tumors[187, 189] will provide opportunities for the development of novel CSC-targeted antiangiogenic therapies with advantage over currently available therapies.[188, 197]

Stem cell markers

The existence of stem cells in rodent mammary glands was first demonstrated by Kordon and Smith (1998).[195, 200] Consequently, human mammary stem cells have been identified and purified based on their surface antigen expression.[191, 201] Human breast cancers are reported to contain a subpopulation of cancer cells similar to epithelial stem cells, the "cancer stem cells".[185, 190, 192] Studies have shown that human breast cancers and cell lines contain a subpopulation of cells characterized by CD44+/CD24$^{-/low}$/Lin- cell surface markers, and these cells have stem cell properties.[190, 202] Breast cancer stem cells which expressed a combined CD44+/CD24$^{-/low}$/ALDH1+ phenotype showed an especially high tumorigenic

capacity.[194] Also, in a recent study of 33 breast cell lines derived from human breast cancers and normal breast tissue, the results indicated that 23 of the cell lines contained functional cancer stem cells with metastatic capacity.[203] In addition to increased aldehyde dehydrogenase 1 (ALDH1) expression, BMI-1 expression has been reported as stem cell marker.[194, 204, 205]

BMI-1 expression

BMI-1 expression, a putative stem cell marker,[204] is one of the several polycomb genes (PcG) which have been identified as oncogenes.[206, 207] It was first identified as an oncogene that co-operates with *c-myc* in the generation of mouse pre B-cell lymphomas.[208] It is a transcriptional repressor which acts as a key regulator of self-renewal activity in both normal and tumorigenic human mammary stem cells.[209, 210] The PcG play a role in maintenance of cellular identity and contribute to regulation of the cell cycle by preserving gene silencing after cell division. Thus, dysregulation of this gene silencing machinery can lead to cancer,[211-213] and BMI-1 has been implicated in breast cancer carcinogenesis, tumor progression and metastasis.[206, 207, 214]

Aldehyde dehydrogenase 1

ALDH1 is another stem cell marker which is considered to be an indicator of both normal and malignant stem and progenitor cells in the breast.[194, 205] ALDH is a family of cytosolic isoenzymes responsible for oxidizing intracellular aldehydes, leading to oxidation of retinol to retinoic acid in early stem cell differentiation, which is important for proliferation, differentiation and survival.[215-217] ALDH1 (also known as ALDH1A1) is the predominant ALDH isoform in mammals,[217-220] and it is highly expressed in the hematopoietic progenitors and in intestinal crypt cells as well as in breast tumor cells.[205, 221, 222] In breast cancer, ALDH1 expression has been associated with poor clinical outcome, resistance to chemotherapy and

the basal-like phenotype of breast cancer.[193, 194, 205] Also, in a recent study, in both *in vitro* and xenografts, the results showed that invasion and metastasis in inflammatory breast cancer are mediated by a CSC component that displays ALDH enzymatic activity,[223] and ALDEFLUOR-positive cells were found to be responsible for mediating metastasis in a study involving 33 cell lines derived from breast tissues.[203]

MOLECULAR PHENOTYPES OF BREAST CANCER

Gene clustering analyses have indicated that breast cancer can be divided into two broad categories; ER+ and ER− groups which can further be subdivided into additional biologically different and clinically significant subgroups. Thus, five different sets of intrinsic gene clusters were recognized (luminal A, luminal B, the HER2+ subtype, the basal-like and the normal breast-like category) with different prognosis in multiple independent studies.[224-228] Although gene expression profiling is the gold standard for molecular classification of breast cancer, its large scale clinical use or use in retrospective studies is limited by the strict tissue requirements (fresh and frozen tissue) and by issues of cost, complexity and technical feasibility.[229, 230] Consequently, in an attempt to develop a molecular classification that is clinically significant, technically simple, reproducible, and readily available, investigators have proposed an immunohistochemical-based classification.[229] These biomarkers can define the molecular subgroups in the routine and readily available formalin-fixed, paraffin embedded tissues by way of immunohistochemical staining. Although some of the proposed IHC markers have been validated using a 930-case tissue microarray,[231, 232] there is, however, still no consensus on these definitions,[233] and overlapping categories exist. By using the immunohistochemical classification, four similar major subgroups have emerged as well as the unclassified tumors (Figure 11) which encompass the normal breast-like class of breast cancer that is still poorly characterized immunohistochemically.[233] Of these, the basal-like

breast cancer (also known as basal-like phenotype or basal-like subtype) and the HER2+ subtype are of particular interest since they have a poor prognosis.[226]

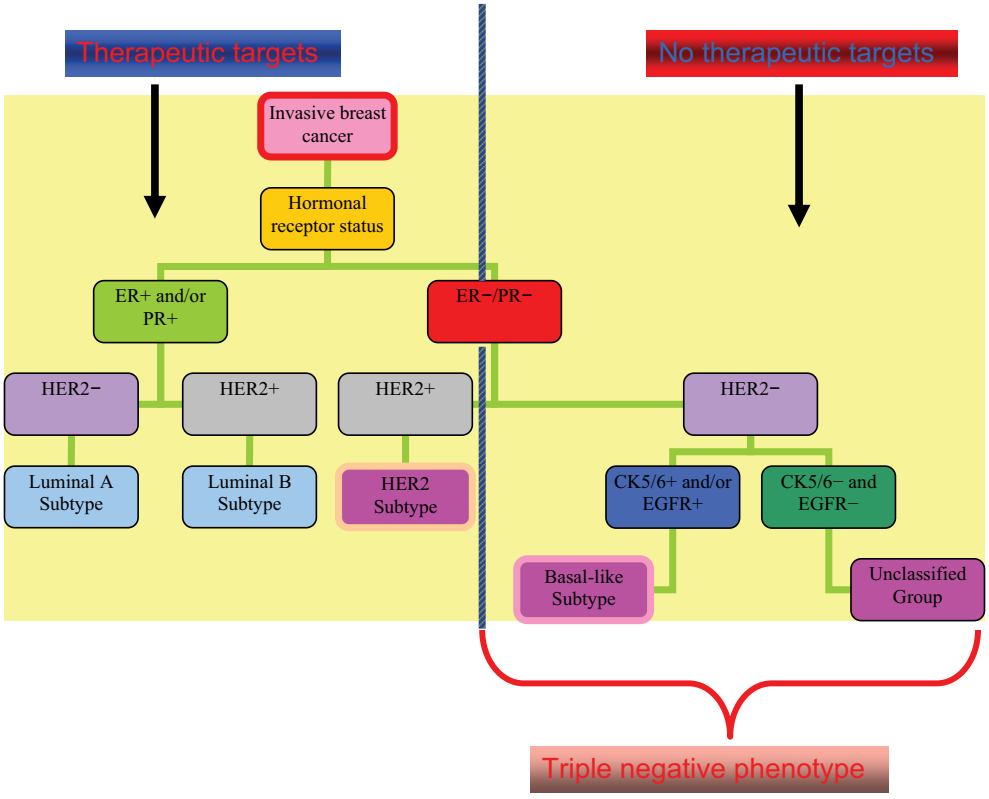

Figure 11: ***The immunohistochemical subclassification of breast cancer (simplified)***

Basal-like subtype

There is no consensus on how to define this subgroup. Basically, these tumors might be defined on the basis of expression of various basal markers. Alternatively, negativity for ER, HER2 and eventually PR might be added to obtain more composite basal-like profiles. According to the latter, basal-like breast carcinomas usually lack ER and HER2 and express genes characteristic of basal or myoepithelial cells such as basal cytokeratins (CK5, CK14, CK17) and other genes characteristic of basal-like cells of the breast.[233-237] In addition to

structural roles, many of the basal-like gene products have been implicated in cellular proliferation, suppression of apoptosis, cell migration and invasion, all *hallmarks of cancer*.[36, 228, 233] Indeed, gene expression studies have further shown that a majority of basal-like tumors express the activated wound-response signature,[238] which represents important processes likely to contribute to cancer invasion and metastasis such as matrix remodeling, cell motility and angiogenesis.

Further, it has been suggested that different subtypes of breast cancer might originate from breast stem or progenitor cells at distinct stages of lineage differentiation, with basal-like tumors arising from the most-primitive ER-negative stem cells.[239-241] Gene expression profile studies of basal-like tumors have suggested a less differentiated breast stem cell or progenitor cell of origin for these tumors[241] and several gene products in the basal cluster are also expressed in stem cells of various tissue types.[241, 242] Given the central role of *BRCA1* in normal mammary development,[243] Foulkes (2004)[40] proposed that *BRCA1* regulates differentiation of breast stem cells, and defects in the *BRCA1* pathway might arrest further differentiation of these cells leading to cancer. Subsequent studies have provided some evidence that basal-like breast cancers originate in stem cells with maturation defects and genomic instability caused by *BRCA1* mutations.[41, 194]

The basal-like breast carcinomas contribute about 8-25% of all breast cancers as defined using gene expression or IHC surrogate criteria.[233, 241, 244] They express basal markers such as basal cytokeratins in addition to other makers like EGFR, P-cadherin, p63 and c-kit.[231, 232, 236, 245-247] However, unexpectedly, basal-like tumors might also co-express luminal cytokeratins CK8 and CK18.[241] The basal-like subgroup partially overlaps with the so called triple negative tumors defined as being ER−/PR−/HER2−, as well as the *BRCA1* associated breast cancers (Figure 12).[233, 248] A majority (82%) of basal-like breast tumor were found to contain *p53* mutations.[226]

Clinically, the basal-like tumors have been associated with younger age (< 40 years) and are more likely premenopausal African-American women in some studies.[231, 249] Studies have shown than the basal-like subtype seems to differ by race and age, whereas other major subtypes do not seem to show a clear difference.[229, 231, 249] Also, previous reports have indicated that the hormonal receptor negative tumors as well as the basal-like subtype are overrepresented in women from African population.[120]

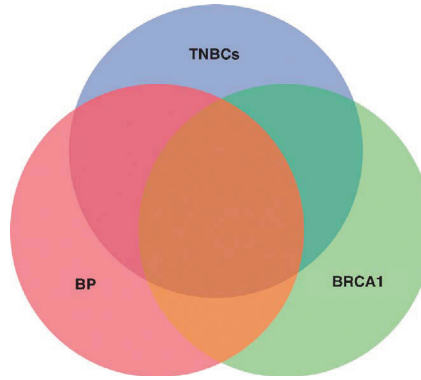

Figure 12: ***The interrelationships of the basal like (BP), the triple negative (TNBCs) and the BRCA1 associated breast carcinomas. Adapted from Diaz et al. (2007)*** [248]

Morphologically, a majority of basal-like breast cancers are usually of high histologic grade and invasive ductal carcinomas. The basal-like tumors are seen as sheets of cells with minimal tubule formation which are more likely to have a pushing non-infiltrative tumor border, higher degree of stromal lymphoplasmacytic infiltration and larger zones of geographic necrosis than the non basal-like tumors. These characteristics represent medullary features.[250] Morphologic characteristics of basal-like breast cancers that have been confirmed in a number of independent studies, although not in all, are listed in Table 2, but however they may also be found in other grade 3 non basal-like tumors. The high proliferative rate of basal-like tumors which has been reported in some reports[248, 251] may explain their

overrepresentation among so called interval breast cancers.[252] Further, Foulkes et al. (2004)[253] observed that glomeruloid microvascular proliferation (GMP), a histologic marker of an aggressive angiogenic phenotype in human cancer,[254] was significantly more frequent in the basal-like subgroup of breast cancer.

Table 2: ***Morphologic characteristics of basal like breast cancer***[233, 248, 250]

Characteristic
Pushing invasion border
Central scar or sclerosis
Geographic tumor necrosis
Marked cellular pleomorphism
High nuclear grade
High mitotic count (average 45 mitoses per 10 high power fields)
High nuclear-cytoplasmic ratio
Vesicular chromatin
Prominent nucleoli
Lack of tubule formation
Frequent apoptotic cells
Spindled tumor cells
Metaplastic features such as squamous cell metaplasia
Scant stromal content
Exaggerated stromal lymphoplasmacytic response

Interestingly, the basal-like subgroup is reported to have a specific pattern of metastatic spread with reduced lymphatic metastases and increased hematogenic spread to sites associated with poor prognosis.[250, 255] They show relatively increased propensity for the

lungs and brain metastases whereas they have a decreased propensity for bone and liver metastases.[250] Thus it has been proposed that basal-like breast cancer posses a distinct mechanism of metastatic spread.[230]

Regarding the therapeutic implications of the molecular subtypes, a recent report has suggested that some chemotherapeutic agents might have different mechanisms of action in different subtype of breast cancer.[256] The basal-like breast cancers are resistant to currently available therapeutic targets for breast cancer, although they may be responsive to EGFR targeted therapy.[229, 257, 258] Also, studies have shown that ER negative tumors benefit twice as much from chemotherapy than the ER positive tumors. A number of studies,[259-261] although not all,[262] have indicated that basal-like tumors have a higher response rate to chemotherapy both as adjuvant and neoadjuvant regimens compared to the luminal subtype.

The current challenge is to identify novel target molecules and pathways for the basal-like subtype which is frequently triple negative. Some possible targets that have been proposed include EGFR and VEGF.[258, 263] C-kit which is expressed in a high proportion of basal-like tumors might also be a suitable target.[264] However, c-kit positive breast tumors have been shown to lack activating c-kit mutations which conveys sensitivity to imatinib, a c-kit inhibitor.[229, 233] The biologic similarities between *BRCA1* associated and basal-like tumors have suggested that strategies like PARP inhibitors targeting DNA-repair defects of the BRCA1 pathway dysfunction in basal-like tumors might be effective.[265, 266]

Other subtypes

In general, the HER2+ subtype has been defined as ER−/PR− and HER2 positive tumors.[229, 231, 233] The HER2+ tumors express high levels of genes located in the HER2 amplicon including HER2 and the GATA4 transcription factor. They lack expression of ER and GATA3. Current literature shows that it contributes about 8-12% of the breast cancers.[124,

[231, 244, 267] This is an aggressive subtype which has been associated with high histologic grade and reduced survival.[78] Fortunately, the clinical outcome of patients with HER2 positive tumors has been greatly improved by development of HER2 targeted therapy like trastuzumab which is now routine treatment for breast cancer. Thus, HER2 expression is a predictive factor currently in use.[268]

Luminal tumors are ER positive tumors that express ER responsive genes and other genes that encode characteristic proteins of luminal epithelial cells such as PR, GATA3, BCL-2 and the luminal cytokeratins 8 and 18.[241] They contribute about 50-70% of breast cancers.[229] Luminal tumors are usually associated with increasing age, low histologic grade, they are less aggressive and have a good prognosis and will respond to hormonal therapy. The luminal A subtype is most frequent and has a better prognosis than luminal B tumors which are more frequently ER+/PR−,[229] and have a higher tumor cell proliferation.

The normal breast-like subtype is also a predominantly ER negative group.[241] It has relatively high expression of many genes known to be expressed by adipose tissue and other non epithelial cell types as well as strong expression of genes in the basal cluster but low expression of luminal epithelial genes.[226, 229] However, some reports have suggested that it may potentially be due to normal tissue contamination.[229, 269] This group is still poorly characterized, most IHC studies have not included this subtype because of its complex expression patterns which can not be summarized into a simple 5-marker panel.[229, 270]

PROGNOSTIC AND PREDICTIVE FACTORS

Currently, histopathologic evaluation of breast cancer includes a detailed description of morphologic patterns and biologic parameters of the tumor, including prognostic and predictive factors.[271, 272] A prognostic marker might be related to molecular mechanisms involved in tumor growth, progression, invasion and metastasis and gives significant

information on clinical outcome for groups of patients. A predictive factor is a clinical, pathologic or biologic feature that is used to estimate the likelihood of response to a particular type of adjuvant therapy.[42, 273] Hence, the use of prognostic and predictive factors has mainly three reasons,[274] to identify patients:

- who may not require adjuvant therapy after local surgery
- whose prognosis is poor enough to warrant a more aggressive adjuvant therapy
- whose tumors are more likely to be responsive or resistant to particular types of therapy

Several potentially useful prognostic and predictive factors have been suggested and can broadly be categorized into clinico-pathologic factors and biologic factors including tumor biomarkers as shown in Table 3. The College of American Pathologists[275] has categorized such factors into 3 groups. Category I, are factors with prognostic importance being useful in clinical management of patients; Category II includes factors that have been extensively studied, but whose importance remains to be validated in statistically robust studies; Category III includes all other markers not sufficiently studied to demonstrate their prognostic value.

Table 3: ***Useful and potential prognostic and predictive parameters in breast cancer.***[42]

Parameter
Patient related factors
Age at diagnosis
Ethnicity/race
Histopathologic features
Tumor size
Tumor differentiation
Histologic type
Histologic grade
Lymph nodes status (stage)
Vascular invasion
Cell cycle and proliferation
Mitotic count/Mitotic index
Ki-67/MIB-1
DNA S-phase fraction (flow cytometry)
DNA/ploidy (flow cytometry)
Cyclin E
Steroid Receptors
ER/PR
Growth factors and receptors
HER2
EGFR
Tumor suppressor genes
TP53
Measures of invasiveness
Cathepsin D
Plasminogen activator inhibitor-1 (PAI-1)
Urokinase plasminogen activator (uPA)
Laminin receptors
Angiogenesis
MVD
VEGF
Multiparameter gene expression analysis
Onco*type* DX assay
MammaPrint
Rotterdam signature
Breast Cancer Gene Expression ratio
Composite prognostic factors
Nottingham Prognostic Index
TNM and pTNM classification
Others
Tumor necrosis
Stromal fibrosis /elastosis
Basal-like phenotype
Triple negativity
Stem cell markers

Clinical factors

Age at diagnosis is one of the useful prognostic indicator in breast cancer.[42] Several independent studies have shown that young breast cancer patients (≤35 years) have more aggressive biologic characteristics and poorer prognosis.[276-278] Consequently, age (<35 years) is one of the parameters which was recommended by the St Gallen 2007 conference, used to determine the risk category of patients.[279, 280] On the other hand, the older patients (>70 years) also exhibit poor survival or higher mortality due to other factors.[278] Interestingly, breast cancer in African and African-American women is diagnosed about 10-15 years earlier than in women from Caucasians populations.[281, 282]

Related to this, race and ethnicity is another patient-related factor that has been proposed as a prognostic marker although it is still a matter of debate, and numerous independent studies have shown that breast cancer in Africans and African-American has poorer prognosis than in Caucasians.[33, 282-286] Indeed, compared with Caucasian women, African-American women, regardless of age presented with higher histologic grade for each stage of breast cancer and tumor size above 1 cm in a study by Henson et al in 2003.[284]

Histopathologic factors

Histologic grade is one of the most widely used prognostic factors. Using traditional morphologic features (tumor glands, nuclear pleomorphism and mitotic frequency), by careful examining of breast cancer specimens, can provide significant prognostic information required for therapeutic stratification. For accurate evaluation, good fixation and specimen preparation are very important in assessing these features. The traditional factors which are the most widely used prognostic markers and have the greatest value in clinical management of patients include; histologic type, histologic grade, tumor diameter, lymph node status, and vascular invasion,[42, 278, 287, 288] as well as distance to resection margins.

Assessment of tumor differentiation (histologic type, histologic grade) gives an indication of the underlying biology within a given tumor. The prognostic value of certain histologic types of invasive carcinoma has been well-established and may be grouped into four categories ranging from excellent to very poor prognosis.[278, 288] However, in multivariate analysis, histologic grade is a more powerful prognostic factor than histologic type.

Several studies have shown significant correlations between histologic grade and survival of breast cancer patients,[271] although a significant concern has been the reproducibility of grading. Currently, two grading systems are widely used, the Nottingham method (modified Scarff-Bloom-Richardson) and the Fisher nuclear grading method. The Nottingham system with its more objective criteria has good to excellent reproducibility when used by experienced pathologists,[289, 290] hence, it is the most widely used and is currently recommended. It evaluates glandular differentiation, nuclear pleomorphism and mitotic counts ultimately generating three tumor grades.[42, 291]

Tumor diameter is one of the strongest prognostic indicators even after 20 years of follow-up.[278] Consequently, it has become an important quality assurance measure for breast screening programs. However, for its prognostic correlation it should be assessed on pathologic specimens (pathologic tumor size), and the greatest diameter is considered as the final tumor size.[288]

Multiple studies have shown that histologically determined axillary lymph node status is one of the strongest independent prognostic factors in breast cancer.[42, 275, 291] However, there is still some debate about the use of axillary clearance or sentinel lymph node biopsy[291] although the latter is frequently used. At a recent St Gallen meeting (2009), the use of sentinel node biopsy was considered as standard care for patients with clinically negative axilla.[292] Nevertheless, it is generally recommended that, in order to obtain accurate histologic evaluation of lymph node stage, several blocks from each node submitted for examination

should be examined.[275, 291, 293] The St Gallen conference 2005 identified nodal status including sentinel node status as the most important feature for defining risk category in patient with breast cancer which was reaffirmed in a subsequent meeting.[280, 294] The absolute number of nodes involved is useful in determining the thresholds for treatment modalities in the same group of patients.[292]

Closely related to lymph node status is vascular invasion. Presence of vascular invasion correlates closely with lymph node involvement, and it has been suggested as a surrogate for lymph node status in cases where nodes have not been removed for examination.[291] Vascular invasion is a powerful predictor of local recurrence following surgery and a prognostic factor for reduced overall survival. It has been recommended that vascular invasion should be assessed in routinely processed tissue with extra care to avoid artifacts of retraction spaces.[275, 291] Interobserver variability about the topographical patterns of vascular invasion still exists[275] and is a matter of debate.

Regarding the composite prognostic factors, the UICC TNM classification which evaluates the primary tumor size (T), regional lymph node status (N) and presence of distant metastasis (M) is commonly used for breast cancer patients at the time of diagnosis (Figure 13). The tumor stage at the time of diagnosis is one of the strongest prognostic factors in breast cancer. The pTNM classification requires examination of the primary cancer with no gross tumor at the margins of resection and is similarly categorized as pT (corresponds to T category), pN and pM (corresponds to M category). The pN classification requires the resection and examination of at least the lower axillary (level 1) lymph nodes. However, sentinel nodes may be used, but even then the grading should be designated (sn) for sentinel node, for example pN1(sn).[2]

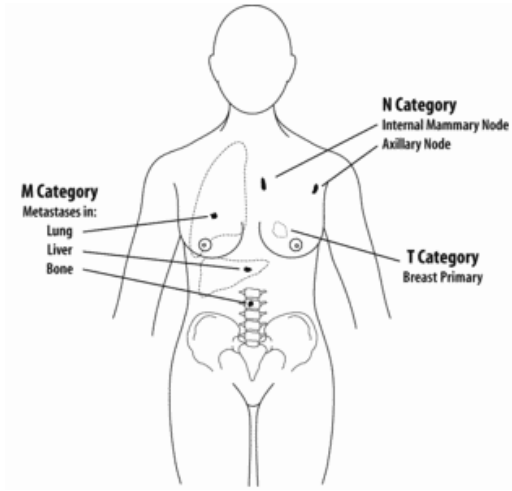

Figure 13: ***TNM classification. The TNM classification is an anatomically based system that records the primary and regional nodal extent of the tumor and the absence or presence of distant metastases. T category describes the primary tumor site, N category describes the regional lymph node involvement, and M category describes the presence or absence of distant metastases.***[295]

The Nottingham Prognostic Index (NPI), another composite parameter which includes nodal status, tumor diameter and histologic grade, is a strong prognostic assessment method, although inclusion of tumor biomarkers like ER status and HER2 expression to the NPI offers additional information about selection of patients for systemic adjuvant therapy.[296]

In 2007, prognostic factors such as histologic tumor grade, lymph node status, peritumoral vascular invasion (PVI), pathologic tumor size and patients age in addition to biomarkers were the criteria used to determine the risk category of patients by the St Gallen's conference.[294] Addition of proliferation assessed either by Ki-67 or mitotic count to the pathologic factors was later used to determine the algorithm for threshold of treatment modalities by the recent St Gallen' conference 2009.[292]

Tumor biomarkers and biologic factors

Over the years, researchers have continued to identify and propose several biomarkers as putative prognostic and predictive factors in breast cancer (Table 3) that might help to better stratify patients to various treatment regimens as well as targeted therapies. These novel biomarkers reflect alterations in genes that regulate development and proliferation of tumors.[42] However, three biomarkers (ER/PR and HER2 expression) have become standard measurements in the management of breast cancer patients. In addition, some factors have recently been recommended for clinical use.[272, 297] The uPA and PAI-1 which should be measured by ELISA may be used to determine prognosis in node negative breast cancer,[272] although the St Gallen conference did not accept uPA/PAI-1 as a useful prognostic factor.[292] In addition, the Onco*type* Dx multiparameter gene expression analysis may be used to predict the risk of recurrence in patients with ER positive breast cancer who are treated with tamoxifen[272] if readily available.[292]

Estrogen receptor status is a widely applied factor that is used to predict response to hormonal therapy in both early and metastatic disease.[272, 297] Prediction of response can be refined further by combing ER and PR assays.[271] Also, it has been suggested that absence of PR may indicate increased signaling of HER2 and may help clinicians decide between using aromatase inhibitor or tamoxifen.[298] In addition, ER expression has been shown to predict the long-term outcome of hormonal therapy,[278] and is associated with improved overall survival although its use as a prognostic factor is limited.[42, 272, 299]

HER2 expression has been associated with poor prognosis in breast carcinoma[78, 84] including poor response to both chemotherapy and hormonal therapy.[268] Studies have shown that HER2 status may be used to predict resistance to tamoxifen or cyclophosphamide-based therapy and enhanced response to anthracycline-based therapy in early breast cancer. However, its current clinical use is limited to predicting the response to HER2 targeted

therapy and selection of patients for treatment with trastuzumab.[272, 297] Other utilities for HER2 status are still undergoing further evaluation.

Gene expression studies have shown that proliferation is the most important component in many prognostic signatures.[300] Cell proliferation plays a major role in the behavior of breast cancer, and increased proliferation correlates strongly with prognosis irrespective of the methodology used.[51] Several methods of assessing proliferation have been studied (Table 3), but mitosis counting provides the most reproducible and independent prognostic information.[51, 301] Currently, it is recommended by the College of American Pathologists,[275] that assessment of cell proliferation should be performed routinely in evaluation of breast cancers, and mitotic figure counting might be sufficient enough for this purpose. It was found to be the most important prognostic component of the Nottingham grading system,[51] and the mitotic activity index (MAI) was validated as the strongest independent and well reproducible prognosticator in lymph node negative patients.[302] Assessment of other proliferation markers such as Ki-67 is currently optional.[275]

Ki-67 is a nuclear antigen that was identified by Gerdes et al. (1991),[303] and is expressed only in the proliferative phases of the cell cycle G_1, S, G_2 and M but absent in G_0. It can be used to stratify patients into good or poor prognostic groups.[275, 304] However, results from different groups are still conflicting and therefore its use in routine clinical management of breast cancer is still undetermined.[305] The Norwegian Breast Cancer Group has recently (February 2010) recommended its use in subgroups of breast cancer. The St Gallen conference in 2009 considered Ki-67-labelling index a useful factor that could be used to indicate the potential value of adding adjuvant chemotherapy in patients with receptor positive disease.[292]

For the *TP53* gene, mutation status and gene expression profiles have been suggested as powerful prognostic markers in breast cancer.[272, 306, 307] In addition, p53 expression has

been associated with poor prognostic factors and poor survival,[308, 309] and may be a prognostic marker in nodal negative breast cancer patients.[42, 275, 310] In addition, it can help to identify patients likely to response to chemotherapy or radiotherapy,[42, 275, 310] but its use as a prognostic or predictive factor is still controversial. Present data are insufficient to recommend clinical use of p53 in breast cancer patients. Moreover, the IHC detection of p53 expression is variable and, does not detect all *TP53* mutations. A consensus on how to assess the staining has not yet occurred.[42, 272, 275]

EGFR expression has been reported as one of the biomarkers which may be a candidate for clinical application in the near future.[94] Several independent studies have shown that EGFR expression in breast cancer is associated with features of aggressive tumors and poor response to tamoxifen.[42, 311] Present data suggest that some patient groups with breast cancer could benefit from EGFR-targeted therapy.[94, 258, 312] EGFR status might possibly have a predictive role for response to such therapy,[313] although detection and interpretation of EGFR is controversial and still needs to be standardized.[94, 311] Indeed, results from a trial in which patients were treated on the basis of EGFR expression are promising.[94, 314] On the contrary, different studies have provided conflicting results concerning the prognostic and predictive significance of EGFR, and its routine value in clinical management of breast cancer patients is still undetermined.[42, 311, 315]

Growth, invasion and metastasis of breast carcinoma depend on angiogenesis, and thus tumor-associated angiogenesis has attracted much attention and has been extensively studied as a possible prognostic or even predictive factor in breast cancer.[271, 316, 317] Several independent studies, although not all, have proposed that tumor angiogenesis is an independent prognostic factor and is associated with the risk of distant metastases and poor survival.[316, 318-320] However, the prognostic significance of angiogenesis remains somewhat controversial. This is mainly due to the variability in measurement of angiogenesis by

assessing the microvessel density (MVD) as a surrogate marker of the degree of angiogenesis.[139, 275] The assessment of MVD within a selected tumor area (hot spot) is too variable to be clinically useful.[317, 319, 321]

Another surrogate marker of angiogenesis with prognostic significance is VEGF expression, which was also found to correlate with MVD in some studies.[318] VEGF has been reported as an independent marker of poor prognosis in some studies,[42, 308, 322] and high expression of VEGF can identify a subgroup of patients who may benefit from selective anti-angiogenic therapy.[42, 153] However, the clinical use of VEGF is still undetermined.

In addition to the above factors, other parameters which might become of clinical importance in breast cancer include but are not limited to the triple negative phenotype, the basal-like subtype and cancer stem cell markers.[292, 294] The triple negative phenotype (TNP) is characterized by lack of ER/PR/HER2 and has recently been recognized as a group with therapeutic implications. It lacks targeted therapy whereas it is frequently resistant to standard chemotherapeutic regimens.[248] Multiple studies have reported the poor prognosis associated with TNP,[323] although the use of TNP as a prognostic factor is still not well studied.[248] Further, molecular predictive signatures will enable characterization of triple negative breast cancers better and design of optimal treatment modalities.[324] Indeed, the recent St Gallen's conference (2009)[292] recommended the use of triple negativity as a parameter that can be used to select some patients for chemotherapy.

Similarly, the basal-like phenotype which partially overlaps with the TNP and *BRCA1* associated breast cancer,[65, 248] has a poor prognosis with clinical importance.[248, 325] It is associated with the shortest relapse-free and overall survival,[226, 230] although its use as a prognostic factor is still not well studied.[305] It has been suggested that pathologists should routinely identify the BLP in breast cancer,[325] and incorporate the specific morphological features associated with the basal-like tumors (Table 2) with standard biomarkers such as

ER/PR and HER2 which might aid clinicians in developing optimal therapeutic strategies for this group.[248]

The presence of cancer stem cells might have prognostic and therapeutic implications in breast cancer.[191, 193, 194] Studies in cell lines have indicated that cancer stem cells have a drug-resistant phenotype,[326, 327] and express drug-resistance proteins such as ABCG2 (breast cancer resistance protein). In addition, breast cancer stem cells displayed resistance to both radiotherapy and tamoxifen treatment at clinically relevant doses.[191, 328, 329] Further, it has been found that breast cancer stem cells metastasize to the bone marrow in early-stage breast cancer.[330] Hence, it might be difficult to eradicate such drug-resistant stem-like cells from the bone marrow of patients using traditional chemotherapy only. The ability to identify cancer stem cells has facilitated the elucidation of pathways that regulate their growth and survival,[190, 191] and has provided a deeper understanding of the natural evolution of cancer as well as clinical behavior and response to treatment.

Studies *in vitro* and in xenografts have indicated that breast cancer stem cells display distinct molecular signatures; the 413-gene CSC signature[203] and the hNMSC signature,[184] which might be potential prognostic parameters. The hNMSC signature could be used to predict biologic and molecular features of breast cancer.[184] Stem cell markers like ALDH1 expression have been associated with poor prognosis,[193, 194] and studies have also suggested that levels of ALDH1 expression in primary breast cancer can be used to predict the response to chemotherapy.[193, 222, 331] Also, in the inflammatory breast cancer ALDH1 expression was found to be an independent prognostic marker that can predict metastasis and poor patient outcome,[223] whereas in pancreatic adenocarcinoma ALDH1 expression was associated with a worse survival.[332] However, the clinical significance of breast cancer stem cells and stem cell markers is still undetermined.

DIAGNOSIS AND TREATMENT

Optimal breast cancer management requires a multidisciplinary or interdisciplinary team approach involving surgeons, oncologists, radiologists, pathologists, geneticists,[333, 334] and possibly psychosocial specialists. Accurate diagnosis is a necessary step in the management of breast cancer and ideally every patient should have a pathologic diagnosis of breast cancer before definitive treatment can be given.[335] Accurate diagnosis confirms the presence or absence of breast cancer and thus, avoids unnecessary treatment in patients with benign conditions, and in addition provides prognostic and predictive features of the cancer, which help in planning treatment and counseling of the patient with breast cancer.[335]

A fundamental principle in evaluation of breast cancer patients is the triple-test diagnosis of breast masses which has been identified as a critical practice in diagnosing breast cancer.[335] The triple test entails a correlation of clinical, pathologic and imaging findings. Regarding pathologic diagnosis, fine needle aspiration biopsy (FNAB) has been recognized as the most cost-effective procedure with short turnaround time,[336] although core needle biopsy and standard surgical biopsy might be used. However, the choice among these three is influenced by availability of the tools and expertise in a limited-resource setting.[335, 336]

In addition to diagnosis, staging of breast cancer, to determine the extent of a disease is necessary for proper breast cancer management as well as providing useful information about the current status of cancer detection and management, and the success of breast health programs.[35] The TNM classification of breast cancer is widely used and has been recommended by a number of regulatory bodies including the UICC among others.[35, 295] Unfortunately, in limited-resource countries, breast cancer is commonly diagnosed at late stages[19, 282, 337] and is therefore characterized by high mortality.[338] Therefore, breast cancer staging in such countries could provide revealing epidemiological information about opportunities for initiating or improving breast health care programs.[35, 339]

Regarding treatment of breast cancer, conservative surgery is currently being promoted.[294] According to the St Gallen conference in 2009,[292] the use of surgical procedures developed to allow a wide excision with satisfactory results (oncoplastic surgery) were considered standard. The sentinel node biopsy was identified as standard of care for patients with clinically negative axilla; axillary node dissection could be avoided in all patients with a negative sentinel node, and in selected patients with micrometastasis in the sentinel node. However, the definition of adequate surgical margin remains controversial[292] and no detailed specific recommendation on this matter was given.[292]

Other treatment modalities for breast cancer include use of radiation therapy, endocrine therapy, anti-HER2 therapy and the cytotoxic chemotherapy.[280, 292, 340] The St Gallen conference 2009 recommended radiation therapy after local excision of ductal carcinoma *in situ* (DCIS) as standard[292] and postmastectomy radiation in invasive cancer, for women with four or more axillary lymph node involved. It could also be used in particularly young patients with one to three nodes and in those with poor prognostic features.[292] Generally, it should be avoided in elderly patients and those with low-grade DCIS and clearly negative margins.[292] In addition, the recommendations of the American Society for Clinical Oncology or the European Society of Mastology may be used to guide radiation treatment choice.[335]

For endocrine therapy, anti-HER2 therapy and the cytotoxic chemotherapy, the St Gallen 2009 conference gave a detailed algorithm for the thresholds for these treatment modalities, although adherence to the therapeutic guidelines is greatly affected by the resources available in various geographic settings.[292] Briefly, it recommended endocrine therapy for all patients whose tumors show any presence of ER, anti-HER2 therapy for patients with HER2 positive disease,[341] and chemotherapy for patients receiving anti-HER2 therapy and as the mainstay of adjuvant treatment of most patients with triple negative

tumors. However, the threshold for use of cytotoxic chemotherapy for some tumor groups was recognized as the most difficult to define.[292]

Briefly, the Breast Cancer Guidelines for Uganda[342] recommended breast self-examination as a way of early detection, the triple assessment approach for diagnosis, the TNM classification for staging and surgery as the mainstay of treatment for breast cancer except in metastatic disease. Also, tumor-free margin should not be less than 10 mm "at surgery", adjuvant radiation therapy after surgery for most patients groups and adjuvant systemic treatment (chemotherapy and hormonal) for all patients in Uganda, with few exceptions (DCIS, lobular carcinoma *in situ* (LCIS) and Paget's disease) were recommended. Hormonal therapy alone was recommended for LCIS. Neoadjuvant chemotherapy could be given to down-stage the tumor before local treatment is offered.[342]

BACKGROUND AND AIMS OF THE STUDY

Background

Over the years, numerous biomarkers have been proposed as putative prognostic and predictive factors in breast cancer that might help to stratify patients to various treatment regimens and targeted therapies.[42, 272, 275, 305] Previous reports have described the prognostic biomarker profiles of breast cancer in African and African-American women,[119, 282, 343] and striking similarities in breast cancer biology between the two groups have been reported.[281] In Uganda, there is a paucity of reports about prognostic and molecular biomarkers in breast cancer, whereas a previous study analyzed HER2 oncoprotein expression in breast cancer.[344] In this population, where the incidence of breast cancer is increasing, early diagnosis remains a challenge and the clinical outcome continues to be poor.[19]

Consequently, in 2000, the Uganda Breast Cancer Working Group launched Breast Cancer Guidelines for the management of breast cancer.[345] Its goal was to improve the quality of life of breast cancer patients and their families. Specifically, it aimed at standardizing and harmonizing diagnosis and treatment of breast cancer. In addition, one aim was to enable early detection of the disease with an ultimate goal of improving survival of the patients. The success of such a program needs to be augmented by studies specifically designed to elucidate the nature, behavior, basic processes and prognosis of breast cancer in this setting. Further, there was a need for a study to identify significant clinico-pathological parameters that might assist to achieve some of the objectives of the guidelines.

Aims of the study

General aim

On this background, the aim of this study was to explore the molecular markers in breast cancer with special focus on molecular subtypes, angiogenesis and stem cells in an African population.

Specific aims

Accordingly, the specific aims of the study included:

1. To explore the expression of selected basal-like markers in a series of breast cancers from native Ugandan women in the Kyadondo County, and to determine their frequency and relationship to other prognostic indicators.
2. To evaluate the expression of EGFR and c-kit in relation to the basal-like phenotype and other prognostic factors in breast cancers from an African population.
3. To explore the expression of candidate stem cell markers ALDH1 and BMI-1 in breast cancers from an African population and their associations with the basal-like phenotype (BLP) and other molecular markers.
4. To explore tumor-associated angiogenesis in relation to the basal-like phenotype, the triple negative phenotype and other tumor characteristics in an African population as well as a non-African population.

MATERIALS AND METHODS

STUDY SITES AND STUDY POPULATIONS

This study was carried out as part of the collaboration between the Department of Pathology at Makerere University College of Health Sciences (MUCHS) in Uganda and Section for Pathology, The Gade Institute, University of Bergen in Norway. Data collection was done at Makerere University while laboratory analysis and writing were done at The Gade Institute. The Department of Pathology at MUCHS is a research and teaching centre, for both undergraduate and postgraduate (average 2; range 1-5 per year) students. In addition, it offers histopathologic diagnostic services for the national referral hospital and the other main hospitals from all over the country which had an estimated population of about 32,4 million in 2009 (Figure 14).[346]

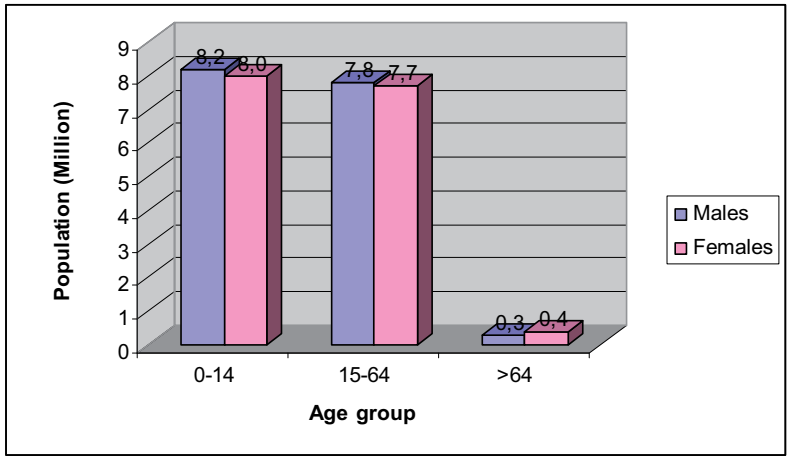

Figure 14: ***The population of Uganda by age group as of 2009***

Furthermore, it also houses the Kampala Cancer Registry (KCR), a population-based registry that was established in 1951 with an aim of determining incidence of cancers in the population of Kyadondo County.[13] The Registry covers an area of about 1914 km^2, which

comprises of Kampala, the capital city of Uganda, and the neighboring urban and semi-urban areas,[18] with an estimated population of 1.7 million (2002);[347] the female population above 15 years old is about 530,000. The annual incidence of breast cancer is 22/100,000 in this population.[13] The Baganda are the largest ethnic group in the county, but all the other ethnic groups are represented. Analysis of the breast cancer cases recorded at the Department of Pathology from 1990-2000 showed that cases from Kyadondo County contributed about 28% of the histologically confirmed breast cancers in females in Uganda. No significant differences were observed in distributions of female breast cancer patients by ethnic groups represented by regions as well as the age structure of cancer patients from Kyadondo County (mean age = 46 years) and those from other counties (mean age = 46 years) (P = 0.697).

The region is served with one 900 bed national referral hospital with attached oncology and radiotherapy units plus three other 100-bed missionary hospitals and hospice. Mammography is available at the national referral hospital but is limited to diagnostic purposes, and no routine screening is available.[342] The study population mainly included female patients with breast cancer who were registered at the Kampala Cancer Registry or presented at the three missionary hospitals in Kyadondo County. The registry methods of collecting data and results have been previously described.[348] A small number of study cases were drawn from the general population (39/192) (Figure 15).

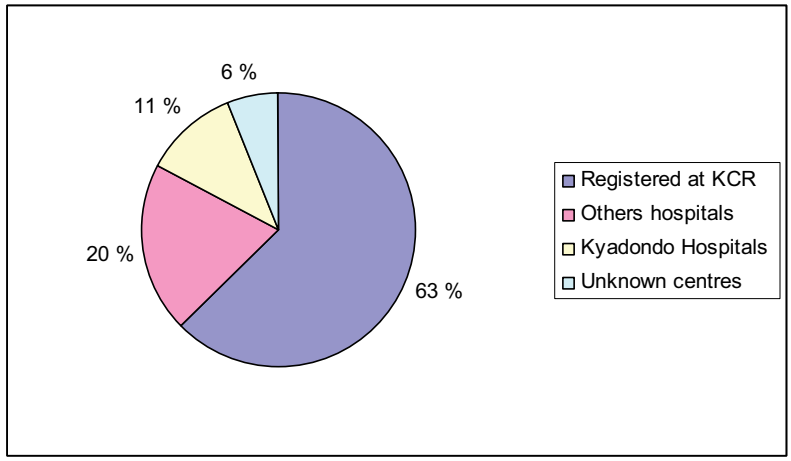

Figure 15: The distribution of the study population in Uganda

In addition to the above population, a second study population included female Ashkenazi Jewish women in North America with breast cancer who were registered in the medical records at the Sir Mortimer B. Davis Jewish General Hospital, Montreal, Quebec, Canada.

PATIENT SERIES AND TISSUES

Series 1

This was the Series on which **Paper I** and **Paper II** were based. Cases of female patients with histologically confirmed breast carcinoma were consecutively compiled from the records of the Kampala Cancer Registry. Altogether, 120 cases from the period 1993-2002 were included whereby a total of 65 cases were identified from which suitable paraffin blocks were available and retrieved from the departmental archives and analyzed. Duplicated cases due to repeated biopsies and subsequent mastectomies were included only once. These blocks were originally obtained from mastectomy, incisional, excisional and core needle biopsy specimens that were submitted to the department. Twenty other cases with inadequate tissue available plus 35 cases which were untraceable were excluded.

Series 2

This series was the basis for **Paper III** and part of **Paper IV** (referred to as Series I in **Paper IV**). Cases of female patients with histologically confirmed breast carcinoma were consecutively compiled from the records at the Department of Pathology in addition to cases registered at the Kampala Cancer Registry. Altogether, 314 cases from the period 1990-2002 were included whereby a total of 192 cases (Table 4) with suitable retrieved paraffin blocks were eventually identified. Altogether, 122 (39%) other cases were excluded. These included cases with inadequate tissue available; 24 metastases, 11 ductal carcinoma in situ, 7 benign, 26 with insufficient material, 1 poorly preserved sample, 18 cases where no tumor tissue was identified and 35 cases where the tissue blocks could not be located.

Table 4: ***Number of cases studied per year (Series 2)***

Year	Frequency	Percent
1990	37	19.3
1991	37	19.3
1992	56	29.2
1993	8	4.2
1994	5	2.6
1995	14	7.3
1996	6	3.1
1998	5	2.6
1999	7	3.6
2000	4	2.1
2001	7	3.6
2002	6	3.1
Total	**192**	**100**

Clinical information on the included cases was obtained from the histology request forms. The mean age was 46.2 years (range 18-80 years) for Series 2 (n=192 cases) and 49.8 years (range 27-89 years) for the Series 1 (n=65 cases). Duration of clinical symptoms as reported by a total of 127 patients at the time of presentation ranged from 0.5-108 months with and average of 17.1 months. The stage of the disease at the time of presentation was

available in only 22 patients and the majority 12 (54.5%) were in stage 4, 8 (36.4%) were in stage 3. Tumor size was recorded in 31 cases and average size was 5 cm (range 1-12 cm).

The histologic type and grade of tumor were available in 181/192 (94%) and 107/192 (56%) cases respectively, however these were not according to the recommended criteria. Therefore, all cases were histologically re-typed according to the World Health Organization[2] and re-graded in accordance with the Nottingham criteria.[291] Nuclear grade and mitotic count was also recorded as separate variables according to the same criteria.

Series 3

This was part of the series on which **Paper IV** was based. For **Paper IV**, a second independent patient series (referred to as Series II in **Paper IV**) was included. Cases from the ethnically restricted single hospital-based retrospective cohort study as previously described were used in this series.[65] The study was approved by the hospital's Institutional Review Board. Briefly, the patients were consecutive cases of Ashkenazi Jewish women aged $\leq$ 65 years diagnosed with a primary, non-metastatic, invasive breast cancer during 1980-1995 at the Sir Mortimer B Davis-Jewish General Hospital, Montreal, Quebec, Canada. In total, 239 cases were included for the analysis and 70 other cases were excluded due to unavailable or unsuitable material, repeated unsatisfactory staining, or inability to amplify DNA after several attempts. Among the 239 cases, 24 *BRCA1* and 6 *BRCA2* mutation carriers were included. All cases were re-examined histologically and typed according to World Health Organization[2] and grading was performed in accordance with the Nottingham criteria.[291] In all, 181 cases (76%) were treated by breast conserving therapy (BCT), while 58 cases (24%) received mastectomy. Adjuvant chemotherapy was given to 45% of the BCT cases and to 54% of those treated by mastectomy. Radiotherapy was given to 85% of the cases treated by BCT and 7% of those receiving mastectomy. Hormone therapy was given to 56% of hormone receptor

positive patients and 22% of receptor negative cases. The median follow-up time of those who did not die of breast cancer was 9.3 years (n=168), and there were 69 breast cancer related deaths during the follow-up period.

TISSUE MICROARRAY

Altogether, 192 archival tissues (Series 2) were assembled on tissue microarray (TMA) blocks according to Kononen et al. (1998).[349] Representative tumor areas with the highest histologic tumor grade preferably at the periphery of the tumor were identified on H&E-stained slides, and tissue cylinders with a diameter of 1 mm were punched from selected areas of the donor blocks and mounted into the receipt paraffin blocks using a custom made precision instrument (Beecher Instruments, Silver Spring, MD). To account for intratumoral heterogeneity and to reduce the problem of drop-outs, a minimum of 3 tissue cores were punched from the selected areas. Five μm thick sections of the resulting TMA blocks were made by standard technique. Serial sections were stained with antibodies as shown in Table 5. Also, a total of 230 cases from Series 3 were available on tissue microarray constructed as previously described,[101] and 4 μm pre-cut slides were made available for staining with EGFR antibody as described.[101] Of these, 223 cases with previously registered results were used for further analysis.

IMMUNOHISTOCHEMICAL METHODS

For series 1 and 2, immunohistochemical staining was performed on 5 μm thick sections of both the conventional and TMA slides. After sections were deparaffinized in xylene and alcohols, heat induced epitope retrieval methods were used for all antibodies except EGFR where proteinase kinase pre-digestion was used. As shown in Table 4, the antigen retrieval time, the antibody dilution and incubation were optimized for each antibody

used. The staining was performed in a Dako Autostainer for all antibodies except the BMI-1 antibody, a non-commercial monoclonal antibody that was kindly provided by Dr Arie P. Otte (The University of Amsterdam, The Netherlands). The main detection system was Dako EnVision+ enzyme labeled polymer with 3,3'-diaminobenzidine (DAB+) as chromogen. For BMI-1, the Catalyzed Signal Amplification II (CSA II) kit (Dako, K1497) which is a biotin-free tyramide signal amplification system was used. Hematoxylin was used as a counter stain. All incubations were done at room temperature. Cases of breast carcinoma (ER, PR CK5/6, P-cadherin, ALDH1 and BMI-1), colonic carcinoma (Ki-67 p53 and EGFR), prostate carcinoma (Factor VIII) and gastrointestinal stromal tumor (GIST) (c-kit) with known immunoreactivity for the respective markers were used as positive controls. Normal breast tissue was used as a control for p63. Replacing the primary antibody with buffer solution served as the negative control.

For evaluation of microvessel density and vascular proliferation, 5 µm conventional sections (**Paper IV**: Series 2; 192 cases; Series 3; 239 cases) were used. We employed the dual staining procedure with Factor VIII and Ki-67 for endothelial cell proliferation.[350] Sections were incubated with a cocktail of polyclonal rabbit anti-human Factor VIII (A0082) and monoclonal mouse antihuman Ki-67 antigen (Table 5). A secondary goat anti-mouse antibody (Dako E0433) with streptavidin alkaline phosphatase (Lab Vision) and Ferangi Blue chromogen kit (Biocare Medical) (Series 2) or StreptABComplex/AP (Dako K0391) and Fast Blue (Series 3) was used for visualization of Ki-67. For, Factor VIII staining, visualization was achieved by using EnVision+ and AEC+ (3-amino-9-ethylcarbazole) in both series. No contrast staining was applied in this protocol.

Table 5: ***Immunohistochemical staining protocols used in the present study***

Antibody	Provider	Dilution	Antigen Retrieval	Incubation (minutes)	Detection system
ER	Dako/1D5	1:50	MW: 15 min in TE9 buffer	30	EnVision
PR	Dako/ PgR 636	1:150	MW: 15 min in TE9 buffer	30	EnVision
HER2	Dako/ Polyclonal	1:500	MW: 15 min in TE9 buffer	60	EnVision
CK5/6	Dako/D5/16B4	1:200	MW: 15 min in TE9 buffer	30	EnVision
P-cadherin	BD/56	1:400	MW: 15 min in TE9 buffer	60	EnVision
Ki-67/MIB-1	Dako/ MIB-1	1:50	MW: 30 min in TE9 buffer	60	EnVision
EGFR	Zymed/31G7	1:30	Proteinase K	30	EnVision
c-kit	Dako/ Polyclonal	1:200	MW: 15 min in TE9 buffer	30	EnVision
p63	Dako/4A4	1:300	MW: 15 min in TE9 buffer	30	EnVision
p53	Dako/DO-7	1:1000	MW: 15 min in TE9 buffer	60	EnVision
BMI-1	Dr Otte/6C9	1:1	MW: 25 min in TE9 buffer	60	CSA-kit
ALDH1	BD/44	1:250	MW: Citrate buffer pH 6	60	EnVision
Factor VIII and Ki-67 (Series 2)	Dako/ Polyclonal and MIB-1	1:800 and 1:50	MW: 30 min in TE9 buffer	60	EnVision and AP/HRP
Factor VIII and Ki-67 (Series 3)	Dako/ Polyclonal and MIB-1	1:400 and 1:200	MW: 20 min in TE9 buffer	60	EnVision and AP/HRP

BD=BD Transduction, MW=microwave, TE9=Tri EDTA buffer pH=9, AP=Alkaline phosphatase, HRP=Horseradish peroxidase

Evaluation of staining

In Series 2, tumors (2.6%-4.7%) without interpretable cores because of insufficient tumor tissue were omitted from the analysis. In total, 183-187 could be evaluated for the various markers (Table 6). For all biomarkers, evaluation was done by qualitative and quantitative visual assessment, and criteria for evaluation is given in Table 6. For ALDH1, nuclear staining alone was considered non specific and was not included in the analysis. Tumors with any mild to strong staining in at least 10% of cells were considered as positive

staining for ER, PR and c-kit. Regarding EGFR, tumors with weak to strong cell membrane staining, whether complete or incomplete, and observed in more than 1% of the tumor cells were considered positive in accordance to the Dako criteria.[101, 351]

Staining index

To evaluate p53, p63, CK 5/6, P-cadherin, ALDH1 and BMI-1 expressions, a staining index (SI) (values=0-9) was determined by multiplying the score for intensity of staining (none=0, weak=1, moderate=2, and strong=3) with the score for proportion of immunoreactive cells (<10%=1, 10%-50%=2, >50%=3).[65, 246, 252, 352, 353] Cut-off points for the various markers, determined based on median or upper quartile SI for Series 2 in consideration of the frequency distribution curve, the size of the subgroups and the number of events in each subgroup are shown in Table 6.

Evaluation of Ki-67 expression

Ki-67 proliferative rate was determined as a proportion (%) of positively stained tumor cell nuclei out of 500 tumor cells examined at high power (x400) using an eyepiece grid. In total, 7 cases with fewer than 500 cells were counted (small tumors). The cut-off point for high tumor cell proliferation by Ki-67 expression was set at 15.4% (Series 1) and 20.0% (Series 2) based on the median values (Table 6).

Table 6: ***Evaluation criteria and cut-off points for the biomarkers in the present study***

Marker	Interpretable cores	Staining pattern	Cut-off point	Positivity rate (%)
ER	187	Nuclear	10% positive	39
PR	187	Nuclear	10% positive	28
HER2	187	Cell membrane	HercepTest criteria[354]	17
Ki-67	189	Nuclear	Median; ≥ 15.4%* or ≥ 20.0%** =high	51
p53	187	Nuclear	upper quartile >4 = positive[353]	29
Cytokeratin 5/6	186	Cell membrane and cytoplasm	Median; 1-9 = positive	15
P-cadherin	187	Cell membrane and cytoplasm	Median; >3 = positive	27
EGFR	185	Cell membrane	>1% stained =positive (Dako criteria)	20
c-kit	186	Cytoplasm and/or cell membrane	10% positivity[355]	4
p63	187	Nuclear	Median; 2-9= positive	17
ALDH1	183	Cytoplasm	Median; 3-9 = positive	48
BMI-1	186	Nuclear	Median; 1-9 = positive	25

Regarding Series 3, detailed information about staining methods of the various markers (ER, PR, HER2, p53 CK5/6, P-cadherin, and EGFR) and evaluation of the markers was available from previous publications.[65, 101, 246, 356, 357] For EGFR, a total of 201 were evaluated for the EGFR staining in accordance to the Dako criteria (EGFR-DA)[351] as previously described;[101] 22 tumors (9.9%) with uninterpretable cores on TMA were omitted.

Evaluation of Microvessel Density (MVD)

The average microvessel density (MVD) in a selected tumor area was assessed in accordance to previous studies.[319, 350, 358, 359] Sections were first scanned at low magnifications

(x50 and x100) to identify the most vascularized area (hot spot) of the tumor. Then 10 consecutive high power fields at x250 (field size: Series 2; 0.45 mm^2, Series 3; 0.42 mm^2) from the hot spot were examined, except in a few cases with small tumors where less than 10 fields were examined in Series 2 (n=18). All positively stained vessels (red) were counted including vessels without microlumina according to Weidner's approach,[319] and clusters of endothelial red cells that were clearly separate from the adjacent microvessels were also counted. In cases with vascular nests (glomeruloid proliferations) or long winding vessels and branching vessels, individual lumina or segments were counted to account for increased angiogenic response. When no clear hot spot was identified, vessels were counted in the most cellular area of the tumor periphery. Areas close to necrosis were excluded in the counting. In Series 2, 11 (5.7%) poorly stained (weak staining or excessive background staining) tissues and 4 (2%) cases with insufficient tumor tissue were excluded leaving 177 cases for analysis, whereas 239 cases were fit for analysis in Series 3. The MVD was then determined as the average number of microvessels counted in the 10 fields expressed as microvessels per mm^2.

Vascular proliferation

Similarly, within the same fields as used for the MVD, the number of microvessels containing positive proliferating endothelial cells were counted. The dividing endothelial cells were recognized by showing distinct Factor VIII/Ki-67 co-expression; red cells with blue nuclei (Figure 16). Positive nuclei outside the endothelial cell layer or within the vessel lumen were not counted. The average number of vessels with proliferating endothelial cells per mm^2 (pMVD) was determined and the Vascular Proliferation Index (VPI) was determined as the ratio of pMVD (mm^2) to the MVD (mm^2) given as a percentage.

The cut-off points for high MVD, high pMVD and high VPI were determined as 80.4/mm^2, 1.7/mm^2 and 3.1% for Series 2 and as 92.3/mm^2, 1.9/mm^2 and 3.3% for Series 3,

respectively based on the upper quartile values for the particular series. Thus, tumors that had a pMVD ≥ 1.7/mm^2 (Series 2) or ≥ 1.9/mm^2 (Series 3) were considered to have a high pMVD, and those with VPI ≥ 3.1% (Series 2) or ≥ 3.3% (Series 3) were considered to have a high VPI. .

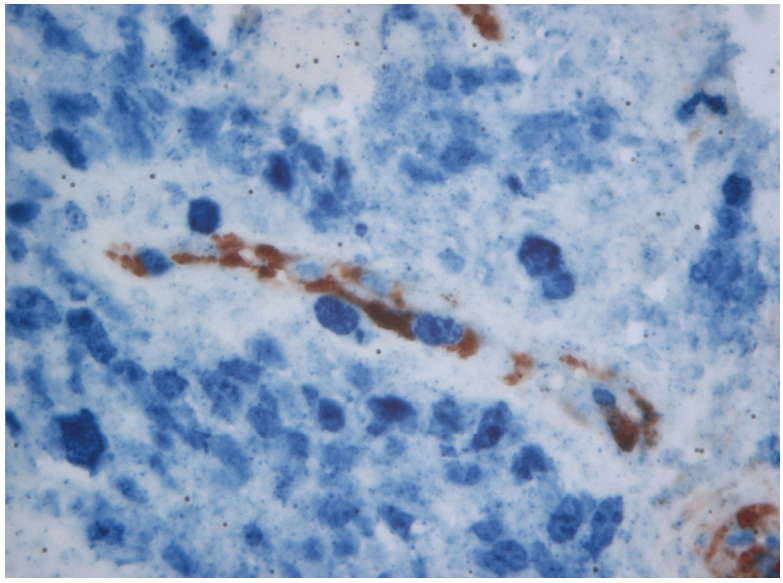

Figure 16: ***Microvessel containing positive proliferating endothelial cells as seen on dual staining x400. The dividing endothelial cells are recognized by distinct Factor VIII/Ki-67 co-expression; red cells with blue nuclei were counted at x250 HPF in 10 consecutive fields in a selected hot spot***

Molecular phenotype sub-classification

There is no consensus on how to define different molecular subtypes of breast cancer by immunohistochemical markers,[233] and overlapping categories exist. We used criteria based on current literature[231, 244, 267] for sub-classification into molecular subtypes. In accordance with Carey et al. (2006),[231] we defined the luminal A (ER+ and/or PR+, HER2−), luminal B (ER+ and/or PR+, HER2+), HER2+ subtype (ER−, PR−, HER2+) and the basal-like subtype (ER−, PR−, HER2−, CK 5/6+ and/or EGFR+) subgroups (Table 7). Tumors negative for all the 5 markers (ER, PR, HER2, CK 5/6 and EGFR) were considered as unclassified. This

definition for luminal B tumors does not identify all luminal B tumors because only 30% to 50% are HER2+ and the rest are classified with the luminal A. We therefore merged luminal A and luminal B into the luminal subtype. Further, in accordance with our previous studies, we included P-cadherin staining in some of the definitions of the basal-like phenotype.[246, 252]

By using the Arnes et al. (2008)[204] criteria, we defined the BLP profiles (Table 7) as follows: BLP1: concurrent ER−, HER2− and CK 5/6+; BLP2: concurrent ER−, HER2− and P-cadherin+; BLP3: concurrent ER−, HER2− and EGFR+; BLP4: concurrent ER−, HER2− and CK 5/6+ and/or EGFR+; BLP5: concurrent ER−, HER2− and positivity for one or more basal markers (CK 5/6, P-cadherin and EGFR). BLP4 is identical to the core basal phenotype (CBP) as defined by Nielsen et al. (2004)[232] and Tischkowitz et al. (2007).[360]

Regarding **Papers I** and **II**, tumors that expressed CK 5/6 and/or P-cadherin were considered to have a basal-like phenotype (BLP) in accordance to previous studies.[246, 252]

Table 7: ***Criteria for molecular subgroup classification of breast cancer and basal-like phenotypic definitions as used in this study***

Subgroup	**Biomarker-criteria**
Subtype	
Luminal A	ER+/and/or PR+/HER−
Luminal B	ER+/and/or PR+/HER+
HER2 subtype	ER−/PR−/HER+
Basal-like subtype	ER−/PR−/HER−/CK5/6+ and/or EGFR+
Unclassified	ER−/PR−/HER−/CK5/6−/EGFR−
Basal-like phenotype profiles	
BLP1	ER−/HER−/CK5/6+
BLP2	ER−/HER−/P-cadherin+
BLP3	ER−/HER−/EGFR+
BLP4	ER−/HER−/CK5/6+ and/or EGFR+
BLP5	ER−/HER−/CK5/6+ and/or P-cadherin+ and/or EGFR+

ETHICAL CONSIDERATIONS

This study was approved by the Institutional Review Board at Makerere University College of Health Sciences.

STATISTICAL METHODS

Statistical analysis was performed using the SPSS 15.0 statistical package. We used the t-test or Mann-Whitney U test to compare continuous variables between different groups. We evaluated associations between categorical variables using the Pearson's χ^2 test or Fisher's exact test. Differences between variables were considered statistically significant when the p-value for any statistical test used was <0.05.

MAIN FINDINGS

In **Paper I**, we found that the basal-like markers were expressed in 34% of the series of breast cancer from an African population, and they were significantly associated with features of aggressive tumors including high histologic grade, high nuclear grade, high mitotic count, and ER/PR negativity.

In **Paper II**, we focused on the expression of tyrosine kinase growth factors (EGFR and c-kit) in relation to basal-like breast carcinoma. We found a strong and significant association between EGFR and/or c-kit expression and the basal-like phenotype. In addition, EGFR and/or c-kit expression was significantly associated with poor prognostic features; histologic grade, nuclear grade, mitotic count, and ER−/PR−/HER2− (triple negativity).

In **Paper III**, we explored the expression of candidate breast cancer stem cell markers, ALDH1 and BMI-1 and their associations with the basal-like phenotype and other molecular characteristics. We found a high prevalence of ALDH1 expression in the series of breast carcinoma from an African population as well as a more extensive ALDH1 staining in cases that were positive, compared to Caucasian and Asian populations from the literature. Expression of ALDH1 was significantly associated with the basal-like phenotype and basal markers as well as features of aggressive tumors (high histologic and nuclear grades, high mitotic count, ER/PR negativity and p53 expression). On the other hand, BMI-1 expression was associated with good prognostic features, low histologic grade and ER positivity whereas it was inversely associated with ALDH1 staining.

In **Paper IV**, we determined vascular proliferation as a marker of tumor angiogenesis, and found that the basal-like subtype had increased tumor vascular proliferation compared to the luminal subtype in two independent breast cancer series. Also, we found that increased angiogenesis was associated with TNP, EGFR, p53 and p63 expression.

DISCUSSION

METHODOLOGICAL CONSIDERATIONS

Patient series

Series 1 and 2

This was a retrospective study with some limitations. The quality of data obtained depends on the accuracy of the records at the Kampala Cancer Registry. The registry methods of collecting data and results have been previously described.[348] However, completeness and accuracy of data in studies done by cancer registries is of major concern particularly in Africa.[18]

Nevertheless, the Kampala Cancer Registry as a population-based registry was established in 1951 with an aim of determining the incidence of cancers in the population of Kyadondo County,[13] and it is one of the longest standing cancer registries in the African continent.[18] Some of the cases included in Series 1 as was well as Series 2 were part of the 174 breast cancer patients enrolled for the survival study at the KCR,[18] which included incident cases diagnosed and registered between 1993-1997. Of these, 109 cases (63%) were histologically or cytologically confirmed breast cancers. However, as indicated by Gondos et al. (2005),[18] collection of follow-up data was particularly challenging, and a large number of patients could not be included in their study. For our study, reduced availability of archival tissue presented another limitation since a number of eligible cases were not evaluated; the 35 tissue blocks on record for the year 1997 were not available.

We eventually identified histologically confirmed breast carcinoma cases and were able to include 65 of 120 cases in Series 1 (1993-2002) whereas 192 of 314 cases were included for Series 2 (1990-2002) upon incorporating cases recorded at the departmental records. Altogether, 122 cases including the 35 tissue blocks on record for the year 1997 were not available and were not analyzed. Therefore, the unavailability of some archival tissues

could cause a selection bias. In addition, 31% (87 of 279) of the retrieved samples were not analyzed due to inappropriate tumor tissue.

A majority of samples used in Series 1 and 2 were from patients who presented to the national referral hospital, and this might also represent a selection bias. However, analysis of the records from 1990-2000 at the department revealed that the patients from Kyadondo County contributed about 28% of the breast cancer patients in the whole country. Therefore, our results might be an indication of the general population. In addition, analysis of breast cancer patients from the population of Kyadondo County compared with patients from the other counties revealed no significant difference in the age at diagnosis.

Series 3

This series is a single hospital and retrospective cohort series from a major teaching clinic in Montreal, Canada. The main objective in recruiting patients was to study the impact of *BRCA*-mutations on breast cancer. Given that the frequency of *BRCA*-mutations are rare in the general population, a restricted population was considered with regard to the feasibility of conducting large-scale genetic analysis as well as the clinical impact of the study. Ashkenazi Jewish women in North America have a well-known high frequency of *BRCA*-mutations, and these can be attributed to a few dominant founder-mutations, highly simplifying the detection of mutations. For that reason, the study recruited only Ashkenazi women with an age restriction of <65 years based on the observation that breast cancers in older Ashkenazi women had similar frequency of *BRCA*-mutations as the general population.

The study originally included 309 patients, of which 17 patients (5.5%) were immediately excluded due to the inability to locate tissue blocks, lack of invasive carcinoma in the available blocks or repeated inability to amplify DNA for mutation analysis, leaving 292 cases. Eventually, a total of 239 cases where available after excluding 4 cases that lacked

follow-up information in our data base and another 49 which lacked tissue blocks or had inadequate staining. Of these 230 cases were available on TMA blocks. Although this reduction in numbers may be considerable compared to the original series we consider the available cases as a random selection. No significant differences in the means of the included cases and the complete series were detected concerning tumor size, histologic grade, axillary nodal status or patient age at diagnosis (data not shown).

Clinical-pathological variables

We obtained much of the clinical information by carefully evaluating the histology request forms. The Department of Pathology at MUCHS has a vast number of archival tissue as well as accompanying histology request forms and reports. In collaboration with Centre for Disease Control and Prevention (CDC), all records available at the department up to 2000 were computerized. This made it easy to identify duplicate cases as well as identify more cases for Series 2 in comparison with the records at the KCR.

However, relative lack of clinical information was a limitation of this study. Accurate information about prognostic variables like tumor size was available in only a few cases (31/192; 16%), while the stage of the disease at time of diagnosis was also available in a limited number of cases (22/192; 12%). In addition, lack of outcome or follow-up information for most of the patients on record caused limitations in assessing the actual prognostic significance of the studied variables.

Use of archival tissue

The main advantage in using archival tissue for research lies in the availability of large tissue archives in pathology laboratories as well as long follow-up of a large patient series. This presents an invaluable tool for research and it has been demonstrated that most proteins

retain their antigenicity for more than 60 years.[361] This study is based on material from the University teaching hospital in Uganda and a major Canadian research clinic originating from 1980. However, variations in tissue handling including fixation time and methods, sampling techniques and storage conditions are some of the major disadvantages as these would reduce immunoreactivity of some antigens.[362, 363] The large archival repositories have been supplemented by the use of tissue microarray.

Tissue microarray (TMA)

The use of tissue microarrays is an efficient approach in studies of most biomarkers and significantly reduces costs and time as well as conserving research tissues has facilitated the use of large archives. The tissue microarray technique has been validated in several studies[361, 364, 365] since its introduction in 1998[349] and has been well established in our research group at The Gade Institute since 2000. After a few practice sessions, precision and accuracy was achieved and we used this technique to assemble the 192 cases (Series 2) for analysis. Similarly, 230 cases from Series 3 were assembled and made available to our group for staining. However, regarding evaluation of angiogenesis (**Paper IV**), conventional 5 μm thick tissue sections were used as well as in **Paper I** and **II** with 65 cases.

Further, we used pre-cut sections for both conventional slides and the TMA slides which were stored at −20^{o} C, and recent studies have indicated that stored slides can still be valuable for research purposes in spite of the aging effects.[366]

Use of immunohistochemical methods

Immunohistochemistry is widely used to study expression of specific proteins in human malignancies and is an invaluable tool in assessing prognostic and predicative biomarkers in breast cancer.[367] Although formalin fixation retains tissue morphology, antigens

may be masked or even lost during fixation. Thus, the fixed form of proteins must be adequately retrieved to be recognized by antibodies. Antigen retrieval is one of the most important factors for achieving accurate and consistent results for biomarker studies by IHC. Consequently, different methods of antigen retrieval have evolved as an approach to standardize immunohistochemistry protocols for formalin fixed archival tissue, with enzymatic digestion and heat-induced retrieval being the best described.[368] The requirements for optimal staining results vary with the choice of antibody as well as the antigen of interest, in terms of the pH of retrieval buffer, the heating time and the temperature. We used a test battery approach in staining some of the study cases as well as control tissues including different retrieval buffers with different boiling schemes, different antibody dilutions and different incubation periods.

However, antigen retrieval of the archival tissue presented one of the biggest challenges of this study. Whereas prolonged formalin fixation is rarely a problem in some institutes,[369] it might be a major concern at our department at MUCHS. There is a considerable variation in the fixation times in formalin at our department (MUCHS) which might induce a bias towards prolonged fixation considering the years that were included. Moreover, the actual fixation time is difficult to determine. Studies have shown that prolonged formalin fixation might lead to decreased antigenicity of some antigens.[362, 363] These factors might have contributed to the difficulty in antigen retrieval, although, this was overcome by determining the appropriate antigen retrieval times for each antibody under consideration[362] as described above.

The choice of antibody clones used was done in consideration of the current literature for the particular antigen in question and considering widely used antibodies. We generally preferred antibodies that had been previously used by our group for consistency and adjusted for differences in reactivity between various tissues by modifying the staining protocols.

Accordingly, for the simultaneous dual staining we used Factor VIII which has been used in breast cancer and other cancers by multiple studies in our laboratory (The Gade Institute) with good results and is now well established,[254, 350, 370-372] although CD31 and CD34 has been considered to be good alternatives.[373] Previous reports have also shown that the staining results using the three antibodies (Factor VIII, CD31 and CD34) are comparable.[374] In addition to these three widely used pan-endothelial cell marker, another antibody CD105 has been used in breast cancer and other tumors.[375-378] CD105 is reported as a highly a specific marker for endothelial cells and has been shown to bind only to activated endothelial cell,[375, 378] and may be better marker of angiogenesis in tumors[378-380] although a number of studies in breast cancer have mainly used fresh or frozen tissue.[375, 378, 381] Also, the non-commercial BMI-1 antibody (donated by Dr Arie Otte, The University of Amsterdam, The Netherlands) used in this study, has been used in various previous studies and was validated in our laboratory;[382] Western blot analysis supported specificity and correlations with the commercial BMI-1 antibody. For candidate antibodies, the supplied data sheets from the manufacturers and literature studies provided information on their reactivities and were tested accordingly before the main staining.

Appropriate positive controls were selected from well-known sections that expressed the antigen of interest and were included for every round of staining. Negative control was achieved by omitting the primary antibody.

Regarding the detection method, whenever possible we used the EnVision system which on top of reducing the cost of staining, reduces the assay time and the workload.[383] Also, we employed the Dako Autostainer which ensures equal staining conditions for all samples in every run, for all antibodies except the BMI-1 antibody.

Evaluation of staining: staining index

We employed the staining index (SI) (values=0-9) as the method of assessing immunohistochemical staining for most markers in the study. This is a semi-quantitative and subjective grading system which considers both the intensity of staining and proportion of cells stained, obtained as described in the methods section. This method of evaluation is now a well established robust and reproducible parameter and has been validated by several studies carried out in our team at The Gade Institute.[252, 382, 384-387]

Selection of cut-off points used to create dichotomous variables was based mainly on median values and quartiles of the staining index, considering the frequency distribution curve, the size of the subgroups and the number of events in each subgroup. A selected cut-off point, once chosen, was then used consistently in all analyses involving that marker.

Estimation of microvessel density

Angiogenic activity is heterogeneous within a given tumor, and MVD assessment within the most vascularized selected tumor area (hot spot) has been the most widely used technique to quantify intratumoral angiogenesis in breast cancer, since its introduction in 1991.[319, 321] The major drawback, however, has been lack of standardization of the method used to assess MVD,[321] although the method used by Weidner et al. (1991)[319] has been generally accepted with some modifications.[350, 358, 359] The variability is mainly due to issues like choice of antibody and "hot spot" based counts versus "global" counts as well as automated versus manual counts. It is now generally agreed that the vessel counts should be in the hot spots preferably at the tumor periphery.[317] In accordance to previous studies at our institute, we used a double staining with Factor VIII/Ki-67 for endothelial cell proliferation,[350, 370] and we evaluated tumor angiogenesis in hot spots counting vessels in 10 fields at x250 magnification and eventually determined MVD per mm^2. MVD estimation by

Weidner's method is fairly rapid, but is influenced by the training and experience of the investigator. Thus, after initial discussion of the criteria and some basic training, a training set (n=25) was scored twice for intra-observer variability with good results (κ=0.83). The inter-observer variability (HN, JBA) using the same set was also good (κ=0.64).

In addition, availability of a representative tissue block[359] for estimation of MVD in the archival tissue depends on the initial tumor sampling technique that was done by both the clinician and at the department since it is not uncommon for the clinicians to submit just a small part of the tumor of a mastectomy specimen (personal observation from Uganda). However, previous studies have reported a concordance rate of 71-78% between different blocks sampled from the same tumor;[388] thus, use of archival tissue did probably not greatly affect the estimated microvessel count.

Vascular proliferation index

Vascular proliferation determined by the number of vessels with evidence of dividing endothelial cells given by dual immunohistochemical staining may be a better indicator of angiogenesis than MVD which is more of a marker for the metabolic demand of tissue rather than angiogenesis.[138] Studies from our laboratory have shown that counting vessels with actively dividing endothelial cells revealed significantly stronger associations with clinico-pathologic phenotype and patient prognosis in endometrial and prostate cancers.[350, 389] In this present study we accordingly determined vascular proliferation as a marker of angiogenesis.

Molecular sub classification

Determining the molecular subtype of breast cancer by immunohistochemical markers has some limitations.[233] There is no consensus on how to define basal-like breast cancer and overlapping categories exist. Although a majority of the basal-like breast cancers are triple negative, ER or HER2 expression has been reported in about 15%-45% of the basal-like cancers.[226, 232, 233] Therefore, the obvious limitation of using IHC to define subtypes is that it might result into misclassification bias of some tumors. In this study, we used criteria based on current literature.[231, 244, 267] In this classification, the definition of basal-like breast cancer as ER−/PR−/HER−/CK5/6+ and/or EGFR+ encompasses the criteria used by Nielsen et al. (2004)[232] which showed a sensitivity of 76% and specificity of 100% to identify the basal-like phenotype as defined by expression profiling analysis. In addition, we used individual basal markers for this subgroup such as cytokeratin 5/6, P-cadherin and EGFR as well as composite definitions such as basal-like subtype, various basal-like phenotypic profiles (BLP1-5) and the triple negative phenotype which overlaps significantly with the basal-like category (see Figure 12).

Comparison with previous studies

There is no uniformly accepted method for the registration of IHC staining that can be applied for most biomarkers. This presents a challenge in interpretation and comparison of results from different research groups, and this was a major source of concern according to the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK).[390] Hence, the report encouraged transparency and complete reporting to facilitate usefulness and reproducibility of scientific data in the field of tumor biomarker research.

DISCUSSION OF MAIN FINDINGS

Molecular subtypes of breast cancer

Microarray studies have indicated that breast cancers may be divided into five major subtypes.[225, 227] Defined by IHC staining, the luminal A subtype is the most prevalent contributing 47-69% of all breast cancers. The other subtypes contribute 8-25% (basal-like), 6-17% (luminal B), 6-10% (HER2 subtype) while the unclassified accounts for 1-7%.[231, 241, 244, 267] The normal breast-like subtype originally identified by gene profiling studies[226] is still poorly characterized immunohistologically.[229, 233] The basal-like and the HER2 subtypes are of particular interest because of their prognostic implications.[226] The basal-like phenotype partially overlaps with the triple negative phenotype (ER−/PR−/HER2−),[360] which is another category of breast cancer that has attracted attention as an easily recognizable prognostic group with therapeutic implications.[391] It contributes about 10-24% of all cases.[391, 392]

Studies have reported that both the basal-like and the TNP are overrepresented in young African-American and African women,[119, 120, 231, 393, 394] and striking similarities have been reported in features of breast cancer in the two groups.[281] Therefore, an important aspect of our studies was to determine the prevalence of the basal-like phenotype and its associations with other tumor characteristics with focus on breast cancer in an African population.

In agreement with these previous reports, we found a high prevalence (34%) of the basal-like differentiation in the series of tumors from an African population (**Paper I**) using two individual basal markers CK5/6 and P-cadherin. This is comparable to what Carey et al. (2006)[231] found (39%) in premenopausal African-American women. Further, in **Paper III**, with more cases included, the prevalence of the basal-like subtype (22%) as defined according to Carey et al's definition, using composite criteria, was again comparable to the overall prevalence of BLP in African-American women (26%) reported in that study. Studies in Caucasians have reported a prevalence of 8-16%.[231, 244, 267] In the same vein, we found a

higher prevalence of triple negative tumors (40-41 %) (**Papers II** and **III**) compared to previous reports (10-21%) among Caucasians.[392, 393, 395, 396] The high frequency of both the BLP and TNP in patients of an African population compared to Caucasians provides further support that breast cancer in women of African ancestry might be, to some extent biologically different from that in Caucasians.[282, 343, 397-399] Indeed, a recent gene expression profile study has indicated that differences beyond the knowledge of current markers might exist in tumor biology of African-American compared to Caucasians.[400] Moreover, breast cancer in the black and African-American women occurs at a much younger age (10-15 years younger on average) than in Caucasians.[281, 282]

However, the higher frequency (40-41%) of TNP (**Papers II** and **IV**) in our study compared to some studies in African-American (21-25%)[393, 394, 396] might indicate that other factors besides ethnical and racial background are important in breast cancer, although others reported comparable frequency (47%).[395] Further, more comparative studies are required to answer this.

Basal-like subtype and expression of EGFR and c-kit

We found a significant association (**Paper II**) between EGFR or EGFR and/or c-kit expression and the basal-like category[232, 233, 245, 360] as well with the triple negative phenotype[401] in accordance with other studies. This provides more support to the stipulation that the basal-like[258] and triple negative tumors[312] represent a group of breast cancers that might potentially benefit from EGFR-targeted therapies in addition to chemotherapy. Whether these tumors will actually respond to such therapies is yet to be known[392, 402] from the results of on-going phase II clinical trials in the basal-like subtype as well as triple negative tumors.[403, 404] Studies in cell lines have shown that basal-like cells are sensitive to EGFR targeted therapy in combination with chemotherapy[405] and the two act synergistically. Basal-

like cells are more sensitive to growth inhibition by dasatinib, a multi-targeted kinase inhibitor, compared to the luminal cell lines.[264]

Moreover, we (**Paper II**) and others[232, 244] found that 23-31% of basal-like breast cancers express c-kit, whereas about 50% of BLP express either EGFR or c-kit, or both (**Paper II**). However, results from phase II trials with a potent inhibitor of tyrosine kinases including c-kit are still negative.[406]

Basal-like subtype and tumor-associated angiogenesis

We found that tumor angiogenesis was increased in the basal-like tumors compared to the luminal subtype (**Paper IV**) in two independent breast cancer series. This is in agreement with a previous report which found that glomeruloid microvascular proliferation was significantly more frequent in the basal-like phenotype.[253] In the present study, vascular proliferation was significantly associated with multiple basal markers (such as CK 5/6, P-cadherin and EGFR). To support this, gene expression studies have indicated that the majority of basal-like tumors express the activated wound-response signature, which represents important processes associated with angiogenesis among others.[238]

Anti-angiogenesis treatment has now been approved for breast cancer and other tumor types,[407] although it is presently not clear whether this treatment should be given to certain subgroups of malignant tumors.[160] Our findings therefore suggest that anti-angiogenic therapy might be a possible target in the basal-like subgroup. Further clinical trials targeting different pathways will give more insight on this.[160]

Similarly, the significant association between TNP and increased tumor angiogenesis which we found in our study (**Paper IV**), in accordance with previous reports,[52, 320] supports the rational of phase II clinical trials that are currently accessing the potential benefit of triple negative tumors from a combination of anti-angiogenic therapy and chemotherapy.[233] Current

studies should also address whether markers of BLP or TNP might be used to predict the response to anti-angiogenic therapy in breast cancer.

Basal-like subtype and poor prognosis

Basal-like breast cancer is an aggressive phenotype, but the underlying biology is still poorly understood.[233] It has been reported to have a poor prognosis[226] with a specific pattern of metastatic spread to sites associated with decreased survival,[255] like hematogenous metastases.[230] Several independent studies have established that angiogenesis plays a central role in tumor development and subsequent metastases.[139, 144, 153] To speculate, increased angiogenesis in basal-like breast cancer might partly explain the frequent metastasis reported in this subgroup. In general, the prognostic significance of increased angiogenesis as determined by MVD has been confirmed in several independent studies.[136, 316-318, 320]

Additionally, we found (**Paper I**) that the basal-like phenotype was associated with features of aggressive tumors in accordance with previous reports[231, 245, 267, 408] as well as EGFR expression (**Paper II**). Given that, both EGFR expression[232] and increased angiogenesis are associated with poor prognosis, and our findings provide further evidence that the basal-like breast cancer exhibit multiple features of aggressive tumors which, in part, explains the poor prognosis.[226]

Cancer stem cells and the basal-like subtype

Our results (**Paper III**) showed that the basal-like subtype was significantly associated with ALDH1 expression which has been associated with poor clinical outcome in breast cancer.[193, 194] Also ALDH1 expression was associated with the different BLP profiles, as well as with individual basal markers CK 5/6, P-cadherin and EGFR (**Paper III**), similar to what others have reported.[194] In breast cancer mouse models, breast cancer stem cells which

expressed a combined CD44+/CD24$^{-/low}$/ALDH1+ phenotype showed an especially high tumorigenic capacity.[194]

Further, others have suggested that the proportion of cancer stem cells within breast tumors may correspond to the risk of distant metastases[185, 186] and the heterogeneous phenotypic and molecular traits of breast cancers are a function of their CSC content.[184] In the integrative model of breast cancer metastasis it has been proposed that oncogenic mutations which occur in the breast stem cells might generate 'poor prognosis' metastatic breast cancers.[181] In these cancers, the resulting breast cancer stem cells under the influence of stromal fibroblasts have the ability to metastasize, and variants of the cancer stem cells which express different tissue-specific profiles determine the tissue selectivity for metastasis.[181] The frequent hematogenous metastases as well as specific pattern of metastatic spread that have been reported in the basal-like subtype might relate to the significant association found between ALDH1 expression and the BLP. Moreover, we also found more extensive ALDH1 staining in cases that were positive, compared to reports among Caucasians and Asians populations.[194, 409] More studies are required to understand the biology of cancer stem cells in the development and clinical behavior of basal-like breast cancer.

ALDH1 expression has been associated with resistance to chemotherapy[193, 222] in breast cancers. Also, there is some evidence that the limitation of chemotherapy and radiation treatment may be associated with the inability to target breast cancer stem cells.[191, 202, 328, 329, 410] To speculate, our findings might be related to the aggressive behavior and therapy resistant features of the basal-like breast cancer subtype.[202, 226, 410, 411]

Cancer stem cell markers in African breast cancer

Our result indicate a higher prevalence of ALDH1 expression (48%) in a series of breast cancer from an African population (**Paper III**) compared to 19% and 30% in two different Caucasian populations[194] and 10% and 19% in Asian populations.[193, 409] We also found more extensive staining in positive cases.[194, 409] Further, in comparison with data derived from breast tumors in Caucasian and Asian populations[193, 194, 409] regarding ALDH1 positivity rate in tumors with similar characteristics (histologic grade, ER, HER2, Ki-67), we observed that tumors from our study (**Paper III**) stained in a higher percentage of cases in poor prognosis categories (such as high histologic grade, ER negative cases, and tumors with high Ki-67 expression). Thus, apart from methodological discrepancies, biologic differences might be present when comparing breast cancers from African and Caucasian populations.[282, 284, 343, 397, 398, 400] In line with this, a difference in the spectrum of tumor characteristics and prognostic features such as the presence of tumor necrosis, low ER positivity rate, high HER2 positive rate, p53 expression, overexpression (p16 and cyclin E) as well as low expression (cyclin D) of cell-cycle regulatory proteins and a high frequency of basal-like features have been reported in African and African-American patients when compared with breast cancers among Caucasians.[32, 119, 231, 282, 396, 398] To speculate, our findings might indicate that poor prognosis of breast cancer in Africans and African-American is a preordained event. In support of this, a high prevalence of TNP in young premenopausal African-American was a contributory factor to the poor outcome which was reported in that group,[394] whereas other independent studies have reported poor prognosis in African and African-American patients.[19, 281] Indeed, a poorer outcome of breast cancer has been observed in the two populations compared to Caucasians.[282, 285, 412]

Additionally, we found that ALDH1 expression was significantly associated with features of poor prognosis including high histologic grade, high nuclear grade, high mitotic

count, p53 expression and ER/PR negativity (**Paper III**). Also, ALDH1 expression was associated with a short duration of symptoms. Moreover, we found a significant association between ALDH1 expression and the triple negative tumors, a group whose poor prognosis has been widely reported.[323] Our results provide further support that ALDH1 status might be an indicator of poor prognosis in breast cancer.[194, 409]

To support this, BMI-1 expression, a candidate stem cell marker,[204] which has been associated with features of good prognosis such as low grade, low mitotic count, ER positivity and absence of TNP in breast cancer in our study and in previous reports,[204, 214, 413] was inversely associated with ALDH1 expression (**Paper III**). As expected, the frequency of BMI-1 was lower (25%) in the series of breast cancers from an African population compared to breast cancer from Caucasian and Asian populations (43-62%).[204, 214] Others have found different results, BMI-1 being associated with poor prognosis.[186, 214, 414] In addition, Glinsky et al. (2005)[186] found that the expression of a BMI-1 driven 11 gene signature was associated with the risk of metastases in breast carcinoma. The reason of this inverse relationship is not known. To speculate, given that, BMI-1 has been implicated in breast carcinogenesis and is involved in stem cell activation and self-renewal,[210] whereas ALDH1 is reported to have a role in early stem cell differentiation, proliferation and survival,[215-217] this might suggest a regulatory relationship between ALDH1 and BMI-1 during carcinogenesis. Hence more studies are required to understand the regulatory pathways and biology of cancer stem cells in breast cancer carcinogenesis.

Finally, to speculate, the significant association between a basal-like phenotype and increased angiogenesis, EGFR expression or ALDH1 expression might indicate that patients with basal-like breast cancer may benefit from combined therapies targeting pathways involved in cell proliferation, angiogenesis as well as cancer stem cell activation and proliferation. These hypotheses still require further exploration.

CONCLUSIONS

1. The basal-like phenotype was frequent in the series of African breast cancer from the Kyadondo County in Uganda and is strongly associated with features of poor prognosis (**Paper I**).
2. There was a high frequency of tyrosine kinase growth factor (EGFR and c-kit) expression in basal-like breast carcinoma in the series of breast cancer from Uganda, and their expression was associated with features of aggressive tumors. (**Paper II**).
3. There was a high frequency of ALDH1 expression in the series of invasive breast carcinomas from Uganda which was significantly associated with a basal-like phenotype and with features of aggressive tumors (**Paper III**).
4. Tumor-associated angiogenesis was increased in basal-like breast cancer in two independent series of breast cancer and was associated with the triple negative phenotype, EGFR expression, p53, p63 and with features of aggressive tumors (**Paper IV**).

CONCLUDING REMARKS AND FUTURE PROSPECTS

Breast cancer is a highly heterogeneous disease in terms of tumor biology, clinical behavior, prognosis and response to treatment.[45] It is believed that this is due to molecular differences even within histologically similar tumors.[178, 224, 261] Interestingly, race appears to be one of the contributory factors to the final outcome.[9, 33, 281, 286, 399, 415-417]

In line with the aim of this study, we have explored novel molecular markers that could be relevant for the understanding and management of breast cancers in African patients. We observed that breast cancer is occurring at a young age (mean 46 years) during the most productive period of the women. This might have implications on the socio-economic status of the families affected.[342]

Additionally, we found a high frequency of poor prognosis features in the series of breast cancers from Ugandan women including a high prevalence of basal-like breast cancer, the TNP and high ALDH1 expression. These factors might influence the final clinical outcome of breast cancer in this population.[18, 19] The prognostic significance of the high and extensive ALDH1 expression deserves further investigations.

Our results have indicated another potential therapy target[258] for the basal-like subtype, since tumor angiogenesis was found to be increased in this category. Thus, well-controlled clinical trials of targeted therapy combining multiply pathways are urgently needed especially in a population where early diagnosis and effective treatment is especially challenging.[19] However, standardization of the method used to assess tumor angiogenesis is still required.[321]

Regarding immunohistochemistry, optimal antigen retrieval is a major concern in both research and the routine setting. The continuing need for routine assessment of hormonal receptors and HER2 to select high-risk patients and provide valuable information on treatment options for breast cancer patients in Uganda cannot be overemphasized. Fortunately, the establishment of routine IHC staining for breast cancer is under way, and personnel are available and have been trained. However, lack of adequate infrastructure is still a problem in some areas.

On the basis of the literature and the present study, we suggest the routine assessment of hormonal receptors and HER2 for treatment of breast cancer patients in African populations. The histologic evaluation of breast carcinoma specimens, including the fixation, should be standardized according to guidelines and specified in the pathology report. This will ensure an improved quality as a basis for better treatment. Regarding research, more clinico-pathologic studies are required to determine the significance of specific entities such as basal-like breast cancer and triple negative tumors in Uganda and how to best stratify patients

towards traditional treatment as well as novel targeted therapies. Other molecular markers in translational breast cancer research also need to be explored and validated in African populations. Ultimately, clinical studies should also be performed in this setting.

In a country like Uganda, with limited resources, and where breast cancer incidence is increasing rapidly, health policies have to be designed to promote early diagnosis in order to improve clinical outcome. National diagnosis and treatment guidelines for breast cancer in line with the WHO recommendations, considering the limited resources, will help promote equity of health care delivery. At the policy level, our results underscore the need to establish routine assessment of ER/PR and HER2 in breast cancer patients in addition to the currently used prognostic factors. This will improve the quality of medical care offered to patients by the health care system.

ERRATA

Introduction: Page 46, paragraph 2, line 6: "…experienced pathologists,[289, 290]…" should read "…experienced pathologists,[289, 290, 291]…".

Page 47, paragraph 1, line 1: "…be examined.[275, 291, 293]…" should read "…be examined.[271, 275, 293]…", paragraph 2, line 4: "…examination.[291]…" should read "…examination.[271]…", paragraph 2, line 7: "…retraction spaces.[275, 291]" should read "…retraction spaces.[271, 275]" and paragraph 3, line 6-7: "…categorized as pT (corresponds to T category), pN and pM (corresponds to M category)…" should read "…categorized as pT; primary tumor (the pT categories correspond to the T categories), pN; regional lymph nodes and pM; distant metastasis (the pM categories correspond to the M categories)…".

Page 55, paragraph 2, line 10: "…choice.[335]" should read "… choice.[294]"

Materials and methods: Page 64, paragraph 3, line 4: "..Table 4.." should read "..Table 5...".

Discussion: Page 77, paragraph 2, line 2: "…research tissues has facilitated…" should read "…research tissues and has facilitated…".

Page 87, paragraph 2, line 11: "…BLP" should read "…BLP in our study (**Paper III**).".

Page 88, paragraph 1, line 14: "…(cyclin D) of cell-cycle regulatory proteins…" should read "…of cell-cycle regulatory proteins (cyclin D)...".

Paper II: Page 517, Statistical methods, paragraph 1, line 3: "…G^2..." should read "…χ^2 …".

Paper III: Page 370, Patient series, paragraph 1, line 3: "…Cancer Registry at the…" should read "…Cancer Registry and at the…" and Page 371, Molecular subtypes, paragraph 1, line 8-9: "…(ER−, HER2− and CK 5/6 and/or EGFR+)…" should read "…(ER−, PR−, HER2− and CK 5/6 and/or EGFR+)…".

Paper IV: Page 6, Patient series, paragraph 1, line 3: "…Cancer Registry at the… " should read "…Cancer Registry and at the…" and Page 16, Discussion, paragraph 1, line 3: "…Our previous study..." should read "…A previous study...".

REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA: a cancer journal for clinicians*. 2005;**55**:74-108.

2. Tavassoli FA, Devilee P. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs*. Lyon: IARC Press; 2003.

3. Stewart BW, Kleihues P, editors. *World Cancer report*. Lyon: IARC Press; 2003.

4. Lee JH, Yim SH, Won YJ, et al. Population-based breast cancer statistics in Korea during 1993-2002: incidence, mortality, and survival. *Journal of Korean medical science*. 2007;**22 Suppl**:S11-16.

5. Murthy NS, Agarwal UK, Chaudhry K, Saxena S. A study on time trends in incidence of breast cancer - Indian scenario. *European journal of cancer care*. 2007;**16**:185-186.

6. Tarabeia J, Baron-Epel O, Barchana M, et al. A comparison of trends in incidence and mortality rates of breast cancer, incidence to mortality ratio and stage at diagnosis between Arab and Jewish women in Israel, 1979-2002. *Eur J Cancer Prev*. 2007;**16**:36-42.

7. Yeole BB. Trends in cancer incidence in female breast, cervix uteri, corpus uteri, and ovary in India. *Asian Pac J Cancer Prev*. 2008;**9**:119-122.

8. Louwman WJ, Voogd AC, van Dijck JA, et al. On the rising trends of incidence and prognosis for breast cancer patients diagnosed 1975-2004: a long-term population-based study in southeastern Netherlands. *Cancer Causes Control*. 2008;**19**:97-106.

9. Shavers VL, Harlan LC, Stevens JL. Racial/ethnic variation in clinical presentation, treatment, and survival among breast cancer patients under age 35. *Cancer*. 2003;**97**:134-147.

10. Sant M, Francisci S, Capocaccia R, Verdecchia A, Allemani C, Berrino F. Time trends of breast cancer survival in Europe in relation to incidence and mortality. *Int J Cancer*. 2006;**119**:2417-2422.

11. Adebamowo CA, Adekunle OO. Case-controlled study of the epidemiological risk factors for breast cancer in Nigeria. *Br J Surg*. 1999;**86**:665-668.

12. Parkin DM, Sitas F, Chirenje M, Stein L, Abratt R, Wabinga H. Part I: Cancer in Indigenous Africans--burden, distribution, and trends. *The lancet oncology*. 2008;**9**:683-692.

13. Wabinga HR, Parkin DM, Wabwire-Mangen F, Nambooze S. Trends in cancer incidence in Kyadondo County, Uganda, 1960-1997. *Br J Cancer*. 2000;**82**:1585-1592.

14. Amir H, Kaaya EE, Kwesigabo G, Kiitinya JN. Breast cancer before and during the AIDS epidemic in women and men: a study of Tanzanian Cancer Registry Data 1968 to 1996. *J Natl Med Assoc*. 2000;**92**:301-305.

15. Guth AA. Breast cancer and human immunodeficiency virus infection: issues for the 21st century. *J Womens Health (Larchmt)*. 2003;**12**:227-232.

16. Sitas F, Pacella-Norman R, Carrara H, et al. The spectrum of HIV-1 related cancers in South Africa. *Int J Cancer*. 2000;**88**:489-492.

17. Nalwoga H. *Histopathological features overexpression of c-erbB-2 oncoprotein and survival of breast cancer patients in Kyadondo County, Uganda* [Master's degree]. Kampala: Makerere University; 2002.

18. Gondos A, Brenner H, Wabinga HR, Parkin DM. Cancer survival in Kampala, Uganda. *Br J Cancer*. 2005;**92**:1808-1812.

19. Gakwaya A, Kigula-Mugambe JB, Kavuma A, et al. Cancer of the breast: 5-year survival in a tertiary hospital in Uganda. *Br J Cancer*. 2008;**99**:63-67.

20. Howe GR, Hirohata T, Hislop TG, et al. Dietary factors and risk of breast cancer: combined analysis of 12 case-control studies. *J Natl Cancer Inst*. 1990;**82**:561-569.

21. Palma M, Ristori E, Ricevuto E, Giannini G, Gulino A. BRCA1 and BRCA2: the genetic testing and the current management options for mutation carriers. *Crit Rev Oncol Hematol*. 2006;**57**:1-23.

22. Oldenburg RA, Meijers-Heijboer H, Cornelisse CJ, Devilee P. Genetic susceptibility for breast cancer: how many more genes to be found? *Crit Rev Oncol Hematol*. 2007;**63**:125-149.

23. Palacios J, Robles-Frias MJ, Castilla MA, Lopez-Garcia MA, Benitez J. The molecular pathology of hereditary breast cancer. *Pathobiology*. 2008;**75**:85-94.

24. Okobia M, Bunker C, Zmuda J, et al. Case-control study of risk factors for breast cancer in Nigerian women. *Int J Cancer*. 2006;**119**:2179-2185.

25. Donegan LW. *Diagnosis of breast cancer*. In: Donegan LW, Spratt SJ, editors. Cancer of the breast. 5th ed. St. Louis Elsevier Science 2002. p. 317-319.

26. Cady B, Stone MD, Schuler JG, Thakur R, Wanner MA, Lavin PT. The new era in breast cancer. Invasion, size, and nodal involvement dramatically decreasing as a result of mammographic screening. *Arch Surg*. 1996;**131**:301-308.

27. Coburn NG, Chung MA, Fulton J, Cady B. Decreased breast cancer tumor size, stage, and mortality in Rhode Island: an example of a well-screened population. *Cancer Control*. 2004;**11**:222-230.

28. Ernst MF, Roukema JA, Coebergh JW, et al. Breast cancers found by screening: earlier detection, lower malignant potential or both? *Breast Cancer Res Treat*. 2002;**76**:19-25.

29. Ernster VL, Barclay J. Increases in ductal carcinoma in situ (DCIS) of the breast in relation to mammography: a dilemma. *J Natl Cancer Inst Monogr*. 1997:151-156.

30. Hall FM, Storella JM, Silverstone DZ, Wyshak G. Nonpalpable breast lesions: recommendations for biopsy based on suspicion of carcinoma at mammography. *Radiology*. 1988;**167**:353-358.

31. Frost FJ, Tollestrup K, Trinkaus KM, et al. Mammography screening and breast cancer tumor size in female members of a managed care organization. *Cancer Epidemiol Biomarkers Prev*. 1998;**7**:585-589.

32. Bird PA, Hill AG, Houssami N. Poor hormone receptor expression in East African breast cancer: evidence of a biologically different disease? *Annals of surgical oncology*. 2008;**15**:1983-1988.

33. Furberg H, Millikan R, Dressler L, Newman B, Geradts J. Tumor characteristics in African American and white women. *Breast Cancer Res Treat*. 2001;**68**:33-43.

34. Mbonde MP, Amir H, Schwartz-Albiez R, Akslen LA, Kitinya JN. Expression of estrogen and progesterone receptors in carcinomas of the female breast in Tanzania. *Oncol Rep*. 2000;**7**:277-283.

35. Singletary SE, Connolly JL. Breast cancer staging: working with the sixth edition of the AJCC Cancer Staging Manual. *CA: a cancer journal for clinicians*. 2006;**56**:37-47; quiz 50-31.

36. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. 2000;**100**:57-70.

37. Bhardwaj N. Harnessing the immune system to treat cancer. *J Clin Invest*. 2007;**117**:1130-1136.

38. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. 2002;**3**:991-998.

39. Baar J. Biological therapy of breast cancer: recent clinical applications. *Curr Opin Investig Drugs*. 2007;**8**:987-995.

40. Foulkes WD. BRCA1 functions as a breast stem cell regulator. *J Med Genet*. 2004;**41**:1-5.

41. Liu S, Ginestier C, Charafe-Jauffret E, et al. BRCA1 regulates human mammary stem/progenitor cell fate. *Proc Natl Acad Sci U S A*. 2008;**105**:1680-1685.

42. Morabito A, Magnani E, Gion M, et al. Prognostic and predictive indicators in operable breast cancer. *Clinical breast cancer*. 2003;**3**:381-390.

43. Pavelic K, Gall-Troselj K. Recent advances in molecular genetics of breast cancer. *J Mol Med*. 2001;**79**:566-573.

44. Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA, Bender HG. Multistep carcinogenesis of breast cancer and tumour heterogeneity. *J Mol Med.* 1997;**75**:429-439.

45. Cuny M, Kramar A, Courjal F, et al. Relating genotype and phenotype in breast cancer: an analysis of the prognostic significance of amplification at eight different genes or loci and of p53 mutations. *Cancer Res*. 2000;**60**:1077-1083.

46. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif*. 2003;**36**:131-149.

47. Fernandez PL, Jares P, Rey MJ, Campo E, Cardesa A. Cell cycle regulators and their abnormalities in breast cancer. *Mol Pathol*. 1998;**51**:305-309.

48. Gillett CE, Barnes DM. Demystified ... cell cycle. *Mol Pathol*. 1998;**51**:310-316.

49. Michalides RJ. Cell cycle regulators: mechanisms and their role in aetiology, prognosis, and treatment of cancer. *J Clin Pathol*. 1999;**52**:555-568.

50. Gompel A, Chaouat M, Hugol D, Forgez P. Steroidal hormones and proliferation, differentiation and apoptosis in breast cells. *Maturitas*. 2004;**49**:16-24.

51. van Diest PJ, van der Wall E, Baak JP. Prognostic value of proliferation in invasive breast cancer: a review. *J Clin Pathol*. 2004;**57**:675-681.

52. de Jong JS, van Diest PJ, van der Valk P, Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol*. 1998;**184**:53-57.

53. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature*. 1998;**396**:643-649.

54. Buchholz TA, Wazer DE. Molecular biology and genetics of breast cancer development: a clinical perspective. *Semin Radiat Oncol*. 2002;**12**:285-295.

55. Oliveira AM, Ross JS, Fletcher JA. Tumor suppressor genes in breast cancer: the gatekeepers and the caretakers. *Am J Clin Pathol*. 2005;**124 Suppl**:S16-28.

56. Frankish H. BRCA1 has a pivotal role in repairing DNA. *Lancet*. 2001;**357**:1678-1678.

57. Liu YL, West SC. Distinct functions of BRCA1 and BRCA2 in double-strand break repair. *Breast Cancer Research*. 2002;**4**:9-13.

58. Paull TT, Cortez D, Bowers B, Elledge SJ, Gellert M. Direct DNA binding by Brca1. *Proc Natl Acad Sci U S A*. 2001;**98**:6086-6091.

59. MacLachlan TK, Somasundaram K, Sgagias M, et al. BRCA1 effects on the cell cycle and the DNA damage response are linked to altered gene expression. *J Biol Chem*. 2000;**275**:2777-2785.

60. Dobrovic A, Simpfendorfer D. Methylation of the BRCA1 gene in sporadic breast cancer. *Cancer Research*. 1997;**57**:3347-3350.

61. Yoshikawa K, Honda K, Inamoto T, et al. Reduction of BRCA1 protein expression in Japanese sporadic breast carcinomas and its frequent loss in BRCA1-associated cases. *Clinical Cancer Research*. 1999;**5**:1249-1261.

62. Thompson ME, Jensen RA, Obermiller PS, Page DL, Holt JT. Decreased Expression of Brca1 Accelerates Growth and Is Often Present During Sporadic Breast-Cancer Progression. *Nature Genetics*. 1995;**9**:444-450.

63. Lambie H, Miremadi A, Pinder SE, et al. Prognostic significance of BRCA1 expression in sporadic breast carcinomas. *Journal of Pathology*. 2003;**200**:207-213.

64. Niwa Y, Oyama T, Nakajima T. BRCA1 expression status in relation to DNA methylation of the BRCA1 promoter region in sporadic breast cancers. *Japanese Journal of Cancer Research*. 2000;**91**:519-526.

65. Foulkes WD, Stefansson IM, Chappuis PO, et al. Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. *J Natl Cancer Inst*. 2003;**95**:1482-1485.

66. Honeth G, Bendahl PO, Ringner M, et al. The CD44+/CD24- phenotype is enriched in basal-like breast tumors. *Breast Cancer Res*. 2008;**10**:R53.

67. Seery LT, Knowlden JM, Gee JMW, et al. BRCA1 expression levels predict distant metastasis of sporadic breast cancers. *International Journal of Cancer*. 1999;**84**:258-262.

68. Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. *Oncogene*. 2007;**26**:2157-2165.

69. Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell*. 1997;**88**:323-331.

70. Buchholz TA, Weil MM, Story MD, Strom EA, Brock WA, McNeese MD. Tumor suppressor genes and breast cancer. *Radiat Oncol Investig*. 1999;**7**:55-65.

71. Wyllie AH. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer Metastasis Rev*. 1992;**11**:95-103.

72. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*. 1993;**74**:609-619.

73. Gasco M, Shami S, Crook T. The p53 pathway in breast cancer. *Breast Cancer Res*. 2002;**4**:70-76.

74. Feki A, Irminger-Finger I. Mutational spectrum of p53 mutations in primary breast and ovarian tumors. *Crit Rev Oncol Hematol*. 2004;**52**:103-116.

75. Soong R, Iacopetta BJ, Harvey JM, et al. Detection of p53 gene mutation by rapid PCR-SSCP and its association with poor survival in breast cancer. *Int J Cancer*. 1997;74:642-647.

76. Osborne C, Wilson P, Tripathy D. Oncogenes and tumor suppressor genes in breast cancer: potential diagnostic and therapeutic applications. *Oncologist*. 2004;**9**:361-377.

77. Al-Kuraya K, Schraml P, Torhorst J, et al. Prognostic relevance of gene amplifications and coamplifications in breast cancer. *Cancer Res*. 2004;**64**:8534-8540.

78. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human-Breast Cancer - Correlation of Relapse and Survival with Amplification of the Her-2 Neu Oncogene. *Science*. 1987;**235**:177-182.

79. Looi LM, Cheah PL. C-erbB-2 oncoprotein amplification in infiltrating ductal carcinoma of breast relates to high histological grade and loss of oestrogen receptor protein. *Malays J Pathol*. 1998;**20**:19-23.

80. Lovekin C, Ellis IO, Locker A, et al. C-erbB-2 Oncoprotein Expression in Primary and Advanced Breast-Cancer. *British Journal of Cancer*. 1991;**63**:439-443.

81. Soomro S, Shousha S, Taylor P, Shepard HM, Feldmann M. C-erbB-2 Expression in Different Histological Types of Invasive Breast-Carcinoma. *Journal of Clinical Pathology*. 1991;**44**:211-214.

82. Oldenhuis CN, Oosting SF, Gietema JA, de Vries EG. Prognostic versus predictive value of biomarkers in oncology. *Eur J Cancer*. 2008;**44**:946-953.

83. Stern DF. ERBB3/HER3 and ERBB2/HER2 duet in mammary development and breast cancer. *J Mammary Gland Biol Neoplasia*. 2008;**13**:215-223.

84. Yamashita H, Nishio M, Toyama T, et al. Coexistence of HER2 over-expression and p53 protein accumulation is a strong prognostic molecular maker in breast cancer. *breast cancer res*. 2004;**6**:R24-R30.

85. Giatromanolaki A, Koukourakis MI, Simopoulos C, et al. c-erbB-2 related aggressiveness in breast cancer is hypoxia inducible factor-1alpha dependent. *Clin Cancer Res*. 2004;**10**:7972-7977.

86. Klapper LN, Kirschbaum MH, Sela M, Yarden Y. Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. *Adv Cancer Res*. 2000;**77**:25-79.

87. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell*. 1990;**61**:203-212.

88. Alroy I, Yarden Y. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett*. 1997;**410**:83-86.

89. Roussidis AE, Theocharis AD, Tzanakakis GN, Karamanos NK. The Importance of c-Kit and PDGF Receptors as Potential Targets for Molecular Therapy in Breast Cancer. *Curr Med Chem*. 2007;**14**:735-743.

90. Bhargava R, Gerald WL, Li AR, et al. EGFR gene amplification in breast cancer: correlation with epidermal growth factor receptor mRNA and protein expression and HER-2 status and absence of EGFR-activating mutations. *Mod Pathol*. 2005;**18**:1027-1033.

91. Quintela I, Corte MD, Allende MT, et al. Expression and prognostic value of EGFR in invasive breast cancer. *Oncol Rep*. 2005;**14**:1655-1663.

92. Woodburn JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther*. 1999;**82**:241-250.

93. Normanno N, Ciardiello F, Brandt R, Salomon DS. Epidermal growth factor-related peptides in the pathogenesis of human breast cancer. *Breast Cancer Res Treat*. 1994;**29**:11-27.

94. Faratian D, Bartlett J. Predictive markers in breast cancer--the future. *Histopathology*. 2008;**52**:91-98.

95. Fox SB, Smith K, Hollyer J, Greenall M, Hastrich D, Harris AL. The epidermal growth factor receptor as a prognostic marker: results of 370 patients and review of 3009 patients. *Breast Cancer Res Treat*. 1994;**29**:41-49.

96. Klijn JG, Berns PM, Schmitz PI, Foekens JA. The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr Rev*. 1992;**13**:3-17.

97. Heldin CH, Westermark B. Growth factors: mechanism of action and relation to oncogenes. *Cell*. 1984;**37**:9-20.

98. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol*. 1995;**19**:183-232.

99. Tsutsui S, Ohno S, Murakami S, Hachitanda Y, Oda S. Prognostic value of epidermal growth factor receptor (EGFR) and its relationship to the estrogen receptor status in 1029 patients with breast cancer. *Breast Cancer Res Treat*. 2002;**71**:67-75.

100. Kim ES, Khuri FR, Herbst RS. Epidermal growth factor receptor biology (IMC-C225). *Curr Opin Oncol*. 2001;**13**:506-513.

101. Arnes JB, Begin LR, Stefansson I, et al. Expression of epidermal growth factor receptor in relation to BRCA1 status, basal-like markers and prognosis in breast cancer. *J Clin Pathol*. 2009;**62**:139-146.

102. Nicholson RI, McClelland RA, Finlay P, et al. Relationship between EGF-R, c-erbB-2 protein expression and Ki67 immunostaining in breast cancer and hormone sensitivity. *Eur J Cancer*. 1993;**29A**:1018-1023.

103. Nicholson S, Sainsbury JR, Halcrow P, Chambers P, Farndon JR, Harris AL. Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer. *Lancet*. 1989;**1**:182-185.

104. Nicholson S, Wright C, Sainsbury JR, et al. Epidermal growth factor receptor (EGFr) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure. *J Steroid Biochem Mol Biol*. 1990;**37**:811-814.

105. Deming SL, Nass SJ, Dickson RB, Trock BJ. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. *Br J Cancer*. 2000;**83**:1688-1695.

106. Fallenius AG, Franzen SA, Auer GU. Predictive value of nuclear DNA content in breast cancer in relation to clinical and morphologic factors. A retrospective study of 227 consecutive cases. *Cancer*. 1988;**62**:521-530.

107. Ingvarsson S. Genetics of breast cancer. *Drugs Today (Barc)*. 2004;**40**:991-1002.

108. Horlings HM, Lai C, Nuyten DS, et al. Integration of DNA copy number alterations and prognostic gene expression signatures in breast cancer patients. *Clin Cancer Res*.**16**:651-663.

109. Ross JS, Linette GP, Stec J, Ross MS, Anwar S, Boguniewicz A. DNA ploidy and cell cycle analysis in breast cancer. *Am J Clin Pathol*. 2003;**120 Suppl**:S72-84.

110. King RJ. William L. McGuire Memorial Symposium. Estrogen and progestin effects in human breast carcinogenesis. *Breast Cancer Res Treat*. 1993;**27**:3-15.

111. van Agthoven T, Timmermans M, Foekens JA, Dorssers LC, Henzen-Logmans SC. Differential expression of estrogen, progesterone, and epidermal growth factor receptors in normal, benign, and malignant human breast tissues using dual staining immunohistochemistry. *Am J Pathol*. 1994;**144**:1238-1246.

112. Horwitz KB. Mechanisms of hormone resistance in breast cancer. *Breast Cancer Res Treat*. 1993;**26**:119-130.

113. Doisneau-Sixou SF, Sergio CM, Carroll JS, Hui R, Musgrove EA, Sutherland RL. Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. *Endocr Relat Cancer*. 2003;**10**:179-186.

114. Foster JS, Henley DC, Ahamed S, Wimalasena J. Estrogens and cell-cycle regulation in breast cancer. *Trends Endocrinol Metab*. 2001;**12**:320-327.

115. Platet N, Cathiard AM, Gleizes M, Garcia M. Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. *Crit Rev Oncol Hematol*. 2004;**51**:55-67.

116. Skildum A, Faivre E, Lange CA. Progesterone receptors induce cell cycle progression via activation of mitogen-activated protein kinases. *Mol Endocrinol*. 2005;**19**:327-339.

117. Elledge RM, Osborne CK. Oestrogen receptors and breast cancer. *Bmj*. 1997;**314**:1843-1844.

118. Lal P, Tan LK, Chen B. Correlation of HER-2 status with estrogen and progesterone receptors and histologic features in 3,655 invasive breast carcinomas. *Am J Clin Pathol*. 2005;**123**:541-546.

119. Amend K, Hicks D, Ambrosone CB. Breast cancer in African-American women: differences in tumor biology from European-American women. *Cancer Res*. 2006;**66**:8327-8330.

120. Ikpatt OF, Xu J, Kramtsov A, Ndoma-Egba R, Perou C, Olufunmilayo Ol, editors. Hormone receptor negative and basal-like subtypes are overrepresented in invasive breast carcinoma from women of African ancestry. AACR Annual Meeting; 2005 April 18; Orange County, California: Proc Amer Assoc Cancer Res; 46:[Abstract 2550].

121. Mbonde MP, Amir H, Akslen LA, Kitinya JN. Expression of oestrogen and progesterone receptors, Ki-67, p53 and BCL-2 proteins, cathepsin D, urokinase plasminogen activator and urokinase plasminogen activator-receptors in carcinomas of the female breast in an African population. *East Afr Med J*. 2001;**78**:360-365.

122. Nyagol J, Nyong'o A, Byakika B, et al. Routine assessment of hormonal receptor and her-2/neu status underscores the need for more therapeutic targets in Kenyan women with breast cancer. *Anal Quant Cytol Histol*. 2006;**28**:97-103.

123. Adebamowo CA, Famooto A, Ogundiran TO, Aniagwu T, Nkwodimmah C, Akang EE. Immunohistochemical and molecular subtypes of breast cancer in Nigeria. *Breast Cancer Res Treat*. 2008;**110**:183-188.

124. Awadelkarim KD, Arizzi C, Elamin EO, et al. Pathological, clinical and prognostic characteristics of breast cancer in Central Sudan versus Northern Italy: implications for breast cancer in Africa. *Histopathology*. 2008;**52**:445-456.

125. Krajewski S, Krajewska M, Turner BC, et al. Prognostic significance of apoptosis regulators in breast cancer. *Endocr Relat Cancer*. 1999;**6**:29-40.

126. Yang Q, Sakurai T, Yoshimura G, et al. Prognostic value of Bcl-2 in invasive breast cancer receiving chemotherapy and endocrine therapy. *Oncology Reports*. 2003;**10**:121-125.

127. Murphy KL, Kittrell FS, Gay JP, Jager R, Medina D, Rosen JM. Bcl-2 expression delays mammary tumor development in dimethylbenz(a)anthracene-treated transgenic mice. *Oncogene*. 1999;**18**:6597-6604.

128. Haldar S, Negrini M, Monne M, Sabbioni S, Croce CM. Down-regulation of bcl-2 by p53 in breast cancer cells. *Cancer Res*. 1994;**54**:2095-2097.

129. Daidone MG, Luisi A, Veneroni S, Benini E, Silvestrini R. Clinical studies of Bcl-2 and treatment benefit in breast cancer patients. *Endocrine-Related Cancer*. 1999;**6**:61-68.

130. Dowsett M, Archer C, Assersohn L, et al. Clinical studies of apoptosis and proliferation in breast cancer. *Endocr Relat Cancer*. 1999;**6**:25-28.

131. Cummings MC, Winterford CM, Walker NI. Apoptosis. *Am J Surg Pathol*. 1997;**21**:88-101.

132. Hartwell LH, Kastan MB. Cell cycle control and cancer. *Science*. 1994;**266**:1821-1828.

133. Liu S, Edgerton SM, Moore DH, 2nd, Thor AD. Measures of cell turnover (proliferation and apoptosis) and their association with survival in breast cancer. *Clin Cancer Res*. 2001;**7**:1716-1723.

134. Lipponen P, Aaltomaa S, Kosma VM, Syrjanen K. Apoptosis in breast cancer as related to histopathological characteristics and prognosis. *Eur J Cancer*. 1994;**30A**:2068-2073.

135. Lipponen P. Apoptosis in breast cancer: relationship with other pathological parameters. *Endocr Relat Cancer*. 1999;**6**:13-16.

136. Sauer G, Deissler H. Angiogenesis: prognostic and therapeutic implications in gynecologic and breast malignancies. *Current opinion in obstetrics & gynecology*. 2003;**15**:45-49.

137. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;**285**:1182-1186.

138. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst*. 2002;**94**:883-893.

139. Morabito A, Sarmiento R, Bonginelli P, Gasparini G. Antiangiogenic strategies, compounds, and early clinical results in breast cancer. *Crit Rev Oncol Hematol*. 2004;**49**:91-107.

140. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nature reviews*. 2003;**3**:401-410.

141. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*. 1996;**86**:353-364.

142. Naumov GN, Bender E, Zurakowski D, et al. A model of human tumor dormancy: an angiogenic switch from the nonangiogenic phenotype. *J Natl Cancer Inst*. 2006;**98**:316-325.

143. Watnick RS, Cheng YN, Rangarajan A, Ince TA, Weinberg RA. Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. *Cancer Cell*. 2003;**3**:219-231.

144. Folkman J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst*. 1990;**82**:4-6.

145. Fidler IJ, Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell*. 1994;**79**:185-188.

146. Gasparini G. Clinical significance of determination of surrogate markers of angiogenesis in breast cancer. *Crit Rev Oncol Hematol*. 2001;**37**:97-114.

147. Fox SB, Generali DG, Harris AL. Breast tumour angiogenesis. *Breast Cancer Res*. 2007;**9**:216.

148. Skobe M, Rockwell P, Goldstein N, Vosseler S, Fusenig NE. Halting angiogenesis suppresses carcinoma cell invasion. *Nat Med*. 1997;**3**:1222-1227.

149. Ferrara N, Houck K, Jakeman L, Leung DW. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev*. 1992;**13**:18-32.

150. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol*. 2002;**20**:4368-4380.

151. Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med*. 1999;**5**:1359-1364.

152. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol*. 1995;**146**:1029-1039.

153. Gasparini G. Prognostic value of vascular endothelial growth factor in breast cancer. *Oncologist*. 2000;**5 Suppl 1**:37-44.

154. Zhang J, Lu A, Beech D, Jiang B, Lu Y. Suppression of breast cancer metastasis through the inhibition of VEGF-mediated tumor angiogenesis. *Cancer therapy*. 2007;**5**:273-286.

155. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Molecular and cellular biology*. 2001;**21**:3995-4004.

156. Kumar R, Yarmand-Bagheri R. The role of HER2 in angiogenesis. *Seminars in oncology*. 2001;**28**:27-32.

157. Volpert OV, Stellmach V, Bouck N. The modulation of thrombospondin and other naturally occurring inhibitors of angiogenesis during tumor progression. *Breast Cancer Res Treat*. 1995;**36**:119-126.

158. Mukhopadhyay D, Tsiokas L, Sukhatme VP. Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res*. 1995;**55**:6161-6165.

159. Ravi R, Mookerjee B, Bhujwalla ZM, et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev*. 2000;**14**:34-44.

160. Schneider BP, Miller KD. Angiogenesis of breast cancer. *J Clin Oncol*. 2005;**23**:1782-1790.

161. Horak ER, Leek R, Klenk N, et al. Angiogenesis, assessed by platelet/endothelial cell adhesion molecule antibodies, as indicator of node metastases and survival in breast cancer. *Lancet*. 1992;**340**:1120-1124.

162. Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst*. 1992;**84**:1875-1887.

163. Wittekind C, Neid M. Cancer invasion and metastasis. *Oncology*. 2005;**69 Suppl 1**:14-16.

164. McSherry EA, Donatello S, Hopkins AM, McDonnell S. Molecular basis of invasion in breast cancer. *Cell Mol Life Sci*. 2007;**64**:3201-3218.

165. Wang W, Goswami S, Sahai E, Wyckoff JB, Segall JE, Condeelis JS. Tumor cells caught in the act of invading: their strategy for enhanced cell motility. *Trends Cell Biol*. 2005;**15**:138-145.

166. Chang HY, Sneddon JB, Alizadeh AA, et al. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS Biol*. 2004;**2**:E7.

167. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature*. 2007;**449**:557-563.

168. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature*. 2001;**411**:375-379.

169. Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. *Nature reviews*. 2003;**3**:362-374.

170. Wolf K, Mazo I, Leung H, et al. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol*. 2003;**160**:267-277.

171. Brooks PC, Stromblad S, Sanders LC, et al. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell*. 1996;**85**:683-693.

172. Semb H, Christofori G. The tumor-suppressor function of E-cadherin. *Am J Hum Genet*. 1998;**63**:1588-1593.

173. Huber MA, Kraut N, Beug H. Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol*. 2005;**17**:548-558.

174. Geiger TR, Peeper DS. Metastasis mechanisms. *Biochim Biophys Acta*. 2009;**1796**:293-308.

175. Weinberg RA. *Moving out: Invasion and Metastasis*. *The biology of cancer*. New York: Garland Science, Taylor & Francis Group, LLC; 2007. p. 616.

176. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nature reviews*. 2002;**2**:442-454.

177. Schneider C, Fehr MK, Steiner RA, Hagen D, Haller U, Fink D. Frequency and distribution pattern of distant metastases in breast cancer patients at the time of primary presentation. *Arch Gynecol Obstet*. 2003;**269**:9-12.

178. van 't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*. 2002;**415**:530-536.

179. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell*. 2003;**3**:537-549.

180. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nature reviews*. 2002;**2**:161-174.

181. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: markers and models. *Nature reviews*. 2005;**5**:591-602.

182. Weigelt B, Peterse JL, van't Veer LJ. Breast Cancer Metastasis: Markers and Models Clinical Features of Breast Cancer Metastasis. *Nature Publishing Group*. 2005; http://www.medscape.com/viewarticle/510221_2.

183. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. 2008;**133**:704-715.

184. Pece S, Tosoni D, Confalonieri S, et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. *Cell*. 2010;**140**:62-73.

185. Abraham BK, Fritz P, McClellan M, Hauptvogel P, Athelogou M, Brauch H. Prevalence of CD44+/CD24-/low cells in breast cancer may not be associated with clinical outcome but may favor distant metastasis. *Clin Cancer Res*. 2005;**11**:1154-1159.

186. Glinsky GV, Berezovska O, Glinskii AB. Microarray analysis identifies a death-from-cancer signature predicting therapy failure in patients with multiple types of cancer. *J Clin Invest*. 2005;**115**:1503-1521.

187. Calabrese C, Poppleton H, Kocak M, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell*. 2007;**11**:69-82.

188. Eyler CE, Rich JN. Survival of the fittest: cancer stem cells in therapeutic resistance and angiogenesis. *J Clin Oncol*. 2008;**26**:2839-2845.

189. Bao S, Wu Q, Sathornsumetee S, et al. Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res*. 2006;**66**:7843-7848.

190. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A*. 2003;**100**:3983-3988.

191. Cariati M, Purushotham AD. Stem cells and breast cancer. *Histopathology*. 2008;**52**:99-107.

192. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science*. 1977;**197**:461-463.

193. Tanei T, Morimoto K, Shimazu K, et al. Association of breast cancer stem cells identified by aldehyde dehydrogenase 1 expression with resistance to sequential Paclitaxel and epirubicin-based chemotherapy for breast cancers. *Clin Cancer Res*. 2009;**15**:4234-4241.

194. Ginestier C, Hur MH, Charafe-Jauffret E, et al. ALDH1 Is a Marker of Normal and Malignant Human Mammary Stem Cells and a Predictor of Poor Clinical Outcome. *Cell Stem Cell*. 2007;**1**:555-567.

195. Kakarala M, Wicha MS. Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. *J Clin Oncol*. 2008;**26**:2813-2820.

196. Damonte P, Hodgson JG, Chen JQ, Young LJ, Cardiff RD, Borowsky AD. Mammary carcinoma behavior is programmed in the precancer stem cell. *Breast Cancer Res*. 2008;**10**:R50.

197. Frank NY, Schatton T, Frank MH. The therapeutic promise of the cancer stem cell concept. *J Clin Invest*. 2010;**120**:41-50.

198. Ginestier C, Liu S, Diebel ME, et al. CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts. *J Clin Invest*.**120**:485-497.

199. Marcato P, Dean CA, Giacomantonio CA, Lee PW. Oncolytic reovirus effectively targets breast cancer stem cells. *Mol Ther*. 2009;**17**:972-979.

200. Kordon EC, Smith GH. An entire functional mammary gland may comprise the progeny from a single cell. *Development (Cambridge, England)*. 1998;**125**:1921-1930.

201. Dontu G, Abdallah WM, Foley JM, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev*. 2003;**17**:1253-1270.

202. Fillmore CM, Kuperwasser C. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res*. 2008;**10**:R25.

203. Charafe-Jauffret E, Ginestier C, Iovino F, et al. Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature. *Cancer Res*. 2009;**69**:1302-1313.

204. Arnes JB, Collett K, Akslen LA. Independent prognostic value of the basal-like phenotype of breast cancer and associations with EGFR and candidate stem cell marker BMI-1. *Histopathology*. 2008;**52**:370-380.

205. Douville J, Beaulieu R, Balicki D. ALDH1 as a Functional Marker of Cancer Stem and Progenitor Cells. *Stem Cells Dev*. 2008.

206. Dimri GP, Martinez JL, Jacobs JJ, et al. The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells. *Cancer Res*. 2002;**62**:4736-4745.

207. Datta S, Hoenerhoff MJ, Bommi P, et al. Bmi-1 cooperates with H-Ras to transform human mammary epithelial cells via dysregulation of multiple growth-regulatory pathways. *Cancer Res*. 2007;**67**:10286-10295.

208. Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM. Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. *Cell*. 1991;**65**:753-763.

209. Caldas C, Aparicio S. Cell memory and cancer--the story of the trithorax and Polycomb group genes. *Cancer Metastasis Rev*. 1999;**18**:313-329.

210. Liu S, Dontu G, Mantle ID, et al. Hedgehog signaling and Bmi-1 regulate self-renewal of normal and malignant human mammary stem cells. *Cancer Res*. 2006;**66**:6063-6071.

211. Jacobs JJ, van Lohuizen M. Polycomb repression: from cellular memory to cellular proliferation and cancer. *Biochim Biophys Acta*. 2002;**1602**:151-161.

212. Orlando V. Polycomb, epigenomes, and control of cell identity. *Cell*. 2003;**112**:599-606.

213. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature*. 1999;**397**:164-168.

214. Kim JH, Yoon SY, Jeong SH, et al. Overexpression of Bmi-1 oncoprotein correlates with axillary lymph node metastases in invasive ductal breast cancer. *Breast*. 2004;**13**:383-388.

215. Balicki D. Moving Forward in Human Mammary Stem Cell Biology and Breast Cancer Prognostication Using ALDH1. *Cell Stem Cell*. 2007;**1**:485-487.

216. Chute JP, Muramoto GG, Whitesides J, et al. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 2006;**103**:11707-11712.

217. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nature reviews*. 2008;**8**:755-768.

218. Sophos NA, Vasiliou V. Aldehyde dehydrogenase gene superfamily: the 2002 update. *Chemico-biological interactions*. 2003;**143-144**:5-22.

219. Yoshida A, Rzhetsky A, Hsu LC, Chang C. Human aldehyde dehydrogenase gene family. *European journal of biochemistry / FEBS*. 1998;**251**:549-557.

220. Retinal dehyrogenase 1- Homo sapiens (Human). *UniProt Consortium*. 2008 http://www.uniprot.org/uniprot/P00352.

221. Kastan MB, Schlaffer E, Russo JE, Colvin OM, Civin CI, Hilton J. Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells. *Blood*. 1990;**75**:1947-1950.

222. Sladek NE, Kollander R, Sreerama L, Kiang DT. Cellular levels of aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) as predictors of therapeutic responses to cyclophosphamide-based chemotherapy of breast cancer: a retrospective study. Rational

individualization of oxazaphosphorine-based cancer chemotherapeutic regimens. *Cancer Chemother Pharmacol*. 2002;**49**:309-321.

223. Charafe-Jauffret E, Ginestier C, Iovino F, et al. Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor clinical outcome in inflammatory breast cancer. *Clin Cancer Res*. 2010;**16**:45-55.

224. Sotiriou C, Neo SY, McShane LM, et al. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci USA*. 2003;**100**:10393-10398.

225. Sorlie T, Tibshirani R, Parker J, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci USA*. 2003;**100**:8418-8423.

226. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA*. 2001;**98**:10869-10874.

227. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature*. 2000;**406**:747-752.

228. Sorlie T, Wang Y, Xiao C, et al. Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different plarforms. *BMC Gemonics*. 2006;**17**:127.

229. Tang P, Skinner KA, Hicks DG. Molecular classification of breast carcinomas by immunohistochemical analysis: are we ready? *Diagn Mol Pathol*. 2009;**18**:125-132.

230. Rakha EA, El-Sayed ME, Reis-Filho J, Ellis IO. Patho-biological aspects of basal-like breast cancer. *Breast Cancer Res Treat*. 2009;**113**:411-422.

231. Carey LA, Perou CM, Livasy CA, et al. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *Jama*. 2006;**295**:2492-2502.

232. Nielsen TO, Hsu FD, Jensen K, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. *Clin Cancer Res*. 2004;**10**:5367-5374.

233. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol*. 2008;**26**:2568-2581.

234. Ribeiro-Silva A, Ramalho LN, Garcia SB, Brandao DF, Chahud F, Zucoloto S. p63 correlates with both BRCA1 and cytokeratin 5 in invasive breast carcinomas: further evidence for the pathogenesis of the basal phenotype of breast cancer. *Histopathology*. 2005;**47**:458-466.

235. Barbareschi M, Pecciarini L, Cangi MG, et al. p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells of the human breast. *American Journal of Surgical Pathology*. 2001;**25**:1054-1060.

236. Matos I, Dufloth R, Alvarenga M, Zeferino LC, Schmitt F. p63, cytokeratin 5, and P-cadherin: three molecular markers to distinguish basal phenotype in breast carcinomas. *Virchows Arch*. 2005;**447**:688-694.

237. van de Rijn M, Perou CM, Tibshirani R, et al. Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. *Am J Pathol*. 2002;**161**:1991-1996.

238. Chang HY, Nuyten DS, Sneddon JB, et al. Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. *Proc Natl Acad Sci U S A*. 2005;**102**:3738-3743.

239. Bocker W, Moll R, Poremba C, et al. Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. *Lab Invest*. 2002;**82**:737-746.

240. Dontu G, El-Ashry D, Wicha MS. Breast cancer, stem/progenitor cells and the estrogen receptor. *Trends Endocrinol Metab*. 2004;**15**:193-197.

241. Yehiely F, Moyano JV, Evans JR, Nielsen TO, Cryns VL. Deconstructing the molecular portrait of basal-like breast cancer. *Trends Mol Med*. 2006;**12**:537-544.

242. Stingl J, Eirew P, Ricketson I, et al. Purification and unique properties of mammary epithelial stem cells. *Nature*. 2006;**439**:993-997.

243. Xu X, Wagner KU, Larson D, et al. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat Genet*. 1999;**22**:37-43.

244. Sihto H, Lundin J, Lehtimaki T, et al. Molecular subtypes of breast cancers detected in mammography screening and outside of screening. *Clin Cancer Res*. 2008;**14**:4103-4110.

245. Kim MJ, Ro JY, Ahn SH, Kim HH, Kim SB, Gong G. Clinicopathologic significance of the basal-like subtype of breast cancer: a comparison with hormone receptor and Her2/neu-overexpressing phenotypes. *Hum Pathol*. 2006;**37**:1217-1226.

246. Arnes JB, Brunet JS, Stefansson I, et al. Placental cadherin and the basal epithelial phenotype of BRCA1-related breast cancer. *Clin Cancer Res*. 2005;**11**:4003-4011.

247. Rodriguez-Pinilla SM, Sarrio D, Honrado E, et al. Prognostic significance of basal-like phenotype and fascin expression in node-negative invasive breast carcinomas. *Clin Cancer Res*. 2006;**12**:1533-1539.

248. Diaz LK, Cryns VL, Symmans WF, Sneige N. Triple negative breast carcinoma and the basal phenotype: from expression profiling to clinical practice. *Advances in anatomic pathology*. 2007;**14**:419-430.

249. Millikan RC, Newman B, Tse CK, et al. Epidemiology of basal-like breast cancer. *Breast Cancer Res Treat*. 2008;**109**:123-139.

250. Fadare O, Tavassoli FA. The phenotypic spectrum of basal-like breast cancers: a critical appraisal. *Advances in anatomic pathology*. 2007;**14**:358-373.

251. Seewaldt VL, Scott V. Images in clinical medicine. Rapid progression of basal-type breast cancer. *N Engl J Med*. 2007;**356**:e12.

252. Collett K, Stefansson IM, Eide J, et al. A Basal epithelial phenotype is more frequent in interval breast cancers compared with screen detected tumors. *Cancer Epidemiol Biomarkers Prev*. 2005;**14**:1108-1112.

253. Foulkes WD, Brunet JS, Stefansson IM, et al. The prognostic implication of the basal-like (cyclin E high/p27 low/p53+/glomeruloid-microvascular-proliferation+) phenotype of BRCA1-related breast cancer. *Cancer Res*. 2004;**64**:830-835.

254. Straume O, Chappuis PO, Salvesen HB, et al. Prognostic importance of glomeruloid microvascular proliferation indicates an aggressive angiogenic phenotype in human cancers. *Cancer Res*. 2002;**62**:6808-6811.

255. Hicks DG, Short SM, Prescott NL, et al. Breast cancers with brain metastases are more likely to be estrogen receptor negative, express the basal cytokeratin CK5/6, and overexpress HER2 or EGFR. *Am J Surg Pathol*. 2006;**30**:1097-1104.

256. Troester MA, Hoadley KA, Sorlie T, et al. Cell-type-specific responses to chemotherapeutics in breast cancer. *Cancer Res*. 2004;**64**:4218-4226.

257. Charafe-Jauffret E, Ginestier C, Monville F, et al. Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene*. 2006;**25**:2273-2284.

258. Siziopikou KP, Cobleigh M. The basal subtype of breast carcinomas may represent the group of breast tumors that could benefit from EGFR-targeted therapies. *Breast*. 2007;**16**:104-107.

259. Carey LA, Dees EC, Sawyer L, et al. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. *Clin Cancer Res*. 2007;**13**:2329-2334.

260. Goldstein NS, Decker D, Severson D, et al. Molecular classification system identifies invasive breast carcinoma patients who are most likely and those who are least likely to achieve a complete pathologic response after neoadjuvant chemotherapy. *Cancer*. 2007;**110**:1687-1696.

261. Rouzier R, Perou CM, Symmans WF, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clin Cancer Res*. 2005;**11**:5678-5685.

262. Rody A, Karn T, Solbach C, et al. The erbB2+ cluster of the intrinsic gene set predicts tumor response of breast cancer patients receiving neoadjuvant chemotherapy with docetaxel, doxorubicin and cyclophosphamide within the GEPARTRIO trial. *Breast*. 2007;**16**:235-240.

263. Ribeiro-Silva A, Ribeiro do Vale F, Zucoloto S. Vascular endothelial growth factor expression in the basal subtype of breast carcinoma. *Am J Clin Pathol*. 2006;**125**:512-518.

264. Finn RS, Dering J, Ginther C, et al. Dasatinib, an orally active small molecule inhibitor of both the src and abl kinases, selectively inhibits growth of basal-type/"triple-negative" breast cancer cell lines growing in vitro. *Breast Cancer Res Treat*. 2007;**105**:319-326.

265. De Soto JA, Deng CX. PARP-1 inhibitors: are they the long-sought genetically specific drugs for BRCA1/2-associated breast cancers? *Int J Med Sci*. 2006;**3**:117-123.

266. Farmer H, McCabe N, Lord CJ, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature*. 2005;**434**:917-921.

267. Yang XR, Sherman ME, Rimm DL, et al. Differences in risk factors for breast cancer molecular subtypes in a population-based study. *Cancer Epidemiol Biomarkers Prev*. 2007;**16**:439-443.

268. Ferretti G, Felici A, Papaldo P, Fabi A, Cognetti F. HER2/neu role in breast cancer: from a prognostic foe to a predictive friend. *Current opinion in obstetrics & gynecology*. 2007;**19**:56-62.

269. Peppercorn J, Perou CM, Carey LA. Molecular subtypes in breast cancer evaluation and management: divide and conquer. *Cancer Invest*. 2008;**26**:1-10.

270. Abd El-Rehim DM, Ball G, Pinder SE, et al. High-throughput protein expression analysis using tissue microarray technology of a large well-characterised series identifies biologically distinct classes of breast cancer confirming recent cDNA expression analyses. *Int J Cancer*. 2005;**116**:340-350.

271. Elston CW, Ellis IO, Pinder SE. Pathological prognostic factors in breast cancer. *Critical Reviews in Oncology Hematology*. 1999;**31**:209-223.

272. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol*. 2007;**25**:5287-5312.

273. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol*. 1998;**11**:155-168.

274. Clark GM. Do we really need prognostic factors for breast cancer? *Breast Cancer Res Treat*. 1994;**30**:117-126.

275. Fitzgibbons PL, Page DL, Weaver D, et al. Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med*. 2000;**124**:966-978.

276. Anders CK, Hsu DS, Broadwater G, et al. Young age at diagnosis correlates with worse prognosis and defines a subset of breast cancers with shared patterns of gene expression. *J Clin Oncol*. 2008;**26**:3324-3330.

277. Walker RA, Lees E, Webb MB, Dearing SJ. Breast carcinomas occurring in young women (< 35 years) are different. *Br J Cancer*. 1996;**74**:1796-1800.

278. Soerjomataram I, Louwman MW, Ribot JG, Roukema JA, Coebergh JW. An overview of prognostic factors for long-term survivors of breast cancer. *Breast Cancer Res Treat*. 2008;**107**:309-330.

279. Goldhirsch A, Glick JH, Gelber RD, Senn HJ. Meeting highlights: International Consensus Panel on the Treatment of Primary Breast Cancer. *J Natl Cancer Inst*. 1998;**90**:1601-1608.

280. Goldhirsch A, Glick JH, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Meeting highlights: international expert consensus on the primary therapy of early breast cancer 2005. *Ann Oncol*. 2005;**16**:1569-1583.

281. Fregene A, Newman LA. Breast cancer in sub-Saharan Africa: how does it relate to breast cancer in African-American women? *Cancer*. 2005;**103**:1540-1550.

282. Ikpatt OF, Kuopio T, Ndoma-Egba R, Collan Y. Breast cancer in Nigeria and Finland: epidemiological, clinical and histological comparison. *Anticancer Res*. 2002;**22**:3005-3012.

283. Curtis E, Quale C, Haggstrom D, Smith-Bindman R. Racial and ethnic differences in breast cancer survival: how much is explained by screening, tumor severity, biology, treatment, comorbidities, and demographics? *Cancer*. 2008;**112**:171-180.

284. Henson DE, Chu KC, Levine PH. Histologic grade, stage, and survival in breast carcinoma: comparison of African American and Caucasian women. *Cancer*. 2003;**98**:908-917.

285. Henson DE, Patierno SR. Breast cancer aggressiveness and racial disparity. *Breast Cancer Res Treat*. 2004;**87**:291-296.

286. Newman LA, Griffith KA, Jatoi I, Simon MS, Crowe JP, Colditz GA. Meta-analysis of survival in African American and white American patients with breast cancer: ethnicity compared with socioeconomic status. *J Clin Oncol*. 2006;**24**:1342-1349.

287. Galea MH, Blamey RW, Elston CE, Ellis IO. The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res Treat*. 1992;**22**:207-219.

288. Ellis IO, Galea M, Broughton N, Locker A, Blamey RW, Elston CW. Pathological Prognostic Factors in Breast-Cancer .2. Histological Type - Relationship with Survival in a Large Study with Long-Term Follow-Up. *Histopathology*. 1992;**20**:479-489.

289. Dalton LW, Page DL, Dupont WD. Histologic grading of breast carcinoma. A reproducibility study. *Cancer*. 1994;**73**:2765-2770.

290. Robbins P, Pinder S, de Klerk N, et al. Histological grading of breast carcinomas: a study of interobserver agreement. *Hum Pathol*. 1995;**26**:873-879.

291. Elston CW, Ellis IO. Pathological prognostic factors in breast-cancer .1. The value of histological grade in breast-cancer - experience from a large study with long-term follow-up. *Histopathology*. 1991;**19**:403-410.

292. Goldhirsch A, Ingle JN, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Thresholds for therapies: highlights of the St Gallen International Expert Consensus on the primary therapy of early breast cancer 2009. *Ann Oncol*. 2009;**20**:1319-1329.

293. Perry N, Broeders M, de Wolf C, Tornberg S, Holland R, von Karsa L. European guidelines for quality assurance in breast cancer screening and diagnosis. Fourth edition-summary document. *Ann Oncol*. 2008;**19**:614-622.

294. Goldhirsch A, Wood WC, Gelber RD, Coates AS, Thurlimann B, Senn HJ. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. *Ann Oncol*. 2007;**18**:1133-1144.

295. UICC. How to use the TNM classification international union against cancer. 2010; http://www.uicc.org/index.php?option=com_content&task=view&id=14296&Itemid=428.

296. Lee AH, Ellis IO. The nottingham prognostic index for invasive carcinoma of the breast. *Pathol Oncol Res*. 2008;**14**:113-115.

297. Sturgeon CM, Duffy MJ, Stenman UH, et al. National Academy of Clinical Biochemistry laboratory medicine practice guidelines for use of tumor markers in testicular, prostate, colorectal, breast, and ovarian cancers. *Clin Chem*. 2008;**54**:e11-79.

298. Osborne CK, Schiff R, Arpino G, Lee AS, Hilsenbeck VG. Endocrine responsiveness: understanding how progesterone receptor can be used to select endocrine therapy. *Breast*. 2005;**14**:458-465.

299. Grann VR, Troxel AB, Zojwalla NJ, Jacobson JS, Hershman D, Neugut AI. Hormone receptor status and survival in a population-based cohort of patients with breast carcinoma. *Cancer*. 2005;**103**:2241-2251.

300. Desmedt C, Sotiriou C. Proliferation: the most prominent predictor of clinical outcome in breast cancer. *Cell Cycle*. 2006;**5**:2198-2202.

301. van Diest PJ, Brugal G, Baak JP. Proliferation markers in tumours: interpretation and clinical value. *J Clin Pathol*. 1998;**51**:716-724.

302. Baak JP, van Diest PJ, Voorhorst FJ, et al. Prospective multicenter validation of the independent prognostic value of the mitotic activity index in lymph node-negative breast cancer patients younger than 55 years. *J Clin Oncol*. 2005;**23**:5993-6001.

303. Gerdes J, Li L, Schlueter C, et al. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. *Am J Pathol*. 1991;**138**:867-873.

304. Gasparini G, Pozza F, Meli S, Reitano M, Santini G, Bevilacqua P. Breast cancer cell kinetics: immunocytochemical determination of growth fractions by monoclonal antibody Ki-67 and correlation with flow cytometric S-phase and with some features of tumor aggressiveness. *Anticancer Res*. 1991;**11**:2015-2021.

305. Tewari M, Krishnamurthy A, Shukla HS. Predictive markers of response to neoadjuvant chemotherapy in breast cancer. *Surg Oncol*. 2008;**17**:301-311.

306. Langerod A, Zhao H, Borgan O, et al. TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. *Breast Cancer Res*. 2007;**9**:R30.

307. Takahashi S, Moriya T, Ishida T, et al. Prediction of breast cancer prognosis by gene expression profile of TP53 status. *Cancer Sci*. 2008;**99**:324-332.

308. Linderholm B, Lindh B, Tavelin B, Grankvist K, Henriksson R. p53 and vascular-endothelial-growth-factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. *Int J Cancer*. 2000;**89**:51-62.

309. Lacroix M, Toillon RA, Leclercq G. p53 and breast cancer, an update. *Endocr Relat Cancer*. 2006;**13**:293-325.

310. Kroger N, Milde-Langosch K, Riethdorf S, et al. Prognostic and predictive effects of immunohistochemical factors in high-risk primary breast cancer patients. *Clin Cancer Res*. 2006;**12**:159-168.

311. Chan SK, Hill ME, Gullick WJ. The role of the epidermal growth factor receptor in breast cancer. *J Mammary Gland Biol Neoplasia*. 2006;**11**:3-11.

312. Siziopikou KP, Ariga R, Proussaloglou KE, Gattuso P, Cobleigh M. The challenging estrogen receptor-negative/ progesterone receptor-negative/HER-2-negative patient: a promising candidate for epidermal growth factor receptor-targeted therapy? *Breast J*. 2006;**12**:360-362.

313. Baselga J, Albanell J, Ruiz A, et al. Phase II and tumor pharmacodynamic study of gefitinib in patients with advanced breast cancer. *J Clin Oncol*. 2005;**23**:5323-5333.

314. Burris HA, 3rd, Hurwitz HI, Dees EC, et al. Phase I safety, pharmacokinetics, and clinical activity study of lapatinib (GW572016), a reversible dual inhibitor of epidermal growth factor receptor tyrosine kinases, in heavily pretreated patients with metastatic carcinomas. *J Clin Oncol*. 2005;**23**:5305-5313.

315. Rampaul RS, Pinder SE, Wencyk PM, et al. Epidermal growth factor receptor status in operable invasive breast cancer: is it of any prognostic value? *Clin Cancer Res*. 2004;**10**:2578.

316. Tsutsui S, Kume M, Era S. Prognostic value of microvessel density in invasive ductal carcinoma of the breast. *Breast Cancer*. 2003;**10**:312-319.

317. de Jong JS, van Diest PJ, Baak JP. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. *Histopathology*. 2000;**36**:306-312.

318. Toi M, Inada K, Suzuki H, Tominaga T. Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression. *Breast Cancer Res Treat*. 1995;**36**:193-204.

319. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med*. 1991;**324**:1-8.

320. Nieto Y, Woods J, Nawaz F, et al. Prognostic analysis of tumour angiogenesis, determined by microvessel density and expression of vascular endothelial growth factor, in high-risk primary breast cancer patients treated with high-dose chemotherapy. *Br J Cancer*. 2007;**97**:391-397.

321. Uzzan B, Nicolas P, Cucherat M, Perret GY. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. *Cancer Res*. 2004;**64**:2941-2955.

322. Gasparini G, Toi M, Gion M, et al. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. *J Natl Cancer Inst*. 1997;**89**:139-147.

323. Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*. 2007;**13**:4429-4434.

324. Kang SP, Martel M, Harris LN. Triple negative breast cancer: current understanding of biology and treatment options. *Current opinion in obstetrics & gynecology*. 2008;**20**:40-46.

325. Rakha EA, El-Rehim DA, Paish C, et al. Basal phenotype identifies a poor prognostic subgroup of breast cancer of clinical importance. *Eur J Cancer*. 2006;**42**:3149-3156.

326. Doyle LA, Yang W, Abruzzo LV, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A*. 1998;**95**:15665-15670.

327. Patrawala L, Calhoun T, Schneider-Broussard R, Zhou J, Claypool K, Tang DG. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. *Cancer Res*. 2005;**65**:6207-6219.

328. Cariati M, Naderi A, Brown JP, et al. Alpha-6 integrin is necessary for the tumourigenicity of a stem cell-like subpopulation within the MCF7 breast cancer cell line. *Int J Cancer*. 2008;**122**:298-304.

329. Phillips TM, McBride WH, Pajonk F. The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst*. 2006;**98**:1777-1785.

330. Balic M, Lin H, Young L, et al. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clin Cancer Res*. 2006;**12**:5615-5621.

331. Sreerama L, Sladek NE. Cellular levels of class 1 and class 3 aldehyde dehydrogenases and certain other drug-metabolizing enzymes in human breast malignancies. *Clin Cancer Res*. 1997;**3**:1901-1914.

332. Rasheed ZA, Yang J, Wang Q, et al. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. *J Natl Cancer Inst*.**102**:340-351.

333. Anderson BO, Carlson RW, Kaufman CS, Kiel KD. Ensuring optimal interdisciplinary breast care in the United States. *Breast J*. 2009;**15**:569-570.

334. Boyle P. Global summit on mammographic screening. *Ann Oncol*. 2003;**14**:1159-1160.

335. Vargas HI, Anderson BO, Chopra R, et al. Diagnosis of breast cancer in countries with limited resources. *Breast J*. 2003;**9 Suppl 2**:S60-66.

336. Masood S, Vass L, Ibarra JA, Jr., et al. Breast pathology guideline implementation in low- and middle-income countries. *Cancer*. 2008;**113**:2297-2304.

337. Anderson BO, Braun S, Lim S, Smith RA, Taplin S, Thomas DB. Early detection of breast cancer in countries with limited resources. *Breast J*. 2003;**9 Suppl 2**:S51-59.

338. Igene H. Global health inequalities and breast cancer: an impending public health problem for developing countries. *Breast J*. 2008;**14**:428-434.

339. Anderson BO, Yip CH, Ramsey SD, et al. Breast cancer in limited-resource countries: health care systems and public policy. *Breast J*. 2006;**12 Suppl 1**:S54-69.

340. Eniu A, Carlson RW, Aziz Z, et al. Breast cancer in limited-resource countries: treatment and allocation of resources. *Breast J*. 2006;**12 Suppl 1**:S38-53.

341. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol*. 2007;**25**:118-145.

342. Gakwaya A, Galukande M, Luwaga A, et al. Breast cancer guidelines for Uganda (2nd Edition 2008). *Afr Health Sci*. 2008;**8**:126-132.

343. Jones BA, Kasl SV, Howe CL, et al. African-American/White differences in breast carcinoma: p53 alterations and other tumor characteristics. *Cancer*. 2004;**101**:1293-1301.

344. Nalwoga H, Odida M, Wabinga H. c-*erb*B-2 oncoprotein over-expression in breast cancer and its relationship to histology type and grade in a Uganda population. *East Afr Med J*. 2006;**83**:411-415.

345. Breast cancer guidelines for Uganda. *Afr Health Sci*. 2003;**3**:47-50.

346. The World Factbook. Washington, DC: Central Intelligence Agency. 2009; https://www.cia.gov/library/publications/the-world-factbook/geos/ug.html.

347. Population Council. Uganda 2000-2001: results from the Demographic and Health Survey. *Studies in family planning*. 2004;**35**:70-74.

348. Wabinga HR, Parkin DM, Wabwire-Mangen F, Mugerwa JW. Cancer in Kampala, Uganda, in 1989-91: changes in incidence in the era of AIDS. *Int J Cancer*. 1993;**54**:26-36.

349. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med*. 1998;**4**:844-847.

350. Stefansson IM, Salvesen HB, Akslen LA. Vascular proliferation is important for clinical progress of endometrial cancer. *Cancer Res*. 2006;**66**:3303-3309.

351. DakoCytomation. EGFR pharmDx Interpretation Manual DakoCytomation. 2006; http://pri.dako.com/08052_egfr_pharmdx_interpretation_manual.pdf.

352. Stefansson IM, Salvesen HB, Akslen LA. Prognostic impact of alterations in P-cadherin expression and related cell adhesion markers in endometrial cancer. *J Clin Oncol*. 2004;**22**:1242-1252.

353. Engelsen IB, Stefansson I, Akslen LA, Salvesen HB. Pathologic expression of p53 or p16 in preoperative curettage specimens identifies high-risk endometrial carcinomas. *American journal of obstetrics and gynecology*. 2006;**195**:979-986.

354. Ellis IO, Bartlett J, Dowsett M, et al. Best Practice No 176: Updated recommendations for HER2 testing in the UK. *J Clin Pathol*. 2004;**57**:233-237.

355. Ulivi P, Zoli W, Medri L, et al. c-kit and SCF expression in normal and tumor breast tissue. *Breast Cancer Res Treat*. 2004;**83**:33-42.

356. Chappuis PO, Donato E, Goffin JR, et al. Cyclin E expression in breast cancer: predicting germline BRCA1 mutations, prognosis and response to treatment. *Ann Oncol*. 2005;**16**:735-742.

357. Yuan ZQ, Begin LR, Wong N, et al. The effect of the I1307K APC polymorphism on the clinicopathological features and natural history of breast cancer. *Br J Cancer*. 1999;**81**:850-854.

358. Heimann R, Ferguson D, Gray S, Hellman S. Assessment of intratumoral vascularization (angiogenesis) in breast cancer prognosis. *Breast Cancer Res Treat*. 1998;**52**:147-158.

359. Vermeulen PB, Gasparini G, Fox SB, et al. Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. *Eur J Cancer*. 1996;**32A**:2474-2484.

360. Tischkowitz M, Brunet JS, Begin LR, et al. Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. *BMC cancer*. 2007;**7**:134.

361. Camp RL, Charette LA, Rimm DL. Validation of tissue microarray technology in breast carcinoma. *Lab Invest*. 2000;**80**:1943-1949.

362. Battifora H, Kopinski M. The influence of protease digestion and duration of fixation on the immunostaining of keratins. A comparison of formalin and ethanol fixation. *J Histochem Cytochem*. 1986;**34**:1095-1100.

363. Arnold MM, Srivastava S, Fredenburgh J, Stockard CR, Myers RB, Grizzle WE. Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. *Biotech Histochem*. 1996;**71**:224-230.

364. Ruiz C, Seibt S, Al Kuraya K, et al. Tissue microarrays for comparing molecular features with proliferation activity in breast cancer. *Int J Cancer*. 2006;**118**:2190-2194.

365. Sapino A, Marchio C, Senetta R, et al. Routine assessment of prognostic factors in breast cancer using a multicore tissue microarray procedure. *Virchows Arch*. 2006;**449**:288-296.

366. Mirlacher M, Kasper M, Storz M, et al. Influence of slide aging on results of translational research studies using immunohistochemistry. *Mod Pathol*. 2004;**17**:1414-1420.

367. Diaz LK, Sneige N. Estrogen receptor analysis for breast cancer: current issues and keys to increasing testing accuracy. *Advances in anatomic pathology*. 2005;**12**:10-19.

368. Shi SR, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. *J Histochem Cytochem*. 2001;**49**:931-937.

369. Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. *Am J Clin Pathol*. 2003;**120**:86-92.

370. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. Expression of bFGF/FGFR-1 and vascular proliferation related to clinicopathologic features and tumor progress in localized prostate cancer. *Virchows Arch*. 2006;**448**:68-74.

371. Halvorsen OJ, Haukaas S, Hoisaeter PA, Akslen LA. Independent prognostic importance of microvessel density in clinically localized prostate cancer. *Anticancer Res*. 2000;**20**:3791-3799.

372. Paulsen T, Aas T, Borresen AL, Varhaug JE, Lonning PE, Akslen LA. Angiogenesis does not predict clinical response to doxorubicin monotherapy in patients with locally advanced breast cancer. *Int J Cancer*. 1997;**74**:138-140.

373. Vermeulen PB, Gasparini G, Fox SB, et al. Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. *Eur J Cancer*. 2002;**38**:1564-1579.

374. Siitonen SM, Haapasalo HK, Rantala IS, Helin HJ, Isola JJ. Comparison of different immunohistochemical methods in the assessment of angiogenesis: lack of prognostic value in a group of 77 selected node-negative breast carcinomas. *Mod Pathol*. 1995;**8**:745-752.

375. Kumar S, Ghellal A, Li C, et al. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. *Cancer Res*. 1999;**59**:856-861.

376. Lopes N, Sousa B, Vieira D, Milanezi F, Schmitt F. Vessel density assessed by endoglin expression in breast carcinomas with different expression profiles. *Histopathology*. 2009;**55**:594-599.

377. Minhajat R, Mori D, Yamasaki F, Sugita Y, Satoh T, Tokunaga O. Organ-specific endoglin (CD105) expression in the angiogenesis of human cancers. *Pathol Int*. 2006;**56**:717-723.

378. Wang JM, Kumar S, Pye D, Haboubi N, al-Nakib L. Breast carcinoma: comparative study of tumor vasculature using two endothelial cell markers. *J Natl Cancer Inst*. 1994;**86**:386-388.

379. Behrem S, Zarkovic K, Eskinja N, Jonjic N. Endoglin is a better marker than CD31 in evaluation of angiogenesis in glioblastoma. *Croat Med J*. 2005;**46**:417-422.

380. Tanaka F, Otake Y, Yanagihara K, et al. Evaluation of angiogenesis in non-small cell lung cancer: comparison between anti-CD34 antibody and anti-CD105 antibody. *Clin Cancer Res*. 2001;**7**:3410-3415.

381. Dales JP, Garcia S, Bonnier P, et al. CD105 expression is a marker of high metastatic risk and poor outcome in breast carcinomas. Correlations between immunohistochemical analysis and long-term follow-up in a series of 929 patients. *Am J Clin Pathol*. 2003;**119**:374-380.

382. Bachmann IM, Puntervoll HE, Otte AP, Akslen LA. Loss of BMI-1 expression is associated with clinical progress of malignant melanoma. *Mod Pathol*. 2008;**21**:583-590.

383. Sabattini E, Bisgaard K, Ascani S, et al. The EnVision++ system: a new immunohistochemical method for diagnostics and research. Critical comparison with the APAAP, ChemMate, CSA, LABC, and SABC techniques. *J Clin Pathol*. 1998;**51**:506-511.

384. Aas T, Borresen AL, Geisler S, et al. Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat Med*. 1996;**2**:811-814.

385. Bachmann IM, Halvorsen OJ, Collett K, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. *J Clin Oncol*. 2006;**24**:268-273.

386. Collett K, Eide GE, Arnes J, et al. Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. *Clin Cancer Res*. 2006;**12**:1168-1174.

387. Straume O, Akslen LA. Alterations and prognostic significance of p16 and p53 protein expression in subgroups of cutaneous melanoma. *Int J Cancer*. 1997;**74**:535-539.

388. Van Hoef ME, Knox WF, Dhesi SS, Howell A, Schor AM. Assessment of tumour vascularity as a prognostic factor in lymph node negative invasive breast cancer. *Eur J Cancer*. 1993;**29A**:1141-1145.

389. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. Proliferation of immature tumor vessels is a novel marker of clinical progression in prostate cancer. *Cancer Res*. 2009;**69**:4708-4715.

390. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst*. 2005;**97**:1180-1184.

391. Rakha EA, Ellis IO. Triple-negative/basal-like breast cancer: review. *Pathology*. 2009;**41**:40-47.

392. Reis-Filho JS, Tutt AN. Triple negative tumours: a critical review. *Histopathology*. 2008;**52**:108-118.

393. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative

invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. *Cancer*. 2007;**109**:1721-1728.

394. Ihemelandu CU, Leffall LD, Jr., Dewitty RL, et al. Molecular breast cancer subtypes in premenopausal and postmenopausal African-American women: age-specific prevalence and survival. *J Surg Res*. 2007;**143**:109-118.

395. Harris LN, Broadwater G, Lin NU, et al. Molecular subtypes of breast cancer in relation to paclitaxel response and outcomes in women with metastatic disease: results from CALGB 9342. *Breast Cancer Res*. 2006;**8**:R66.

396. Morris GJ, Naidu S, Topham AK, et al. Differences in breast carcinoma characteristics in newly diagnosed African-American and Caucasian patients: a single-institution compilation compared with the National Cancer Institute's Surveillance, Epidemiology, and End Results database. *Cancer*. 2007;**110**:876-884.

397. Elledge RM, Clark GM, Chamness GC, Osborne CK. Tumor biologic factors and breast cancer prognosis among white, Hispanic, and black women in the United States. *J Natl Cancer Inst*. 1994;**86**:705-712.

398. Porter PL, Lund MJ, Lin MG, et al. Racial differences in the expression of cell cycle-regulatory proteins in breast carcinoma. *Cancer*. 2004;**100**:2533-2542.

399. Cunningham JE, Butler WM. Racial disparities in female breast cancer in South Carolina: clinical evidence for a biological basis. *Breast Cancer Res Treat*. 2004;**88**:161-176.

400. Martin DN, Boersma BJ, Yi M, et al. Differences in the tumor microenvironment between African-American and European-American breast cancer patients. *PLoS One*. 2009;**4**:e4531.

401. Rakha EA, El-Sayed ME, Green AR, Lee AH, Robertson JF, Ellis IO. Prognostic markers in triple-negative breast cancer. *Cancer*. 2007;**109**:25-32.

402. Phase II Trial of Cetuximab Alone and in Combination With Carboplatin in ER-Negative, PR-Negative, HER-2 Nonoverexpressing Metastatic Breast Cancer. Chapel Hill, North Calorina National Cancer Institute (NCI). 2005; http://www.clinicaltrials.gov/ct/show/NCT00232505.

403. Phase II Combined Treatment of Cetuximab and Paclitaxel in Basal Like Breast Carcinoma. NCT00353717. 2006; http://www.clinicaltrials.gov/ct2/show/NCT00353717.

404. Randomized Phase II trial with Cetuximab and Cisplatin in the Treatment of "Triple Negative" (Estrogen Receptor Negative, Progesterone Receptor Negative, and HER2 Negative) Metastatic Breast Cancer ("Basal Like"). 2007; http://www.clinicaltrials.gov/ct2/show/NCT00463788.

405. Hoadley KA, Weigman VJ, Fan C, et al. EGFR associated expression profiles vary with breast tumor subtype. *BMC Genomics*. 2007;**8**:258.

406. Chew HK, Barlow WE, Albain K, et al. A phase II study of imatinib mesylate and capecitabine in metastatic breast cancer: Southwest Oncology Group Study 0338. *Clinical breast cancer*. 2008;**8**:511-515.

407. Sessa C, Guibal A, Del Conte G, Ruegg C. Biomarkers of angiogenesis for the development of antiangiogenic therapies in oncology: tools or decorations? *Nat Clin Pract Oncol*. 2008;**5**:378-391.

408. Abd El-Rehim DM, Pinder SE, Paish CE, et al. Expression of luminal and basal cytokeratins in human breast carcinoma. *J Pathol*. 2004;**203**:661-671.

409. Morimoto K, Kim SJ, Tanei T, et al. Stem cell marker aldehyde dehydrogenase 1-positive breast cancers are characterized by negative estrogen receptor, positive human epidermal growth factor receptor type 2, and high Ki67 expression. *Cancer Sci*. 2009;**100**:1062-1068.

410. Li X, Lewis MT, Huang J, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. *J Natl Cancer Inst*. 2008;**100**:672-679.

411. Banerjee S, Reis-Filho JS, Ashley S, et al. Basal-like breast carcinomas: clinical outcome and response to chemotherapy. *J Clin Pathol*. 2006;**59**:729-735.

412. Wojcik BE, Spinks MK, Optenberg SA. Breast carcinoma survival analysis for African American and white women in an equal-access health care system. *Cancer*. 1998;**82**:1310-1318.

413. Choi YJ, Choi YL, Cho EY, et al. Expression of Bmi-1 protein in tumor tissues is associated with favorable prognosis in breast cancer patients. *Breast Cancer Res Treat*. 2009;**113**:83-93.

414. Silva J, Garcia V, Garcia JM, et al. Circulating Bmi-1 mRNA as a possible prognostic factor for advanced breast cancer patients. *Breast Cancer Res*. 2007;**9**:R55.

415. Newman LA, Bunner S, Carolin K, et al. Ethnicity related differences in the survival of young breast carcinoma patients. *Cancer*. 2002;**95**:21-27.

416. Chu KC, Lamar CA, Freeman HP. Racial disparities in breast carcinoma survival rates: seperating factors that affect diagnosis from factors that affect treatment. *Cancer*. 2003;**97**:2853-2860.

417. Chen VW, Correa P, Kurman RJ, et al. Histological characteristics of breast carcinoma in blacks and whites. *Cancer Epidemiol Biomarkers Prev*. 1994;**3**:127-135.