High Incidence of Triple-Negative Tumors in Sub-Saharan Africa: A Prospective Study of Breast Cancer Characteristics and Risk Factors in Malian Women Seen in a Bamako University Hospital

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Key Words

Woman • Breast cancer • Triple-negative tumor • Mali • Sub-Saharan Africa • Risk factors

Abstract

Objective: Few studies have been conducted on breast cancer in Sub-Saharan Africa and their results have been suspected to be impaired by artefacts. This prospective study was designed to determine tumor and patient characteristics in Mali with control of each methodological step. These data are necessary to define breast cancer treatment guidelines in this country. **Methods:** Clinical and tumor characteristics and known risk factors were obtained in a consecutive series of 114 patients. Each technical step for the determination of tumor characteristics [histology, TNM, grade, estrogen (ER) and progesterone receptors (PR), HER2, and Ki67] was controlled. **Results:** Patients had a mean age of 46 years. Most tumors were invasive ductal carcinomas (94%), T3-T4 (90%) with positive nodes (91%), grade III (78%), and ER (61%) and PR (72%) negative. HER2 was overexpressed in 18% of

cases. The triple-negative subgroup represented 46%, displaying a particularly aggressive pattern (90% grade III; 88% Ki67 >20%). **Conclusion:** This study demonstrates the high incidence of aggressive triple-negative tumors in Mali. Apart from a higher prevalence of premenopausal women, no significant difference in risk factors was observed between triple-negative tumors and other tumors. The hormonal therapy systematically prescribed therefore needs to be revised in light of this study.

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Background

According to demographic projections, Africa will face an unprecedented growth of cancer burden in the next decades [1]. In this context, there is evidence to suggest an emerging epidemic of breast cancer in Sub-Saharan Africa which already constitutes a major health issue [1, 2]. Breast cancer is the second leading cancer after cervical cancer and is the leading cancer in Nigerian women

[3, 4]. The age-specific breast cancer incidence rate has been evaluated to be between 20 and 25 per 100,000 in West African women and it has been estimated that almost 44,000 women die each year from breast cancer in Sub-Saharan Africa [5, 6]. Although the incidence is lower than in developed countries, a significant increase in breast cancer is expected in African countries as a result of urbanization, with changing patterns of risks factors and increasing life expectancy [1, 2].

Compared to developed countries, few studies have been conducted on the patient and biological characteristics of breast cancer in Sub-Saharan Africa and no data are currently available in Mali, a country with a population of 14.5 million [7], apart from a previously published retrospective study [8]. The few published epidemiologic studies have suggested that breast cancer exhibits specific features in this region of the world compared to the USA and Europe [9–12], as breast cancer in African women is reported to usually occur at a younger age and to present more aggressive features, such as high-grade tumors, estrogen receptor (ER)-negative and/or triple-negative phenotypes [ER-/progesterone receptor (PR)-/human epidermal growth factor receptor 2 (HER2)-] [13–16]. However, the high frequency of ER-negative or triple-negative tumors in Africa has recently been questioned [2], as data derived from some studies have been considered to be possibly related to technical artefacts and more systematic studies have provided contradictory results [2]. Processing of pathological samples is often inadequate due to a lack of resources, with no access to immunohistochemistry, and most studies are retrospective with possibly unsatisfactory storage conditions [17].

For all of these reasons, we decided to set up a prospective study on breast cancer in patients referred to the Department of Medical Oncology and Hematology of the Hôpital du Point G of Bamako, Mali. The primary objective was to determine the tumor characteristics with control of each methodological step from biopsy to immunohistochemistry and fluorescent in situ hybridization (FISH) studies. The secondary objective was to investigate the association of tumor patterns with various risk factors, such as age, BMI, age of menarche, number of pregnancies, and breast-feeding.

Patients and Methods

Patients and Tumor Sampling

This study included a prospective consecutive series of 114 patients referred between October 2008 and March 2011 for breast cancer treatment to the Department of Medical Oncology and

Hematology of the Hôpital du Point G university hospital in Bamako. For each patient, the systematic work-up included family history of breast cancer, parity, age at menarche, age at first full-term birth and duration of breast-feeding, use of hormonal contraceptives, height and weight, and evaluation of BMI. Breast and axillary nodes were systematically evaluated by clinical examination, mammography, and ultrasonography (US). Usual blood tests, chest and bone X-rays, liver US, and, when necessary, chest or liver computerized tomography (CT) were routinely performed. Finally, TNM classification and staging were assessed. Patients were reviewed every 3 months for the first year and every 6 months thereafter, but their results will not be reported or discussed in this study, which is primarily designed to evaluate tumor characteristics at the time of diagnosis.

Core biopsies were performed under 1% xylocaine local anesthesia, without US guidance for tumors >1 cm, by physicians after a training period using a Bard® Magnum® device with 14G, 16G, or 18G biopsy needles (Bard MC 1410 and MC 1416; Voisins-le-Bretonneux, France). Four biopsies were taken from each patient, smears were performed for cytological examination, two biopsies were fixed in 10% formalin to be paraffin embedded, and two others were frozen and stored at –80°C. Paraffin-embedded tissue samples were sent twice a month to the Hôpital Tenon Pathology Department in Paris, France (Prof. P. Callard and Prof. I. Broche-riou) for histological examination and immunohistochemistry. Tumors were classified according to the WHO classification of breast tumors and graded with the Elston and Ellis histoprognostic grade (EE) [18].

The study was approved by the Institutional Ethics Committee of the Faculty of Medicine and Dentistry of Bamako, University of Bamako, Mali (No. 0887/FMPOS), and an informed consent form was signed by all patients.

Immunohistochemistry

A similar process was performed for all primary antibodies used. Paraffin-embedded specimens were cut into 3-μm sections, and slides were deparaffinized in xylene for 30 min and then rehydrated in graded alcohols. Antigen unmasking was obtained by 30 min of incubation in a pH 6 citrate buffer (Target Retrieval Solution, reference: S1699; DAKO, Trappes, France) in a 100°C waterbath. After cooling to room temperature, immunohistochemical assays were performed using a NexES® (Roche Ventana®, Illkirch, France) immunostainer for 30 min of incubation at 37°C with antibodies diluted according to the manufacturer's recommendations (table 1). Slides were subsequently incubated with an I View™ DAB Detection Kit (reference: 760-91; Roche Ventana) containing hydrogen peroxide to block endogenous peroxidase activity, biotinylated secondary antibody, streptavidin peroxidase complex, and chromogen. For ER and PR expression, an amplification step using the Roche Ventana Kit (amplification kit reference: 760-080) was added.

Finally, the sections were counterstained with Harris hematoxylin. Internal positive controls, i.e. nuclear labeling after incubation with anti-ER and/or anti-PR antibodies of epithelial cells from normal nonneoplastic ducts, were checked for each section. Controls for HER2 were sections of referenced positive tumors and negative normal epithelial cells. For Ki67, it has been verified that the percentage of immunolabeled cells was always greater than the percentage of mitotic cells observed on HES-stained sections. Immunostainings were also controlled accord-

ing to the external quality program (AFAQAP; France) [19]. Immunostaining results were scored semiquantitatively based on the percentage of cells labeled after immunohistochemical reaction. ER and PR were considered to be positive when more than 10% of the nuclei of carcinomatous cells were labeled [20, 21]. A proliferative state was concluded when more than 20% of nuclei were labeled with the Ki67 antibody [22]. A scoring system was used for HER2 immunostaining: a score of 3 when more than 30% of cells exhibited marked peripheral membranous staining, a score of 2 when moderate peripheral staining was observed in more than 10% of cells, and a score of 1 or 0 when staining was observed in less than 10% of cells and/or in the presence of weak staining. Only score 3 was considered to be significant for assessing overexpression. Score 2 specimens were tested by FISH for HER2 amplification [23].

Fluorescent in situ Hybridization

FISH was performed using a ZytoLightSPEC HER2/CEN17 Dual Color Probe Kit (ZytoVision, Bremerhaven, Germany), which includes two directly labeled DNA probes: a locus-specific probe for the HER2 gene labeled with Zygreen similar to FITC and an alpha satellite probe targeting the centromere region of chromosome 17 (CEP17) labeled with Zorange similar to rhodamine. The assay was performed according to the manufacturer's instructions. Briefly, following deparaffinization, slides were incubated in the pretreatment solution (98°C, 15 min), rinsed, and then digested with pepsin (37°C, 15 min). Slides were rinsed and then dehydrated with graded alcohols and air dried. The ZytoLightSPEC HER2/CEN17 Dual Color Probe cocktail (10 µl) was applied, a coverslip was sealed, and after a 10-min denaturation step at 75°C the specimens were hybridized overnight at 37°C. Slides were then washed and counterstained with 30 µl DAPI/antifading solution for 15 min. The presence of the two probes was evaluated using appropriate band-pass filters on an epifluorescence microscope. Average copy numbers of HER2 and CEP17 from 100 tumor cell nuclei were counted and the signal ratios were calculated. Specimens with a HER2/CEN17 ratio greater than or equal to 2.2 were considered to be amplified (FISH positive), while those with a HER2/CEN17 ratio of less than 2.2 were considered to be nonamplified (FISH negative) [23]. For specimens that were clearly nonamplified (signal ratio less than 1.8) or clearly amplified (signal ratio greater than 2.2) after assessment of 100 cells, no further counting was performed. For specimens with borderline signal ratios of 1.8–2.2, the correlation with immunohistochemistry was controlled in order to reveal potential heterogeneity, and an additional set of 20 cells was counted and the signal ratio was recalculated.

Statistics

Statistical analysis was performed to compare the clinical and biological characteristics of patients with triple-negative or hormonal receptor (ER and/or PR)-negative tumors and other tumors, i.e. non-triple-negative or hormonal (ER and/or PR)-positive tumors. Quantitative variables were tested using a Student t test for sample sizes greater than 29 and a Mann-Whitney test for smaller sample sizes. Qualitative variables were tested with a χ^2 test or Fisher's exact test (table 4). StatView software (V5.0; SAS Institute) was used. Some parameters were grouped into two classes as follows: T: 1 + 2 and 3 + 4; N: 0 and 1 + 2 + 3; stage: 1 + 2 and 3 + 4, and SBR grade: 1 and 2/3. Due to test repetition, the significance level was set at 0.002.

Table 1. Monoclonal antibodies used for immunohistochemistry

Antibodies	Clone	Dilution	Manufacturer
ER	6F11	1/50	Novocastra (NCL-ER 6 F11)
PR	16	1/50	Novocastra (NCL-PGR312)
Cerb B2/HER2	Polyclonal	1/1,000	DAKO (A0485)
Ki67	Mib1	1/150	DAKO (M7240)

Results

Patients and Clinical Data

As shown in table 2, the youngest patient of the series was 25 years old and the oldest patient was 82 years old (mean: 46; median: 45). Most patients (90%) had a tumor larger than 5 cm at diagnosis, i.e. cT3-T4 with clinical enlarged axillary lymph nodes (99/111; 89%), and 49/114 (43%) patients had metastatic disease at diagnosis (liver: 18, bone: 9, and lung: 22) (table 3).

As shown in table 2, the mean and median BMI were 25 and 24, respectively (normal between 18.5 and 25). The mean and median ages of menarche were 14.5 and 15 years, respectively. Most women (105/114; 92%) had already had children (average of 5 children), the mean and median ages of the first pregnancy were 17 and 18 years, respectively, and they usually breastfed their babies for a mean and median duration of 17 and 18 months, respectively. Twelve patients (10.5%) used oral contraception and 7 (6%) had a family history of breast cancer.

Tumor Characteristics

The biological characteristics of the tumors are reported in table 3. It should be emphasized that most tumors (86/110; 78%) had a high EE grade 3, with more than 20% of Ki67-labeled cell nuclei in 78/102 cases (76%). ER and PR were expressed on 39 and 29% of cells, respectively. CerbB2/HER2, considered to be positive for an immunohistochemical score of 3 or 2 or marked amplification of the HER2 gene locus, was observed in 20/113 cases (18%); 53/114 (46%) of tumors failed to meet any of the above criteria and were considered to be triple negative. When considering the 66 patients aged ≤ 45 years, 24 tumors (51.5%) were triple negative, 4 tumors were ER+/HER2+ and/or PR+/HER2+ (3.5%), 14 tumors were ER+/HER2- and/or PR+/HER2- (12.5%), and 10 tumors were ER-/PR-/HER2+ (9%).

Table 2. Clinical characteristics of the 114 patients

Characteristics	Overall (n = 114)	Triple-negative tumors (n = 53)	Non-triple-negative tumors			
			Total (n = 61)	RH+HER- (n = 33)	RH+HER+ (n = 15)	Others ^a (n = 13)
Age, years	46 (25–82)	44.24 (28–73)	48.03 (25–82)	50.27 (25–82)	44.6 (28–60)	46.31 (25–65)
Number of patients <50 years of age	74	42	32	16	10	6
Number of patients >50 years of age	40	11	29	17	5	7
Height, cm	164 (142–188)	164.7 (154–180)	164.1 (142–188)	163.09 (146–175)	166.8 (145–188)	163.92 (142–182)
BMI	25 (15–40)	24.11 (15–39)	25.77 (16–40)	25.48 (16–39)	26.6 (18–40)	25.54 (18–33)
Age of menarche, years	14.5 (10–20)	14.40 (10–18)	14.6 (13–20)	14.53 (13–16)	15.07 (13–16)	14.53 (13–16)
Number of full-term pregnancies	5 (1–15)	5.3 (0–14)	4.63 (0–15)	5 (0–12)	3.46 (0–7)	5.07 (0–15)
Breast-feeding duration, months	17 (6–36)	17.81 (0–36)	16.57 (0–24)	16.09 (0–24)	16.6 (0–24)	17.76 (0–24)

Values are presented as means (range) unless otherwise stated.

^a This subgroup included 5 RH–HER2+ tumors and tumors for which immunohistochemistry results were not available for 1 of the 3 markers.

Table 3. Clinical and biological characteristics of tumors

Characteristics	n (%)
Age	
<50 years	74 (68)
≥50 years	40 (35)
TNM classification	
T1–T2	12/114 (11)
T3	11/114 (10)
T4	91/114 (80)
N0	10/114 (9)
N1	43/114 (38)
N2	38/114 (33)
N3	23/114 (20)
M1	49/114 (43)
Histology	
Invasive ductal carcinoma	107/114 (94)
Other	6/114 (6)
EE grading ^a	
Grade 1	4/110 (4)
Grade 2	20/110 (18)
Grade 3	86/110 (78)
Hormonal receptors ^b	
ER <10%	69/113 (61)
ER ≥10%	44/113 (39)
PR <10%	81/112 (72)
PR ≥10%	31/112 (28)
HER2 expression ^c	
HER2 negative	93/113 (82)
HER2 positive	20/113 (18)
RH+HER2–	33/113 (29)
RH+HER2+	15/113 (13)
RH–HER2+	5/113 (4)
Triple negative	53/114 (46)
Ki67 >20%	78/102 (76)

^a According to Elston and Ellis. ^b Evaluated by immunohistochemistry. ^c Evaluated by immunohistochemistry and/or FISH.

Comparison of Characteristics and Risk Factors between Triple-Negative and Non-Triple-Negative Tumors

The clinical characteristics and risk factors of patients according to the various subgroups of tumors are shown in table 2 and comparative results are presented in table 4. As indicated in Materials and Methods, the significance level was set at 0.002 due to test repetition. No significant difference was observed between patients with triple-negative tumors and patients with other tumors in terms of height, BMI, age of menarche, number of full-term pregnancies, duration of breast-feeding, and TNM or stage. However, a significant difference was observed for age, as the triple-negative group comprised significantly more patients under the age of 50 years ($p = 0.0028$). Moreover, more patients in the triple-negative group had received oral contraception ($p = 0.03$), but this difference was not considered to be significant at the limit of $p \leq 0.002$. The main significant differences concerned tumor EE grade and Ki67 labeling ($p \leq 0.0001$).

Discussion

This prospective consecutive series of 114 patients with breast cancer with well-documented clinical and biological characteristics is the first series concerning women living in Mali. As already reported for other Sub-Saharan countries, patients were younger (mean age: 46 years) than European or North American Caucasian patients, among whom breast cancer is mostly observed in older women (e.g. in France, the incidence of breast can-

Table 4. Statistics comparing clinical and biological characteristics of triple-negative tumors versus other tumors and hormonal receptor-negative tumors versus other tumors

	Triple-negative (n = 53) versus others (n = 61)	Estrogen receptor-negative (n = 66) versus others (n = 48)
Age ^a (years)	p = 0.102	p = 0.183
Height ^a (cm)	p = 0.704	p = 0.704
BMI ^a	p = 0.137	p = 0.380
Age of menarche ^a (years)	p = 0.181	p = 0.382
Number of full-term pregnancies ^b	p = 0.185	p = 0.299
Breast-feeding duration ^b (months)	p = 0.639	p = 0.225
Oral contraception ^b (yes/no)	p = 0.036	p = 0.055
T ^d (1+2/3+4) ^c	p = 0.796	p = 0.952
N ^d (0/1+2+3) ^c	p = 0.273	p = 0.240
M ^c	p = 0.767	p = 0.648
Stage ^d (1+2/3+4) ^c	p = 0.815	p = 0.614
EE grade ^d (1+2/3) ^c	p = 0.0024	p = 0.0007
Ki67 ^b	p < 0.0001	p < 0.0001

^a Student's t test. ^b Mann-Whitney test. ^c χ^2 test. ^d Two classes were determined for these parameters, as indicated.

cer in women over 50 years is nearly 3 times that observed in women 30–49 years) [24]. Sixty-eight percent of patients from our series were younger than 50 years but, as already emphasized, this finding is probably mainly due to the lower life expectancy in Mali (54.3 years for women, as in most Sub-Saharan African countries, compared to 84.4 years for women in France) [2, 25]. It has been stressed that, due to the low median age of the African population, breast cancer among young women represents a higher proportion of the cases observed in African clinics compared to breast cancer in older women [2, 26]. However, African-American women are also more likely to be diagnosed with breast cancer at a younger age than are white American women [27]. Various explanations for this higher incidence in young women have been proposed, such as socioeconomic differences, but, as quoted by Akarolo-Anthony et al. [2], a 65-year-old urban woman from Kampala Uganda has only a 20% lower risk of developing breast cancer than an age-matched European woman. Breast cancer prevalence in younger women in Mali, as in other Sub-Saharan African countries, is therefore very likely at least partly due to demographic characteristics. This aspect highlights the need for cancer registries to record the age-adjusted prevalence and incidence of breast cancer.

Breast cancer biology in African women, particularly their hormonal receptor status, is a controversial issue. Several studies have reported that African breast cancers are mostly hormone receptor poor [13–16]. However, it has been rightly suggested that this observation could be due to artefacts related to the problems encountered by pathologists in low-income countries [2, 17]. We therefore decided to set up this prospective study with control of each technical step, and all immunohistochemical and FISH studies were performed in the Pathology Department of a French university hospital (Hôpital Tenon, APHP, Paris), a breast cancer pathology reference center. Moreover, nonneoplastic duct cells on the same section expressing endocrine receptors were used as internal controls. In this context, we found that 46% of tumors could be classified as ‘triple negative’ and 58% were considered to be hormone receptor poor. In Caucasian patients, triple-negative or basal-like tumors account for 16% of all breast cancers [27], while in our study 46.5% of tumors were triple negative. This result supports the finding that breast cancer in African woman, and Malian women in the present study, mostly corresponds to hormone receptor-poor tumors. This result is in agreement with data obtained from other African countries. We recently reviewed various studies of breast cancer in African women, most of which reported 40–55% of triple-negative tumors [28]. A high frequency of triple-negative tumors has also been reported in African-American patients. A recent study in premenopausal women shown that the prevalence of triple-negative breast cancer in African-American and white American women was 39 and 16%, respectively [27]. Although a potential risk associated with African ancestry is still under debate, the triple-negative burden in African-American women cannot be explained exclusively by socioeconomic factors [29].

A large number of live births and a younger age at the first full-term pregnancy, as in this series, is usually considered to lower the risk of breast cancer in Western countries. However, Millikan et al. [30] reported that women between 20 and 72 years of age have an increased risk of basal-like tumors, which are triple-negative tumors, associated with these conditions. Various factors have been suggested to be risk factors for triple-negative tumors. In the present series, we therefore compared patients with triple-negative or hormone receptor-poor tumors with other patients. More patients under the age of 50 years presented a triple-negative tumor, which indicates that triple-negative tumors were observed with a higher prevalence in the premenopausal women of our series. Similarly, Carey et al. [27] reported a high prevalence (59% in

their series) of basal-like breast cancer (i.e. triple negative) in premenopausal African-American women. In contrast, the present study did not reveal any significant differences for most of the known risk factors (BMI, age at menarche, number of full-term pregnancies, cumulative duration of breast-feeding, or stage of disease) [29, 31, 32]. Analysis of data from 30 countries has clearly shown that, in developed countries, a longer breast-feeding period has a protective effect against breast cancer, i.e. about a 5% reduction of the relative risk per 12 months of breast-feeding [32]. However, this risk factor was not observed in our study, probably because 92% of the women in our series, like most Malian women, had already had children and all Malian mothers breast-feed their babies. In contrast, hormonal contraceptive use was more frequent among patients with triple-negative tumors, but this difference was not significant at the limit of $p \leq 0.002$. This could be due to the small sample size of this series. The relationship between oral contraceptive use and breast cancer risk has been the subject of extensive research. Analysis of a series of 897 breast cancers in patients aged ≤ 45 years showed a relative risk of 4.2 associated with oral contraceptive use specific for triple-negative tumors in younger patients, but this association was not observed for other breast cancers [29]. The mechanisms by which oral contraceptive use is associated with a high risk of ER-negative breast cancer are still unknown. However, one of them, the promoting role of estrogen on tumors via stromal cells and angiogenesis, has been described [33]. In the specific setting of our study, our results must be considered cautiously and need to be re-evaluated in a larger series taking into account the type of oral contraception, duration, and age of first use.

Comparison of triple-negative or hormone receptor-poor tumors with other tumors of the series did not reveal any significant differences in terms of cTNM or disease stage, but, as reported for this subset of tumors in Europe or the USA, a highly significant relationship was observed with a high histoprognostic grade and a high Ki67 proliferative index. This association between aggressive parameters and triple-negative breast cancer has been described in a large cohort of patients [30]. Triple-negative breast cancer with high Ki67 is associated with more aggressive clinical features and a pattern of early recurrence. These patients therefore require particularly stringent follow-up during the first 3 years after treatment [22, 34, 35].

The results of this study have an impact on the treatment of breast cancer in Mali and other Sub-Saharan African countries in which hormonal therapy is systemati-

cally proposed as the hormonal receptor status is more often not available for treatment decisions. Systematic treatment with tamoxifen is therefore inappropriate for most patients and, in patients with estrogen-independent cancer, could be responsible for harmful side effects such as deep vein thrombosis or osteoporosis with no significant effect on the tumor [36, 37]. Treatment options for triple-negative breast cancer are currently limited to cytotoxic chemotherapy, but effective low-cost chemotherapy needs to be developed for low-income countries [38]. Novel targeted therapies for this subset of tumors are also under development [39].

Conclusion

This prospective study of 114 patients showed that most women with breast cancer managed by physicians and oncologists in Bamako, Mali, are relatively young, with aggressive hormone receptor-poor tumors that require cytotoxic chemotherapy either as adjuvant therapy or as the sole therapy for metastatic patients. In light of this study, the hormonal therapy systematically prescribed to Malian women needs to be revised. Moreover, as already emphasized by Akarolo-Anthony et al. [2], 'hub and spoke regional pathology laboratories with trained technicians' must be urgently developed to ensure good quality immunohistochemical studies in order to determine the hormonal status of each breast cancer.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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