Erratum

Original Article

A Cross-Sectional Study of RankL and Nf-Kβ Levels Among Postmenopausal Breast Cancer Patients Attending Ahmadu Bello University Teaching Hospital, Zaria Nigeria: Preliminary Investigations and Implications on Disease Subtypes, Severity and Therapy

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Abstract

Background: Breast cancer incidence and mortality is characterized by variations in disease pathogenesis and treatment outcomes across racial and ethnic boundaries in which RANKL and NF-κβ levels are central especially among postmenopausal breast cancer patients. **Aims and Objectives:** This study sought to examine if RANKL, NF-κβ and oxidative stress levels were associated with disease subtypes, severity, and chemotherapy courses among the postmenopausal breast cancer patients attending Ahmadu Bello University Teaching Hospital. **Materials and Methods:** We examined RANKL and NF-κβ levels in serum of postmenopausal breast cancer patients attending Ahmadu Bello University Teaching Hospital (ABUTH) using ELISA kits. Sociodemographic characteristic and clinical parameters of the patients were documented using questionnaires. **Results:** The mean age of the respondents was 54.7 ± 6.7 years, while the modal age was 45-50 years. Triple negative breast cancer subtype accounted for 40% of the patients, while 83.3% had invasive carcinoma histological type. Serum RANKL was significantly (P<0.05) higher (40%) in breast cancer patients when compared to apparently healthy control, but lowest in triple negative patients when compared to other subtypes. NF-κβ concentration was significantly (P<0.05) higher (83.7%) in breast cancer patients but highest among triple negative and HER2-enriched patients when compared to apparently healthy. NF-κβ correlated with third chemotherapy course of chemotherapy. Oxidative stress markers were significantly (P<0.05) higher in breast cancer patients when compared to apparently healthy control. A significant (P<0.05) association between RANKL, NF-κβ concentrations and disease severity (stage I to stage IV) was also observed. **Conclusion:** RANKL and NF-κβ levels were associated with disease subtypes, severity, and chemotherapy courses among the postmenopausal breast cancer patients. This could serve as a preliminary source of information for further studies on their prognostic value.

Keywords: Breast Cancer; NF-kB; RANKL; Sociodemographic Parameters; Oxidative Stress

INTRODUCTION

Breast cancer is a type of cancer that develops in the breast cells [1]. Typically, the cancer forms either in the lobules, the ducts, the fatty tissue, or the fibrous connective tissue within the breast. It is now a commonly diagnosed disease even in developing countries with an incidence rate of 50.5 per 100,000 in Nigeria, implying that 1 out of every 2000 women in Nigeria stands the risk of being diagnosed with breast cancer each year [2]. Reports show that there is an increasing rate of mortality among breast cancer patients in Sub-Saharan Africa, and Nigeria contributes greatly to the recorded mortality cases in the region [3]. Accumulating evidence from molecular examination suggests that breast cancers with different histopathological and biological features exhibit varying levels of disease aggressiveness and response to treatment [4]. Breast cancer is a heterogeneous condition consisting of multiple subtypes with distinct morphologies [5]. Breast cancer is subdivided into

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with consistent hormone receptor expression [5] The luminal A tumors have higher expression of estrogen receptor related genes and lower expression of proliferative genes (ER+, PR+, HER2-) when compared to luminal B cancers (ER+, PR+, HER2-) [6]. Tumors classified as HER2 overexpressing are a group of aggressive breast cancers that are associated with poor prognosis, expressed only HER2 and termed as HER2-enriched [7]. Basal like tumors are tumors that originate from mammary basal myo-epithelium and are classified as group of aggressive breast cancers that are characterized by negative expression of hormone receptors (ER-, PR-, and HER2-) thus termed Triple negative breast cancer [8]. Triple negative breast cancer follows an aggressive clinical course with difficulty of standard targeted systemic therapy[8]. It is characterized by rapid growth and is commonly seen among African women and women of African descent [9]. The tumors are reported to be larger than other subtypes and metastasis among patients is seen to have a tendency towards extending to visceral organs [8].

Several breast cancer-predisposing factors have been identified, prominent among which are age, early menarche, genetics, breast feeding, exogenous hormone exposure, cigarette smoking, alcohol use and obesity [10]. However, these predisposing factors can vary based on individuals, ethnicity, and geographical location [11]. Breast cancer is more prevalent among post-menopausal women, as women aged 60 – 70 years have the highest risk of developing breast cancer [12]. An increased risk for developing breast cancer is also associated with the number of first-degree relatives of an individual who have been diagnosed with breast cancer[13]. Early menarche and late menopause have also been identified as a risk factor for breast cancer, due to longer duration of circulating estrogen in this category of women [14]. Women with no history of breast feeding are also at risk of developing breast cancer because they usually have continuous circles of estrogen synthesis [15].

As a signaling intermediary biomolecule, RANKL has also been implicated in mammary cell proliferation, breast cancer initiation and metastasis to bone [16]. Receptor Activator of Nuclear factor Kappa beta Ligand (RANKL) is an important molecule that plays crucial role not only in bone metabolism and osteoporosis [17], but also in the development of the mammary gland during pregnancy [18]. Downstream of RANKL, Nuclear Factor kappa Beta (NF-kB) is a transcription factor that controls physiological functions that are observably altered during breast cancer [19]. NF-kB contributes to breast cancer via mechanisms involving mammary cell proliferation, inflammation, resistance to apoptosis and metastasis [20]. Altered NF-kB expression has been associated with larger tumor size and aggressive disease progression in several breast cancer patients [21,22]. Research carried out in our laboratories have previously examined biochemical alterations associated with breast carcinogenesis in *in vivo* models [23,24] and the ameliorative potential of phytonutrients [25–28]. However, studies are needed to further understand biochemical alterations related to inflammatory processes, response to therapy and their possible association with socio-demographic factors among postmenopausal breast cancer patients in the sub-region. This study was carried out at the Ahmadu Bello University Teaching Hospital (ABUTH) which is the largest teaching hospital in North-Western Nigeria, the largest geopolitical zone in Nigeria with a population of about 39,000,000 people [29].

MATERIALS AND METHODS

Study Population

This cross-sectional study was carried out at the surgical outpatient clinic of Ahmadu Bello University Teaching Hospital (ABUTH) Zaria between June 2019 and December 2020. The respondents were breast cancer patients aged 46 – 80 years. A convenient sampling technique was used for the study. Validated semi-structured questionnaires were employed in assessing the socio-demographic characteristics of the patients. Sixty (60) breast cancer patients and thirty (30) apparently healthy individuals were used for the study. Ethical approval (ABUTHZ/HREC/W41/2020) was obtained from the health research and ethics Committee of the ABUTH, Zaria, in accordance with the Helsinki Declaration, and a voluntary written informed consent was obtained from each participant before recruitment into the study.

All female breast cancer patients diagnosed with bone disorders, cardiovascular diseases, HIV, or other forms of cancers and patients that are alcoholics and cigarette smokers were excluded. Patients with induced menopause and those aged 46 years but still menstruating were also excluded from the study. The selected breast cancer patients were categorized into four (luminal A, luminal B, HER2-enriched, triple negative) based on their receptor status as determined by immunohistochemical studies.

Blood Sample Collection

About 5ml of blood sample from each participating patient was collected separately into sterilized dry plain tubes and allowed to clot for about 30 minutes. It was then centrifuged at 3500 rpm for 15 minutes and the serum obtained was stored in the refrigerator at -20° until further analysis.

Measurement of Serum RANKL

RANKL concentration was determined in serum samples using ELISA Kit (Wuhan Fine Biotech Co., Ltd, China, Batch No. H0313F033). According to Manufacturer's instruction. Briefly all reagents and samples were allowed to equilibrate to room

temperature (18-26^{0}C) and then mixed well. Exactly 100µl of the prepared standard was aliquot into the standard wells while 100µl of sample dilution buffer was added into the blank well and 100µl of the sample was then properly added into the test sample wells. The plate was sealed with a cover and incubated at 37°C for 90 minutes. After incubation, the content of the plate was discarded and washed twice with wash buffer, and then 100µl of biotin-labeled antibody was added into the wells, the plate was then covered and incubated for 60 minutes at 37°C. Later the content of the plate was discarded and washed thrice with wash buffer, then 100µl of HRP-streptavidin conjugate (SABC) working solution was pipetted into each well, which was then covered and incubated at 37°C for 30 minutes. After the incubation, the plate was washed with a wash buffer five times, allowing the wash buffer to stay for two minutes each time in the well. Next, 90µl of TMB substrate was added into each well, the plate was sealed and incubated at 37°C in the dark for fifteen minutes. Then, 50µl of acidic stop solution was added into each well. The color changed from blue to yellow immediately after the addition of the substrate. The absorbance was read in a microplate reader at 450nm immediately after adding the stop solution. Concentrations were extrapolated using a standard curve.

Serum phosphorylated-NF-κβ quantification

Serum Human phosphorylated nuclear factor kappa B (NF-**κ**B) concentrations were determined using ELISA Kit obtained from Wuhan Fine Biotech Co., Ltd, China (Batch No. H1950F033). All reagents and samples were allowed to equilibrate to room temperature (18-26^{0}C) and then shaken well. Each microtiter well was washed twice by dispensing 250µL of diluted wash buffer. Exactly 100µl of the prepared standard was aliquot into the standard wells while 100µl of sample dilution buffer was added into the blank well and 100µl of the sample were properly added into the test sample wells. The plate was sealed with a cover and incubated at 37°C for 90 minutes. After incubation the contents of the plate were discarded and washed twice with wash buffer, and then 100µl of biotin-labeled antibody was added into the wells, the plate was then covered and incubated for 60 minutes at 37°C. Later the contents of the plate were discarded and washed thrice with wash buffer, then 100µl of HRP-streptavidin conjugate (SABC) working solution was pipetted into each well, which was then covered and incubated at 37°C for 30 minutes. After incubation, the plate was washed with a wash buffer five times, allowing the wash buffer to stay in the well for two minutes each time. After that, 90µl of TMB substrate was added into each well, the plate was sealed and incubated at 37°C in the dark for fifteen minutes. Then 50µl of acidic stop solution was added into each well. The color changed from blue to yellow immediately after the addition of the substrate. The absorbance was read in a microplate reader at 450nm immediately after adding the stop solution. The concentrations were extrapolated using standard curve.

Determination of Serum Reduced Glutathione

Reduced glutathione (GSH) concentration was determined according to standard procedures [30,31]. The assay is based on the reaction of 5,5-dithiobisnitrobenzoic acid (DNTB) and reduced Glutathione (GSH). To 150µl of the sample, 1.5ml of 10% trichloroacetic acid (TCA) was added and then centrifuged at 1500 × g for 5 minutes. After which 1000µl of the supernatant was treated with 500µl of Ellman's reagent and then 3ml of phosphate buffer (0.2M, pH 8.0) was added. The absorbance of the mixture was read at 412nm using a spectrophotometer. The concentration of GSH was obtained from the standard curve.

Determination Of Serum Malondialdehyde

Lipid peroxidation is evidenced by formation of TBARS which was measured using the modified method of [32] and described by [33]. Lipid peroxidation generates peroxide intermediates which upon cleavage release malondialdehyde (MDA), a product which reacts with thiobarbituric acid (TBA), forming a MDA-TBA adduct that absorbs strongly at 535nm. Exactly 150µl of the samples was treated with TBA-TCA-HCL reagent (1:1:1 ratio) which was then placed in a water bath at 90°C for 60 minutes, later the mixture was cooled and centrifuged at 3000 rpm for 5 minutes and the absorbance of the pink supernatant (TBA-malondialdehyde complex) was then read at 535nm. Malondialdehyde formed was then calculated using the molar extinction coefficient of $1.56 \times 10^{-5} cm^{-1} M^{-1}$

Data Analysis

Where appropriate, results were presented as the mean ± SD. Data was analyzed using a statistical software package (SPSS for windows, version 21, IBM Corporation, NY, USA). Statistical difference between means was analyzed using one-way ANOVA and Duncan post-hoc test was used to ascertain the degree of significance, while association studies and relationships were analyzed using Chi-square test and correlation analysis respectively. Results are significantly different at $P \leq 0.05$.

RESULTS

Socio-Demographic Characteristics Among Postmenopausal Breast Cancer Patients Attending Surgical Outpatient Clinic, ABUTH, Zaria

The socio-demographic characteristics of the histologically confirmed breast cancer patients showed that the majority (38.3%) of the breast cancer patients were within the age group of 45 – 50 years with mean age of 54.7±6.7 years while 26.67% were within the age group of 51 – 55 years. Among the breast cancer patients, 76.6% were married while 10% were widowed. Forty percent of respondents had tertiary

education followed by secondary education with 30%. Majority of the breast cancer patients (55%) were of Hausa ethnic group and 60% were unemployed (Table 1).

Table 1: Sociodemographic parameter among postmenopausal breast cancer patients attending Surgical Outpatient Clinic ABUTH, Zaria

Socio-demographic characteristics		Frequencies (n = 60)	Percentage (%)
Marital Status	Single	4	6.7
	Married	46	76.6
	Divorced	4	6.7
	Widowed	6	10.0
Ethnicity	Hausa	33	55.0
	Yoruba	8	13.3
	Igbo	7	11.7
	Others	12	20.0
Occupation	Public Servant	14	23.3
	Unemployed	36	60.0
	Business Women	9	15.0
	Others	1	1.7
Educational	Primary	8	13.3
	Secondary	18	30.0
	Qur'anic	10	16.7
	Tertiary	24	40.0
Age	45-50	23	38.3
	51-55	16	26.7
	56-60	10	16.7
	61-65	8	13.3
	≥66	3	5.0

Classification of Breast Cancer Patients into Subtypes

Breast cancer patients were classified into different subtypes based on the immunohistochemistry result (Table 2). Triple negative breast cancer (TNBC) was the most frequent accounting for 40% followed by Luminal A (31.7%), while HER2-enriched and Luminal B were 20% and 8.3% respectively. Hence 40% of the cases were ER-positive (Luminal A and Luminal B), whereas 60% are found to be ER-negative (HER2-enriched and triple negative). The result also showed that 83.3% had invasive carcinoma, no specific type NST-histological type and most of the patients presented at latter stages of the disease; stage III (53.3%) and stage IV (28.3%) (Table 2).

Table 2: Classifications of breast cancer among breast cancer patients attending Surgical Outpatient Clinic ABUTH, Zaria

classification			Frequency (n=60)	Percentage (%)
molecular	**Breast cancer subtypes**	Luminal A*	19	31.7
		Luminal B*	5	8.3
		**HER2-enriched	12	20.0
		**Triple Negative	24	40.0
histopathological	**Histology types**	Invasive carcinoma	50	83.3
		Non-invasive carcinoma	10	16.7
Severity	**Breast cancer stages**	Stage I	2	3.3
		Stage II	9	15.0
		Stage III	32	53.3
		Stage IV	17	28.4

Luminal A: (ER+, PR+, HER2-), **Luminal B**: (ER+, PR+, HER2+), **HER2-enriched**: (ER-, PR-, HER2+), **Triple negative**: (ER-, PR-, HER2-)
*Estrogen receptor positive, **Estrogen receptor negative

Table 3a: Association between Possible Risk Factors and Breast Cancer Subtypes among Postmenopausal Breast Cancer Patients Attending Surgical Outpatient Clinic ABUTH, Zaria

Variables		LA N (%)	LB N (%)	HER2 N (%)	TN N (%)	χ^2-values	*P*-values
Marital Status	Single	2(50.0)	1(25.0)	0 (0.0)	1(25.0)	9.499	0.393
	Married	14(30.4)	4(8.7)	9(19.6)	19(41.3)		
	Divorced	0 (0.0)	0(0.0)	0 (0.0)	4(100.0)		
	Widowed	1(16.7)	1(16.7)	2(33.3)	2(33.3)		
Ethnicity	Hausa	13(39.4)	1(3.0)	4(12.1)	15(45.5)	20.219	0.017*
	Yoruba	0(0.0)	1(12.5)	4(50.0)	3(37.5)		
	Igbo	1(14.3)	3(42.9)	0(0.0)	3(42.8)		
	Others	3(25.0)	1(8.3)	3(25.0)	5(41.7)		
Age	45-50	7(30.43)	2(8.7)	4(17.4)	10(43.5)	12.822	0.382
	51-55	6(37.5)	1(6.3)	0(00.0)	9(56.2)		
	56-60	3(30.0)	1(10.0)	4(40.0)	2(20.0)		
	61-65	1(12.5)	2(25.0)	2(25.0)	3(37.5)		
	≥66	0(00.0)	0(00.0)	1(33.3)	2(66.7)		
Age at menarche	11-12	8(47.1)	2(11.8)	4(23.5)	3(17.6)	9.750	0.371
	13-14	4(22.2)	1(5.6)	3(16.7)	10(55.5)		
	15-16	1(14.3)	1(14.3)	2(28.6)	3(42.8)		
	≥17	0(00.0)	0(00.0)	0(00.0)	2(100.0)		
Birth control pills	Yes	5(21.7)	1(4.4)	5(21.7)	12(52.2)	2.673	0.445
	No	12(32.5)	5(13.5)	6(16.2)	14(37.8)		
Alcohol consumption	Yes	0 (0.0)	1(50.0)	0(0.0)	1(50.0)	4.297	0.231
	No	17(29.3)	5(8.6)	11(19.0)	25(43.1)		
Pregnancy	0	2(50.0)	0(0.0)	1(25.0)	1(25.0)	9.728	0.640
	1-2	5(45.5)	2(18.2)	1(9.0)	3(27.3)		
	3-4	4(21.1)	2(10.5)	2(10.5)	11(57.9)		
	5-6	3(21.4)	2(14.3)	3(21.4)	6(42.9)		
	≥7	3(25.0)	0 (0.0)	4(33.3)	5(41.7)		
BMI	Underweight	0(0.0)	0(0.0)	0(0.0)	0(0.0)	4.300	0.231
	Normal	5(41.7)	1(8.3)	2(16.7)	4(33.3)		
	Overweight/obese	1(7.7)	2(15.4)	2(15.4)	8(61.5)		

*Results are significant at $P<0.05$ (Chi-square test)
LA = Luminal A, **LB** = Luminal B, **TN** = triple negative, **HER2** = human epidermal growth factor receptor 2

Risk Factors Among Postmenopausal Breast Cancer Patients

Association between possible risk factors and breast cancer subtypes showed significant association (χ^2= 20.219, *P*= 0.017) between ethnicity and breast cancer subtypes (Table 3). However, the results showed no association (P>0.05) between breast cancer and marital status, age at menarche, age, alcohol consumption, birth control pills, pregnancy and body mass index (Table 3). The association between possible risk factors and breast cancer severity indicated significant association between breast cancer severity and pregnancy (χ^2= 25.671, *P*= 0.012) as well as breast cancer severity and body mass index (χ^2= 25.278, *P*= 0.001), between breast cancer severity and marital status (χ^2= 18.992, *P*= 0.025) (Table 4). In addition, there was no significant association (*P*>0.05) between breast cancer severity and age at menarche, age, alcohol consumption as well as the use of birth control pills (Table 4).

Table 3b: Association between possible risk factors and breast cancer severity among Postmenopausal Breast Cancer Patients Attending Surgical Outpatient Clinic ABUTH, Zaria

Possible Risk factors		Stage I N (%)	Stage II N (%)	Stage III N (%)	Stage IV N (%)	χ^2-values	*P*-values
Marital status	Single	0(0.0)	2(50.0)	2(50.0)	0(0.0)	18.992	0.025*
	Married	2(4.3)	5(10.9)	29(63.1)	10(21.7)		
	Divorced	0(0.0)	0(0.0)	1(25.0)	3(75.0)		
	Widowed	0(0.0)	2(33.3)	0(0.0)	4(66.7)		
Ethnicity	Hausa	1(3.0)	5(15.2)	15(45.5)	12(36.3)	11.818	0.224
	Yoruba	1(12.5)	1(12.5)	5(62.5)	1(12.5)		
	Igbo	0(0.0)	3(42.9)	3(42.9)	1(14.2)		
	Others	0(0.0)	0(0.0)	9(75.0)	3(25.0)		
Age at menarche	11-12	1(6.0)	3(17.6)	10(58.8)	3(17.6)	9.258	0.414
	13-14	0(0.00)	1(5.6)	11(61.1)	6(33.3)		
	15-16	1(14.2)	2(28.6)	2(28.6)	2(28.6)		
	≥17	0(0.00)	1(50.0)	0(0.00)	1(50.0)		
Age	45-50	1(4.3)	2(8.6)	14(61.0)	6(26.1)	14.973	0.243
	51-55	1(6.2)	1(6.2)	11(68.8)	3(18.8)		
	56-60	0(00.0)	2(20.0)	6(60.0)	2(20.0)		
	61-65	0(00.0)	3(37.5)	1(12.5)	4(50.0)		
	≥66	0(00.0)	1(33.3)	0(00.0)	2(66.7)		
Birth Control	Yes	1(4.4)	4(17.4)	11(47.8)	7(30.4)	1.521	0.677
	No	1(2.7)	10(27.0)	19(51.4)	7(18.9)		
Alcohol consumption	Yes	0 (0.0)	1(50.0)	1(50.0)	0(0.0)	1.182	0.757
	No	2(3.4)	13(22.4)	29(50.0)	14(24.1)		
Pregnancy	0	0(0.0)	3(75.0)	1(25.0)	0(0.0)	25.671	0.012*
	1-2	0(0.0)	1(9.1)	10(90.9)	0(0.0)		
	3-4	1(5.2)	4(21.1)	8(42.1)	6(31.6)		
	5-6	0(0.0)	6(42.9)	6(42.9)	2(14.2)		
	≥7	1(8.3)	0 (0.0)	5(41.7)	6(50.0)		
BMI	Underweight	0(0.0)	0(0.0)	0(0.0)	0(0.0)	25.278	0.001*
	Normal	0(0.0)	2(16.7)	8(66.6)	2(16.7)		
	Overweight	0(0.0)	3(25.0)	7(58.3)	2(16.7)		
	Obese	1(100.0)	0(0.0)	0(0.0)	0(0.0)		

*Results are significant at *P*<0.05 (Chi-square test)

Table 4: Association between possible risk factors and serum levels of RANKL & NF-κB among postmenopausal breast cancer patients attending Surgical Outpatient Clinic ABUTH, Zaria

Possible risk factors	RANKL(pg/ml)		NF-κB (pg/ml)	
	χ^2-values	*P*-value	χ^2-values	*P*-values
Age	3.704	0.157	1.151	0.562
Pregnancy	2.355	0.671	2.233	0.693
Birth Control	0.255	0.613	2.090	0.148
Alcohol consumption	0.500	0.479	0.182	0.669
Age at menarche	0.993	0.803	0.684	0.711
Ethnicity	3.949	0.267	1.906	0.592
Education level	5.910	0.116	6.205	0.102
occupation	4.900	0.086	2.015	0.365
Marital status	1.147	0.766	1.736	0.420

Results are significant at *P*<0.05 (Chi-square test)

NF-κB = Nuclear transcription factor kappa-B, RANKL = Receptor activator of nuclear factor kappa-B ligand

Table 5: Relationship between the levels of oxidative stress biomarkers and pro-inflammatory markers (RANKL & NF-κB) among postmenopausal breast cancer patients attending Surgical Outpatient Clinic ABUTH, Zaria

		NF-κB	RANKL	GSH	MDA
NF-κB	Pearson Correlation	1	0.916**	0.695**	0.834**
	Sig. (2-tailed)		0.001	0.001	0.001
RANKL	Pearson Correlation		1	0.842**	0.939**
	Sig. (2-tailed)			0.001	0.001
GSH	Pearson Correlation			1	0.860**
	Sig. (2-tailed)				0.001
MDA	Pearson Correlation				1
	Sig. (2-tailed)				

**. Correlation is significant at the 0.01 level (2-tailed). (Pearson/Spearman correlation)

GSH = Reduced glutathione, **MDA** = Malondialdehyde, **NF-κB** = Nuclear transcription factor kappa-B **RANKL** = receptor activator of nuclear factor kappa-B ligand

Association Between Breast Cancer and Possible

Serum Levels of Pro-inflammatory Markers (RANKL and NF-κB) among Postmenopausal Breast Cancer Patient

The serum levels of RANKL and NF-κβ within the different molecular subtypes of breast cancer showed that the serum concentrations of RANKL were significantly (*P*<0.05) higher in Luminal A and Luminal B subtypes of when compared to apparently healthy controls (Figure 1a). Also, the concentration of RANKL was significantly (*P*<0.05) higher in HER2-enriched and triple negative subtypes of breast cancer when compared to the apparently healthy control group. Although, there was no significant (*P*>0.05) difference between luminal A subtypes and luminal B subtype as well as between HER2-enriched subtype and triple negative subtype of breast cancer. Generally, RANKL concentration is significantly (*P*<0.05) higher in breast cancer patients when compared to the apparently healthy individuals (Figure 1a).

NF-κβ concentration across the different subtypes of breast cancer, was significantly (*P*<0.05) higher in triple negative and HER2-enriched subtypes as compared to apparently healthy control (Figure 1a). The data also revealed that NF-κβ concentration was significantly (*P*<0.05) higher in luminal A and luminal B subtypes of breast cancer when compared with apparently healthy control. Similarly, NF-κβ concentration was significantly (*P*<0.05) higher in all breast cancer patients when compared to apparently healthy controls (Figure 1a).

The serum concentration of RANKL and NF-κβ across different stages of breast cancer severity demonstrated a significant (*P*<0.05) increase in RANKL across stage I to stage IV (Figure 1b). However, there was no significant (*P*>0.05) difference between stage II and stage III as well as between stage

III and stage IV. The result also revealed a significant (P<0.05) increase in NF-κβ concentration from stage I through stage IV. However, the increase was not significant (P>0.05) with respect to stages I and II as well as between stages II, III and IV (Figure 1b).

The serum RANKL and NF-κβ concentrations across different chemotherapy courses taken by the patients showed a slight decrease in RANKL concentration from 1st course through 6th course though the decrease was not significant (P>0.05) (Figure 1c). The result also showed a significant (P<0.05) decrease

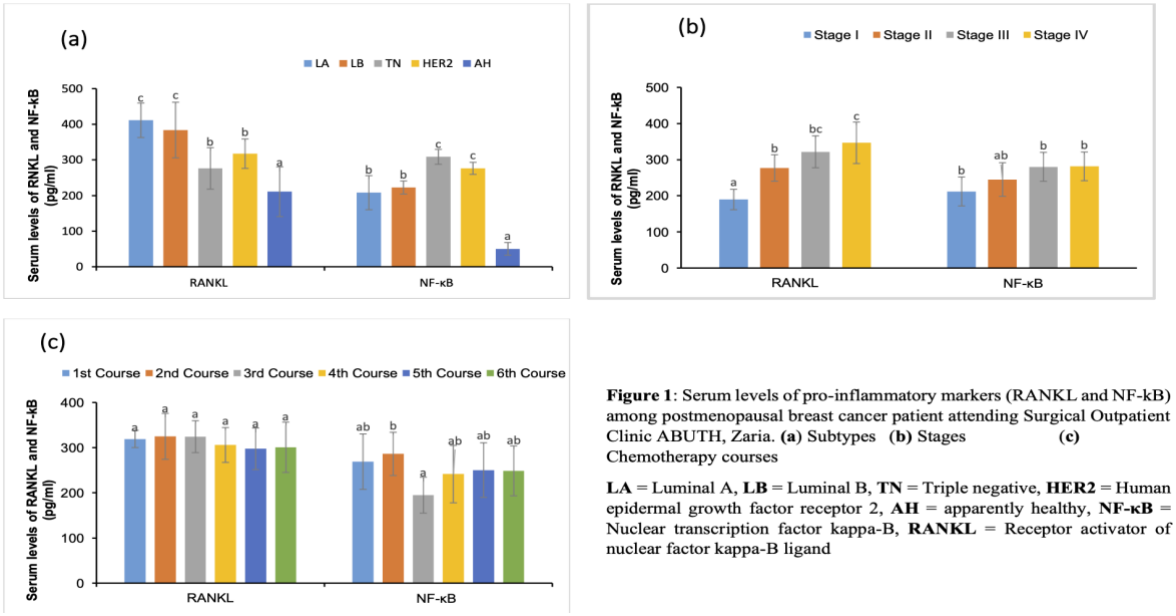

Figure 1: Serum levels of pro-inflammatory markers (RANKL and NF-kB) among postmenopausal breast cancer patient attending Surgical Outpatient Clinic ABUTH, Zaria. **(a)** Subtypes **(b)** Stages **(c)** Chemotherapy courses

LA = Luminal A, **LB** = Luminal B, **TN** = Triple negative, **HER2** = Human epidermal growth factor receptor 2, **AH** = apparently healthy, **NF-κB** = Nuclear transcription factor kappa-B, **RANKL** = Receptor activator of nuclear factor kappa-B ligand

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test (P<0.05)

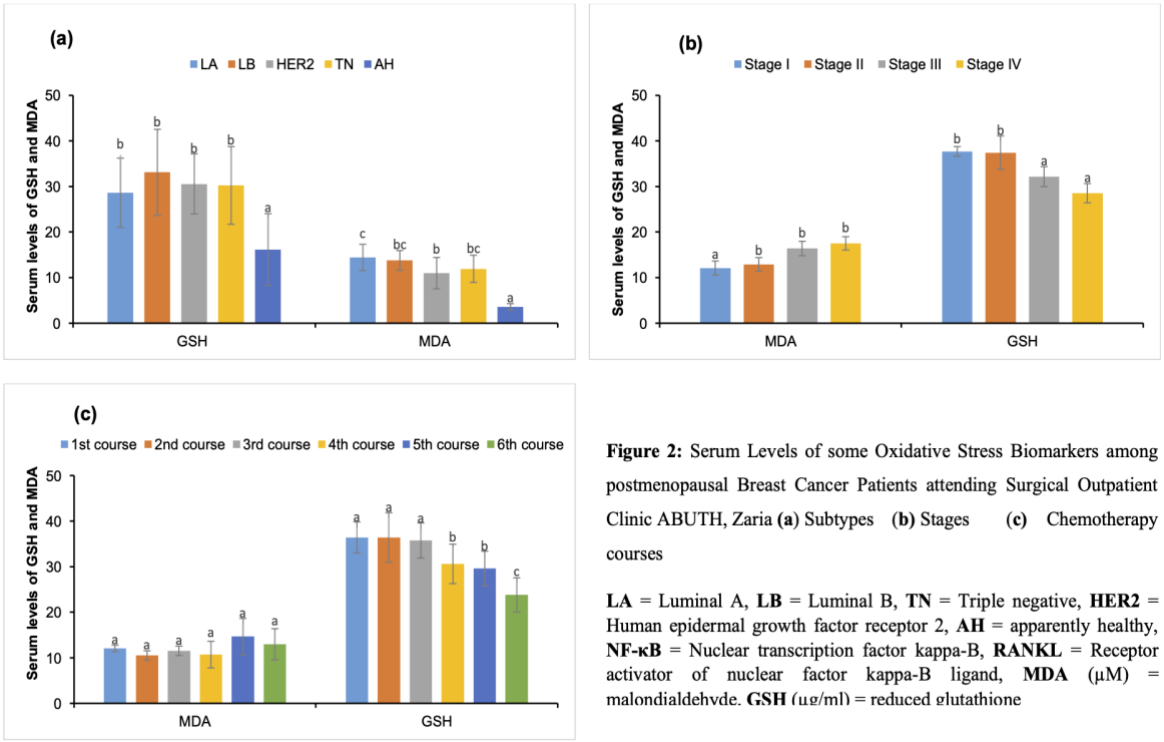

Figure 2: Serum Levels of some Oxidative Stress Biomarkers among postmenopausal Breast Cancer Patients attending Surgical Outpatient Clinic ABUTH, Zaria **(a)** Subtypes **(b)** Stages **(c)** Chemotherapy courses

LA = Luminal A, **LB** = Luminal B, **TN** = Triple negative, **HER2** = Human epidermal growth factor receptor 2, **AH** = apparently healthy, **NF-κB** = Nuclear transcription factor kappa-B, **RANKL** = Receptor activator of nuclear factor kappa-B ligand, **MDA** (μM) = malondialdehyde, **GSH** (μg/ml) = reduced glutathione

Bars with different alphabets are significantly different from each other using one-way ANOVA and Duncan multiple range test (P<0.05)

The results showed no significant (P>0.05) difference among the four subtypes of breast cancer with respect to GSH concentration, but it is significantly (P<0.05) higher in breast cancer patients when compared to apparently healthy individuals. Furthermore, the serum MDA concentration was significantly (P<0.05) higher in Luminal A subtype when compared to HER2-enriched. Although higher Luminal A is not significant (P<0.05) when compared to triple negative as well as Luminal B subtypes of breast cancer. Serum malondialdehyde concentrations were significantly (P<0.05) higher among breast cancer patients when compared to apparently healthy individuals. Serum oxidative stress markers across stages of breast cancer severity was presented in Figure 2b.

The result showed an increase in MDA concentration from stage I to stage IV. However, stages III and IV have significantly (P<0.05) higher MDA level compared to stage I and II. Serum GSH level was decrease from stage I to IV, with Stages III and IV significantly (P<0.05) lower compared to stages I and II.

Figure 2c shows oxidative stress levels across different chemotherapy cycles. There was no significant (P>0.05) difference in MDA concentration across the different chemotherapy courses. GSH concentration decreases from 1st to 6th course. However, the reduction in GSH was significantly (P<0.05) lower in the 6th course compared to 1st, 2nd, 3rd, 4^{th,} and 5th courses. It is also significantly (P<0.05) lower in the 4th and 5th compared to 1st, 2nd, and 3rd courses.

Relationship Between Oxidative Stress, Biomarkers, and Inflammatory Markers Among Postmenopausal Breast Cancer Patients

The relationship between some oxidative stress biomarkers (GSH & MDA) and the pro-inflammatory biomarkers (RANKL & NF-κB) of the breast cancer patients were tested using Pearson's correlation (Table 6). The results showed that there were significant positive correlations between NF-κβ and RANKL (r = 0.916; P=0.001), NF-κβ and GSH (r = 0.695; P=0.001) and NF-κβ and MDA (r = 0.834; P=0.001). In addition, significant positive correlations were also observed between the level of RANKL and GSH (r = 0.842; P=0.001) and between RANKL and MDA (r = 0.939; P=0.001). There was also a significant positive relationship between serum GSH and MDA (r = 0.860; P=0.001).

DISCUSSION

Breast cancer incidence and mortality is characterized by variations in disease pathogenesis and treatment outcomes across racial and ethnic boundaries, pointing to a multifactorial basis for the disease [11]. A number of factors could be responsible for the high mortality rate among breast cancer patients in West Africa and Nigeria in particular, necessitating research into socio-demographic patterns, breast cancer phenotypes and possible biochemical alterations in these patients [3]. The modal age of the respondents from this study was 45 - 50 years, while the mean age was 54.7 ± 6.7 years and is similar to the age of breast cancer patients observed by a study carried out in South-West Nigeria [34]. The immunohistochemistry data showed that triple negative breast cancer was the most common subtype among the patients and agrees with previous studies which showed that women of West African ancestry are more predisposed to the triple negative breast cancer phenotype [35]. Majority of the patients were observed to be in stages III and IV, suggesting that the cancer was aggressive and possibly metastasized which agrees with the aggressive pattern of breast cancer pathogenesis observed in previous studies [34,36].

Interestingly, the data from our study showed no association between breast cancer and marital status, age at menarche, and body mass index which are established risk factors for breast cancer predisposition [37]. This might be due to the fact that some of these risk factors are modifiable. We also found no association between history of alcohol consumption, use of birth control pills and breast cancer risk which disagrees with a previously reported study carried out among breast cancer patients in West Africa [38]. This could be due to the lifestyle of the women in the area as most of them are from local communities and are mostly not exposed to alcohol use as well as birth control pills for personal and religious reasons.

Moreover, the serum level of Receptor Activator of Nuclear factor Kappa beta Ligand (RANKL) among the breast cancer patients was significantly higher when compared to apparently healthy control subjects, agreeing with previous studies which showed that RANKL plays significant roles in mammary epithelial cell proliferation and is dysregulated during breast cancer [39]. The data also showed that among the patients, RANKL was higher in patients with triple negative phenotype compared to apparently control subject which is reportedly associated with worse clinical outcomes and bone metastasis in breast cancer patients with higher RANKL activity [40]. Also, serum NF-kB levels were significantly higher in breast cancer patients when compared to apparently healthy control, agreeing with the observation that NF-kB activity correlates with increased disease severity among breast cancer patients [41]. The serum levels of both RANKL and NF-kB were found to correlate with disease severity and were higher in patients at later stages of the disease, further suggesting that these proteins could have prognostic potential.

Oxidative stress is implicated in breast cancer pathogenesis [42], and our data showed significantly higher level of reduced glutathione (GSH) and higher level of malondialdehyde (MDA) in breast cancer patients when compared to apparently healthy control subjects. MDA was more elevated while GSH was more depleted in stage III and IV patients suggesting

that oxidative stress correlated with disease progression which is similar to previously reported studies [43].

CONCLUSION

Breast cancer patients studied in ABUTH, North-Western Nigeria showed no association between breast cancer and birth control pills and alcohol. However, serum levels of RANKL and NF-kβ were elevated in the patients and were higher as the disease progressed pointing to their strong procarcinogenic activity due to their role in tumor cells proliferation, survival, angiogenesis, inflammation, and metastasis. Malondialdehyde was elevated while glutathione was depleted in the patients and was more evident as the disease progressed, suggesting oxidative stress and a possible need for dietary awareness among the patients. Further cohort studies are needed to examine if similar patterns of RANKL and NF-kβ levels will be observed in patients *viz a viz* disease progression or response to therapy to determine the possible prognostic potential of RANKL and NF-kβ in postmenopausal breast cancer patients.

Abbreviations:

RANKL= receptor activator of nuclear factor kappa-beta ligand, NF-κβ = nuclear factor kappa-beta, GSH = reduced glutathione, MDA = malondialdehyde, ABUTH = Ahmadu Bello University Teaching Hospital

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Conflict of Interest:

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Data Availability Statement

Data will be available and share on request.

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