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Serum Inflammation Biomarkers and Micronutrient Levels in Nigerian Breast Cancer Patients with Different Hormonal Immunohistochemistry Status

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ABSTRACT

Background: The importance and relevance of serum inflammation biomarkers and DNA methylation-dependent micronutrients in breast tumorigenesis is gaining wider acceptance. However, the association of serum inflammation biomarkers and micronutrient status with expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor-2 (HER-2) by the tumor has not been investigated in Nigerian breast cancer patients. The objective of this study was to determine the levels of serum biomarkers of inflammation [Homocysteine, Nitric Oxide (NO), Hydrogen peroxide (H_2O_2), Myeloperoxidase (MPO), Tumor necrosis factor alpha (TNF-α), Interleukins 6 and 8 (IL-6 and IL-8)] and DNA methylation-dependent micronutrients [Zinc (Zn), Folic acid, Vitamin B6 and B12] in breast cancer patients with different hormone receptors (ER, PR and HER-2).

Methods: One hundred and fifteen women (80 with breast cancer and 35 controls) were randomly recruited for this study. Serum levels of homocysteine, folic acid, vitamins B6, vitamin B12, TNF-α, IL-6 and IL-8) were analyzed using ELISA, while the levels of NO, MPO, H_2O_2 and Zn were determined using spectrophotometer in patients with breast cancer and control subjects without breast cancer as well as breast cancer patients with ER, PR and HER-2 expression were determined.

Results: The results showed that mean serum levels of IL-6 (p=0.002), IL-8 (p=0.018) and H_2O_2 (p=0.000) were significantly increased while TNF-α (p=0.014) and NO levels (p=0.044) were significantly decreased in breast cancer patients compared to healthy controls. However, there were no statistically significant differences in the levels of Zn, homocysteine, Vitamin B6, Vitamin B12 and MPO in breast cancer patients and controls. Furthermore, the levels of serum inflammatory biomarkers and methylation-dependent micronutrients were similar in breast cancer patients with HER-2, ER and PR expression.

Conclusion: Systemic inflammation exists in breast cancer patients but the inflammation biomarkers and methylation-dependent micronutrients did not differ among breast cancer patients with PR, ER and HER-2 expression.

INTRODUCTION

Globally, breast cancer is the most commonly diagnosed cancer in women[1] and while progress has been made over the last decades in understanding the biology of breast cancer, the mechanisms for growth and progression of breast cancer with different hormonal phenotypes and therapeutic resistance are still not fully understood.[2,3] Inflammation is an important factor in carcinogenesis; hence, the use of non-steroidal anti-inflammatory drugs such as aspirin in cancer prevention adjuvant therapy[4,5] and micronutrients as supplements was suggested.[6] There

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is evidence that DNA methylation and its associated micronutrients influence incidence of cancers through regulation of inflammatory genes and that the tumor cells produce proinflammatory factors that encourage chronic inflammation and tumor growth.[7,8]

Studies associating circulating homocysteine with overall breast cancer risk are limited and inconsistent.[9] Homocysteine is associated with oxidative damage and metabolic disorders which may lead to carcinogenesis. It is transsulfurated to cysteine or remethylated to methionine using cystathionine β-synthase with vitamin B6 and methionine synthase with vitamin B12 plus folate, respectively.[10] In vitro studies have shown that homocysteine levels were positively associated with proliferation rates of cells in several tumors, including breast cancer.[11,12] Similar associations have been observed with oxidative damage to cells apart from breast cancer cells.[13,14] One case-control study reported a positive association between homocysteine levels and breast cancer risk[15], whereas another cohort study did not observe such association.[9]

Folate, vitamin B6, Zn and vitamin B12 are important in cancer prevention by upholding DNA integrity and regulation of gene expression.[16,17] Observational studies have suggested an inverse association between high intake or blood levels of folate, vitamin B6, and vitamin B12 and increased risk of cancer, particularly colorectal and breast cancers.[18–21] Zinc activates inflammasome[22], induces IL-1β secretion by macrophages[23], reduces IL-6 and TNF-α in human monocytes[24], and neutralizes generation of reactive oxygen species (ROS). Two case-control studies demonstrated statistically significant inverse associations between serum Zn exposure and breast cancer risk.[25,26] However, two nested case-control studies reported no association between zinc in benign breast tissue and breast cancer risk.[27,28] Another case-control study also found no difference in plasma level of zinc between breast cancer cases and controls.[29] Thus, interlinkage of Zn, vitamin B6, B12, folate and homocysteine metabolism seems essential for carcinogenesis but the results are inconsistent.

The importance of steroid hormone receptors to the biology of breast cancer was recognized when human breast cancers were shown to be dependent on estrogen and/or progesterone through their receptors for growth.[30] Breast cancer cells may or may not have estrogen receptor (ER), progesterone receptor (PR), and Human Epidermal Growth Factor Receptor 2 (HER-2). These receptor types have been found to determine breast cancer aggressiveness, treatment and prognosis[31,32] but the basis of this is not yet fully elucidated.

Metastasis involves cellular elements that secrete humoral products, which modulate the behavior of tumor cells in the micro-environment.[33,34] Hence, the presence of hypoxia and abundance of cytokines (Colony Stimulating Factor-1, TNF-α, IFN-γ and MIF in the tumor determines the macrophage subtypes (M1 and M2)[35,36] which affect the clinical course of the tumor. M1 macrophages are 'primed' by the cytokine IFN-γ for activation either by TNF-α or (and more importantly) by activation of toll-like receptors[37]. Activated M1 macrophages secrete pro-inflammatory cytokines such as interferons and interleukins (IL-12, IL-23)[36], generate toxic oxygen species and activate inducible NO synthase (iNOS) gene to produce nitric oxide (NO). Nitric oxide is an intermediate reactive oxygen species and prolonged exposure to NO results in DNA damage that is linked to cancer development.[37] M2 macrophages are activated by cytokines or by immune complexes to induce T-helper 2 immune responses.

Overall, micronutrients and other inflammation factors play important roles in tumor progression. Therefore, knowledge of functions and levels of biomarkers of inflammation, and micronutrients involved in tumorigenesis could provide better understanding of tumor development, prevention and immunotherapy. This study explored the relationship between pro-inflammation factors (IL-6, IL-8, TNF-α, NO, MPO and H_2O_2) and DNA methylation-dependent micronutrients (Zn, folate, vitamin B6, B12 and homocysteine) in breast cancer patients compared with controls in different breast cancer hormone subtypes in Nigerian women.

These markers (pro-inflammation factors and DNA methylation-dependent micronutrients) were evaluated in the participants because they were directly linked with breast cancer immune-pathology and progression.

METHODS

Subjects

Approval for the study was obtained from the ethics committee of UI/UCH, Ibadan. After obtaining informed consent, eighty women with breast cancer who attended the University Teaching Hospital Ibadan, Oyo State, in Nigeria were recruited between March 2016 and June 2019. The mean age of women was 57.9±11.1 years. Breast cancer patients who received any therapy prior to diagnosis (surgery/radiotherapy/chemotherapy) were excluded. Patients who presented with other malignancies, advanced organ failure or active infection were also excluded. The diagnosis of breast cancer was confirmed by histopathological and immunohi-stochemistry examination of the tumor tissue samples. Thirty-five healthy age-matched female consented volunteers (55.9±9.0 years) who had no history or clinical evidence of breast problem or cancer

drawn from the Hospital and University communities were selected as healthy controls. Confounding factors such as age of menarche, ethnicity, age of first birth and parity were not taken into consideration during the study. However, smokers, hormone and alcohol users, and obese participants were excluded. All participants on compulsory medications or on food supplements were also excluded. Blood samples collected from breast cancer patients before treatment and in the healthy controls were allowed to clot, centrifuged at 8000 rpm for 10 minutes, serum separated and stored at -80^0C until analyzed.

Serum IL-6, IL-8 and TNF-α assays

The procedure followed the manufacturer's (ABCAM USA) instructions as previously described.[38] All reagents were brought to room temperature (18-25^{0}C) prior to use. Up to 100µL of each standard and sample was added into appropriate wells. The wells were covered and incubated for 150 minutes at room temperature. The solutions were discarded and washed 4 times, by filling each well with 1X wash solution (300µL) using a multi-channel pipette. Supernatant was completely removed at each step. After the last wash, any remaining wash buffer was removed by decanting and the plate was blotted against clean paper towels. Then, 100µL of 1X biotinylated TNF-α detection antibody was added to each well and incubated for 60 minutes at room temperature with gentle shaking. The solution was decanted and the wash was repeated. Then, 100µL of 1X HRP-Streptavidin solution was added to each well. The plate was incubated for 45 minutes at room temperature with gentle shaking. The solution was decanted and the wells were washed. Then, 100µL of TMB one-step substrate reagent was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking, and 50µL of stop solution was added to each well. The wells were read at 450 nm. A standard curve was constructed for each method using the respective standard and used for the determination of unknown respective serum sample concentrations of IL-6, IL-8 or TNF-α.

Serum nitric oxide (NO) determination

Nitric oxide concentration was determined using Griess reagent (Sulpanilamide and N-1-napthyethylenediamine dihydrochloride) as previously described.[38] The assay was based on a reaction that utilized sulpanilamide and N-1-napthyethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. Nitrite forms colored chromophore with reagent, with an absorbance spectrum maximum at 540nm. The production of nitrite was quantified by comparing the result with absorbance of standard solutions of sodium nitrite.

Serum hydrogen peroxide determination

Hydrogen peroxide concentration was determined as previously carried out. The assay was based on peroxide-mediated oxidation of Fe2+, followed by the reaction of Fe3+ with Xylenol orange to form Fe3+-Xylenol orange complex with an absorbance maximum of 560nm. Plasma H_2O_2 was determined by comparing absorbance with standard solutions of H_2O_2.

Serum myeloperoxidase (MPO) activity determination

MPO activity was determined as previously carried out.[39] The rate of decomposition of H_2O_2 by peroxidase, with guaiacol as hydrogen donor, produced tetraguaiacol which was measured at 436nm and at 25^{o}C.

Vitamin determinations

Vitamins B6, B12, folic acid and homocysteine were determined using Enzyme Linked Immunosorbent assay (ELISA) method as previously carried out.[38,39]

Statistical analysis

Data obtained were analyzed using SPSS version 20, which are presented in the Tables as Means and Standard error of means. The data were evaluated using Student *t*-test for the patient group relative to the control group (Table 1) while Analysis of Variance (ANOVA) was used to compare data between the three groups of breast cancer patients. Student *t*-test was used to compare data between two groups of breast cancer patients in Tables 2, 3, Figures 1, 2, 3 and 4. $P \leq 0.05$ was taken as significant.

RESULTS

The mean values and statistical comparison of TNF-α, IL-6, IL-8, MPO, NO and H_2O_2 in breast cancer patients and the control group are presented in Table 1. As the results show, the mean values of IL-6 (p=0.002), IL-8 (p=0.018) and H_2O_2 (p=0.000) were significantly increased while TNF-α (p=0.014) and NO (p=0.044) were significantly decreased when breast cancer patients were compared with apparently healthy controls. However, there were no statistically significant differences in the levels of MPO, Zn, homocysyteine, vitamin B6 and vitamin B12 when breast cancer patients were compared with controls (Table 1). Further, there were no statistically significant differences in the values of inflammation factors (Table 2) and micronutrients (Table 3) when breast cancer patients with different immunohisto -

chemical hormonal status were compared or when breast cancer patients with double positives, single positive or no hormonal receptors were compared (Figures 1 and 2). There were no statistically significant differences in the values of inflammation factors and micronutrients when breast cancer patients with different receptors were compared (Figures 3 and 4).

Table 1. Comparison (Mean±SEM) of inflammation biomarkers and micronutrients in breast cancer patients and controls.

Inflammation biomarkers	BCa (n=80)	Controls (n=35)	t-values	*P*
H_2O_2(µmol/L)	37.98±1.64	18.18±1.27	9.521	0.000*
IL-6 (pg/mL)	31.72±6.10	11.54±0.37	3.305	0.002*
IL-8 (pg/mL)	29.65±2.48	23.33±0.77	2.438	0.018*
TNF-α (pg/mL)	7.96±1.24	19.49±5.95	2.507	0.014*
NO (µmol/L)	25.32±2.48	32.58±2.54	2.046	0.044*
MPO (U/mL)	1.15±0.20	1.29±0.02	0.695	0.489
Micronutrients				
Zn (µg/dL)	102.66±1.23	104.49±2.00	0.780	0.439
Hcy (µmol/L)	12.69±0.54	12.14±0.81	0.560	0.570
Folate (µg/L)	348.51±14.76	333.39±22.14	0.568	0.572
Vit B6 (ng/mL)	15.86±0.67	15.17±1.01	0.570	0.578
Vit B12 (ng/L)	419.12±17.29	405.73±26.95	0.416	0.677

*Significant at $P<0.05$

BCa= Breast cancer, ER= Estrogen receptor, PR= Progesterone receptor, HER-2= Human Epidermal Growth Factor Receptor 2, Hcy= Homocysteine, NO= Nitric Oxide, H_2O_2=Hydrogen peroxide, MPO= Myeloperoxidase, TNF-α= Tumor necrosis factor alpha, IL-6= Interleukin 6, Zn= Zinc, IL-8= Interleukin 8

Table 2. Comparison (Mean±SEM) of inflammation biomarkers in breast cancer patients with different immunohistochemical hormonal levels.

	H_2O_2 (µmol/L)	IL-6 (pg/mL)	IL-8 (pg/mL)	TNF-α (pg/mL)	NO (µmol/L)	MPO (U/mL)
ER+ve(n=17)	38.6±10.4	26.6±9.4	28.1±2.1	8.1±1.5	24.8±14.9	1.0±0.3
PR+ve (n=16)	35.8±14.0	24.2±8.5	27.3±3.7	8.7±2.3	31.9±32.2	0.8±0.4
HER-2+ve (n=16)	33.0±14.4	17.8±6.4	32.8±18.8	7.9±0.9	18.2±11.3	1.0±0.4
t, p-a	0.415, 0.686	0.497, 0.629	0.498, 0.628	-0.564, 0.584	-0.527, 0.609	0.851, 0.413
t, p-b	0.807, 0.437	1.947, 0.078	-0.669, 0.517	0.424, 0.680	0.879, 0.398	0.017, 0.987
t, p-c	0.335, 0.744	1.475, 0.171	-0.713, 0.492	0.883, 0.398	0.982, 0.349	-0.769, 0.460

a: ER+ve compared withPR+ve
b: ER+ve compared with HER-2+ve
c: PR+ve compared with HER-2+ve
ER=Estrogen receptor, PR=Progesterone receptor, HER-2=Human Epidermal Growth Factor Receptor 2, NO=Nitric Oxide, H_2O_2=Hydrogen peroxide, MPO=Myeloperoxidase, TNF-α=Tumor necrosis factor alpha, IL-6=Interleukin 6, IL-8=Interleukin 8

Table 3. Comparison (Mean±SEM) of micronutrients in breast cancer patients with different immunohistochemical hormonal levels.

	Zn (µg/dL)	**Hcy(µmol/L)**	**Folate (µg/L)**	**Vit B6 (ng/mL)**	**Vit B12 (ng/L)**
ER+ve (n=17)	102.3±9.5	11.3±4.2	310.3±115.1	14.1±5.2	377.6±140.1
PR+ve (n=16)	101.7±7.6	12.4±3.7	339.8±102.1	15.5±4.6	413.5±124.2
HER-2+ve (n=16)	100.3±8.2	12.5±5.2	343.5±143.1	15.6±6.5	418.1±174.1
t, p-a	0.131, 0.898	-0.485, 0.637	-0.485, 0.637	-0.485, 0.637	-0.485, 0.637
t, p-b	0.390, 0.704	-0.465, 0.651	-0.465, 0.651	-0.485, 0.651	-0.485, 0.651
t, p-c	0.287, 0.780	-0.052, 0.960	-0.052, 0.960	-0.052, 0.960	-0.052, 0.960

a: ER+ve compared withPR+ve
b: ER+ve compared with HER-2+ve
c: PR+ve compared with HER-2+ve
ER=Estrogen receptor, PR=Progesterone receptor, HER-2=Human Epidermal Growth Factor Receptor 2, Hcy=Homocysteine, Zn=Zinc, Vit B6=Vitamin B6, Vit B12=Vitamin B12

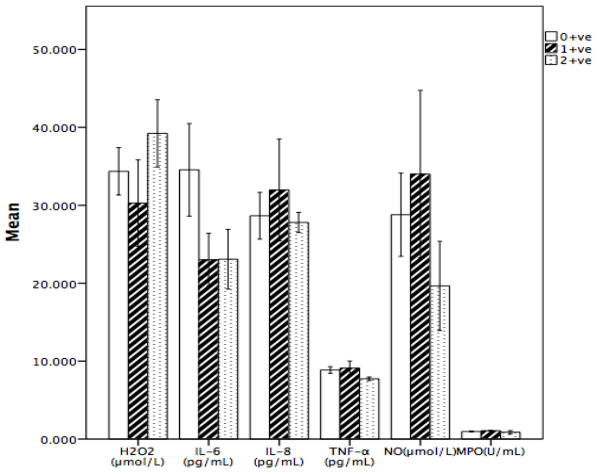

Figure 1. Inflammation biomarkers in breast cancer patients with double positives (2+ve), single positive (1+ve) or no (0+ve) hormonal receptor.
NO=Nitric Oxide, H_2O_2=Hydrogen peroxide, MPO= Myeloperoxidase, TNF-α=Tumor necrosis factor alpha, IL-6=Interleukin 6, IL-8=Interleukin 8

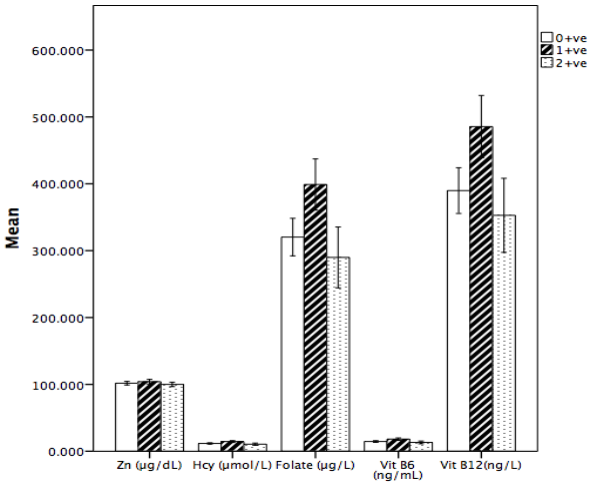

Figure 2. Micronutrients in breast cancer patients having double positives (2+ve), single positive (1+ve) or no (0+ve) hormonal receptor.
Hcy=Homocysteine, Zn=Zinc, Vit B6=Vitamin B6, Vit B12=Vitamin B12

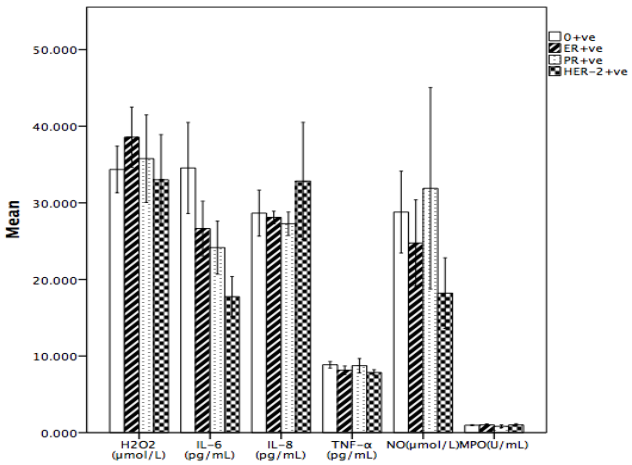

Figure 3. Inflammation factors in breast cancer patients having HER-2+ve, ER+ve, PR+ve with no (0+ve) hormonal receptor.
ER=Estrogen receptor, PR=Progesterone receptor, HER-2=Human Epidermal Growth Factor Receptor 2, Hcy=Homocysteine, NO=Nitric Oxide, H_2O_2=Hydrogen peroxide, MPO=Myeloperoxidase, TNF-α=Tumor necrosis factor alpha, IL-6=Interleukin 6, IL-8=Interleukin 8

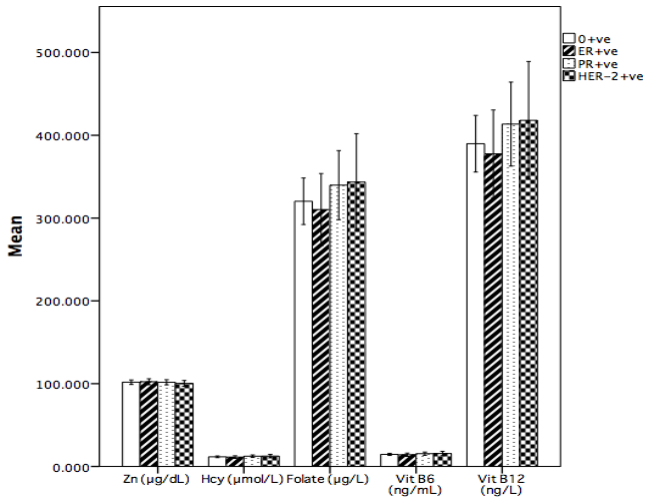

Figure 4. Micronutrients in breast cancer patients having HER-2+ve, ER+ve, PR+ve with no (0+ve) hormonal receptor.
ER=Estrogen receptor, PR=Progesterone receptor, HER-2=Human Epidermal Growth Factor Receptor 2, Hcy=Homocysteine, Zn=Zinc, Vit B6=Vitamin B6, Vit B12=Vitamin B12

DISCUSSION

Globally, breast cancer constitutes a large public health burden among females and is often associated with inflammation. Studies elsewhere proposed the use of non-steroidal anti-inflammatory drugs such as aspirin in cancer prevention and as adjuvant therapies[4,5] or use of micronutrients in reducing cancer progression.[6] Other studies found inverse correlation between high intake or blood level of folate, vitamin B6, and vitamin B12 and risk of breast cancer[18–21,40] while the studies associating circulating homocysteine with breast cancer risk are limited and with inconsistent result.[9] However, the bases of earlier propositions linking serum inflammation factors or micronutrient status with breast cancer hormonal types have not been completely established. This study provides additional insight into the complex role of systemic inflammation and micronutrients involved in DNA methylation pathway in breast cancer etiology and progression.

The present study observed that serum levels of H_2O_2, IL-6 and IL-8, which drive inflammation were elevated in women with breast cancer relative to control subjects. Therefore, control of inflammatory process might have a complementary role in the management of breast cancer patients. Esquivel-Velázquez *et al.* earlier reported that over-production of certain pro-inflammatory cytokines in breast cancer patients correlated with poor prognosis.[41] Serum IL-6 and IL-8 have been implicated in the initiation and progression of ductal carcinoma.[42–47] Our finding of increased IL-8 in breast cancer patients support previous reports that IL-8 is a chemokine that has tumor-promoting role and cancer predictive potential.[44,45] Angiogenesis is an important step in metastasis in which IL-8 plays a critical role.[48] Increased serum IL-8 in our breast cancer patients supports its functions in angiogenicity, neo-vascularization and increased propensity for metastasis as earlier studies suggested.[44,45,48] Derin *et al.* reported high expression of IL-8 receptors on all breast cancer cells; thus, increased IL-8 in the breast cancer patients might be in response to over-expressed IL-8 receptors on breast cancer cells which accelerate angiogenesis.[49] Thus, our data support the use of IL-8 antagonist as a plausible complementary option in the management of breast cancer patients.[48,49]

Numerous cytokines, such as IL-6, IL-8 and TNF-α have been implicated in the initiation and progression of ductal carcinoma.[42–49] IL-6 is a pro-inflammatory cytokine that has multiple functions such as regulation of immune functions and hematopoiesis, inhibition of apoptosis of cancer cells and stimulation of tumor angiogenesis.[24] Serum IL-6 levels were increased in breast cancer patients which correlated with tumor stage and patient survival.[12–16] Therefore, IL-6 has a tumor-promoting role with predictive cancer potential as earlier research pointed out.[44]

Granulocytes produce H_2O_2 via metabolic processes as an immunological response to foreign invaders. Hydrogen peroxide is catabolized to produce hypochlorous acid and hydroxyl radical. These highly reactive oxygen species have been reported to be effective in the killing of intracellular bacteria.[6] Hydrogen peroxide at high concentrations causes membrane damage, increases lactate dehydrogenase leakage, membrane permeability or cell necrosis.[50] A change in membrane permeability disturbs structural integrity, which could lead to the increased entry of toxins into cells and cause cell death at a later stage. Also, hydrogen peroxide has been reported to inactivate superoxide dismutase, which is an antioxidant.[51] Therefore, increased blood levels of H_2O_2 might be responsible for gradual local tissue destruction or attempt to destroy invading micro-organisms in breast cancer patients.

Apart from its role in intracellular killing of pathogens and vascular smooth muscle relaxation,

Nitric oxide (NO) modulates gene expression via the agency of transcription factors, especially Nuclear Factor-κB (NF-κB), which regulates transcription of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6 and IL-8) and enzyme (COX-2).[52] In this present study, serum concentration of NO in breast cancer patients was significantly decreased when compared with controls. This might be one of the mechanisms to reduce NO-induced inflammatory processes in breast cancer patients. Also, NO directly oxidizes DNA, resulting in mutagenic changes and damage to DNA repair proteins.[53] NO is produced by conversion of arginine to citrulline using inducible nitric oxide synthase in phagocytes. Therefore, we speculated that low arginine level might account for decreased NO levels in breast cancer patients, but this requires further investigation.

Breast cancer is distinguished by different molecular subtypes, risk factors, clinical behaviors, and responses to treatment and steroid hormone receptors such as progesterone receptor (PR), estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER-2) on tumors have been shown to play important roles in cancer progression and prognosis.[54,55] Whether there are relationships between the wide spectrum of serum inflammation factors, micronutrients and immunohistochemistry status of breast cancer patients has not been fully addressed.

Furthermore, expressions of ER, PR and HER-2 in breast carcinoma patients were found to correlate with prognosis.[56–59] This necessitates linking pro-inflammatory biomarkers with ER, PR or HER-2 expressions in Nigerian breast cancer patients.

CONCLUSION

In the present study, there were no differences in the levels of serum inflammation biomarkers and micronutrient levels in Nigerian breast cancer patients relative to ER, PR or HER-2 expression. The authors of the present study speculated that cytokine production and micronutrient status were not affected by ER, PR or HER-2 expression. However, recruitment of larger participants will further elucidate this conjecture. A key limitation of this study was the small number of breast cancer patients with different hormonal immunohistochemistry statuses.

CONFLICT OF INTEREST

None.

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