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Relationships Between Circulating Immune Complexes (CIC) and Serum Immunoglobulins (IgG, IgM and IgA) in Malignant and Pre-malignant Disease Conditions of the Breast.

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ABSTRACT

Background: Breast cancer is a major public health burden and the most frequently diagnosed cancer among women globally. Most cancers arise in association with chronic inflammation and contain inflammatory infiltrates. These have a broad impact on tumour initiation, growth, and progression and various immunological factors are expressed in the serum during breast tumorigenesis, and can be of value in the surveillance of the disease. In order to examine the clinical utility of CIC and different classes of immunoglobulin, we evaluated and correlated the serum levels at different disease stages and treatment groups in women with malignant and pre-malignant disease conditions of the breast.
Methods: A total of 59 females (mean age = 48.7± 8.7yrs) with clinically and pathologically confirmed breast cancer were prospectively recruited alongside with 20 patients with benign breast tumour representing patients' control group and 20 apparently healthy age and sex-matched control subjects (mean age = 47.5± 13.4yrs). Breast cancer patients were further grouped into early stage breast cancer (N=25) and advanced stage breast cancer (N=34). Patients were subjected to standard treatment modalities and pre- and post–treatment samples collected at intervals. The CIC, IgG and IgM assays were carried out by immuno-enzymatic methods while immuno-turbidimetric method was used for IgA assay.
Results: The descriptive comparison of CIC and serum immunoglobulins (IgG, IgM, and IgA) showed no significant ($P>0.05$) differences across disease stages and treatment groups. Inconsistent relationships were observed between CIC and immunoglobulins across disease groups and stages of treatment.
Conclusion: Results suggest that serum levels of CIC and immunoglobulins have limited potentials in the surveillance of breast cancer in our environment. There is a need for other immunological factors that may be of clinical utility.

Key words: Breast tumour, CIC, serum immunoglobulins, disease and treatment stages.

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Author's contributions:
This work was carried out and approved in collaboration between all authors. CEF designed the study, sourced for funding, wrote the protocol, contributed in literature search, statistical analysis, wrote the manuscript. CCF did the experiments; managed literature searches, data & statistical analysis, wrote the manuscript, and proofread the manuscript.

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1. INTRODUCTION

Breast cancer is a major public health burden and is the most frequently diagnosed cancer among women globally (1, 2). The WHO (3) estimates that the prevalence of breast cancer could go up to 50% by 2020 current prevalence of 1.2 million worldwide to 1.5 million. The prevalence rate is increasing even among countries that used to have low prevalence.

African countries present with low prevalence, more aggressive, increased mortality, earlier age at presentation (35 – 45 years) and different pattern of gene expression (4). Most of the studies in Nigeria confirm late presentation with advanced disease and poor clinical outcome (5,6). Therefore, study on the current paradigm will be of immense clinical benefit in the management of breast cancer.

Although biological markers are widely recognized as important complementary tools in the differential diagnosis and management of breast cancer and cancers in general (7,8), there are still varied opinions on their clinical utility in diagnosing, staging or managing patients with cancer. Racial/ethnic variations in some of these immunological factors have also been documented (9). Thus, there is need to develop immunological based techniques with diagnostic and or prognostic potentials peculiar to each race.

Human breast cancer cells have long been shown to possess tumour neoantigens (10). The immunogenicity of cancer cells, the possible antibody response in cancer patients and the resultant interaction between specific antibodies and cancer antigens may result in the production of specific circulating immune complexes (11). Circulating immune complexes have been detected in certain cancers of the mouth (12-14), esophagopharyngeal and gastric cancers (15) lung cancer (16), breast cancer (17,18), and colon cancer (19) and their concentrations suggested to have possible relationship to stages of the disease. Circulating immune complexes play a specific role as initiators of mechanism of tissue injury in many infections, autoimmune diseases and neoplastic diseases. A strong correlation is thought to exists between circulating immune complex level and progress to cancer and to some extent in breast cancer. The discovery of circulating immune complexes against specific cancer antigens have been documented (21-22). The association of serum immunoglobulins in human cancers has been reported by various workers such as carcinoma of cervix (23), pancreatic cancer, (24), primary liver carcinoma, (25), squamous cell carcinoma, (26) and breast cancer, (21,27) with varied findings and opinions. In oral cancer patients, previous investigations of serum immunogloblin levels showed an increase in IgM, IgA, IgE, and IgG when compared with normal individuals (12). The work of Kemp *et al.*, (23), contradicted the earlier finding and observed that serum IgG and IgA are not significantly elevated in oral cancers and hence not to be used as a good surrogate marker for diagnosis and prognosis.

In our quest for immunological factors that may be of use in the diagnosis, surveillance and management of breast cancer in our environment, we had previously studied the clinical utility of serum cancer antigens and pro-inflammatory cytokines (28-31). There is need therefore to study the immunoglobulins and their correlative association with circulating immune complexes. Previous reports of immunoglobulin levels in breast cancer patients are conflicting (32-37) and numerous responses of immune disturbances have been associated with breast cancer due to possible defect in immune mechanisms (38). A study observed marked increased

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IgM in breast fluid of breast cancer patients prior to mastectomy with decrease in IgA levels (35). Similar significant reductions in IgA and IgG in breast tissues were observed by Robert *et al.,* (32). Total immunoglobulin levels and IgG levels were found to correlate with plasma cell infiltration. The previous studies neither discussed the staging of the breast cancer patients, the effect of treatment nor used age-matched controls. Our study was undertaken to compare the major serum immunoglobulin levels (IgA, IgG, and IgM) in patients with malignant and pre-malignant breast conditions of the breast across disease stages and treatment groups that were of similar age.

2. MATERIALS AND METHODS

2.1: Subjects and sampling

This is a prospective longitudinal study conducted between February 2015 and April; 2018 on treatment-naïve female patients age range from 30 to 68 years with breast tumour and referred to the Oncology Units of Departments of Surgery of University of Nigeria Teaching Hospital, Enugu and Federal Teaching Hospital, Abakaliki (both in Eastern-Nigeria). Diagnosis and staging were clinically and pathologically confirmed. The studied individuals were grouped into three.

Group I: **Patients group** – included 59 breast cancer patients; due to small sample size this group was further divided into early stage breast cancer (stages 1 & 2, N=24) and advanced stage breast cancer (stages 3 & 4, N=35) based on Tumour Node Metastasis (TNM) classification.

Group II: Patients control – included 20 patients with benign breast tumour (BBT).

Group III: Normal control – 20 apparently healthy age/sex matched (AHMC) female volunteers without history or clinical evidence of breast lesion drawn from hospital and university communities.

Sampling was by self-selection consequent to the approval of the study protocol by the respective Hospitals Ethical Committee, informed written consent obtained from the individuals and exclusion criteria applied. Structured questionnaire on socio-demographic factors were served, explained and interpreted in local Igbo language for the patients that are not literate. Breast tumour patients who received any therapy prior to diagnosis (surgery/radiotherapy/chemotherapy), previous history of malignancy and history of any other medical illness, which would otherwise limit the survival of the patient in the absence of malignancy, were excluded. All patients underwent standard treatment modalities (neoadjuvant or adjuvant chemotherapy, radiotherapy, chemoradiation, and/or surgery; depending on the stage of presentation. In breast cancer (BC) patients and benign breast tumour (patients control groups), blood samples were collected before any form of treatment and two more samples at 3 and 6 months interval. In apparently healthy sex/age-matched control, one blood sample was taken from each participant. The samples were allowed to clot and retract, centrifuged at 5000rpm, serum separated and stored at -20°C until analyzed.

2.2: Assay Methods

Circulating immune complexes and serum immunoglobulins (IgG and IgM**)** assays were carried out by immunoenzymatic methods (kits sourced from abcamR; ab178665, Diagnostic Automation/Cortz Diagnostics, Inc., AccuDiagTM Cat# 1803-9, Calabasa, CA 91302, USA) respectively. The molecules of CIC-C3d, IgG or IgM respectively are sandwiched between two monoclonal antibodies; one coated to the bottom of the wells of microtiter plates and the other linked to the horseradish peroxidase (enzyme conjugate). After incubation and washing, the enzymatic reaction develops a colour which is proportional to the amount of CIC-C3d, IgG or IgM molecules present in the assay.

The immunoturbidimetric assay method was adopted for the estimation of total serum IgA using URIT – 810 Chemistry Analyzer (kit was

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sourced from Randox Laboratories, UK; Cat #1A2447). The principle is based on the turbidimetric specific reaction which occurs between the anti-IgA polyclonal antiserum and its corresponding antigen in optimal pH conditions and in the presence of polyethyleneglycol polymer (PEG). The change in turbidity of the immunocomplex (as measured spectrometrically) is proportional to the concentration of the analyte in the sample.

2.3: Statistical analysis

Data were analyzed using statistical package for Social Sciences (SPSS) version 20 software. Statistical significance was set at $p < 0.05$. The descriptive statistics were presented in tables. Comparison of relationships across disease and treatment groups were done using Pearson's correlation and Dunn's multiple comparison tests. GraphPad prism version 6.0 (by GraphPad, USA) was used for the graphs

3. RESULTS

The descriptive comparison of CIC, IgG, IgM and IgA is presented in Table 1. No significant differences in CIC were found between treatment and disease groups except between 3 months ESBS and Pre-BBT ($P<0.05$). The highest level in mean CIC values were seen in advanced stage breast cancer though comparatively insignificant with other disease groups (Fig. 1).

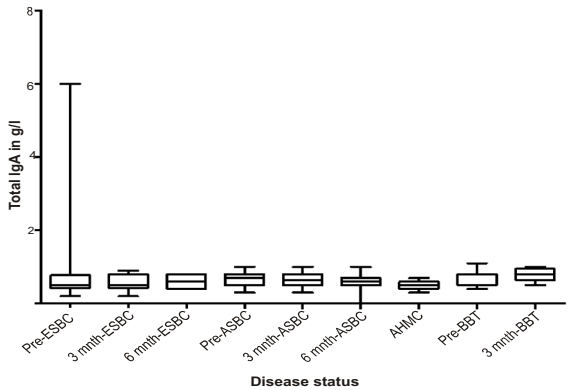

Fig. 4 Comparison of total serum IgA levels across the disease and treatment groups (P value = 0.11).

ESBC-Early stage breast cancer. ASBC-Advanced stage breast cancer. AHMC-Apparently healthy control.

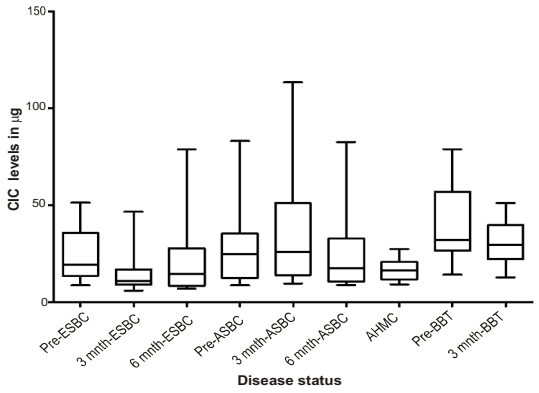

Fig 1. Comparison of CIC levels across disease and treatment groups (P value = 0.0058).

ESBC-Early stage breast cancer. ASBC-Advanced stage breast cancer. AHMC-Apparently healthy control. BBT=Benign breast tumour.

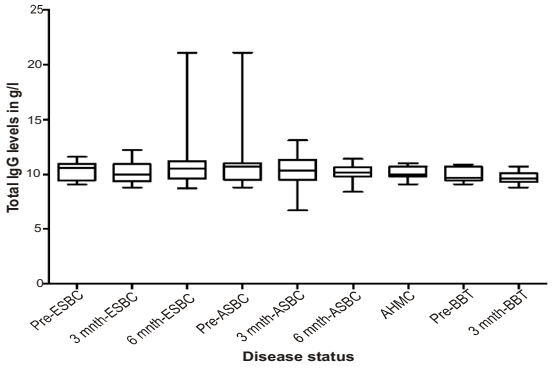

Fig 2. Comparison of total serum IgG levels across the disease and treatment groups (P value = 0.57).

ESBC-Early stage breast cancer. ASBC-Advanced stage breast cancer. AHMC-Apparently healthy control. BBT=Benign breast tumour.

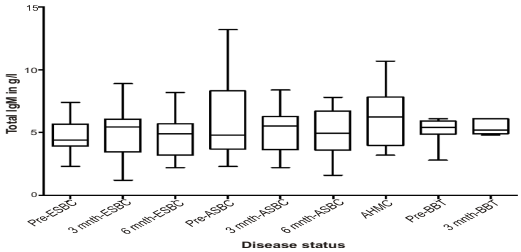

Fig 3. Comparison of total serum IgM levels across the disease and treatment groups (P value = 0.86).

ESBC-Early stage breast cancer. ASBC-Advanced stage breast cancer. AHMC-Apparently healthy control. BBT=Benign breast tumour.

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Table 1 Summary of Statistics for immunoglobulins level across disease and treatment groups

Category	Var	Mean	SE	Median	Std. Dev.	Min	Max	25% Per	75% Per	LCI	UCI
Pre-treatment ESBC	IgG	10.43	0.13	10.70	0.70	9.10	11.60	9.93	10.95	10.30	10.56
	IgM	4.61	0.25	4.20	1.32	2.30	7.40	3.90	5.48	4.37	4.86
	IgA	0.64	0.06	0.50	0.33	0.20	2.00	0.50	0.80	0.58	0.71
3 months post-treatment ESBC	IgG	9.97	0.17	9.70	0.88	8.80	12.20	9.40	10.53	9.81	10.14
	IgM	5.04	0.43	5.25	2.26	1.20	8.80	3.40	6.08	4.61	5.47
	IgA	0.58	0.05	0.50	0.25	0.10	1.10	0.40	0.80	0.53	0.62
6 months post-treatment ESBC	IgG	10.03	0.17	10.30	0.85	8.60	11.20	9.50	10.70	9.86	10.19
	IgM	4.84	0.34	5.00	1.75	2.10	8.50	3.20	5.73	4.50	5.19
	IgA	0.63	0.03	0.70	0.16	0.40	0.80	0.48	0.80	0.60	0.66
Pre-treatment ASBC	IgG	11.29	0.52	10.70	3.31	8.80	21.10	9.50	11.00	10.76	11.81
	IgM	5.94	0.46	4.80	2.90	2.30	13.20	3.68	8.33	5.48	6.40
	IgA	0.65	0.03	0.70	0.19	0.30	1.00	0.50	0.80	0.62	0.67
3 months post-treatment ASBC	IgG	10.14	0.19	10.20	1.23	6.70	11.70	9.50	11.08	9.94	10.33
	IgM	5.26	0.30	5.50	1.88	2.20	8.40	3.63	6.28	4.96	5.56
	IgA	0.67	0.03	0.65	0.20	0.30	1.00	0.50	0.80	0.64	0.70
6 months post-treatment ASBC	Igg	10.19	0.13	10.15	0.70	8.40	11.40	9.80	10.60	10.05	10.32
	IGm	5.03	0.38	4.95	1.99	1.60	7.80	3.90	6.50	4.65	5.40
	IgA	0.62	0.03	0.60	0.16	0.40	1.00	0.50	0.70	0.59	0.65
Pre-treatment BBT	IgG	10.15	0.16	9.95	0.64	9.10	11.30	9.83	10.70	9.99	10.31
	IgM	6.12	0.59	6.20	2.35	3.20	10.70	3.68	7.60	5.53	6.71
	IgA	0.51	0.04	0.50	0.17	0.30	0.90	0.40	0.60	0.46	0.55
3 months post-treatment BBT	IgG	10.12	0.15	9.95	0.59	9.10	11.00	9.83	10.70	9.97	10.27
	IgM	5.91	0.51	6.20	2.02	3.20	8.50	3.68	7.60	5.41	6.42
	IgA	0.51	0.04	0.50	0.17	0.30	0.90	0.40	0.60	0.46	0.55
AHMC	IgG	9.74	0.12	9.60	0.58	8.80	10.80	9.40	10.13	9.61	9.86
	IgM	5.42	0.11	5.20	0.54	4.80	6.30	4.98	6.10	5.31	5.54
	IgA	0.82	0.04	0.80	0.20	0.50	1.30	0.70	0.93	0.78	0.86

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Table 2: Relationship between CIC and immunoglobulins across disease and treatment groups

Ig.	Stages	Parameters	R	Equation	P-value
IgG	ESBC	CIC pre	0.016	=28.263 – 0.368 (IgG)	0.935
		CIC 3 months	0.124	=-1.563 + 1.869 (IgG)	0.530
		CIC 6 months	0.160	=14.382 + 0.477 (IgG)	0.76
IgM	ESBC	CIC pre	0.026	=25.883 – 0.319 (IgM)	0.895
		CIC 3 months	0.458	=3.532 + 2.688 (IgM)	0.014
		CIC 6 months	0.082	=15.154 + 0.896 (IgM)	0.763
IgA	ESBC	CIC pre	0.262	=16.242 + 12.707 (IgA)	0.178
		CIC 3 months	0.455	=3.386 + 23.812 (IgA)	0.015
		CIC 6 months	0.270	=-0.523 + 31.731 (IgA)	0.182
IgG	ASBC	CIC pre	0.046	=24.538 + 0.289 (IgG)	0.780
		CIC 3 months	0.109	=58.679 – 2.383 (IgG)	0.505
		CIC 6 months	0.643	=248.338 – 21.700 (IgG)	0.000
IgM	ASBC	CIC pre	0.015	=28.465 – 0.112 (IgM)	0.780
		CIC 3 months	0.308	=57.759 – 4.417 (IgM)	0.053
		CIC 6 months	0.347	=6.605 + 4.117 (IgM)	0.000
IgA	ASBC	CIC pre	0.174	=15.093 + 19.701 (IgA)	0.283
		CIC 3 months	0.429	=-4.137 + 57.705 (IgA)	0.006
		CIC 6 months	0.257	=50.933 – 38.019 (IgA)	0.186
IgG	BBT	CIC pre	0.082	=10.182 + 2.899 (IgG)	0.710
		CIC 3 months	0.328	=93.519 – 6.621 (IgG)	0.136
		CIC 6 months	-	-	-
IgM	BBT	CIC pre	0.249	=12.557 + 5.117 (IgM)	0.710
		CIC 3 months	0.039	=33.686 – 0.855 (IgM)	0.863
		CIC 6 months	-	-	-
IgA	BBT	CIC pre	0.368	=58.894 – 31.352 (IgA)	0.084
		CIC 3 months	0.561	=2.405 + 32.566 (IgA)	0.007
		CIC 6 months	-	-	-
IgG	AHM	CIC	0.160	=30.696 – 1.375 (IgG)	0.553
IgM	AHM	CIC	0.278	=20.695 – 0.646 (IgM)	0.553
IgA	AHM	CIC	0.277	=21.276 – 8.953 (IgA)	0.299

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In comparison; the serum immunoglobulins (IgG, IgM and IgA) showed no significant difference across disease and treatment groups Figs 2, 3 and 4).

No definite pattern of relationships was observed between CIC and serum immunoglobulins. At early stage breast cancer (ESBC), there were significant ($P<0.05$) positive relationship between CIC and (IgM and IgA) at 3 months post-treatment respectively. There were no relationships in other stages (Table 2). At advanced stage breast cancer (ASBC), the CIC showed negative significant relationship with IgG and positive significant relationship with IgM at 6 months post-treatment while the IgA presented positive significant relationship at 3 months post-treatment. In benign breast tumour stage (BBT), the CIC only correlated significantly with IgA at 3 months treatment. The 6 months post-treatment values of BBT were not determined because of poor sample size due to loss of patients to follow-up. The CIC values of apparently healthy control group correlated positively between the three immunoglobulin classes. These however were not statistically significant ($P>0.05$).

Results also showed that 78% of the cases did not have any history of cancer in their families. Also, majority (63%) of the cases had body mass index values suggestive of obesity ($> 30kg/m^2$). Based on our findings, it could also be concluded that cigarette smoking and social sophistication are not among the risk factors to cancer in this part of the world.

4. DISCUSSION

Biological markers are widely recognized as important tools in the evaluation and management of patients with cancer. An especially wide array of body fluid markers have been investigated for clinical utility in diagnosing, staging or managing patients with cancer (8). Clinical uses of biomarkers include screening and diagnosis of breast cancer; staging and monitoring response to therapy. Human cancer cells have long been shown to possess tumour neoantigens (10). The immunogenicity of cancer cells, the possible antibody response in cancer patients and the resultant interaction between specific antibodies and cancer antigens may result in the production of specific circulating immune complexes (11). Circulating immune complexes have been detected in certain cancers of the mouth (12,13), esophagopharyngeal cancer (39), lung cancer (16), breast cancer (40), colon cancer, Burkitts lymphoma and their concentrations suggested to have possible relationship to stages of the disease. Circulating immune complexes when deposited on tissues play a specific role as initiators of mechanism of tissue injury in many infections, autoimmune dseases and neoplastic diseases. A strong correlation is thought to exists between circulating immune complex levels and progress to cancer and to some extent in breast cancer. As one of the tumour markers, increased CIC levels is indicative of tumour burden and serum levels of CIC could be a prognostic indicator in cancers (41).

Some authors had reported that there is positive correlation between CIC and acute phase of the disease and that remission phase is often preceded by decrease in CIC (24,40,42,43). These studies nevertheless were done in western countries with different pattern in proteomics as well general health and environmental factors. Our previous works (28, 30) and studies by other researchers (14, 16) in breast cancer however, never associated CIC with disease stages and treatment. This suggests that CIC has no clinical utility in breast cancer in our racial/ethnic group. The present study considered the possible elevation in CIC among patients with malignant and pre-malignant disease conditions of the breast by measurement of CIC-C3d immunocomplex in sera. Since it is known that the activated C3 molecule is attached to the CIC by a covalent thiolester bond; the specific binding of C3 fragments may be adopted in detecting circulating immune complexes in different diseases.

Although in this study, some patients with breast cancer (28%) as well as patients with benign breast tumour presented with elevated CIC activities, the results did not differ significantly in course of treatment, hence the diagnostic utility is queried. This could possibly be due to

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increased triggers for immunocomplex formation in this environment- infections (especially parasitic and bacteria associated with unhealthy environment) and other inflammatory conditions. This study could not possibly exclude all the triggers for the elevation of CIC even in the apparently healthy control groups. For CIC to be of good clinical and diagnostic value there should be marked initial elevation that drops as the treatment of the disease is initiated. Previous studies associated immunoglobulin classes and subclasses with varied diseases (44,45) and suggested correlation of CIC with immunoglobulin classes and possible implication in the immunopathogenesis of these diseases. The fact that there were no significant variations in serum immunoglobulins across the disease and treatment groups as well as controls could not possibly associate CIC with breast tumour. More so the trend in correlative assessment between the CIC and immunoglobulin classes never followed any definite pattern.

Changes in serum immunoglobulins in human cancers had been reported by various workers such as carcinoma of cervix (23), pancreatic cancer, (24), primary liver carcinoma, (25), squamous cell carcinoma, and breast cancer, (32-35), with varied findings and opinions. Increased IgM, IgA, IgE and IgG were reported in oral cancer patients. The works of Kemp *et al.*, (23), however, contradicted the earlier finding and observed that serum IgG and IgA are not significantly elevated in oral cancers and hence not to be used as a good surrogate marker for diagnosis and prognosis. This is in parity with this study though in different racial/ethnic groups with different environmental and social factors.

Numerous responses of immune disturbances have been associated with breast cancer and CIC suggested to correlate with the immunoglobulins (32). During cancer progression, the level of immunoglobulins and complements are altered markedly to compensate for changing environment of cancer cells. It was documented that patients who have undergone modified radical mastectomy for breast cancer showed alterations in serum immunoglobulin concentration after receiving full cycle of chemotherapy. This is in disparity with the present findings. The fact that some immunoglubulins positively correlated with CIC at some stages of treatment cannot be used to generalize decrease due to immunocomplex formation. For correlative assessment of each class of immunoglobulin with CIC there should be negative correlations in treatment groups (both at 3 and 6 months, post-treatment). In this study there is no strong and definite pattern of relationship between the CIC and immunoglobulins. This could be attributed to changes in individual and other immunological factors.

Immunoglobulin subclasses were not however analyzed in this study to ascertain possible association with progression of breast cancer. There is need therefore for further work on the clinical utility of immunoglobulin subclasses in the management of breast cancer. The 6 months post-treatment, patient's group in benign breast tumour (BBT) could not be evaluated because most of the patients at this stage were lost to follow-up. That was a prime limitation. Nigerians have the habit of absconding to medical appointments once the sickness has subsided. This was more pronounced in benign breast tumour patients. Few breast cancer patients also were lost to follow-up as some patients resorted to unorthodox and spiritual healing and some traveled overseas for treatment. Need for well-equipped special cancer treatment and research centers and trained personnel are advocated.

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