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# Evaluation of Immune Complexomes and Possible Pathological Influences at Various Stages of Breast Cancer

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## Abstract

Staging of breast cancer is important in the reading of cancer progression and level of treatment required. Certain underlying factors may be determinant and consequent to prognostic values at various stages. These underlying factors are considered to include circulating immune complexes which has the capability to mediate molecular expression and could give insight into possible progressive remission developments at various stages of Breast cancer.

**Methods:** We recruited 50 female participants including 10 with benign tumour, 15 apparently healthy participants and 25 with malignant tumour and at stages: 2 (No.6); 3b (No.8); 3c (No.7) and 4 (No.4). Prospective observational analytical study was conducted to identify changes in certain inflammatory molecules, DNA oxidation and sex hormonal molecules in different stages, with respect to increased or normal level of circulating immune complexes. Serum samples were assayed for circulating immune complexes using Polyethylene Glycol (PEG) immuno-precipitation and quantification. Isolated cell free DNA (cfDNA) (FitAmp blood kit (Epigentek, USA) was assayed for nuclear factor kappa B (NFkB), while serum samples were assayed for Tumour necrosis factor alpha (TNF-$\alpha$), immunoglobulin G (IgG), 8-Hydroxy-2-Deoxy Guanosine (8-OH2DG), estrogen and progesterone, using Enzyme Linked Immunosorbent Assay. Mean differences in expression of the molecules in health, tumour and malignancy, were recorded as point reference to determine the effect of immune complexes at various stages.

**Result:** The result recorded only stages 2, 3b 3c and 4. Serum levels of CIC were significantly increased in all the stages and benign tumour (P=0.000), under this influence, expression of NFkB, (P=0.006), TNF-$\alpha$, (P=0.000), 8-OH2DG (P=0.010) were significantly increased, while expression of progesterone was significantly reduced (P=0.014). Specifically, significant increases in expression of the molecules are indicated as follows: CICs: benign tumour- p=0.017, stage 3b- P=0.012 and 3c-P=0.000; NFkB: 3c-P=0.030; TNF-$\alpha$: benign tumour-P=0.040, stage 3b-P=0.000, 3c-P=0.000 and 4-P=0.019; 8OH2DG: stage 3b-0.045 only. However significant decrease in progesterone was found only in stage 3b compared to levels found in healthy subjects (P=0.048). The degree to which the expression of one molecule, influence another molecule was determined.

**Conclusion:** Serum levels of CICs increase in all stages of breast cancer and benign tumor. Presence of CIC could be a leading circumstance mediating inflammatory molecular expression at various stages of cancer. It was observed that, the degree of expression of one molecule could positively predict the expression of another, suffice it to say that there could be collaborating influence of CIC on DNA oxidative damage and inflammatory molecules at stages of breast cancer.

**Keywords:** Breast cancer immune complexome • Cancer Stages • Inflammatory molecules • Cancer associated molecules

## Introduction

Staging of breast cancer is important in the reading of cancer progression and level of treatment required. Certain underlying factors may be determinant and consequent to prognostic values at various stages. These underlying factors are considered in this study to include circulating immune complexes which has the capability to retain antigenic molecules capable of mediating chronic immune response and persistent homeostatic imbalance and ensuing inflammation. We therefore considered that presence of such molecules as immune complexes could induce characteristic biomarker as well as give insight into possible progressive development at various stages of Breast cancer. Sequel to our discovery of immune complexes as important clinical burden in breast tumours we deemed it fit to conducted observational analytical study on various stages of breast cancer to understudy molecular interactions and expressions at various stages of breast cancer in female participants in other to explore CIC involvement in cancer progression. Considering the capability of immune complexes to persist in the system and continue to mediate immune response which could degenerate into pathological responses due to persistent activation of immune cells and possible polarization of these cells to pro tumour activities, immune complexome was considered to be of clinical importance in various stages of breast cancer.

Our attention was on inflammatory molecules because of fundamental mediation mechanisms of CICs. Therefore this observational cohort study

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becomes important in other to access the burden of immune complexes at various stages of breast cancer if any and identify some biomarkers based on patterns of molecular expression at various stages. Without experimental exposure and or intervention in the participants, we believed this to be a natural phenomenon that could reveal the natural occurrences or developments that could reveal some underlying good or poor prognostic values in breast cancer. Immune complex(IC) is formed when antigen binds specifically with its corresponding antibody to form a complex, thus giving non-covalent perfect interactions between the epitopes of the antigen and the paratopes of the antibody. Sources of antigenic components of immune complexes could be foreign (exogenous) or self-antigens (endogenous), found in healthy individuals but can affect various tissues, thus creating many pathological phenomena [1]. In healthy subjects, ICs are systematically removed from the bloodstream, they are basically opsonized with C3b fragments of complement protein and under this condition, they are recognized by complement receptor CR1 on erythrocytes, which binds the complex and aid its transport to the reticulo-endothelial system (RES), either the spleen or liver. The immune complex is taken up at this stage by the macrophage with help of complement receptor 3 or 4 (CR3, 4) molecules on the resident macrophage. The transporting erythrocytes remain undestroyed in this process and returns into circulation [2].

However, immune complex may persist and thus mediating chronic or unhealthy inflammatory response. This informs the clinical importance of IC removal [2]. Immune complex could form in any part of the body system and thus deposit their or could be carried in circulation through the vasculature causing vasculitis and to other sites to cause damages [2]. Indeed, the accumulation of CIC could provide a novel source of biomarkers for the diagnosis and the follow-up of a pathological condition. The use of CIC as disease biomarkers was specifically studied for autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis [3] , but it was also observed in other pathologies, including infectious diseases and acquired thrombotic thrombocytopenic purpura [4,5]. Of note, the level of CIC increases in the presence of several malignancies including Pancreatic Ductal Adenocarcinoma (PDA). Earlier, CICs were found in melanoma, osteogenic sarcoma, lymphoma and carcinomas of the colon, lungs, breast, prostate, pancreas, stomach, esophagus and uterine cervix [6,7]. Additionally a systematic analysis of CIC was provided only in 2013 in uveitis, using pull-down assays to separate IgG-IC [8]. This current study, evaluates the existence of CICs at stages of breast cancer and we considered immune complex as underlying factor in cancer prognosis in which research exploration is required to determine its contribution in prevention, invasion, progression and cancer therapy.

We live with immune complexes as product of normal progressive immune response, response required by human health condition. However, the existence of immune complex turns inimical to life when it is not properly removed and accumulates due to increasing source of formation. Its existence manifests a lot of inflammatory response in other wards it could be a potent source of inducing inflammation or stirring inflammatory immune response to ameliorate cancer progress. On the other hand, its persistence may stirrup chronic smoldering or damaging inflammation favorable for cancer development and possible metastasis. Therefore thorough exploration of CIC is required to understand its boon and or bane effects in tumours.

# Materials and Methods

We recruited 50 female participants including 10 with benign tumour, 25 with malignant tumour and 15 apparently healthy participants. Only 4 different stages were encountered at the malignant level including, stage 2 (6); stage 3b (8); stage 3c (7) and stage 4 (4). Prospective observational cohort study was conducted to identify changes in certain inflammatory, DNA oxidative and sex hormonal molecules in different stages of breast cancer, with respect to increased or normal level of circulating immune complexes in health and in tumour/cancer cases. Serum samples from the participants were assayed for circulating immune complexes using Polyethylene Glycol (PEG) immuno-precipitation and quantification. Isolated cell free DNA (cfDNA) (Fit Amp blood kit (Epigentek, USA) was assayed for Translocated nuclear factor kappa B (NFkB), while serum samples were assayed for Tumour necrosis factor alpha (TNF-$\alpha$), immunoglobulin G (IgG), 8-Hydroxy-2-Deoxy Guanosine (8-OH2DG), estrogen and progesterone, using Enzyme Linked Immunosorbent Assay. Mean differences in expression of the molecules in health, tumour and malignancy, were recorded as point reference to determine the effect of immune complexes at various stages. The participants with benign and malignant breast tumours were attending clinic at the surgical unit of Nnamdi Azikiwe University Teaching Hospital Nnewi, Anambra State. The cutoff values were drawn from 100 healthy female volunteers.

The healthy participants were confirmed free from breast tumours by clinical examination using physical breast examination by Surgeon Ravi C and Rodrigues G [9], while the diagnosis of breast tumour was established by histo-pathological examination of biopsy and detection of cancer associated antigen 15-3 (CA 15-3). Staging of cancer was done according to American Joint Committee for Cancer (Tumour Node Metastasis (TNM) classification). None of the participants had received any form of treatment (chemotherapy, surgery, radiotherapy or immunotherapy) for breast tumour prior to the study. All the subjects were screened clinically and biochemically to exclude any autoimmune diseases and Human Immunodeficiency Virus. Approval for the study was obtained from the ethics committee of Nnamdi Azikiwe University Teaching Hospital. Informed consent was obtained from the subjects before participation. All the subjects were administered questionnaire to obtain medical history and demographic information.

## Inclusion criteria

Female subjects with benign or malignant breast tumour. Female subjects without tumour and are not under cytotoxic or hormonal drug or any anti-immune therapy such as in auto-immune disease.

## Exclusion criteria

Immuno-deficient patients or patients with HIV infection, Female subjects without cancer but are under cytotoxic drug or any anti-immune therapy such as in auto-immune disease. Female subjects with breast tumour and had received any form of treatment (chemotherapy, surgery, radiotherapy or immunotherapy) for breast tumours.

Assessment was done by:

Evaluation of Immune complex at various stages

The serum level of immune complexes at various stages

Inflammatory molecules being expressed at high and normal levels of immune complex

Sex hormone molecules being Expressed

Level of DNA damage at various stages based on expression of Oxidative DNA stress marker with respect to pathological level of CICs

## Specimen collection

Ten (10) ml of fasting blood sample was drawn by veni-puncture from all the participants. Serum was obtained from the fasting blood samples. Criteria for blood sample collection were made to suit the various parameters required to be tested in this study. The blood sample was put in a plain vacutainer tube and allowed to clot at room temperature, for 30 minutes. The retracted clot was removed by centrifugation (Sorvall RC5C HS-4 rotor at 1500 ×g for 15 min) at room temperature and the formed serum was carefully pipetted into another tube. Immune complex precipitation and DNA extraction were carried out from the serum immediately for the quantification CICs levels and detection Nuclear Factor kappa B (NF-kB) respectively. The remaining sera were stored at -20°C in aliquots until used for the analysis of, Immunoglobulin G (IgG), Tumour Necrosis Factor-alpha (TNF-$\alpha$), 8-hydroxy-2-deoxyguanosine (8-OH2DG), Estrogen (Estradiol 2), Progesterone and CA15-3 antigen.

## Statistical methods

SPSS version 23 (IBM Inc., USA) was used to analyze the data. Descriptive statistics (mean ± SD) were performed for distribution patterns. Kruskal Walis Mean Ranking was used where homogeneity or equal variance was not met and Mann Whitney was applied instead for post Hoc analysis. Mean +2SD (at 95% confidence interval) method was used to determine the reference values in healthy subjects [10]. The P values lower than 0.05 were considered statistically significant. Linear regression was applied to assess correlation of the molecules.

# Result

Breast cancer was presenting at stages 2, 3b, 3c and 4 only, indicating limited early cancer detection and confirming persistent problem of late presentation of cases to the clinic. Serum levels of CIC were significantly increased in all the

**Table 1.** Kruskal-Walli mean ranking of various molecules expressed at various stages of cancer, benign tumour and control group.

| | CIC/µgeq/ml | NFkB/ng/ml | IgG/mg/ml | TNFα/pg/ml | 8OH2DG/ng/ml | Estrogen pg/ml | Progest ng/ml |
|---|---|---|---|---|---|---|---|
| Benign N=10 | 28.15 | 19.60 | 28.00 | 28.85 | 21.75 | 30.20 | 31.35 |
| Stage 2 N=6 | 30.92 | 22.08 | 26.50 | 27.33 | 29.50 | 19.08 | 20.75 |
| Stage 3b N=8 | 33.63 | 36.44 | 27.13 | 34.94 | 32.00 | 24.50 | 14.00 |
| Stage 3c N=7 | 33.21 | 40.57 | 33.14 | 35.86 | 35.71 | 31.1 | 22.21 |
| Stage 4 N=4 | 34.75 | 31.63 | 29.00 | 36.38 | 34.13 | 31.25 | 23.75 |
| Control N=15 | 11.17 | 16.30 | 18.07 | 9.77 | 15.87 | 21.30 | 31.63 |
| (P-value) | (0.001) | (0.001) | (0.263) | (0.000) | (0.015) | (0.397) | (0.065) |

**Table 2.** Mann Whitney showing statistically significant and non-significant differences in various molecule expressions including CICs between control group and various stages of cancer and benign tumour.

| | CIC/µgeq/ml | NFkB/ng/ml | IgG/mg/ml | TNF-α/pg/ml | 8OH2DG/ng/ml | Estrogen pg/ml | Progest ng/ml |
|---|---|---|---|---|---|---|---|
| Benign *vs.* control gp | 0.001 | 0.892 | 0.103 | 0.001 | 0.428 | 0.129 | 0.531 |
| Stage 2 *vs.* control gp | 0.008 | 0.519 | 0.235 | 0.003 | 0.066 | 0.850 | 0.205 |
| Stage 3b *vs.* control gp | 0.001 | 0.000 | 0.169 | 0.000 | 0.003 | 0.776 | 0.013 |
| Stage 3c *vs.* control gp | 0.000 | 0.000 | 0.021 | 0.000 | 0.004 | 0.091 | 0.210 |
| Stage 4 *vs.* control gp | 0.006 | 0.020 | 0.307 | 0.001 | 0.020 | 0.469 | 0.411 |

stages and benign tumor (P=0.000)

## Circulating immune complexes

Mean ranking of these macromolecules assayed at the unit value of µgeq/ml, were highly elevated. in participants with benign tumour (28.15) and more so in different stages of malignancy: stage 2 (30.92); stage 3b (33.63); 3c (33.21); stage 4(34.71), while the healthy control group recorded mean ranking serum level of 11.17 slightly below the reference value of .056 µgeq/ml. This increase in serum level of the molecules was highly significant in participants with benign tumour (P=0,001); Stage 2 (P=0.008); Stage 3b (P=0.001); stage 3c (0.000) and stage 4 (P=0.006).

Simple linear regression calculated to determine the degree to which CIC predicts other molecules showed a positive significant regression coefficient with 8-OH2DG in stage 3b. Regression equation: (F (1 and 6) = (6.340), p<0.045), with an $R^2$ of 0.514. Therefore in stage 3b only, 8-OH2DG increased 75.977 for each µgeq/ml of CIC. No significant regression coefficient was found on the degree to which CIC predicts other molecules tested in other groups of the participants except in stage 3b.

## Nuclear factor kappa B (NFkB)

Mean ranking of these transcription factor assayed at the unit value of ng/ml, were also highly elevated in different stages of malignancy: Stage 2 (22.08); stage 3b (36.44); 3c (40.57); stage 4(31.63), while participants in healthy control group and benign tumour recorded mean ranking serum level of 16.30 and 19.60 respectively, below the reference value of .328 ng/ml. This increase in cfDNA level of the NFkB molecule was highly significant in participants at Stage 3b (P=0.000); stage 3c (0.000) and stage 4 (P=0.020) only.

Simple linear regression calculated to determine the degree to which NFkB predicts other molecules, showed significant positive regression coefficient with 8-OH2DG in stage 3c: Regression equation (F (1 and 5) = (10.119), *p*<0.025), with an $R^2$ of 0.669. Therefore in stage 3c, 8-OH2DG increased 219.040 for each ng/ml of NFkB. No significant regression coefficient was found on the degree to which NFkB predicts other molecules tested in other groups of the participants (Tables 1 and 2).

## Immunoglobulin G (IgG)

Mean ranking of these immunoglobulin molecule assayed at the unit value of mg/ml, were elevated in stage 3c (33.14); while participants in stage 2 (26.50); stage 3b (27.13); stage 4 (29.00); healthy control (18.07) and benign tumor (28.60) recorded mean ranking equivalent or below the reference value of 24mg/ml. This increase in immunoglobulin G molecule in participants at Stage 3c was statistically significant (P=0.021)

Simple linear regression calculated to determine the degree to which IgG predicts other molecules, showed strong negative significant regression coefficient with progesterone in stage 3c. Regression equation: (F(1 and 5)= (9.318), *p*<0.028), with an $R^2$ of 0.651. Therefore in stage 3c, progesterone decreased .045 for each mg/ml of IgG. No significant regression coefficient was found on the degree to which IgG predicts other molecules tested in other groups of the participants.

## Tumour necrosis factor alpha (TNF-ϕ

Mean ranking of these pro-inflammatory molecule assayed at the unit value of pg/ml, were highly elevated in participants with benign tumour (28.85) and more so in different stages of malignancy: stage 2 (27.33); stage 3b (34.94); 3c (35.86); stage 4(36.38), while the healthy control group recorded mean ranking serum level of 9.77 below the reference value of 12.2 pg/ml. This increase in serum level of the inflammatory molecule was highly significant in participants with benign tumour (P=0,001); Stage 2 (P=0.003); Stage 3b (P=0.000); stage 3c (0.000) and stage 4 (P=0.001).

Simple linear regression calculated to determine the degree to which TNF-α predicts other molecules showed a positive significant regression coefficient with progesterone. Regression equation: (F (1 and 4) = (7.893), p<0.045), with an $R^2$ of 0.664. Therefore in stage 2b, progesterone increased -0.110 for each pg/ml of TNF-α. No significant regression coefficient was found on the degree to which TNF-α predicts other molecules tested in other groups of the participants except in stage 2b.

## 8-Hydroxydeoxyguanosine (8-OHdG)

Mean ranking of these DNA oxidative marker molecule assayed at the unit value of ng/ml, were slightly elevated in participants with benign tumour (21.75) and highly elevated in different stages of malignancy: stage 2 (29.50); stage 3b (32.00); 3c (35.71); stage 4(13), while the healthy control group recorded mean ranking serum level of 15.87 below the reference value of 14.1 ng/ml. This increase in serum level of the DNA oxidative marker molecule was highly significant in participants at Stage 3b (P=0.003); stage 3c (0.004) and stage 4 (P=0.020). Simple linear regression calculated to determine the degree to which 8-OH2DG predicts other molecules showed no significant positive regression coefficient with estrogen. Regression equation: (F (1 and 6) = (4.892), p<0.078), with an $R^2$ of 0.495. Therefore in stage 3c, estrogen increased 0.434 for each ng/ml of 8-OH2DG. No significant regression coefficient was found on the degree to which 8-OH2DG predicts other molecules tested in other groups of the participants.

# Discussion

The transitory half-life of Circulating Immune Complexes (CICs), must have made it unpopular and easily neglected in pathogenesis of many disease conditions especially cancer. Research on immune complexes is re-emanating and we considered it here as unattended, underlying and perturbing molecule on the frontline of boon and bane of any disease especially cancer. Generally, we found abnormal/pathological increase in level of CICs in all the stages of breast cancer and benign tumour and under this influence, expression of Nuclear Factor kappa B, tumour Necrosis Factor- Alpha, 8 OH2DG were significantly high, while

expression of progesterone was significantly low. We highlighted that presence of CICs could mediate increased systemic expression of the above mentioned tumour associated molecules, thus modulating inflammatory responses at various stages of cancer. Considering the nature of the participants, modulation at this state could mean mediation of acute and or chronic inflammatory responses or pro and anti-inflammation. Specifically, molecular expressions were found to differ at various stages of breast cancer. While NFkB expression prevails in stage 3c, TNF-$\alpha$ was prominent in benign tumour, stages 3b, 3c and 4 and it is considered in this study as the most prominent inflammatory molecule in breast cancer considering its significant increased expression in all stages of the cancer. Meanwhile, DNA oxidative marker 8-OH2DG was high in stage 3b, against the levels in healthy participants.

This is an indication that inflammation and oxidative stress are concerned phenomena at various stages of cancer even in benign tumour, involving different physiologic and or immunologic activities and could carry lots of basic diagnostic and therapeutic interpretations with positive or negative information about the disease condition. This is in line with report that both infectious and non-infectious agents and cell damage activate inflammatory cells and trigger inflammatory signaling pathways, most commonly the NF-$\kappa$B, MAPK and JAK-STAT pathways [11]. For example, decrease in progesterone found only in stage 3b could be an insight into the tumor cell/immune activities and development compared to stages where progesterone and or estrogen were found to be high, considering their ability to initiate cell growth. The increasing cases of breast cancer in Nigeria and worldwide initiates the attention to this work and little or no work has been done on the implication of strong potent immune activator such as immune complexes existing in breast tumours.

Positive correlation of CIC and 8-OH2DG molecules in stage 3b gives room for concern, this is because persistence of CICs at that stage may continue to mediate DNA oxidation and further DNA damages even under therapy. This study discovered that in stage 3c, at each ng/ml of NFkB, 8-OH2DG also increased, which is a significant metastatic stage of the cancer, indicating further increase in DNA damage at this stage under the influence of CICs. This also falls in line with the report of Wang W, et al. [12] that inhibition of NFkB, results in an increase in the sensitivity of cancer cells to the apoptotic effects of chemotherapeutic agents and radiation and restoring hormone sensitivity, which is correlated with increased disease-free survival in patients with breast cancer, thus indicating that presence of NFkB is a factor to decrease in sensitivity of cancer cells to apoptotic chemotherapeutic agents. In the same vein, Sarkar DK, et al. [13] showed that NF-$\kappa$B/p65 expression implies aggressive biological behaviour of breast cancer & this study validates significant association of NF-$\kappa$B/p65 overexpression with large tumour size, negative estrogen & progesterone receptor status and overexpression of c-erbB2 oncoprotein. These earlier research reports could also justify the increased presence of 8-OH2DG alongside NFkB molecular expression, considering that 8-OH2DG is a serious marker of DNA oxidative damages. This calls for serious attention on stage 3c of breast cancer during therapies.

The association between progesterone levels and IgG is not well understood, however, this study suggests that presence of IgG may have a suppressive effect on progesterone expression or vice versa in stage 3c breast cancer under pathological level of CICs. Involvement of IgG in this study is due to its abundance in blood circulation and ability to retain immune complex and verse majority of immune activation. Zaytseva OO, et al. [14] reported that Glycosylation of the IgG fragment crystallizable (Fc) region is shown to vary in different physiological and pathological states. Fc *N*-glycan composition can alter the effector functions of IgG by modulating its affinity for ligands, such as Fc$\gamma$ receptors (Fc$\gamma$Rs), thus, altering the properties and functionality of these molecules includes changes in antibody affinity and stability. Earlier, Prados M, et al. [15] provided evidence that (STT3 Oligosaccharyltransferase Complex Catalytic) isoforms can be hormonally modulated, with marked consequences on IgG N-glycosylation. They found that progesterone induces a switch of STT3 isoform expression, increasing IgG N-glycosylation. This is in line with the strong negative correlation between IgG and progesterone detected in this study. Of note is that mammalian cells can express two isoforms of the oligosaccharyltransferase catalytic subunit (STT3-A and STT3-B), which are endowed with distinct enzymatic properties. The enzymes catalyze the Polypeptide N-linked glycosylation in the lumen of the endoplasmic reticulum. Progesterone (P4) has earlier been implicated in modulation of inflammation by hindering pro-inflammatory cytokine production, regulation of local and systemic inflammation [16].

In peripheral blood leukocytes, P4 appears to dampen pro-inflammatory cytokine production, which consequently reduces T-helper subtype differentiation and proliferation [17]. They reported that exposure of maternal T cells to progesterone at physiological doses induced a unique skewing of the cytokine production profile of CD4(+) and CD8(+) T cells, with reductions not only in potentially deleterious IFN-$\gamma$ and TNF-$\alpha$ production but also in IL-10 and IL-5. Conversely, production of IL-4 was increased. In other words, significant increase in progesterone would have been an indicator for immune suppression and possible tumour progression. In this study, the down regulation of progesterone in stage 3c could be a reversed immune response to induce inflammatory process to suppress cancer progression or metastasis. If this is the case, the emanating questions include: could it be that at this stage, immune system is still competent to fight cancer progression?, could it be that presence of pathological level of CICs mediates inflammatory response considering the possible increased and available level of IgG and their Fc binding molecules, in other words capable of reenacting acute inflammatory response against the cancer progression?. However, this is contrary to the line of reported from Fabre A, et al. [18] who reported that risk of breast cancer has not been noted from taken physiological progesterone alone. They emphasized on taken only the physiological kind of hormone human body makes but not the progestin or progesterone and estrogen in combination, thus maintained that treatment of breast tenderness or heavy flow that are likely from menstruating and midlife women with progestin, imposes breast cancer risk, but taking cyclic or daily progesterone would be safer and better treatment for breast tenderness or heavy premenopausal flow.

In support of this line of argument, more recent study indicated that women with a combination of high progesterone and low estradiol levels experience a lower breast cancer risk: In the nurses' health study population, the data reinforce the context-specificity of progesterone effect, which appears to require a minimal estrogen presence to initiate the biologic consequences that promote cancer development [19]. In our own study, emphasis is on physiological progesterone because the participants in this study were not under any form of hormonal or cancer therapy. Therefore this finding is considered an update on the role of progesterone in women and in breast cancer development as well as in different stages of breast cancers, thus highlighting the possibilities of physiological progesterone exposing participant to cancer, based on possible induction of immunological alterations to alter inflammatory responses as indicated above. On the other hand, the contrary increase in estrogen level in association with 8-OH2DG, in the same stage 3c left much to border about over activities going on at that stage and may have contributed immensely as a combination effect with progesterone to induce cancer progression and or metastasis. The strong positive correlation of estrogen and expression of 8-OH2DG found in this study has a backup as reported by Singh B, et al. [20], that Estrogen metabolism-mediated oxidative stress is suggested to play an important role in estrogen-induced breast carcinogenesis and that antioxidants, vitamin C and butylated hydroxyanisole (BHA) inhibit 17$\beta$-estradiol (E2)-mediated oxidative stress and oxidative DNA damage and breast carcinogenesis in female August Copenhagen Irish (ACI) rats Singh B, et al. [20]. Furthermore, 8-Hydroxydeoxyguanosine (8-OHDG) is one of the most commonly formed markers of DNA lesions produced in response to E2-induced oxidative stress and is considered as a cellular marker for both oxidative stress and oxidative DNA damage [21].

8-Hydroxydeoxyguanosine in DNA is repaired primarily *via* the DNA base excision repair pathway. Earlier, Ba X and Boldogh I [22] reported that oxidative stress and the resulting damage to genomic DNA are inevitable consequences of endogenous physiological processes and they are amplified by cellular responses to environmental exposures. One of the most frequent reactions of reactive oxygen species with DNA is the oxidation of guanine to pre-mutagenic 8-oXo-7, 8-dihydroguanine (8-oXoG) [22]. In the promoter, 8-oXoG may serve as an epigenetic mark and when tangled with the oxidatively inactivated repair enzyme 8-oxoguanine DNA glycosylase 1, provide a platform for the coordination of the initial steps of DNA repair and the assembly of the transcriptional machinery to launch the prompt and preferential expression of redox-regulated genes. Deviations/variations from this artful coordination may be the etiological links between guanine oxidation and various cellular pathologies and diseases during ageing processes [22]. Naturally, estrogen causes cells to grow or proliferate. If this is not controlled, estrogen can promote genetic errors and possibly cancer. On the other hand, progesterone decreases proliferation and cell growth while encouraging cells to become more specialized or mature progesterone's actions decrease cancer risk [23]. When progesterone is present, its receptors make the estrogen receptor no longer able to cause breast cells to grow or proliferate [24].

The alternating increase and decrease in progesterone and estrogen levels in stage 3c is an indication of modulatory and regulatory effects going on, of which we suggest that presence of immune complexes could be a serious factor

in the process because of the availability of the Fc portion of the immunoglobulin molecules especially in IgG as detected in this study. Treatment with E2 also had also been found to increase St6Gal1 ((Beta-galactoside alpha-2,6-sialyltransferase 1) expression in mouse and human antibody-producing cells, providing a mechanistic explanation for the increase in IgG-Fc sialylation [25]. In postmenopausal women with rheumatoid arthritis (RA), treatment with E2 significantly increased the Fc sialylation of IgG. Their inference is that E2 induces anti-inflammatory effector functions in IgG by inducing St6Gal1 expression in antibody-producing cells and by increasing Fc sialylation, thus provides a mechanistic explanation for the increased risk of rheumatoid arthritis (RA) in conditions with low estrogen levels such as menopause [25]. While this is taking place in stage 3c, progesterone was found to be increasing in stage 2 in association with TNF-alpha. On the other hand, the report of Escalante Gómez C and Mora SQ [26] showed that 8-OH-2dG levels were significantly lower in women who received combined Hormone replacement therapy (HRT) compared to women who did not receive HRT and that lipid oxidation was significantly lower in women on HRT compared to women taking no HRT. We opined that this may not be applicable in cancer situation where the normal cellular mechanism has been overtaken. Negative correlation of IgG and progesterone in stage 3c may enhance inflammatory response suggesting some immunotherapeutic values, but cancer cells in the same stage may have developed a means of suppressing the proinflammatory response to induce anti-inflammatory actions. This was observed by the positive correlation of estrogen and 8-OH-2dG in the same stage 3c.

Such increase in estrogen may in turn activate the anti-inflammatory response of IgG to favour tumour progression [26]. However, the work of Escalante Gómez C and Mora SQ [26] did not consider the presence and the level of CICs in the participants recruited for their study. One major and common tumour development and progressive pathway noted in this study and at various stages with pathological level of CICs, is DNA oxidative damage indicated by increased expression of 8-OH-2dG. Suffice it to say that CICs retention in tumour is synonymous with DNA oxidative damages as observed between participants with benign tumour who have pathological levels of CICs and apparently healthy participants who have normal or low levels of CICs.

## Conclusion

Serum levels of CICs increased in all stages of breast cancer and benign tumour. Presence of CIC could be a leading circumstance mediating or fanning inflammatory molecular expression at various stages of cancer. The degree of expression of one molecule, could positively predict the expression of another as indicated in the result, thus indicting CICs as a regulator of inflammatory molecules and DNA oxidative stress which are determinants in cancer prognosis. However, ascertaining the specific mechanism behind this regulatory influence is beyond the scope of this study. Suffice to say that expression CICs and NFkB could positively associate with the levels of 8-OH2DG, while expression of IgG and TNF-a, could positively associate with the levels of estrogen and progesterone respectively. The outcome of this work, suggests serious pathological (DNA oxidative damage and chronic inflammation) impact of circulating immune complexes at different stages of breast cancer indicated by increased expression of 8-OH2DG and generation of inflammatory molecules, a condition capable of mediating metastasis and undermining adequate cancer therapy.

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## Conflict of interest

None.

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