

Original Research Paper

Cross Talk between Iron Regulatory Proteins and Proinflammatory Molecules in Ovarian Cancer Patients Based on Menopausal Status

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Abstract: Micronutrient iron is postulated to contribute to the initiation of cancer mainly by the formation of Reactive Oxygen Species (ROS), the key signaling molecules of inflammatory reactions. Iron may also cause elevation of MMP9, a gelatinase responsible for remodeling extracellular matrix in cancer cells through the ROS pathway. Estrogen the hormone which decreases markedly in post menopause is also known to increase the risk of tumorigenesis in ovaries. Hence this study aims to explore alterations in iron metabolism in Ovarian Cancer (OC) patients based on menopausal status. Plasma iron, Total Iron Binding Capacity (TIBC), erythropoietin and ceruloplasmin were estimated spectrophotometrically in 33 premenopausal and 50 postmenopausal OC patients. Proinflammatory cytokine TNF α and MMP9 were determined by ELISA. Plasma iron was markedly lower and erythropoietin was significantly higher in all OC patients compared to normal reference intervals, irrespective of menopausal state indicating severe anemia in OC. TIBC was significantly lower in postmenopausal OC patients compared to their premenopausal counterparts ($p = 0.023$). Plasma ceruloplasmin was significantly higher in postmenopausal OC patients compared to premenopausal patients ($p = 0.022$), implying greater oxidative stress. The increase in TNF α was statistically significant in postmenopausal cancer patients compared to their premenopausal counterparts ($p = 0.005$). A substantial increase in MMP9 suggests the enhanced ability of tumor cells for migration and invasion in postmenopausal OC patients. The results of the present study demonstrate greatly perturbed iron metabolism in postmenopausal OC patients than in premenopausal patients. This higher degree of iron dysregulation may induce persistent ROS production and promote inflammatory processes in OC.

Keywords: Ovarian Cancer, Menopausal Status, TNF α , MMP9, Iron, Ceruloplasmin

Introduction

Ovarian Carcinoma (OC) is one of the leading causes of death due to gynecological malignancies the majority of which are derived from ovarian surface epithelium or cysts. Preclinical research has illustrated various carcinogenic properties of ferrous iron, most importantly by ROS production (Forciniti *et al.*, 2020). Inflammation is the other mechanism linking carcinogenesis and iron metabolism. Several pieces of evidence attribute the etiopathogenesis of OC to the release of inflammatory

mediators. TNF α is implicated in inflammation, ROS production and anemia by increasing iron sequestration by RES and various other tissues (Blaser *et al.*, 2016). This complex relationship may explain the effects of iron on inflammation and cancer. Further, iron may be one of the several factors that cause the elevation of MMP9, a gelatinase responsible for remodeling the extracellular matrix in cancer cells (Kaomongkolgit *et al.*, 2008). Moreover, perturbation of ECM is associated with carcinogenesis and metastasis. Epidemiological evidence suggests that estrogens and progesterone are implicated in

the etiology of ovarian cancer. According to some studies even in the absence of estrogen, estrogen receptors take part in tumor progression (Kyriakidis and Papaioannidou, 2016). Estrogen metabolites generate reactive oxygen species which promote the release of iron from ferritin (Wyllie and Liehr, 1997). Iron metabolism with reference to OC etiology and pathogenesis is yet to be understood fully. Relatively few studies have examined the variation in iron homeostasis between pre and postmenopausal ovarian cancer patients. Hence the study aims to explore alterations in iron metabolism based on menopausal status and try to establish the cross-talk between iron and pro-inflammatory molecules in OC.

Materials and Methods

This is a prospective cross-sectional study conducted ethically as per the declaration of Helsinki on biomedical research involving human beings and was approved by the Institution Ethics Committee (IEC KMC MLR11/2021/345). Permission was sought from the medical superintendents of the government Lady Goshen Hospital and KMC Hospital Mangalore to collect blood samples and get the patient details from the medical records department. Patients enrolled were briefed about the study and the sample was collected only after taking written consent. The research was conducted at the clinical lab of KMC Mangalore on a total of 83 female patients who were histologically confirmed with serous adenocarcinoma of the ovary. Patients with amenorrhea for more than a year and FSH >40 mIU/mL and baseline estrogen <50 pg/mL were classified as postmenopausal and the rest were considered premenopausal. The study population was divided into 2 groups based on their menopausal status which included 33 premenopausal and 50 postmenopausal OC patients. At the time of blood collection (baseline samples), no patient had received any chemotherapy or radiotherapy. None of the patients underwent any surgery. Patients who underwent hysterectomy were excluded from the study. None of the patients included in the study had infections, vaginal bleeding, GI diseases, or hemolytic disorders. Data on clinical presentation and diagnostic tests of the patients were collected from medical records available in the hospital. Baseline data and laboratory findings of the study subjects are depicted in Table 1. The demographic data of the patients is shown in Table 2. All the patients were from the same district of coastal Karnataka and had the same socio-economic background. Fasting blood samples were collected in heparin vacuum tubes by venipuncture and centrifuged at 3000 rpm for 10 min. The plasma collected was divided into 2 aliquots, one was used immediately for spectrophotometric analysis and the other aliquot was stored at -20°C for ELISA. Plasma iron and Total Iron Binding Capacity (TIBC) were

estimated spectrophotometrically using commercially available kits using the ferrozine method in a semi-auto analyzer (Adams *et al.*, 2007).

Plasma iron is reduced to ferrous iron which then complexes with ferrozine to give a violet-coloured complex which is measured at 540 nm. Plasma is treated with excess ferrous iron to saturate iron binding sites of transferrin followed by precipitation, iron content in the supernatant measures TIBC. Plasma ceruloplasmin was determined by its p-phenylene diamine oxidase activity to give a purple-colored complex measured at 560 nm (Prabhakaran *et al.*, 2022). Erythropoietin was calculated using the formula: Erythropoietin [U/L] = 2.5 × (140-hemoglobin [g/L]). Plasma cytokines MMP 9 and TNFα were quantified using commercially available ELISA kits following the sandwich principle (Chen *et al.*, 2015; Jain *et al.*, 2020). The assays were conducted according to the manufacturer's instructions. The kit made use of biotinylated anti-human MMP-9 antibody and biotinylated TNFα along with Avidin-Biotin-Peroxidase Complex. The absorbance of the colored product was read on an ELISA reader using 450 nm wavelengths. The total MMP-9 level and TNFα were expressed in nanograms/ml and picogram/ml respectively.

Statistical analysis was done using SPSS statistical software version 20. Mann-Whitney test was used to evaluate the differences in parameters between the groups. p<0.05 was considered to be statistically significant.

Results

The baseline data of the study population is depicted in Table 1. The demographic data of the study population is represented in Table 2. Plasma iron was considerably lower and erythropoietin was markedly higher in both the groups of OC patients with respect to the normal reference range (Table 3). Both plasma TIBC and ceruloplasmin were significantly higher in postmenopausal patients compared to premenopausal patients (p = 0.02). Figures 1-2 the increase in TNFα was statistically significant (p = 0.005) in postmenopausal patients compared to premenopausal OC patients. Figure 3 Plasma MMP9 was also markedly high in both groups of OC patients compared to the normal reference range (Table 3).

Table 1: Baseline data and laboratory findings of ovarian cancer patients

| | Premenopausal patients N = 33 | Postmenopausal patients N = 50 | p-value |
|--------------|-------------------------------|--------------------------------|---------|
| Menarche | | | |
| Age (yrs) | 8 | 25 | - |
| <14 | 11 | 39 | |
| >14 | | | |
| CA125 (U/mL) | 792±233 | 1235±375 | 0.01 |
| Hb (g/dL) | 10.61±0.2 | 10.59±0.19 | NS |

Table 2: Demographic data of patients with ovarian cancer

| | Premenopausal patients N = 33 | Postmenopausal patients N = 50 | p-value |
|--------------------------|-------------------------------|--------------------------------|---------|
| Age (yrs) | 35-43 | 55-64 | - |
| | <40 (23) | <60 (28) | |
| | >40 (10) | >60 (22) | |
| Weight (Kgs) | 48.1±7.7 | 47.45±7 | NS |
| BMI (kg/m ²) | 17.65±2.4 | 17.39±2.54 | NS |

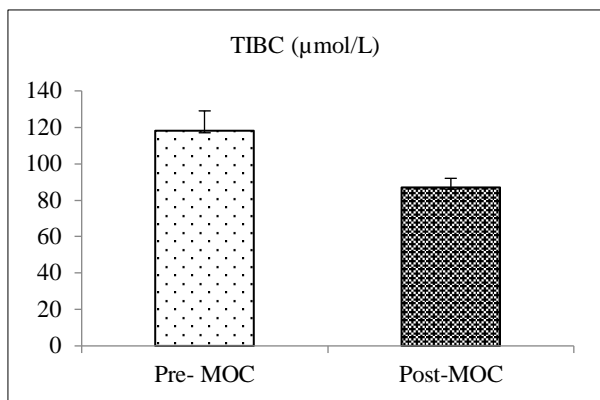


Fig. 1: Comparison of plasma TIBC in pre and post-menopausal OC patients

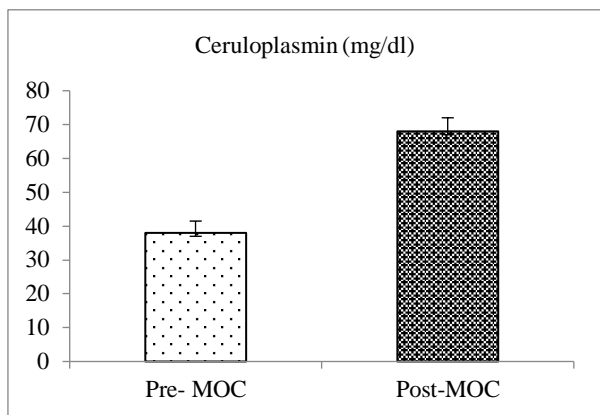


Fig. 2: Comparison of plasma ceruloplasmin in Pre and post-menopausal OC patients

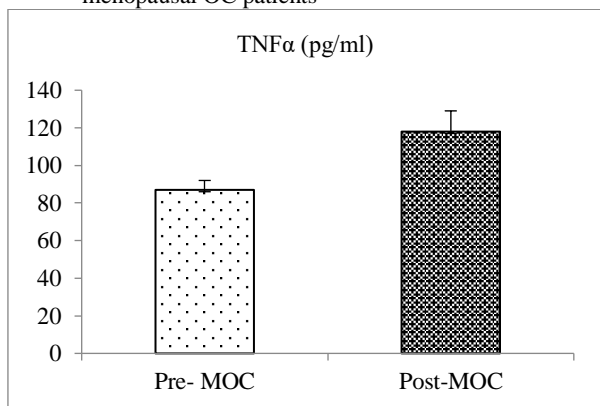


Fig. 3: Comparison of plasma TNFα in Pre and post-menopausal OC patients

Table 3: Evaluation of iron homeostasis, MMP9 and TNFα in premenopausal and postmenopausal OC patients

| | Normal reference range | Premenopausal OC | Postmenopausal OC | p-value |
|-------------------------|------------------------|------------------|-------------------|---------|
| Iron (µg/dl) | 40-190 | 48±8 | 45±3.7 | 0.135 |
| TIBC (µmol/L) | 42-80 | 118±11 | 87±5 | 0.023 |
| Erythropoietin (mIU/ml) | 4-26 | 85±0.2 | 85±0.19 | 0.262 |
| Ceruloplasmin (mg/dl) | 20-40 | 38±3.5 | 68±4 | 0.022 |
| MMP9 (ng/ml) | 40-60 | 384±41 | 431±64 | 0.882 |
| TNFα (pg/ml) | 43-57 | 87±5 | 118±11 | 0.005 |

Discussion

Iron is an essential micronutrient required for several processes within the cell. Dysregulation of iron homeostasis is postulated to contribute to the initiation of cancer. Ferrous iron by participating in the Fenton reaction induces ROS production a key factor for the development of cancer (Forciniti *et al.*, 2020). Iron deposits were common in endometriotic cysts and in fallopian tubes which are sites of tumorigenesis in females. Ovarian epithelial cells treated with iron elicited cellular proliferation by p53 inactivation and Ras activation thus contributing to neoplastic transformation (Lattuada *et al.*, 2015). Iron treatment in immortalized ovarian surface epithelial cells induced the transformation of precursor cells to cancer cells (Yamaguchi *et al.*, 2010). In the present study, compared to the normal reference range, the serum iron was markedly lower both in pre and post-menopausal OC patients. Generally, in postmenopausal women without OC, serum iron will be twice that of premenopausal women due to cessation of menstrual bleeding. On the contrary, we observed converse findings in women with cancer. Patients of both groups were anemic with a significant increase in erythropoietin levels. Since estrogen increases the transcription of the transferrin gene (Vyhlidal *et al.*, 2002), a notable increase in serum TIBC was seen in premenopausal OC patients compared to postmenopausal patients. TIBC is a measure of functional transferrin concentration in serum. Transferrin is more saturated in postmenopausal women. Transferrin is needed for the uptake of iron by the peripheral tissues. As the requirement of iron is higher for cancer cells, increased levels of transferrin account for increased cellular uptake resulting in low serum iron and, thereby anemia in OC patients (Ivanova *et al.*, 2022). Ceruloplasmin is a ferroxidase that helps the incorporation of iron into transferrin. A significant increase in ceruloplasmin seen in postmenopausal OC patients compared to premenopausal patients implies a greater dysregulation of iron metabolism in the postmenopausal state. Ceruloplasmin is not only an acute-phase protein but also a plasma antioxidant that scavenges ROS generated in these cancer patients (Mukae *et al.*, 2021).

Persistent oxidative stress in the tumor microenvironment promotes harmful inflammatory processes. ROS act as second messengers that stimulate

the production of inflammatory molecules like TNF α (Aboeella *et al.*, 2021). Cytokine TNF α reduces erythropoiesis by decreasing the proliferative response of erythropoietic tissue to erythropoietin (Madeddu *et al.*, 2018). It inhibits iron absorption and is described to be responsible for the pathogenesis of inflammation and anemia in cancer patients (Buck *et al.*, 2009). Elevated serum erythropoietin in both groups of cancer patients points to iron deficiency. Anemia was profound in postmenopausal group patients compared to premenopausal females based on serum iron levels. Further, TNF α upregulates transferrin receptors and enhances its sequestration by RES thereby increasing intracellular iron and its associated toxicity. This could probably be the cause for low serum iron levels in OC patients in general and high intracellular iron appears to be the key causative factor of carcinogenesis through ROS generation. Furthermore, iron administration to human monocytic cell lines induces upregulation of TNF α which justifies coordinated interaction between iron and TNF α (Scaccabarozzi *et al.*, 2000). Anti-inflammatory effects of estrogens in noncancerous cells involve transcriptional repression of cytokines like TNF α (Cvoro *et al.*, 2006). Normally, the decrease in estrogen levels during menopause could be a causative factor for increased TNF α production and therefore inflammatory diseases. A significant increase in serum TNF α further deteriorates iron status in post-menopausal OC patients compared to the premenopausal state. Conversely, TNF α increases estrogen concentration in the breast tumor cells by increasing the activities of enzymes needed for its synthesis (Kamel *et al.*, 2012). Furthermore, estradiol stimulates the expression of the TNF α gene in several types of cancers (To *et al.*, 2014).

A nearly 10-fold increase in serum MMP9 was seen in both pre and postmenopausal cancer patients in the current study compared to the normal reference range. Hydrogen peroxide is known to increase the expression of the MMP9 gene by activating the RAS oncogene. Iron remodels the ECM to enable motility and invasion of cancer cells via the expression of MMPs through ROS. Treatment of ferric ions increased expression of MMP9 in head and neck cancer cell lines by the surge in intracellular reactive oxygen species (Kaomongkolgit *et al.*, 2008). Moreover, TNF α also promotes MMP9 expression in breast cancer increasing the metastatic behavior of tumor cells (Wolczyk *et al.*, 2016). The study points to altered cross-talks between iron and anti-inflammatory molecules as one of the differences between pre and postmenopausal OC patients. The study also signifies that iron dysregulation associated with increased proinflammatory cytokines may add to the morbidity in estrogen-deprived post-menopausal OC patients. Therefore, dietary supplements rich in antioxidants and anti-inflammatory drugs may serve as potential therapeutic mechanisms to decrease morbidity in post-menopausal OC patients.

Conclusion

The results of the present study add to the growing body of literature on changes in iron metabolism in OC suggesting that alterations are marked in postmenopausal state than premenopausal condition. Analysis of a larger population may provide a better insight into whether the changes are strongly associated with age or menopausal status.

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Author's Contributions

Haritha Padannapurath Manoj: Obtained the ethics approval, collected data and performed analysis.

Sudha Kuthethur: Conception of study design and drafted the manuscript.

Reshma Kumarchandra: Approval of final manuscript version.

Neelam Manjunath Pawar: Statistical analysis and Approval of final manuscript version.

Arya Kokkalayil Gopalakrishnakurup: Contributed to data collection and approval of final version of the manuscript.

Sowndarya Kollampare: Approval of final version of the manuscript.

Ethics

The study was approved by the Institution Ethics Committee (IEC KMC MLR11/2021/345).

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