Am J Transl Res 2022;14(9):6563-6573
www.ajtr.org /ISSN:1943-8141/AJTR0143678

Original Article

Analyzing the drivers of cancer relapse: hypocalcemia and iron absorption in hormone-dependent female cancers

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Received April 21, 2022; Accepted July 20, 2022; Epub September 15, 2022; Published September 30, 2022

Abstract: Background: Alterations in the levels of nutrients like calcium, ferritin, and electrolytes play a pivotal role in human physiology and might serve as biomarkers. Ferritin, an iron storage protein is important in various metabolic reactions of both cancer and cancer stem cells (CSCs) and is found to regulate 'stemness' leading to cancer relapse. Interestingly, ferritin levels are found to be regulated by calcium uptake. Several studies have shown that high levels of calcium inhibit absorption of iron, thereby reducing ferritin levels. In the present study, we evaluated and correlated the serum ferritin and calcium levels in pre- and post-treated hormone-dependent female cancers and deciphered their role in tumor recurrence and relapse. Materials and methods: The present retrospective study was approved by the Institutional Ethical Committees (IEC) of GIMSR (No. GIMSR/Admn./Ethics/approval/IEC-3/2021), and Omega cancer hospitals (Reg No: ECR/1486/Inst/AP/2020). Serum from 197 clinical samples diagnosed with breast, cervical, and ovarian cancers (99 pre-and 98 post-treatment) and 10 blood samples were analyzed for ferritin and calcium using auto bioanalyzer and sandwich enzyme-linked immunosorbent assay (ELISA). Results: Ferritin levels were elevated in both pre- and post-treatment hormone-dependent female cancer patients while calcium levels showed gradual decrease. The mean ferritin value for pre-treatment was 0.0409 mg/dL while it was 0.0428 mg/dL for post-treatment hormone-dependent female cancer. Conclusion: Our results suggest that hypocalcaemia in post-treatment cancer patients leads to ferritin accumulation which might make these patients more prone to tumor recurrence and relapse.

Keywords: Cancer relapse, calcium, serum ferritin, stemness, estrogen, cancer stem cells

Introduction

Cancer is one of the most life-threatening cellular impairment disorder which has a major impact on the mortality rate all over the world. The major reasons behind the high mortality are late diagnosis as well as cancer relapse. Although there are various techniques to diagnose cancer, they fail to diagnose it in the early stage. Cancer is a condition where the cell loses its regulation to divide, proliferate, and die. There are various types of cancers which affect various organs. Among them hormonally related cancer plays a vital role in cancer cell proliferation and protects the cells from various death mechanisms. Hormonally related cancers are the type of cancers in which elevated hormone levels, for instance estrogen and progesterone, play a crucial role in cancer cell transformation. Cancers of breast, ovarian, cervical, uterine, vaginal, anal, and pProstrate are the various types that fall under this category. Since the cancer cells are usually immortal, they require nutrients in bulk quantities to protect themselves from cell death mechanisms. For instance, ferritin is an iron storage protein

which is present in bulk quantities in cancer cells than normal cells. Ferritin plays a crucial role in cancer cells by protecting them from ferroptosis, an iron dependent non-apoptotic cell death [1, 2]. Transferrin (Tf) is an iron binding protein which has high affinity to ferritin [3, 4]. The ferritin enters the cells via transferrin-receptor (TfR) mediated endocytosis suggesting that cancer cells upregulate Tf and TfR as well [5]. Interestingly, studies have shown that ferritin levels are elevated in cancer cells and CSCs [6] in vivo. On the other hand, estrogens related to female sex hormones are considered to be human carcinogens. Estrogen regulates growth, development, and physiology of the human reproductive system and also plays a critical role in neuroendocrine, skeletal, adipogenesis, and cardiovascular systems [7, 8]. Cancer relapse is a condition where a patient who was diagnosed with cancer and had undergone cancer therapy is diagnosed for cancer for a second time. Although cancer relapse does not occur in all cancer patients, such incidences are high. One of the key regulators of cancer relapse is the presence of CSCs [9]. Interestingly, CSCs depend on ferritin to proliferate, thereby resulting in cancer relapse [10]. However, there are no studies demonstrating the corelation between ferritin and calcium levels in post-treatment patients and the risk of cancer relapse. This is the first study aiming to detect ferritin levels and ferritin bound transferrin levels in the serum of female cancer patients. In the present study, we hypothesize that ferritin levels might decrease corresponding to the elevation in calcium levels after the therapy and thus draw the correlation between ferritin levels and cancer relapse.

Materials & methods

Sample collection

In the present retrospective study approved by the IEC of GIMSR (No. GIMSR/Admn./Ethics/approval/IEC-3/2021) and Omega Cancer Hospitals, Visakhapatnam (Reg No: ECR/1486/Inst/AP/2020), serum samples from 197 (99 pre-, and 98 post-treatment) cancer patients attending the OPD of Omega Cancer Hospitals, Visakhapatnam and 10 blood samples from healthy volunteers between August, 2021 to February, 2022 were collected. The informed and written consents either in English or in regional language *Telugu* have been taken from all the patients participated in the present study. The format of patient consent is submitted as Supplementary Files 1, 2. Details like age, gender, smoking habits, alcohol consumption, and other medical conditions such as diabetes and hypertension were collected from all the enrolled patients. The proforma of details collected is submitted as Supplementary File 3. Among 99 pre-treatment samples, 33 samples were of carcinoma breast, 33 samples were of cervical cancer and 33 samples were of ovarian cancer, while among 98 post-treatment samples 33 samples were of carcinoma breast, 33 samples were of carcinoma cervix and 32 samples were of carcinoma ovarian. 5 mL of whole blood from each of the pre- and post-treatment patients diagnosed with breast, ovarian, and cervical cancers were collected in red colored evacuated containers. The samples were centrifuged at 1000 g for 10 mins and the obtained serum was collected into sterile 1.5 mL micro centrifuge tubes. The collected serum samples were stored at -20°C till further analysis was carried out.

Inclusion criteria: Female patients with a mean age of 49.27 years whose treatment sessions had been completed, were considered in the study along with pre-treatment patients. Briefly, samples from post-treatment patients were collected immediately after their last therapy sessions (radiotherapy/chemotherapy) which was confirmed by the PET-CT reports. High tumor regression identified in PET-CT reports were considered as pre-treated samples, since it was confirmed orally by the patients that they did not undergo any treatment. Both diabetic and non-diabetic patients were included in the study. However, the age period of 47-52 years was considered as the window period for the menopausal stage, age above 52 years was considered as post menopause and below 47 was considered as pre-menopause.

Exclusion criteria: Patients diagnosed with breast, cervical and ovarian cancer who came for follow-up after three months were excluded from the study. Also, patients with relapsed cancer were excluded from this study. Furthermore, due to the ongoing pandemic, we could not collect control samples and used the

reference ranges declared by World Health Organization (WHO).

Estrogen levels from the patients diagnosed with breast, ovarian and cervical cancers were not assessed, because the experimental and clinical data suggested that estrogens play an important role in the growth and development of breast, cervical, and ovarian cancers. Due to ethical issues, we could not procure a good number of serum samples from healthy volunteers and hence, we took the reference ranges from the available literature and the standardized values from the National Institute of Health (NIH) website.

Estimation of serum ferritin levels and electrolytes

The serum ferritin, sodium, potassium, calcium, magnesium, and phosphate levels of cancer patients are evaluated using an auto bioanalyzer (COBAS C311 ANALYZER) according to the manufacturer's instructions. Briefly, the serum samples were placed in the autoanalyzer containing the respective COBAS autoanalyzer kit and the readings were computed in excel that are displayed on the monitor. For serum ferritin levels briefly, the transferrin-bound ferritin complex was subjected to acidic hydrolysis to yield ferritin. With the help of the enzyme ascorbate, the ferric iron stored in ferritin was reduced to ferrous iron and converted to ferrozine to yield a coloured complex. The color intensity is directly proportional to the amount of ferrous that has been reduced from the ferritin.

Estimation of serum ferritin bound transferrin

Serum levels of ferritin bound transferrin was obtained by performing ELISA. Briefly, the 96-well plates were coated overnight with primary antibody and stored at 4°C. Next day, the primary antibody was removed and the plates were washed with wash buffer (1x TBST) twice. 100 µL of serum samples and known concentration of standards were added to the plate and incubated for 2 hours at room temperature. After incubation, the plates were washed twice with wash buffer. The plates were blocked using 5% BSA for 30 mins at room temperature. Then secondary antibody was added to the plate and incubated for 2 hours at room temperature and was washed twice with wash buffer after the completion of incubation. Then 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was added to the wells and incubated for 1 hour at room temperature. The reaction was stopped by adding the stop buffer (0.5 M NaOH) and the OD was measured at 450 nm using ELISA microplate reader.

Statistical analysis

All the data are represented in terms of geometrical mean. Student's t test was performed to compare differences of ferritin levels in pre and post chemo treated female hormonal related cancers samples. Pearson correlation was used to measure significance between the measured ferritin levels.

Results

Correlation of serum ferritin levels from auto bioanalyzer and ELISA

A total of 197 serum samples were estimated for ferritin levels. Out of the 197 samples, 99 were from pre- treatment group and 98 were from post treatment group. **Figure 1** represents total number of samples. Ferritin levels were estimated using auto bioanalyzer. Ferritin levels in serum were elevated in female hormonal-related cancers with a mean value of 0.0409 mg/dL in pre-treatment patients, while it was 0.0428 mg/dL in post-treatment patients. The results showed that serum ferritin levels in both pre and post-treatment hormone-related cancers were elevated. However, there was not much difference between ferritin levels estimated using auto bioanalyzer and ELISA. Statistical Analysis demonstrated that p value is less than 0.05 which is significant. **Figure 2** represents standard curve of ferritin obtained by performing ELISA.

Figure 3 represents the ferritin levels in pre-treated and post-treated hormone-dependent female cancers evaluated using auto-bioanalyzer, while **Figure 4** represents the correlation of serum ferritin levels analyzed using auto bioanalyzer and ELISA in terms of the obtained mean values.

Estimation of serum calcium, magnesium, phosphate and electrolytes

There is a decreased concentration of calcium levels in both pre and post-treatment cancer

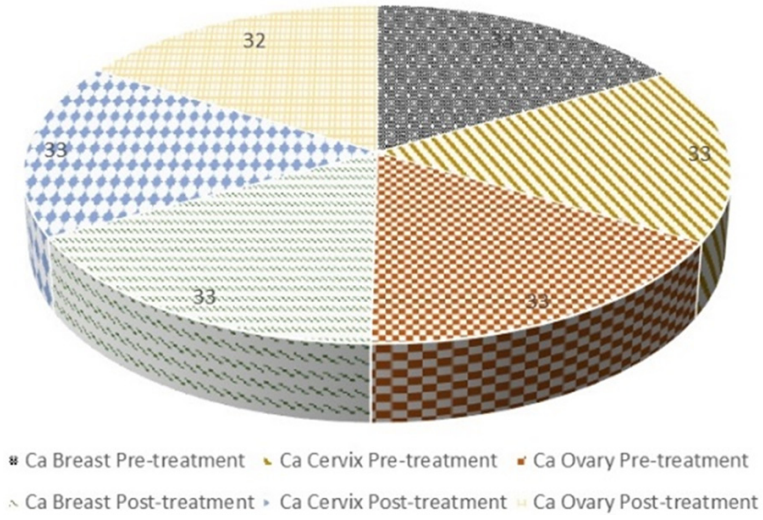

Figure 1. Graphical representation of total number of samples collected during the period of study. The pie-chart represents total number of samples included in the present study. A total of 197 serum samples were collected from female cancer patients diagnosed with breast, ovarian, and cervical. Among them 99 samples were pre-treatment samples, 98 samples were post-treatment. Among pre-treated patients, 33 samples were of carcinoma breast, 33 samples were of cancer cervix and 33 samples were ovarian cancer. While among post-treated patients 33 samples were of carcinoma breast, 33 samples were of cancer cervix, and 32 samples were ovarian cancer.

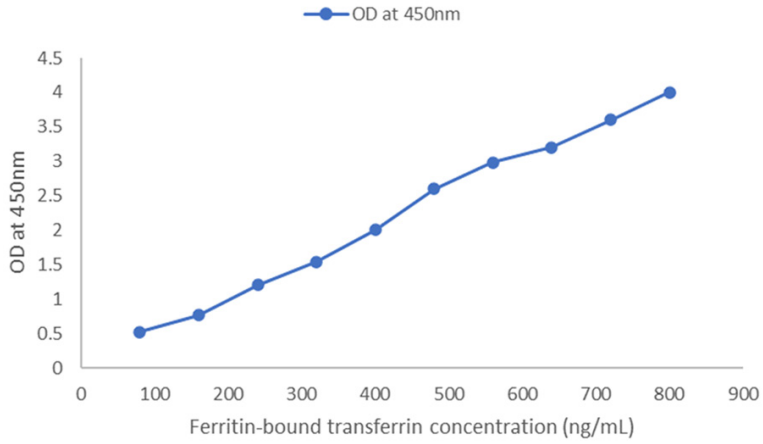

Figure 2. Graphical representation of serum ferritin bound transferrin levels measured using ELISA. **Figure 2** represents the standard curve of ferritin concentrations in ng/mL obtained from ELISA. The standard concentrations were taken from 100-900 ng/mL and the absorbance was measured at 450 nm using a UV-Visible spectrophotometry.

patients. We observed a mean value of 7.41 mg/dL in pre-treated hormone-dependent cancer, while it was 6.51 mg/dL for post-treated hormone-dependent female cancers. However, there was no significant change in phosphorous, magnesium, sodium, potassium, and chloride when compared with the normal range of these electrolytes. **Table 1** represents the recommended normal ranges of various electrolytes. **Figure 5** represents the serum levels of calcium, phosphorous, magnesium, chloride, sodium, and potassium in both pre-treated and post-treated hormone-dependent female cancers, while **Figure 6** represents the mean serum levels of electrolytes, which were evaluated in both pre-treated and post-treated hormone-dependent female cancers.

Correlation between serum ferritin and calcium levels

We hypothesized an inverse correlation between circulating calcium levels and ferritin levels. Interestingly, our results matched the hypothesis. We observed that ferritin levels were increased in both pre- and post-treated cancer patients while calcium levels were decreased. When these results were correlated between pre-treated and post treated hormone dependent female cancers, we observed that the ferritin levels were high in post-treated cancer patients associated with low calcium levels. Statistical Analysis demonstrated that *p* value was less than 0.05 (refer **Table 2** for Pearson's coefficient, T-Statistic, and *P*-value of various hormonally related cancers both pre- and post-treatment).

Discussion

Hormones play an essential role in growth and development of the cell. Particularly sex related hormones are crucial for the development of sexual characteristics such as beard moustache for men, and breast and ovaries for women. Apart from regulating the sexual char-

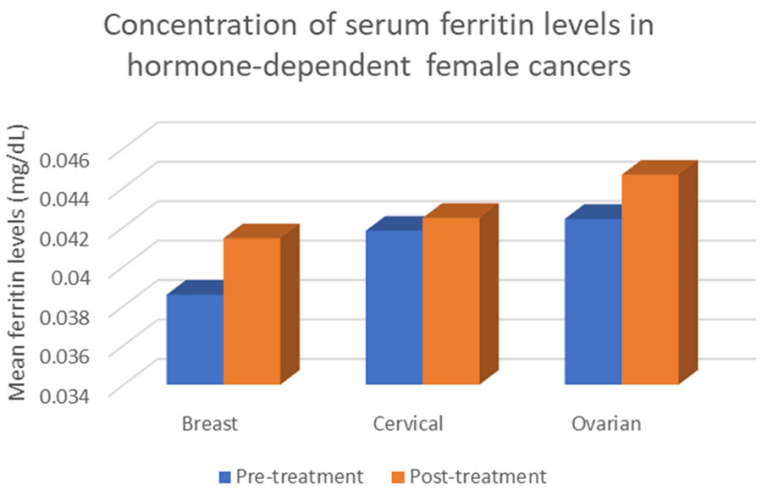

Figure 3. Graphical representation of serum ferritin in female hormonal related cancers before undergoing treatment. The present figure depicts the concentration of serum ferritin level in mg/dL in both the patients group. The serum ferritin levels should be lower in post-treatment cancer patients. Unfortunately, the post-treatment group of patients were observed to have similar levels of serum ferritin when compared with pre-treatment group.

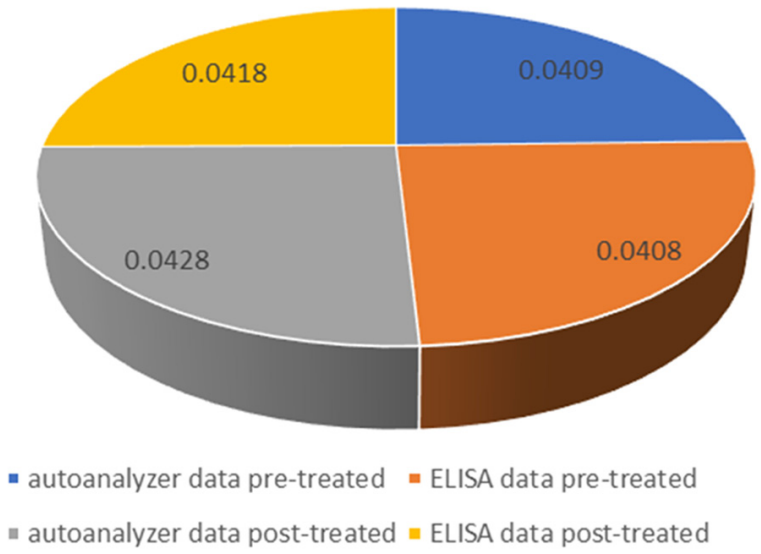

Figure 4. Comparison of serum ferritin levels from auto bioanalyzer data and ELISA data. The graphical representation of serum ferritin levels (ng/mL) in both pre- and post-treatment cancer patients diagnosed with breast, cervical, and ovarian cancers is shown. The data are represented in mean values.

acteristics, sex hormones play an essential role in various metabolic processes. For instance, estrogen, a steroid hormone involved in breast and ovary development, is also essential for the functioning and integrity of the tissues of the urinary tract [11]. Estrogen plays an important role in hormone-dependent cancer. However, as a part of senescence, menstruation status and co-morbidities such as obesity and autoimmune disorders, estrogen levels tend to fluctuate and change throughout the life cycle. These levels are elevated in hormone-dependent cancer. Various studies demonstrate the correlation of estrogen levels, calcium levels, and ferritin levels in blood. For instance, previous studies demonstrated that women using oral contraceptives have higher levels of serum ferritin. However, estrogen replacement therapy in postmenopausal women showed no significant changes in ferritin levels [12]. On the other hand, estrogen plays a key role in calcium absorption. Gallagher in 1980 demonstrated that estrogen therapy improves calcium balance in patients with postmenopausal osteoporosis [13]. Gennari *et al.* suggested that estrogen could affect intestinal calcium absorption by modifying the secretion of parathyroid (PTH) hormone, a peptide hormone produced by the parathyroid glands which control blood calcium levels [14]. Another study done by Nie *et al.* demonstrated that estrogen regulates duodenal calcium absorption through exerting effects on ER α and ER β receptors [15]. All these studies suggest an inverse correlation between estrogen and serum ferritin levels and a direct correlation between estrogen and calcium absorption.

Additionally, iron is one of the essential metals required by the body to carry out various metabolic processes. It is one of the major constituents of hemoglobin whose prime role is to deliver oxygen to various tissues [16]. Ferritin is an iron storing protein that stores excess iron present in the body. The ferritin protein is localised in the cytoplasm as well as in the circulation. High levels of ferritin have been indicated

Table 1. Representation of normal levels of ferritin, calcium, magnesium, sodium, potassium, and chloride according to COBAS autoanalyzer kit

Ferritin (ug/dL)	Calcium (mg/mL)	Phosphorous (mg/mL)	Magnesium (mg/mL)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)
1.2-15	86-102	25-45	16-26	135-147	3.5-5.0	95-110

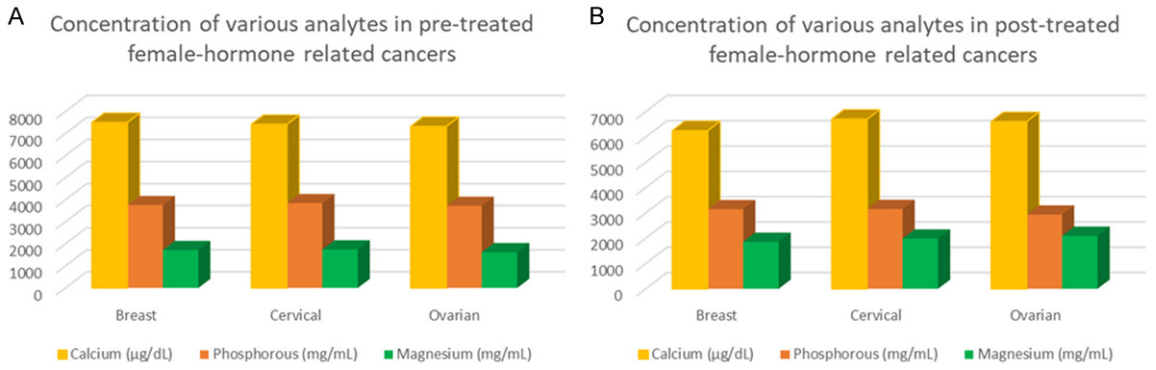

Figure 5. Graphical representation of serum calcium, magnesium, and phosphorous in female hormonal related cancers before undergoing treatment. A. Represents serum levels of calcium (µg/mL), magnesium (mg/mL) and phosphorous (mg/mL) in pre-treated patient group diagnosed with Ca breast, Ca cervical, and Ca ovarian. B. Represents serum levels of calcium (µg/mL), magnesium (mg/mL) and phosphorous (mg/mL) in post-treated patient group diagnosed with Ca breast, Ca cervical, and Ca ovarian.

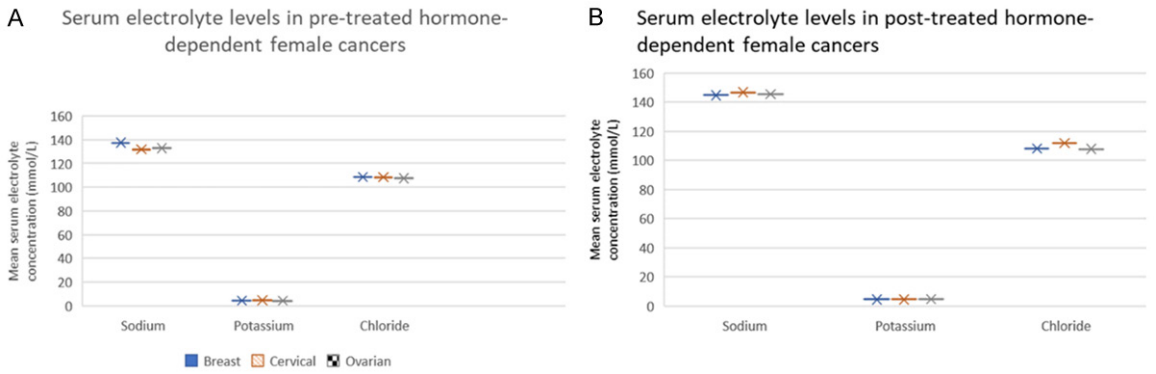

Figure 6. Graphical representation of serum electrolyte levels (mmol/L) in pre- and post-treated female hormone-dependent cancers. A. Represents the mean serum sodium, potassium, and chloride levels in pre-treated female hormone-dependent cancers. B. Represents the mean serum sodium, potassium, and chloride levels in post-treated female hormone-dependent cancers. However, there is no significant change in these levels when compared with the normal levels.

as a risk factor for cancer incidence [17]. Chronic anaemia which is characterized by decreased RBC count or size characterised with high levels of ferritin is thought to be one of the major drivers for elevated ferritin levels, which might be associated with increased risk of cancer incidence. Studies demonstrated that cancer cells have high demand for ferritin due to its importance in nucleotide biosynthesis, cell proliferation, and protection of tumor cells from ferroptosis which is a novel cell death pathway [18-20]. Interestingly CSCs, the major drivers of cancer relapse and recurrence, require more ferritin as they reside in the quiescent stage when compared to cancer cells [6, 21-23]. The demand for cancer cells as well as CSCs for ferritin is because of its protective role that is regulated in two distinct ways: firstly ferritin which is a ferric iron reservoir serves as a co-factor for ribonucleotide reductase, an

Table 2. Representation of mean, standard deviation, Pearson's coefficient, t-Statistics, and *P*-value of both pre- and post-treatment female patients diagnosed with hormonally related cancers

Statistical analysis of pre-treated hormone-dependent female cancer							
	Ferritin (mg/dL)	Calcium (mg/dL)	Phosphorous (mg/mL)	Magnesium (mg/mL)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)
Mean	0.0409	7.4175	3782.012	1693.885	134.0071	4.6461	108.1507
St. Dev	0.0118	1.1296	1205.212	664.3491	11.3007	1.8362	10.2308
Pearson Co-efficient	1	0.448710167	0.24672	0.243562	0.262492	0.267338	0.296331
N	99	99	99	99	99	99	99
df	97	97	97	97	97	97	97
t stat	0	4.9451	2.5074	2.4733	2.6792	2.7324	3.0558
p value	0	0.0000	0.0069	0.0076	0.0043	0.0037	0.0014
Statistical analysis of post-treated hormone-dependent female cancer							
Mean	0.0428	6.5171	3074.701	1970.728	145.6140	4.4321	109.2691
St. Dev	0.0125	1.4890	968.7639	601.6594	12.0518	0.8914	12.3531
Pearson Co-efficient	1	0.196757546	0.22488	0.264249	0.197734	0.182637	0.171102
N	98	98	98	98	98	98	98
df	96	96	96	96	96	96	96
t stat	0	1.9663	2.2613	2.6845	1.9764	1.8201	1.7015
p value	0	0.0261	0.0130	0.0043	0.0255	0.0359	0.0460

enzyme that catalyzes the reduction of ribonucleotides into deoxy-ribonucleotides, thereby contributing to DNA synthesis [24] and secondly, cell requires ferrous iron to promote ferroptosis but ferritin prevents the oxidation of ferric iron [25]. Ferrous iron is very crucial for a cell to undergo ferroptosis [26], because ferrous iron acts as a co-factor for the enzyme lipoxygenase which catalyzes the generation of lipid peroxides leading to cell death. Hence ferritin is highly essential for a cancer cell to sustain [27]. Henceforth, to meet the ferritin requirement, tumor associated macrophages (TAMs) come into picture, which play a major role in ferritin synthesis and secretion. It has been stated that ferritin levels in untreated cancer patients are increased which is regulated by TAMs [28]. To fulfil the ferritin requirement cancer cells and CSCs upregulate the transferrin receptor. The transferrin receptor endocytose the ferritin bound transferrin into cells [29].

Apart from ferritin electrolytes, along with Ca^{2+}, Mg^{2+}, and PO_4^- play an essential role in regulating cell division, proliferation, and apoptosis. For instance, it is evident that high Ca^{2+} levels activate nuclear factor kappa B (NF-κB) gene which is the critical regulator of various genes involved in inflammation, cell proliferation, and is often [30] found to be over expressed in various malignancies [31] by dissociating IκB from NF-κB [32, 33]. Mg^{2+} on the other hand is the critical regulator of glycolysis as it serves as a co-factor for kinases. It is evident that cancer cells elevate anaerobic glycolysis pathway due to the prevailed hypoxic conditions. Karki P et al., showed that low levels of Mg^{2+} inhibited DNA and protein synthesis leading to cell growth arrest and finally apoptosis [34]. In contrast, studies performed by Kardalas *et al.* have demonstrated that high levels of Mg^{2+} showed inverse correlation with breast, ovarian, liver, esophageal, and prostate cancer mortality. Besides these ions, PO_4^- is another important ion that regulates various cellular pathways. Nucleotide biosynthesis is of utmost importance for a cell to carry out all other metabolic pathways. Nucleotide is composed of sugar, nitrogen base, and a phosphate group which indicates that PO_4^- is essential for normal cells as well as for the tumor cells [35]. Additionally, sodium and potassium are also required by the cells to proliferate and to inhibit apoptosis. Studies demonstrated that during apoptosis intracellular potassium ions are lowered in order to protect themselves from apoptosis [36]. Interestingly, intracellular sodium levels have been elevated and coupled with decreased potassium levels during early stage of apoptosis, suggesting that cancer cells have low levels of sodium ions while high levels of potassium ions to abscond apoptosis [37]. All

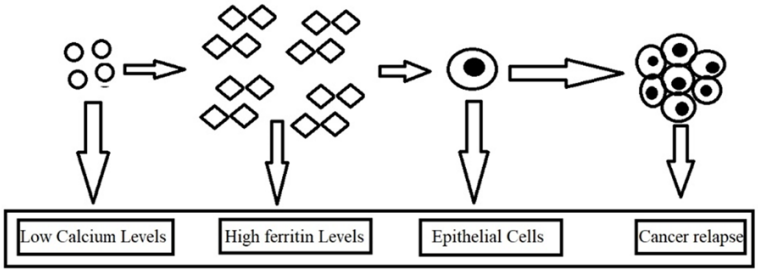

Figure 7. Schematic representation of the hypothesis. The above diagram represents the overall outline of the present study. It represents that when calcium levels are low it leads to high iron absorption thereby leading to high ferritin levels which play a key role in cancer relapse in the post-treated hormone-dependent female cancer patients.

this literature suggest that Ca^{2+}, Mg^{2+}, K^{+}, PO_4^{-}, Na^{+} levels might be altered in various cancer patients. However, our data showed decreased serum Ca^{2+} levels in both pre- and post-treatment cancer patients with increase in serum ferritin levels, while other electrolytes did not show significant change in both pre- and post-treatment patients.

In 1976, Jacob and co-workers reported that breast cancer patients who have undergone chemo/radiotherapy/surgery with high serum ferritin levels have a greater recurrence/relapse than the patients with low serum ferritin levels [38]. On the other hand, studies done by Rosner and Ferraz Gonçalve et al. suggested the role of hypocalcemia in cancer incidence and relapse [35, 39].

Our results hence suggest an inverse correlation between serum calcium and ferritin levels in both pre- and post-treatment patients diagnosed with hormone-dependent cancers. Our results were also matched with various *in vitro* and *in vivo* studies. Kletzein *et al.,* confirmed that $CaCO_3$ inhibits iron absorption in experimental animals [40-43]. It was observed that, ferritin retention and the rate of hemoglobin regeneration were gradually diminished in animal models that were fed with various sources of Calcium (including $CaCO_3$, $CaCl_2$, calcium lactate, calcium phosphate, and bone meal). Chapman *et al.,* and Manis et al., reported individually the inhibitory effect of calcium on elemental iron absorption and the mechanism by which it exerts this effect by radioisotope-labelling in experimental animals [44, 45]. Calcium reduced the uptake of iron by duodenal brush borders thus reducing ferritin levels in the circulation. It also resulted in reduced and delayed uptake of Fe from a $FeCl_2$ solution introduced into isolated gastrointestinal loops *in vivo* in rats. The chief effect seemed to be associated with the initial uptake of iron into mucosal cells and rely primarily on the absolute quantity of calcium present in the duodenal lumen [43]. Hallberg *et al.,* postulated a dose-dependent relationship between calcium content and iron absorption. They conducted a series of experiments where the volunteers were recommended to ingest bread rolls incorporated with calcium chloride. They found inhibition of 300-600 mg of ferritin content at 60% of calcium dosages [46]. All these studies prove that calcium levels regulate iron absorption and thereby ferritin levels in blood. We hypothesized that ferritin levels would be decreased in post-treatment cancer patients with concomitant increase in calcium levels. On the other hand, studies by various scientists revealed the protective effects of calcium supplements in reducing the risk of cancer incidence [47-51]. Based on these studies we hypothesized that ferritin levels would be lowered in association with concomitant increase in calcium levels there by reducing the risk of tumor relapse and recurrence. However, our results demonstrated that ferritin levels are high in both pre- and post-treated cancer patients with low serum calcium levels suggesting that these patients might have increased risk of cancer relapse indicating that low levels of calcium results in accumulation of ferritin which might be associated with high risk of tumor relapse and recurrence. The increased ferritin levels in post-treatment cancer patients might be due to the incidence of chronic anemia induced by chemo/radiotherapy [52]. Finally, based on our results and from available literature studies we conclude that calcium intake might reduce the bioavailability of ferritin levels which finally reduces the risk of cancer relapse. **Figure 7** represents the outline of our present study.

However, there are few limitations in the present study. This is a preliminary study conducted in the heterogenous populations that were

diagnosed with breast, cervical, and ovarian cancers. We could not assess the required parameters in control samples because we could only collect 10 blood samples from the healthy volunteers. However, as a control we took the standard reference ranges provided by the National Institute of Health for calcium, magnesium, phosphorus, and electrolytes while the standard reference range of ferritin bound to transferrin from the study done by Rushton et al., [53].

Acknowledgements

SLP gratefully acknowledges DBT (BT/PR3-0629/BIC/101/1093/2018), New Delhi; UGC (Ref No: No.F.30-456/2018 (BSR)), SERB (Ref No.: PDF/2015/000867), and GITAM-RSG for the financial support. PC gratefully acknowledges DBT (BT/PR30629/BIC/101/1093/2018), New Delhi, for the Junior research fellowship. We thank GITAM Deemed to be University for funding this study through Research Seed Grant (Ref: F.No 2021/0028).

Disclosure of conflict of interest

None.

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