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Evaluation of trace elements associated with antioxidant enzymes in blood of primary epithelial ovarian cancer patients

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ABSTRACT

Epithelial ovarian cancer (EOC) has been associated with oxidative stress (OS) due to epithelial inflammation which makes ovaries more vulnerable to the deleterious effects of reactive oxygen species (ROS). However, antioxidant enzymes (AOEs) such as manganese-superoxide dismutase (Mn-SOD), copper,zinc-superoxide dismutase (Cu,Zn-SOD) and glutathione peroxidase (GPx1) protect cells against the biological damage of ROS-induced OS and support cancer prevention by maintaining normal cell cycle progression, inhibiting proliferation, tumor invasion, angiogenesis, inflammation or inducing apoptosis.

In the present study, we aimed to measure the levels of trace elements [manganese (Mn), copper (Cu), zinc (Zn) and selenium (Se)] which are structurally and/or functionally associated with the AOEs by inductively coupled plasma/mass-spectrometry (ICP/MS) in blood samples of patients with EOC (M, n = 26) and compare the data with healthy subjects (C, n = 46). Serous EOC (M1, n = 18) data were also evaluated according to the tumor grading [well or moderately well differentiated (G 1-2) vs. poorly differentiated or undifferentiated (G3)] and staging of disease [stage I-II (SI-II) vs. stage III (SIII)]. We obtained; i) The Mn and Se levels of M were significantly lower than C, ii) only Mn levels were changed [($G3_{(Mn)}$ < G 1-2 $_{(Mn)}$] in M1, iii) significant correlations were observed between [Cu and Zn levels (r = 0.701, p = 0.036) in G 1-2 and (r = 0.686, p = 0.041) in G3; Cu and Se levels (r = 0.960, p = 0.000) in G3; Mn levels and Mn-SOD expression (r = 0.551, p = 0.006) in M, (r = 0.857, p = 0.007) in G 1-2 and (r = 0.690, p = 0.056) in G3; Se levels and erythrocyte GPx1 activity (r = 0.660, p = 0.053) in G 1-2 ; Se levels and erythrocyte Cu,Zn-SOD activity (r = 0.693, p = 0.038) in G3]. The study revealed that trace elements, particularly low Mn and Se levels along with high Cu/Se ratios might be of value in all histologic subtypes of EOC. Although Mn level was important in terms of discriminating tumor grades, positive correlation between Cu-Se levels was also remarkable in patients with G 1-2 tumors of M1. Moreover, high erythrocyte Cu/Se ratios might be a favourable marker for EOC.

1. Introduction

Carcinogenesis is a multifactorial diease due to genetic and epigenetic alterations facilitated by various risk factors including environmental factors such as toxic heavy metals [arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), nickel (Ni), etc.] and trace elements [manganese (Mn), copper (Cu), zinc (Zn), selenium (Se), iron (Fe), etc]. The carcinogenicity of metals and their compounds/reactive metabolites mostly depends on their bioavailability in biological systems and their interference with (i) cellular redox regulation and antioxidant defense systems resulting in generally reactive oxygen species (ROS)-induced oxidative stress (OS), enhanced lipid peroxidation (LPO),

Abbreviations: EOC, epithelial ovarian cancer; OS, oxidative stress; ROS, reactive oxygen species; AOEs, antioxidant enzymes; Mn-SOD, manganese superoxide dismutase; Cu, Zn-SOD, copper,zinc-superoxide dismutase; GPx1, glutathione peroxidase; Mn, manganese; Cu, copper; Zn, zinc; Se, selenium; As, arsenic; Cd, cadmium; Pb, lead; Hg, mercury; Ni, nickel; Fe, iron; LPO, lipid peroxidation; ICP/MS, inductively coupled plasma/ mass-spectrometry; FIGO, International Federation of Gynecology and Obstetrics; G 1-2, well or moderately well differentiated; G3, poorly differentiated or undifferentiated; SI-II, stage I-II: early stages; SIII, stage III: advanced stage; H_2O_2, hydrogen peroxide; GSH, reduced glutathione; GSSG, oxidized glutathione; SELENBP1, selenium binding protein; MDA, malondialdehyde; ATP, adenosine triphosphate

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changes in calcium and sulphydril homeostasis (ii) DNA repair mechanisms resulting in genomic instability/accumulation of important mutations, (iii) elements of signal transduction pathways such as protein kinases, transcription factors, etc. resulting in deregulation of cell proliferation, (iv) gene expression by activating proto-oncogenes or inactivating cell growth controls such as tumor supressor genes resulting in stimulated cell proliferation, (v) autophagy and related proteins resulting in dysfunction and cellular transformation or inflammation [1–6].

Numerous *in vivo* and *in vitro* studies have pointed out to adverse effects of metals on female reproductive system and scientific consensus reached that high metal exposure/deficiency is associated with some reproductive disorders/diseases [7–14] such as infertility, spontaneous abortions, preterm deliveries, uterine fibroids [15], endometriosis [16], polycystic ovarian syndrome [17] or gynecological cancers [15,18–22]. Ovarian cancer is the leading cause of death among gynecological cancers. Thus, it is a life-threatining disease with respect to its histopathology, clinical characteristics and high incidence/mortality rates increased with age. Epithelial ovarian cancer (EOC) comprises 90% of malignant and 60% of all ovarian tumors and 75% of EOC are characterized by serous histologic subtype [23–26].

Epithelial ovarian tumors are associated with reactive oxygen species (ROS) induced-oxidative stress (OS) due to epithelial inflammation of the ovaries arising from incessant ovulation [27,28]. Excessive ROS have a paradoxical role in tumorigenesis with their deleterious effects to the body such as oxidative damage to biomolecules, genomic instability, uncontrolled growth, and perturbed differentiation [4,29–31] and anti-tumorigenic effects against tumor cells such as induction of cellular senescence and apoptosis [4,32–34]. ROS are involved in all stages of cancer development; initiation, promotion and progression. A disruption in the redox homeostasis due to high ROS levels and depletion or decreased removing capacity of endogeneous antioxidants might lead to cancer [28,30,31,35]. There are various processes in the body to alleviate ROS and counteract detrimental effects of ROS induced-OS on important biomolecules. These mechanisms include antioxidant enzymes (AOEs) which protect aerobic cells against biological damage of ROS and support cancer prevention by modulating signal transduction pathways [4,29–31,35,36].

Some trace elements such as Mn, Cu, Zn, Se and Fe are essential for normal formation or functions of the body and exist in the structure of AOEs. Thus, they play critical roles in important biological processes, but also contribute to the development of many chronic diseases, including cancer with their excessive levels (e.g. Cu, Fe, Se) or loss of antioxidant feature depending on deficiency (e.g. Zn, Se) [2,16–19,37].

The main AOEs associated with trace elements are superoxide dismutases (SODs) in the body. They dismutate superoxide anion into hydrogen peroxide (H_2O_2) by requiring catalytic cycle of redox active transition metals (i.e. oxidized Mn^{+3}/reduced Mn^{+2}) in their active sites. Manganese-superoxide dismutase (Mn-SOD) has one Mn^{+2} ion in its each monomeric subunit, whereas copper,zinc-superoxide dismutase (Cu,Zn-SOD) has two subunits each containing one Cu^{+2} and one Zn^{+2} ions. The Cu^{+2} is a redox active metal and responsible for the catalytic activity of Cu,Zn-SOD with its catalytic cycle (Cu^{+3}/Cu^{+2}), while Zn^{+2} enhances the stability of this enzyme and provides a wide range of pH value for its activity [38–42]. Cu can form complexes with gonadotropin releasing hormones and high levels of FSH and LH are associated with ovarian tumor development [7]. Another group of AOEs is glutathione peroxidase (GPx1) which is Se-dependent and carries 4 atom gram of Se in each molecule in the form of selenocysteine. Organic hydroperoxides, including H_2O_2 are converted and detoxified by these seleno-enzymes mainly through oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) [43–45]. Alterations in the GSH:GSSG ratio may modulate Cu homeostasis in the body due to the critical roles of GSH in cellular redox balance, Cu binding and Cu delivery or flux [40]. Besides this, Se and selenoproteins like GPx1 are elevated in large healthy follicles and pronounced to have a critical role as an antioxidant during late follicular development [46]. Zn has also antioxidant properties as a cofactor of important enzymes, including Cu,Zn-SOD. It modulates total cellular GSH concentration by altering the expression of glutamate-cysteine ligase, inhibits the pro-oxidant enzyme NADPH-oxidase and induces the synthesis of toxic heavy metal binding protein metallothionein [47,48]. Zn also functions as an anti-inflammatory agent by reducing inflammatory cytokine production [48]. The studies pointed out that both Se and Zn deficiencies are in association with disturbed female reproductive system functions such as unexplained infertility, gestational complications, miscarriages, low oocyte maturation or anovulation [49,50].

Therefore, in the present study, firstly we aimed to measure trace element levels (Mn, Cu, Zn, Se) which are structurally and/or functionally associated with the AOEs in blood samples of patients with EOC and then evaluated the possible correlations between these trace elements and their relevant AOEs together with OS-induced LPO markers. This is the first study evaluating trace elements in patients with EOC according to histopathological and clinical characteristics of the disease by identifying also the relationship between these elements and relevant AOEs requiring them for enzymatic activity or stability.

2. Materials and methods

2.1. Chemicals

Chemicals used to measure trace elements were analytical grade and purchased from Sigma-Aldrich Co. Ltd. (St Louis, MO, USA) and Merck (Darmstadt, Germany). Multielement Standard Solution IV for ICP was purchased from Sigma-Aldrich Co. Ltd. (Buchs, Switzerland) and Trace Elements Whole Blood L-1 and L-2 were obtained from Seronorm (Billingstad, Norway).

2.2. Study groups

The study group consisted of patients undergone surgical resection with confirmed pathology of malignant epithelial ovarian tumors [M, n = 26] at the Hacettepe University Adult Hospital, Department of Obstetrics and Gynecology (Ankara, T9URKEY). The group was divided into subgroups; [M1, n = 18 serous adenocarcinomas] and [M2, n = 8 miscellaneous adenocarcinomas (endometrioid, n = 4; mixed, n = 3; clear cell, n = 1)]. Blood samples obtained both from M1 and M2, and the trace elements data were compared to data of a control group (C) comprised of 46 normal healthy individuals (C) without any diease including gynecological cancers. The patients diagnosed for the first time were involved into the study and those receiving radiation therapy for metastases were excluded. The data of patients with serous EOC were also evaluated according to the International Federation of Gynecology and Obstetrics (FIGO) tumor grading [well or moderately well differentiated (G 1-2) vs. poorly differentiated or undifferentiated (G3)] and disease staging [stage I–II (S I–II) vs. stage III (S III)]. Written informed consent was obtained from all the patients before participation and a questionnaire has been carried out to collect demographic data, dietary habits, and smoking history. The study was approved by the Clinical Research Ethics Committee of the Hacettepe University [Project number: GO 17/163].

2.3. Preparation of blood samples

10 ml heparinized and 5 ml whole venous blood samples were obtained in the morning after an overnight fasting. Heparinized blood samples were centrifuged at 3000xg for 15 min. and after discarding plasma the packed cells (**red** blood cells) were washed thrice with cold 0.9% physiological saline. Erythrocye and whole blood samples were kept at −80 °C until the metal analyses conducted.

In our study, we preferred to use whole blood samples to measure Mn levels, as whole blood Mn is the most frequently used indicator that

may better reflect Mn stores in tissues and is 10–20 times higher than plasma. It is also recommended for assessment of Mn status of the body after long term exposure as Mn is known to accumulate in erythrocytes [51]. Additionally, we preferred to use erythrocytes to measure Cu and Zn levels as false positive results might be obtained with serum samples due to its many disadvantages such as hemolysis or ceruloplasmin/albumin levels alterations induced by various drugs or several acute/chronic diseases.

2.4. Measurement of manganese, copper, zinc and selenium levels

The Mn, Cu, Zn and Se levels of blood samples were determined quantitatively by inductively coupled plasma/mass spectrometer (ICP/MS, 7500ce, Agilent, Tokyo, Japan). Whole blood samples were diluted 1:20 for Mn and 1:50 for erythrocyte Cu, Zn and Se assays with fresh trace element buffer solution containing 0.1% triton X-100 which provides trace elements availability in solution and enables washout processes. External calibration solutions of Mn, Cu, Zn and Se were prepared by a serial dilution of "Multielement Standard solution IV for ICP Solution". The same diluent was also used with samples. The concentration intervals for standard solutions were 2–20 µg/L for Mn, 100–500 µg/L for Cu, 500–2500 µg/dL for Zn and 50–250 µg/L for Se. Limits of detection were 0.01 µg/L for Mn, 0.5 µg/L for Cu and Se, 0.5 µg/dL for Zn. Two levels of "Seronorm Trace Elements Whole Blood Control L1 and L2" were used for each element.

The instrument was operated with a Agilent cooled impact bead spray chamber, single-piece quartz torch (2.5 mm ID injector) together with Agilent Nickel (Ni) interface cones and Agilent-ASX-500 Series autosampler (Agilent, Tokyo, Japan). Burgener trace nebulizer was used and the instrument was operated in standard mode with 1500 WRF power, 0.9 l/min nebuliser gas flow, 0.25 l/min auxiliary gas flow, 60 ms dwell time, 30 s sample uptake and 30 s wash time (3 repeats per sample).

2.5. Statistical analyses

Experimental data were analyzed with Shapiro-Wilk, test of normality followed by Student's *t*-test for normally distributed variables and Mann-Whitney U test for non-normally distributed variables using a Statistical Package for Social Sciences Programme (SPSS programme v23.0, SPSS Inc., Chicago, IL). The correlations were assessed using Pearson or Spearman correlation coefficients. The level of significance was defined as ($p < 0.05$). Values were given as mean ± standard deviation (SD) for normally distributed variables and median (min-max) for non-normally distributed variables.

3. Results

In our study, all groups were matched in terms of their mean age, BMI, dietary and smoking habits and existence of chronic diseases. The mean age of M was 55.61 ± 1.75 (41–75 years), M1 was 57.33 ± 2.35 (41–75 years), M2 was 52.75 ± 2.01 (44–60 years) and C was 51.55 ± 0.92 (44–69 years). The patients and healthy sujects were both over 40 years of age (> 40) and the groups were not different with respect to their mean age ($p > 0.05$). Besides this, BMI of M was 28.65 ± 1.15 kg/m^2, M1 was 29.80 ± 1.56 kg/m^2, M2 was 26.35 ± 1.24 kg/m^2 and C was 29.83 ± 0.92 kg/m^2. All the subjects of case and contol groups were overweighted and no significant differences were observed between them ($p > 0.05$). Since, 80% of M1 and 85% of C were never smokers, significant differences were not observed in the distribution of M1 and C in terms of their cigarette smoking.

The distributions were analyzed by using the Shapiro-Wilk test for Mn, Cu, Zn and Se levels along with Cu/Zn, Cu/Se and Zn/Se ratios. Student's *t*-test was used to compare normally distributed data [(Cu and Se levels of M, M1, M2, C), (Cu, Zn, Se levels and Cu/Zn, Cu/Se ratios of G 1-2 and G3)] and Mann-Whitney U test was used for non-normally distributed data [(Mn and Zn levels and Cu/Zn, Cu/Se, Zn/Se ratios of M, M1, M2, C), (Mn levels and Zn/Se ratios of G 1-2 and G3)]. Distributions of Mn and Se levels of M (Fig.1 A1 and A2), and Mn levels of G3 (Fig. 1B) were depicted with box and whisker plots which were significantly different from C and G 1-2, respectively ($p < 0.05$).

Trace element concentrations in blood samples of patients with M according to tumor histologic subtypes (M1 and M2) were summarized in Table 1. The Mn and Se levels of M were significantly lower than C ($p < 0.05$) (Fig. 2.A1). The ratios of trace element concentrations were examined according to tumor histologic subtype and the Cu/Se and Zn/Se ratios of M was found significantly higher than C (Table 2) (Fig. 2.A2).

Moreover, when we evaluated the data of M1 in terms of tumor grading and staging of disease, only Mn levels were significantly changed [($G3_{(Mn)} < G\ 1\text{-}2_{(Mn)}$, 32%, $p < 0.05$] as depicted in Table 3 and Fig. 2B, whereas Cu/Zn, Cu/Se and Zn/Se ratios did not change (Table 4).

In addition, some significant correlations were observed in our study. Pearson correlation coefficients and significance levels were as follows: Cu and Zn levels ($r = 0.701$, $p = 0.036$) in G 1-2, ($r = 0.686$, $p = 0.041$) in G3 of M1; Cu and Se levels ($r = 0.960$, $p = 0.000$) in G3 of M1. When we assessed the relationship between trace elements and their relevant AOEs, we found marked correlations between Mn and Mn-SOD, Se and GPx1, Se and Cu,Zn-SOD [Spearman's rhos and significance levels of Mn levels and Mn-SOD expression ($r = 0.551$, $p = 0.006$) in M, ($r = 0.857$, $p = 0.007$) in G 1-2, ($r = 0.690$, $p = 0.056$) in G3 of M1; pearson correlation coefficients and significance levels of Se levels and erythrocyte GPx1 activity ($r = 0.660$, $p = 0.053$) in G 1-2 of M1; Se levels and erythrocyte Cu,Zn-SOD activity ($r = 0.693$, $p = 0.038$) in G3 of M1]. Although positive linear correlations were observed between erythrocyte Cu and Zn levels of patients with serous G 1-2 tumors [$y = 1.6492x + 6.2061$, $R^2 = 0.4909$] and serous G3 tumors [$y = 0.8455x + 515.38$, $R^2 = 0.4712$], strong correlations were only observed between erythrocyte Cu and Se levels of patients with serous G3 tumors [$y = 0.1844x\text{-}36.775$, $R^2 = 0.9208$] (Fig.3 A1), whole blood Mn level and Mn-SOD expression of patients with serous grade 1-2 (G 1-2) tumors [$y = 0.0307x\text{-}0.1473$, $R^2 = 0.7344$] in M1 (Fig.3 A2). On the other hand, even a moderate statistically significant correlation ($r = 0.551$, $p = 0.006$) was observed between blood Mn level and Mn-SOD expression, residuals did not contradict a linear assumption without histologic classification and grading in M.

4. Discussion

In spite of many toxicological and epidemiological studies searching the role of OS in various cancer types and evaluating the adverse effects of metals on female reproductive system, little is known about their effects on EOC directly or through oxidant-antioxidant alterations. Thus, to our knowledge, this is the first study assessing the blood trace elements (Mn, Cu, Zn, Se) in EOC comprehensively according to histopathological and clinical characteristics of tumors and evaluating the correlations between trace elements and their structurally/functionally associated AOEs and OS-induced LPO.

Trace elements like Mn, Cu, Zn, Se are fundamental cofactors responsible for the modulation of AOEs involved in cellular redox mechanisms. The balance between these elements and toxic heavy metals/non-essential elements is very critical to maintain normal body homeostasis and if any imbalance is observed towards the deficiency of trace elements, the hosts resistance might be impaired against carcinogenic stress due to altered activities and/or expressions of AOEs [52–54]. Therefore, their role on cancer development/inhibition is quite complex depending on their essential/toxic effects on human health.

Manganese plays a critical role in cell survival and death mechanisms as it is a cofactor/activator of many enzymes, including Mn-SOD.

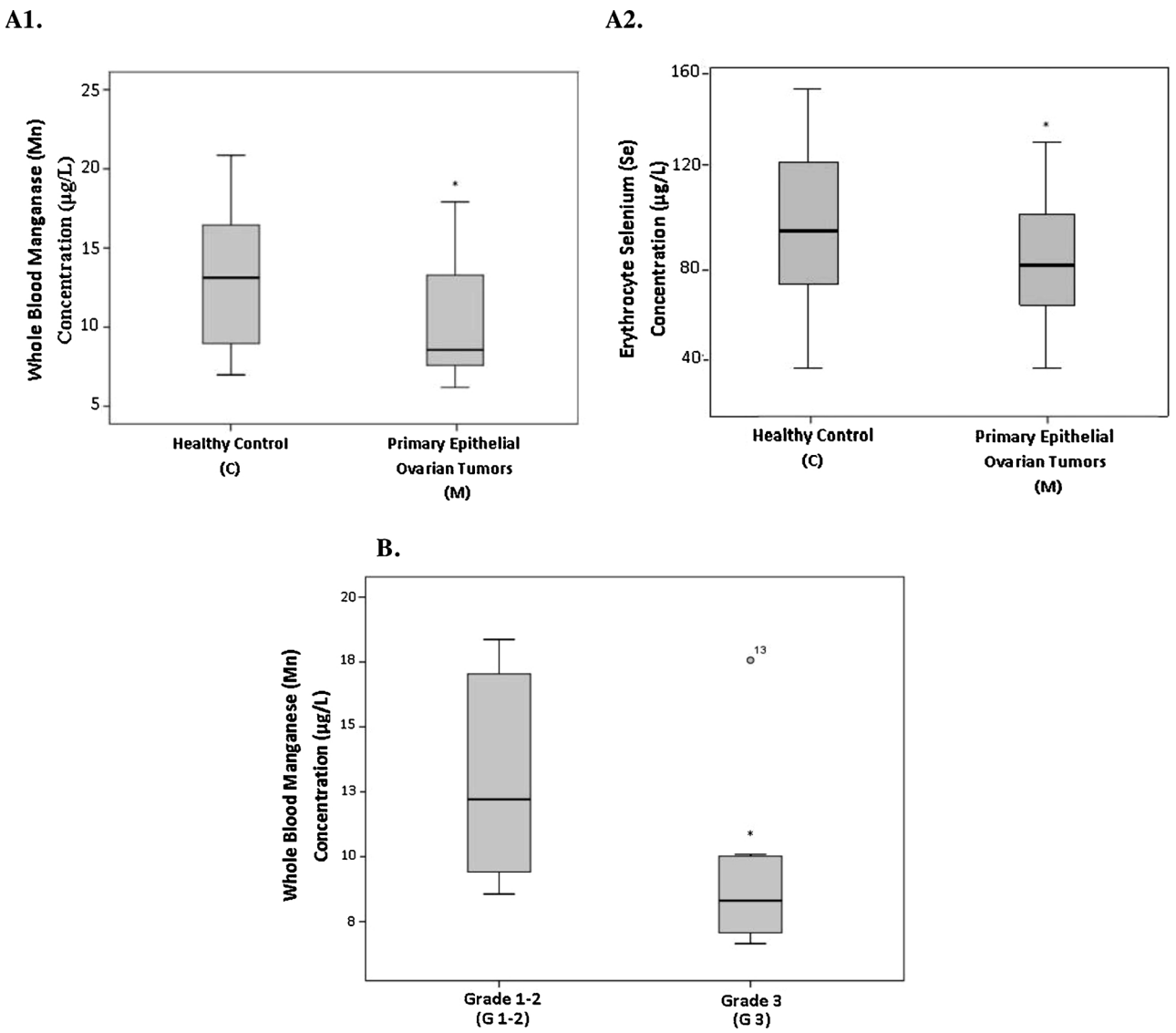

Fig. 1. A1.The box and whisker plot representing the skewed distributions of whole blood manganese (Mn) levels of patients with malignant epithelial ovarian tumors (M) and healthy control (C),*$p < 0.05$. A2. The box and whisker plot representing the symmetric distributions of erythrocyte selenium (Se) levels of patients with malignant epithelial ovarian tumors (M) and healthy control (C),*$p < 0.05$. B. The box and whisker plot representing the skewed distributions of whole blood manganese (Mn) levels of patients with malignant epithelial serous ovarian grade 3 tumors (G3) and grade 1-2 tumors (G 1-2),*$p < 0.05$.

High Mn levels may disturb proper respiration which in turn lead to ROS-induced OS, cellular damage and Mn-dependent caspase-induced apoptosis [42,55,56]. In the literature, SODs were illustrated as mediators of ovulation, steroidogenesis and luteal function [57–59]. Besides this, Mn-SOD activity was found generally lower in cancer cells and a possible association was reported between decreased Mn-SOD

Table 1
Trace element concentrations in whole blood and erythrocyte samples of patients with malignant epithelial ovarian tumors according to tumor histologic subtype.

Variables/ Study Groups	Control (C) (n = 46)	Primary epithelial ovarian tumors (M) (n = 26)	Primary epithelial serous ovarian tumors (M1) (n = 18)	Miscellaneous epithelial ovarian tumors (M2) (n = 8)	p values (M vs C)[a,b]
Whole Blood Manganese Concentration (Mn)(µg/L)	12.55 (7.38-22.14)	9.01 (6.65-18.37)*	9.95 (6.65-18.37)*	8.13 (6.78-16.33)*	0.008[a]
Erythrocyte Copper Concentration (Cu)(µg/L)	655.65 ± 114.30	666.51 ± 128.34	662.03 ± 109.33	676.59 ± 172.13	0.712[b]
Erythrocyte Zinc Concentration (Zn)(µg/dL)	1018.50 (665-2159)	1097.50 (547-1450)	1106.50 (726-1381)	1036.97 (547-1450)	0.336[a]
Erythrocyte Selenium Concentration (Se)(µg/L)	95.36 ± 26.87	82.98 ± 26.15*	81.65 ± 23.47*	85.96 ± 33.01*	0.038[b]

[a] Mann Whitney *U* test was used for non-normally distributed variables (Mn and Zn concentrations); data were given as median (min-max).
[b] Student's *t*-test was used for normally distributed variables (Cu and Se concentrations); data were given as mean ± standard deviation (SD).
* M vs C, M1 vs C, and M2 vs C ($p < 0.05$).

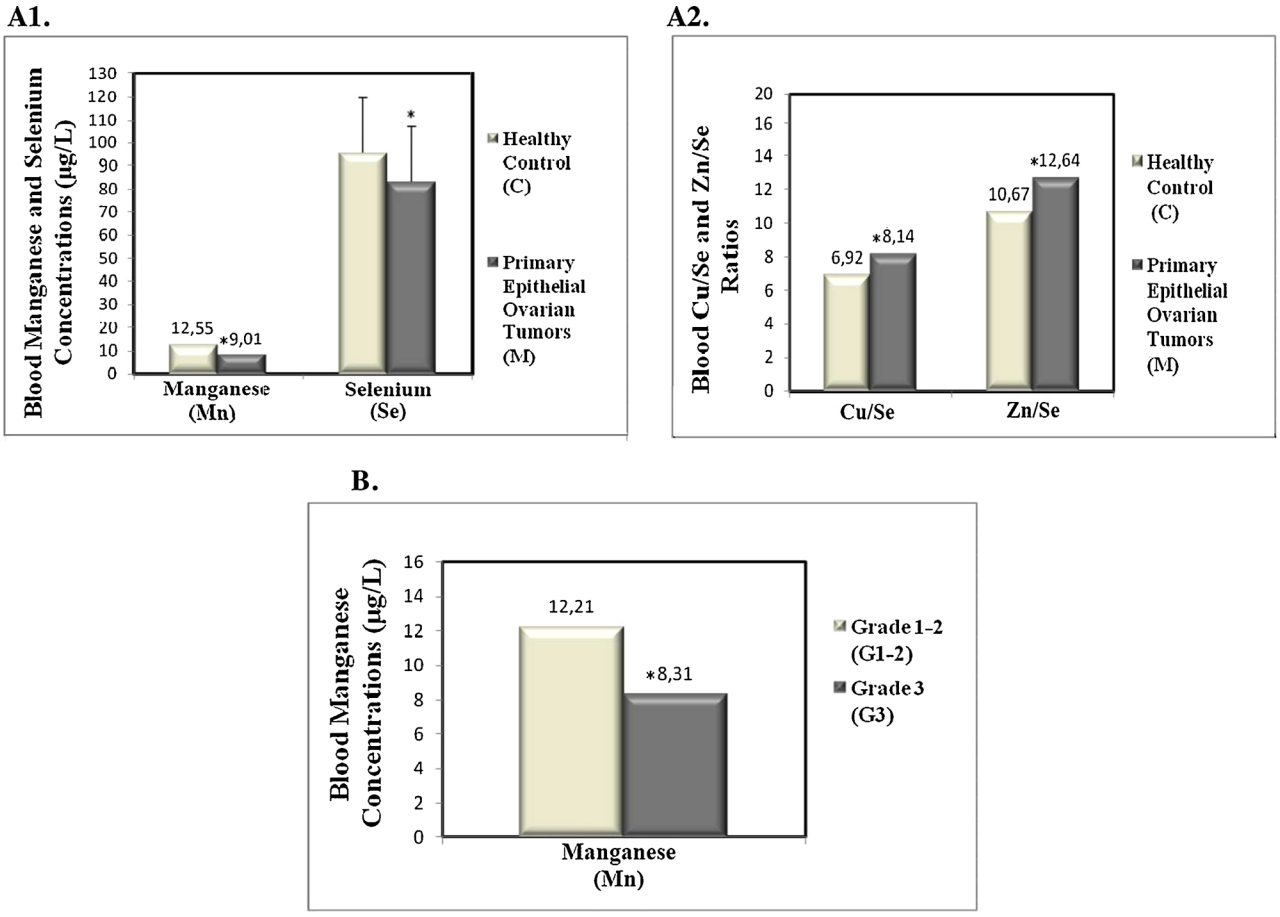

Fig. 2. A1. Whole blood manganese (Mn) levels and erythrocyte selenium (Se) levels of patients with malignant epithelial ovarian tumors (M) were lower than healthy control (C). A2. Blood Cu/Se and Zn/Se ratios of patients with malignant epithelial ovarian tumors (M) were higher than healthy control (C). B. Whole blood manganese (Mn) levels of patients with malignant epithelial serous ovarian grade 3 tumors (G3) were lower than grade 1-2 tumors (G 1-2). Median values were given for non-normally distributed Mn levels and Cu/Se, Zn/Se ratios. Mean values were given as mean ± standart deviation (SD) of triplicate measurements for normally distributed Se levels, * $p < 0.05$.

activity and malignant phenotype in various studies [60], including our previous study with EOC [28]. Mn-SOD overexpression with low Mn-SOD activity in transformed cells was shown to restore cells to a growth pattern. The paradoxical views of Mn-SOD as a tumor suppressor/promoter might be explained by the cellular redox status alterations through H_2O_2 accumulation/detoxification in normal, transformed and metastatic phenotypes [38,60–64].

According to our results, Mn were found significantly lower [$M_{(Mn)} < C_{(Mn)}$] ; [$M1_{(Mn)} < C_{(Mn)}$] ; [$M2_{(Mn)} < C_{(Mn)}$] in whole blood samples of patients with all histologic subtypes of EOC compared to C. In our previous study [28], tumor Mn-SOD expression was higher (%67), but Mn-SOD activity was lower (%42) along with higher malondialdehyde (MDA) levels (127%) in M1 compared to C. Since, Mn would enhance the expression of Mn-SOD [38] and tumor tissue requires more Mn for Mn-SOD overexpression to protect cells against OS-mediated mitochondrial injury and suppress apoptosis, blood Mn might be transferred rapidly to the tissue site. Therefore, decreased blood Mn levels are consistent with the higher tumor Mn-SOD expression observed in M1 and M2. Besides this, some possible post-translational modifications on Mn-SOD protein due to high levels of MDA and low levels of Mn might supress the activity of Mn-SOD in patients with M1.

Unlike normal cells, most transformed cells derive energy from aerobic glycolysis. However, energy supplied from glycolytic processes may not be sufficient during the aggressive growth of cancer cells, particularly in the advanced stages of carcinogenesis. Therefore, aggressive cancer cells switch back from glycolysis to oxidative phosphorylation for their increased energy requirements. Increased Mn-SOD expression may contribute to the repair of mitochondrial function which also allows cells to utilize oxidative phosphorylation again for maximum energy production and to maintain cell survival

Table 2
The ratios of trace element concentrations in erythrocyte samples of patients with malignant epithelial ovarian tumors according to tumor histologic subtype.

Variables/ Study Groups	Control (C) (n = 46)	Primary epithelial ovarian tumors (M) (n = 26)	Primary epithelial serous ovarian tumors (M1) (n = 18)	Miscellaneous epithelial ovarian tumors (M2) (n = 8)	p values (M vs C)[a]
Cu/Zn	0.66 (0.37-1.10)	0.64 (0.42-1.00)	0.61 (0.51-0.83)	0.66 (0.42-1.00)	0.489[a]
Cu/Se	6.92 (3.74-16.06)	8.14 (5.73-16.73)*	8.25 (5.73-16.73)*	8.07 (5.84-13.60)*	0.005[a]
Zn/Se	10.67 (5.39-29.29)	12.64 (7.95-26.13)*	12.82 (7.95-26.13)*	12.06 (9.84-17.56)*	0.002[a]

[a] Mann Whitney *U* test was used for non-normally distributed variables (Cu/Zn, Cu/Se and Zn/Se ratios); data were given as median (min-max).
* M vs C, M1 vs C, and M2 vs C ($p < 0.05$).

Table 3
Trace element concentrations in whole blood and erythrocyte samples of patients with malignant epithelial serous ovarian tumors according to grading and staging.

Variables/ Grading d Staging	Primary Epithelial Serous Ovarian Tumors (M1) (n = 18)					
	Grading			Staging		
	G1-2 (n = 9)	G3 (n = 9)	p values (G3 vs G1-2)[a,b]	S I-II (n = 6)	S III (n = 12)	p values (S III vs S I-II)[a,b]
Whole Blood Manganese Concentration (Mn)(μg/L)	12.21 (8.56-18.37)	8.31 (6.65-17.57)*	0.036[a]	11.62 ± 4.86	11.00 ± 3.77	0.774[b]
Erythrocyte Copper Concentration (Cu)(μg/L)	642.76 ± 79.85	667.90 ± 137.63	0.471[b]	616.80 ± 89.55	675.20 ± 115.30	0.225[b]
Erythrocyte Zinc Concentration (Zn)(μg/dL)	1056.22 ± 187.95	1069.38 ± 162.89	0.767[b]	1050.50 ± 220.37	1077.09 ± 149.28	0.637[b]
Erythrocyte Selenium Concentration (Se)(μg/L)	74.45 ± 19.56	86.04 ± 26.19	0.202[b]	70.40 ± 12.68	85.09 ± 26.06	0.156[b]

[a] Mann Whitney *U* test was used for non-normally distributed variables (Mn); data were given as median (min-max).
[b] Student's *t*-test was used for normally distributed variables (Cu, Zn and Se concentrations); data were given as mean ± standard deviation (SD).

Table 4
The ratios of trace element concentrations in erythrocyte samples of patients with malignant epithelial serous ovarian tumors according to grading and staging.

Variables/Grading and Staging	Primary Epithelial Serous Ovarian Tumors (M1) (n = 18)					
	Grading			Staging		
	G1-2 (n = 9)	G3 (n = 9)	p values (G3 vs G1-2)[a,b]	S I-II (n = 6)	S III (n = 12)	p values (S III vs S I-II)[a,b]
Cu/Zn	0.61 ± 0.08	0.63 ± 0.09	0.749[a]	0.60 ± 0.08	0.62 ± 0.09	0.679[a]
Cu/Se	9.31 ± 3.30	7.86 ± 0.89	0.222[a]	8.94 ± 1.71	8.41 ± 2.81	0.223[a]
Zn/Se	12.72 (11.08-26.13)	12.91 (7.95-16.25)	0.508[b]	14.44 (12.07-20.76)	12.53 (7.95-26.13)	0.190[b]

[a] Student's *t*-test was used for normally distributed variables (Cu/Zn and Cu/Se ratios); data were given as mean ± standard deviation (SD).
[b] Mann Whitney *U* test was used for non-normally distributed variables (Zn/Se ratios); data were given as median (min-max).

and growth [60,63]. Our previous results [28] were in consistent with the knowledge above as high tumor Mn-SOD expression levels were observed in G3 and SIII of M1. Besides this, in this study, blood Mn levels were lower in patients with tumors that tend to grow quickly and more likely to spread (G3) as compared to G 1-2 [$G3_{(Mn)}$ < G $1\text{-}2_{(Mn)}$, 32%] in M1. This might be explained as an adaptive defense mechanism to grow and spread in G3 tumors against Mn-dependent caspase induced apoptosis.

Copper and Zn are key essential trace elements of many cellular functions as they are responsible for the stability and activity of Cu,Zn-SOD. Although Cu levels are under strict homeostatic control mechanisms that regulate its absorption, excretion and bioavailability, any imbalance observed in its bioavailability might cause deficiency/toxicity or major pathological outcomes, including cancer [65–67]. Redox active Cu can induce DNA base/chain breaks leading to tumor growth/proliferation at high concentrations through Fenton's reaction and also facilitate angiogenesis and metastasis [48,66,68,69]. Besides this, Zn is a redox-inert metal, but the decrease in cellular Zn pool is associated with high LPO-induced OS and impaired DNA repair mechanisms resulting in DNA integrity and cancer. In addition, low serum Zn levels were observed in patients with various cancers [68]. Thus, levels of Cu, Zn and the ratio of Cu/Zn are great interest of area for many

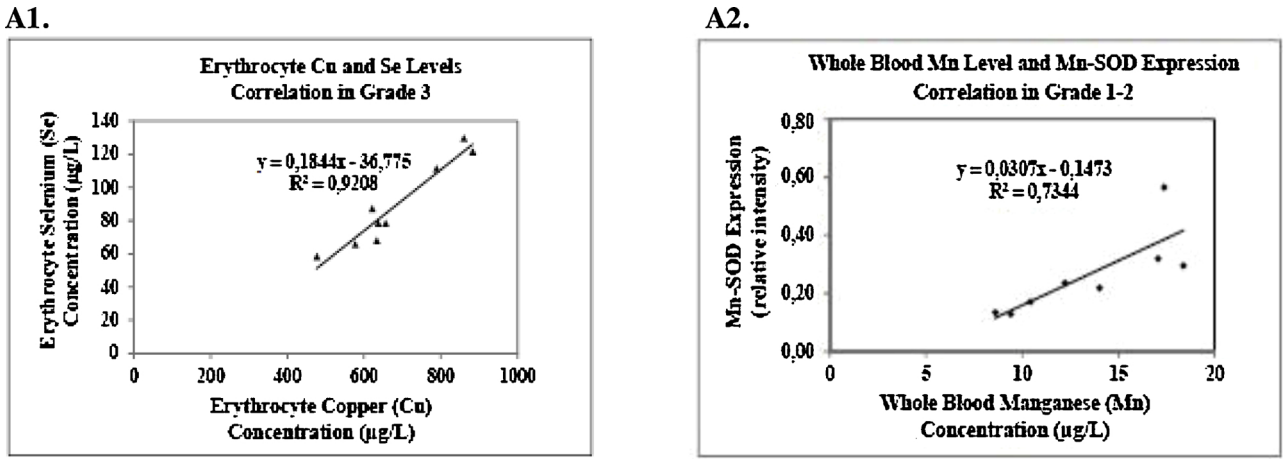

Fig. 3. A1. The correlation between erythrocyte Cu and Se levels of patients with serous grade 3 (G3) tumors, [r:0.960, p:0.000] and [y = 0.1844x-36.775, R^2 = 0.9208]. A2. The correlation between whole blood Mn level and Mn-SOD expression of patients with serous grade 1-2 (G 1-2) tumors, [r:0.857, p:0.007] and [y = 0.0307x-0.1473, R^2 = 0.7344].

years. High Cu/Zn ratio was found to reflect the homeostatic status from juvenile age to older ages and associated with ageing and age-related chronic diseases [70–72]. Besides this, cigarette smoking can also interfere with metal homeostatis in the body and might affect the Cu/Zn ratios [73]. Under inflammatory conditions observed in gynecologic malignant tumors; high Cu levels [22,74,75], low Zn levels [22,76] and high Cu/Zn ratios [8,20,75,77,78] were observed as compared to healthy subjects. However, serum Cu/Zn ratio has a non-specific character as higher levels could also be obtained in another cancer types [15,37,79–81] and acute/chronic infections [69,82]. On the other hand, Swaminathan et al. [83] suggested the measurement of Cu levels together with tumor markers CEA and CA125 might be useful in gynecologic cancers, whereas Nayak et al. [74] recommended high Cu, ceruloplasmin and TBARS levels for the early diagnosis of ovarian cancer.

When we evaluated the studies on Cu and Zn levels/ratios above, at first we preferred to classify the histopathological and clinical characteristics of EOC and match the ages, chronic diseases and smoking history of patients to obtain a more specific, reliable data of Cu, Zn levels and precise interpretations of trace element ratios as a biomarker in our study. Although low tumor Cu,Zn-SOD activity (55%) was measured in M1 compared to C in our previous study [28]; no correlations were observed between Cu, Zn levels and Cu,Zn-SOD activity in M1. Besides this, significant positive correlations were observed between Cu and Zn levels ($r = 0.701$, $p = 0.036$) in G 1-2, ($r = 0.686$, $p = 0.041$) in G3 of M1. Furthermore, Cu and Zn levels were not different among M1 and C; whereas Se levels were found significantly lower in M1 compared to C. Denoyer et al. [66] reported unlike Cu, the levels of Zn and Se are often lower in the serum of cancer patients and emphasized that the Cu/Zn and Cu/Se ratios all appear to be better indicators of the presence of cancer than Cu, Zn or Se levels alone [48,66]. We observed high Cu/Se and Zn/Se ratios in M1 and M2 compared to C ($p < 0.05$). Trace element ratios were not different between G 1-2 and G3; SI-II and SIII, as tumor and erythrocyte Cu,Zn-SOD activities were also did not change according to tumor grading and staging in M1 in our previous study [28]. Thus, the critical and determinative element was Se along with Mn in the present study.

Se is essential for the antioxidant functions of selenoproteins like GPx1 and any alteration observed in GPx1 expression is used as an important biomarker for Se deficiency [84]. In addition, selenoproteins are accepted as potent modifiers of tumorigenesis with their variable actions like inhibition of tumor development by overcoming ROS-induced OS or mutagenesis particularly in inflammatory-driven cancers such as EOC. On the other hand, they can also enhance resistance to apoptosis and chemotherapeutic approaches in tumor cells with higher basal OS. There is a growing body of evidence supporting an association between low Se levels and cancers such as breast, liver, colorectal, etc. [61,84], including ovarian cancer [85]. In contrast, Canaz et al. [21] observed no marked statistical differences between Se concentrations of benign, borderline and malignant ovarian tumors.

In our study; Se levels of M, M1 and M2 were found significantly lower than C. Moreover, erythrocyte GPx1 activity of M1 (27%) and M2 (20%) were lower, whereas tumor GPx1 activity of M1 (60%) was higher than C in our previous study [28]. The low eyrthrocyte Se levels were consistent with low eyrthrocyte GPx1 activity both in M1 and M2. Since high tumoral GPx1 activity was observed in M1, these tissues might have required Se to display their activity and supplied it from erythrocyte Se pools. From a comprehensive point of view, high tumoral Mn-SOD expression with low activity, GPx1 activity and MDA levels [28] with low blood erythrocyte Se levels might be an adaptive response to protect tumor cells against apoptosis due to ROS-induced OS for tumor cell growth in EOC. Besides this, strong positive correlation between erythrocyte Cu and Se levels in G3 might be explained by adapted antioxidant response of Se against contributary role of redox active Cu on tumor growth and metastasis in tumors which tend to grow quickly and more likely to spread.

5. Conclusion

In conclusion, this study revealed Mn and Se levels along with Cu/Se ratios may be of value in patients with all histologic subtypes of malignant epithelial ovarian tumors. Observed decreases in erythrocyte Mn and Se levels might be associated with an adaptive defense mechanism of tumor cells directly or through AOEs such as Mn-SOD and GPx1 to avoid from aggravated OS-induced or caspase-induced apoptosis in inflammatory-driven cancer; EOC. The level of Mn was important in terms of discriminating tumor grades and the loss of Mn might contribute to tumor growth and/or spread in serous EOC. Besides this, observed increase in Cu/Se ratio might be responsible for decreased blood antioxidant capacity and increased inflammatory response due to low Se levels in all histologic subtypes of EOC. Thus, high erythrocyte Cu/Se ratios might be a favourable marker for EOC. However, further clinical researches are needed with a large study population to confirm the endpoints of this study and to identify potential trace element and AOEs relationships which might play a critical role in the pathogenesis of EOC by altering oxidant-antioxidant homeostasis. Furthermore, trace elements structurally or functionally associated with AOEs might also provide an important insight to develop new AOEs-targeted therapies in EOC.

Conflict of interest

All authors declare that they have no competing interests.

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