Dose-Response:
An International Journal
October-December 2020:1-10

DOI: 10.1177/1559325820982172
journals.sagepub.com/home/dos
SAGE

8-Hydroxy-2'-Deoxyguanosine and 8-Nitroguanine Production and Detection in Blood Serum of Breast Cancer Patients in Response to Postoperative Complementary External Ionizing Irradiation of Normal Tissues

Kosmas E. Verigos[1,2], Sofia Sagredou[1], Kyriakos Orfanakos[1,3], Panayiotis Dalezis[1], and Dimitrios T. Trafalis[1]

Abstract

It is widely known that ionizing irradiation is strongly linked to the production of reactive oxygen (ROS) and nitrative species (RNS) through which DNA damage products like 8-hydroxy-2-deoxyguanosine (8-OHdG) and 8-nitroguanine (8-NG) are generated, respectively. In the present study, we aimed to investigate the formation of 8-OHdG and 8-NG upon irradiation and to further explore whether alterations in their concentration levels are related to the administered radiation doses and exposure time. Our research work was conducted in blood serum samples collected from 33 breast cancer patients who received adjuvant radiotherapy. The detection of 8-OHdG and 8-NG was assessed by enzyme-linked immunosorbent assay. Our results suggest that both, 8-OHdG and 8-NG, were formed during the radiation regimen. Significant correlations with radiation dose were also demonstrated by the dose-response curves of 8-OHdG and 8-NG, fitted by logarithmic distribution and polynomial regression, respectively. More precisely, 8-OHdG and 8-NG concentrations (ng/mL) were considerably increased when patients received ionizing radiation up to 30 Gy whereas irradiation over 30 Gy did not induce any further increases. The current study supports a) the production of 8-OHdG and 8-NG during radiotherapy and b) significant correlations between either 8-OHdG or 8-NG levels and radiation doses, indicating a radiation-dose-dependent relationship.

Keywords

radiation therapy, 8-hydroxy-2'-guanosine, 8-nitroguanine, breast cancer, radiation dose, exposure time, dose-response relationships

Introduction

Radiotherapy constitutes an integral part of cancer treatment in most of cancer cases since it fairly reduces the risk of local recurrence, increases the chances of survival and relieves suffering from symptoms. The aim of radiation treatment is the shrinkage of tumor mass as well as frustration of residual tumor cells under the exposure to ionizing radiation, highlighting radiation therapy as a primary or adjuvant treatment to other therapeutic options, as surgery.[1,2]

X- and gamma radiation are the most common types of ionizing radiation which are delivered in cancer patients through radiotherapy.[2] Two methods with which patients can receive radiation therapy are the external beam-radiation and

[1] Laboratory of Pharmacology, Faculty of Medicine, National and Kapodistrian University of Athens, Athens, Greece
[2] Department of Radiation Therapy, 401 General Military Hospital, Athens, Greece
[3] Radiation Therapy Clinic–A, "Metaxa" Cancer Hospital, Piraeus, Greece

Received 16 September 2020; received revised 18 November 2020; accepted 25 November 2020

Corresponding Authors:
Dimitrios T. Trafalis, Experimental and Clinical Pharmacology and Oncotherapy, Laboratory of Pharmacology, Faculty of Medicine, National and Kapodistrian University of Athens, 75 Mikras Asias, Goudi-11527, Athens, Greece.
Email: dtrafal@med.uoa.gr

Sofia Sagredou, Laboratory of Pharmacology, Faculty of Medicine, National and Kapodistrian University of Athens, 75 Mikras Asias, Goudi-11527, Athens, Greece.
Email: ssagredou@med.uoa.gr

internal radiation. With regard to the external beam-radiation, a linear accelerator produces photon beams known as X-rays whereas internal radiation or else brachytherapy, is distributed mainly by gamma-radiation sources, like radioactive isotopes, settled on patient's body. Due to the high electrical potential (4-20 MV) of the external beam radiation, patients receive the radiation dose fractioned in different schemes during the required time interval of radiotherapy. On the other hand, the internal radiation therapy provides focalized radiation with a potential ranging from 0.6 to 1 MV, restricting a potential damage in normal tissues induced by radiotherapy.[3,4]

Ionizing radiation, particularly used in radiotherapy, affects directly or indirectly living cells. More specifically, the absorbed ionizing radiation leads to either direct disruption of atomic structures or to generation of reactive chemical species that in turn cause damage to a range of cellular macromolecules including nucleic acids, proteins and lipids. Pointing to the indirect effect of ionizing radiation, ions that are produced by electrons, released from atoms and molecules, lead to subsequent formation of reactive oxygen and nitrogen species.[5] Radiolysis of water constitutes a major source for the generation of reactive oxygen species (ROS) since water is an essential cellular component occupying 80% of cell volume. More specifically, in an aerobic cellular environment, the absorption of ionizing radiation triggers excitations and ionizations which in turn result in the generation of free radicals as well as molecular products including superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($\bullet OH$) and hydrogen peroxide (H_2O_2).[6,7] Given that DNA belongs to critical molecules that may be attacked by ROS, several DNA alterations arise such as DNA breaks, base damage and destruction of sugars.[8,9] Nevertheless, the most common base lesion, induced by ROS, concerns guanine which is the most prone to oxidation. More specifically, once the guanine molecule has been oxidized, a hydroxyl group (-OH) is added to the eighth position of the purine base leading to the formation of the oxidatively modified product, 8-Hydroxy-2'-deoxyguanosine (8-OHdG). Due to the fact that 8-OHdG is one the predominant forms of the free radical-induced lesions of DNA, its quantification signifies the extent of DNA damage.[10]

Except radiolysis of water, the exposure to ionizing radiation stimulates the activity of the inducible nitric oxide synthase (iNOS), leading this way to the formation of reactive nitrogen species (RNS) such as nitric oxide ($\bullet NO$). Even though $\bullet NO$ is inactive with most of cellular constituents, it can be converted into peroxynitrite anion ($ONOO^-$) through a reaction with $O_2^{\bullet -}$.[11] Due to the high reactivity that $ONOO^-$ displays, it is capable of attacking a wide range of cellular targets including DNA bases.[12,13] Similar to ROS, $ONOO^-$ as RNS entity, interacts with guanine and as consequence it induces nitrative lesions like 8-Nitroguanine (8-NG).[14] Nevertheless, the glycosidic bond between 8-NG and deoxyribose shows significant chemical instability which is responsible for the spontaneous release of this DNA lesion and consequently for the generation of an apurinic site. The resulted apurinic site pairs with adenine during DNA synthesis, leading to G: C to T: A transversions.[15,16] Upon exposure to ionizing radiation, ROS and RNS can be continuously risen for days and months in irradiated cells and tissues, leading to a late tissue injury.[17,18] The most frequent symptoms in chronic radiation-induced reactions, are chronic ulcerations and wounds, tissue damages as a consequence to irradiation induced inflammation, fibrosis, telangiectasias and secondary cancers.[19]

Table 1. Characteristics of Breast Cancer Patients.

Patients characteristics	Values
Patients (n)	33
Stage disease (n, %)	
Stage I	17 (52%)
Stage II	15 (45%)
Stage III	1 (3%)
Menopausal (n, %)	30 (91%)
Premenopausal (n, %)	3 (9%)
Adjuvant cytotoxic therapy (n, %)	27 (82%)
Neoadjuvant cytotoxic therapy (n, %)	4 (12%)
Median Age (years) (range)	57 (48-77)
Median BSA (m^2) (range)	1.77 (1.44-2.02)
Median BSI (range)	28.6 (22.2-31.2)
Median GFR (mL/min) (range)	97.7 (44.8-159.7)
Median HCT (%) (range)	39.7 (35.2-44.9)

The present study is one of the first attempts to explore the formation and production of 8-OHdG and 8-NG and to study the potential correlations between 8-OHdG as well as 8-NG blood serum levels with the radiation dose received postoperatively by patients with breast cancer. Our results reflect the induction of ROS and NOS in normal tissues, in vivo, as response to ionizing irradiation. For the purpose of our work, a study on blood samples obtained by breast cancer patients that received postoperative complementary external irradiation was performed. Ex-vivo determination of the serum 8-OHdG and 8-NG levels was performed and their altered concentration levels were related to radiation dose and exposure time.

Materials and Methods

Radiotherapy Patients

Thirty-three female patients with breast adenocarcinoma (stages I to III) underwent postoperative adjuvant radiotherapy. The age of the recruited patients ranged from 48 to 77 years, with a median age of 57 years. From the total group, 30 patients were menopausal whereas 3 only patients were premenopausal. Additionally, 82% and 12% of patients have received adjuvant and neoadjuvant cytotoxic chemotherapy, respectively. For the purpose of our study, clinical parameters such as body surface area (BSA), body mass index (BMI), glomerular filtration rate (GFR) and hematocrit (HCT) were recorded (**Table 1**). All patients were treated and the blood samples were collected in the Department of Radiation Therapy, 401 General Military Hospital, Athens, Greece.

The study was performed in accordance with the Europe Convention on human rights and biomedicine (law 2919/

1998) and approved by the Hospital Scientific Board, and the Board of Bioethics of NKUA.

Treatment

All patients were firstly subjected to computed tomography scan (CT) and thereby a 3-dimensional conformal radiation therapy was designed. The radiotherapy regimen was performed during the postoperative stage and involved the irradiation of the entire breast including the axillary area as well as the submandibular and subclavian lymph nodes, using opposite tangent fields of 6-MV photons. The total irradiation dose ranged from 46 to 50 Gy while it was increased by 10 Gy in the area of initial disease (tumor bed). The referred radiation doses were delivered at daily doses of 2Gy/5 days for a week.

Sample Collection and Preparation

Blood samples were collected before the initiation of radiation therapy on day 0 (D0), and after the initiation of radiotherapy, during the day 14 (2 weeks; D14), day 28 (4 weeks; D28) and day 56 (8 weeks; D56{2 weeks after the end of radiotherapy}). In order to obtain the samples, disposable and sterile needles were used and blood samples were transferred into vacuum tubes without anticoagulant. The collected samples were centrifuged at 3000 g for 10 minutes straightforward (10-15 min) after blood collection and precipitates were removed. Finally, the supernatant serum was transferred to 1.5 mL labeled centrifuge tubes and stored at −80°C until analysis.

ELISA Measurement of Plasma 8-OHdG

Blood serum 8-OHdG was quantified using the OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation), Trial Size (CELL BIOLABS, Inc., San Diego, USA), a modified competitive ELISA with detection sensitivity range of 100 pg/mL to 20 ng/mL. According to manufacturer's recommendations, the unknown 8-OHdG samples as well as 8-OHdG standards were loaded onto an 8-OHdG-BSA conjugate preabsorbed 96-well plate. Briefly, to coat the 96-well plate with 8-OHdG/BSA conjugate, 1mg/mL of OHdG conjugate was diluted to 1 μg/mL in 1x PBS solution. Then, 100 μL of the 1 μg/mL of 8-OHdG Conjugate were added to each well and the plate was incubated overnight at 4°C. After the incubation time, the 8-OHdG coating solution was discarded and the plate was washed once with distilled H_2O. Hence, 200 μL of Assay Diluent were added to each well and the 8-OHdG Conjugate was blocked for 1 h at room temperature. The plate was transferred to 4°C and the Assay Diluent was removed immediately before use. In order to prepare the 8-OHdG standard curve, serial dilutions of 8-OHdG Standard were carried out covering a concentration range of 0 ng/mL to 20 ng/mL. Afterwards, 50 μL of the tested sample or 8-OHdG standard were added to the corresponding wells of the 8-OHdG Conjugate coated plate and samples were incubated at room temperature for 10 minutes. Then, 50 μL of the diluted anti-8-OHdG antibody were added and samples were incubated at room temperature for 1 h. After the incubation time, all wells were washed 3 times with 250 μL of 1x Wash Buffer and thereafter 100 μL/well of the diluted Secondary Antibody-Enzyme Conjugate were added. All samples were incubated at room temperature for 1 hour and subsequently all wells were washed 3 times as previously described. The addition of 100 μL of Substrate Solution was followed by incubation at room temperature from 2 to 30 min. The enzymatic reaction was terminated by the addition of 100 μL of Stop Solution. The absorbance was immediately measured on an ELISA reader at 450 nm (Versamax, Orleans, USA). The experiment was carried out in triplicates.

ELISA Measurement of Plasma 8-NG

Blood serum 8-NG was quantitated using the OxiSelect™ Nitrosative DNA/RNA Damage ELISA kit (CELL BIOLABS, Inc., San Diego, USA), a competitive ELISA with detection sensitivity of 1 ng/mL. Similar to the 8-OHdG assay, the unknown 8-NG samples as well as 8-NG standards were settled on an 8-NG-BSA conjugate preabsorbed microplate. According to manufacturer's instructions, the 96-well plate had to be coated with 8-NG conjugate which was diluted 1:400 in 1x PBS dilution. Then, 100 μL of the 8-Nitroguanine Conjugate solution were loaded to each well and the plate was incubated overnight at 4°C. The 8-NG coating solution was removed and all wells were washed once with 1x PBS solution. After washing, 200 μL of Assay Diluent were added to each well and the 8-NG conjugate was blocked for 1-2 h at room temperature. The plate was transferred at 4°C and the Assay Diluent was removed immediately before use. A standard curve for 8-NG was established with a concentration range from 0 to 1000 ng/mL. Thereafter, 50 μL of unknown sample or 8-NG standard were added to the 8-NG Conjugate coated plate and samples were incubated for 10 min. After the incubation time, 50 μL of the diluted anti-8-NG antibody were added per well and then samples were incubated for 1 h. The incubation time was followed by 3 washing steps with 250 μL of 1X Wash Buffer to each well. Subsequently, 100 μL of the diluted Secondary Antibody HRP Conjugate were added and samples were incubated for 1 h. The 3 washing steps were repeated as previously described and 100 μL of Substrate Solution were added per well. At this step, the incubation time ranged from 2-30 min. The enzymatic reaction was terminated by adding 100 μL of Stop Solution into each well and the absorbance was measured immediately on an ELISA reader at 450 nm (Versamax, Orleans, USA). The experiment was carried out in triplicates.

Statistical Analysis

Blood serum concentrations of 8-OHdG and 8-NG were associated with dosimetric parameters of the radiotherapy regimen, according to lognormal distribution and polynomial regression, respectively. Furthermore, Pearson correlation coefficient (r) was calculated to evaluate the association between plasma 8-OHdG levels and relevant parameters including age, BSA,

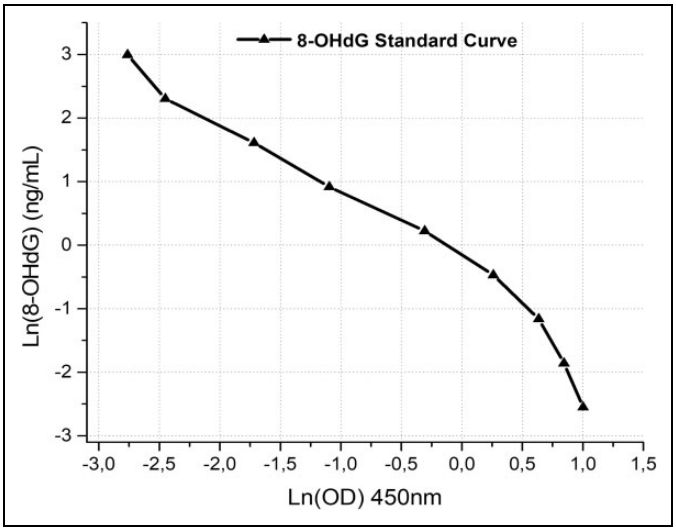

Figure 1. 8-OHdG standard curve fitted by logarithmic model. The standard curve was made by plotting the 8-OHdG concentration (ng/mL) against OD (450 nm) values.

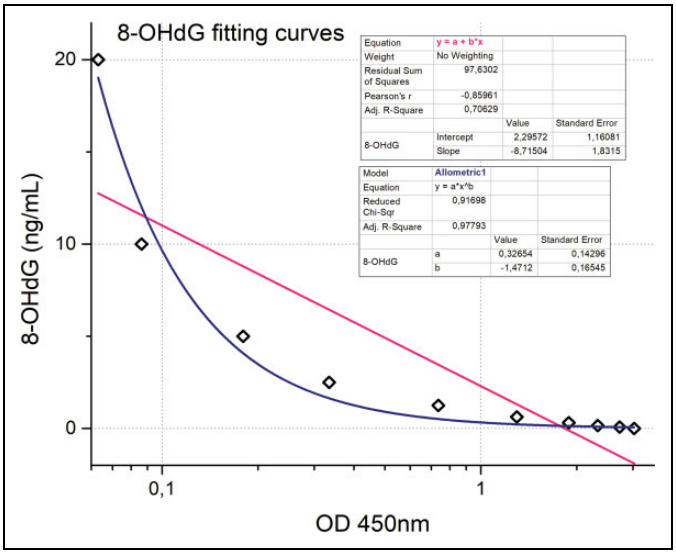

Figure 2. 8-OHdG fitting curves were determined according to the linear and allometric mathematic models. The blue line corresponds to the allometric and the red line to the linear.

BMI, GFR and HCT. Similar to 8-OHdG, Pearson correlation coefficient (r) was also determined so as to estimate the potential association of plasma 8-NG with the referred clinical parameters. Statistical significance was assumed at $p < 0.05$. Microsoft Excel (Microsoft Hellas, Athens, Greece).

Results

Plasma 8-OHdG Levels in Patients Receiving Radiotherapy

The 8-OHdG concentration (ng/mL) in the plasma samples of patients who received radiotherapy was quantitated according to the 8-OHdG standard curve. As **Figure 1** demonstrates, the 8-OHdG standard curve was made by applying the logarithmic model where the 8-OHdG concentration (ng/mL) of the 9 standard samples were plotted against the corresponding absorbance values (OD). Furthermore, according to **Figure 2** the two 8-OHdG fitting curves were performed using the equations of the linear and allometric models. Regarding the linear fitting curve, the utilized equation was y = a + b*x, Pearson's r = −0.85961 ($p < 0.01$) and adjusted R-square = 0.70629. The relevant equation for obtaining the allometric fitting curve was y = a*x^b with adjusted R-square = 0.97773 ($p < 0.001$).

In order to estimate potential alterations in plasma 8-OHdG concentration (ng/mL) during the radiation regimen, data were fitted according to Lognormal model (95% prediction band and 95% confidence band). According to the **Figure 3**, a statistically significant increase of 8-OHdG concentration (ng/mL) was induced during the 2 first weeks (Day5-Day15; 10-20 Gy), followed by a decrease for the next 13 days (Day 15-Day30; 20-45 Gy) and then its concentration levels remained steady for the last 32 days (Day28-Day60; 45-60 Gy) ($p < 0.001$).

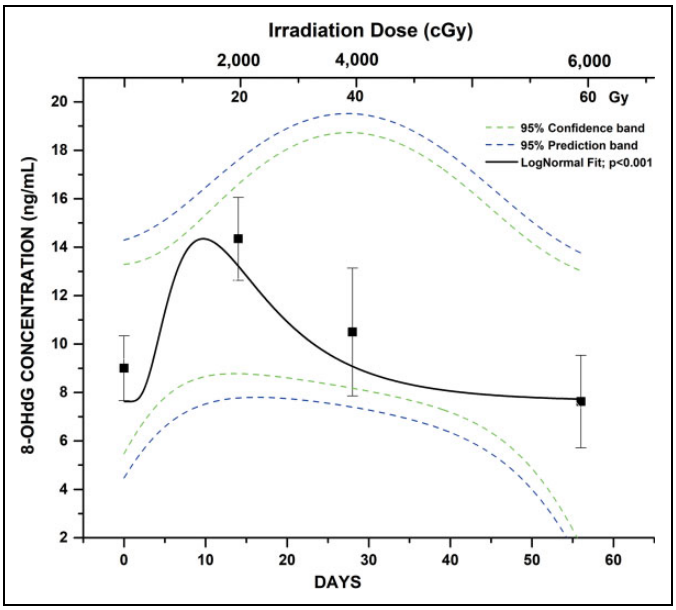

Figure 3. Alterations in plasma 8-OHdG levels associated with the radiation dose and time period of radiotherapy. The radiation dose-response curve was made according to Lognormal distribution (95% prediction band and 95% confidence band; $p < 0.001$), by plotting 8-OHdG concentration (ng/mL) against the range of radiation dose which was received by breast cancer patients (0, 20, 40 and 60 Gy) during 60 days of radiation therapy.

As it is shown in **Figure 4**, no statistically significant correlations ($p > 0.05$) were found between the Pearson's correlation coefficient indexes (r) of patients' clinical characteristics and parameters: Body Mass Index (BMI), Body Surface Area (BSA), Glomerular Filtration Rate (GFR), Hematocrit (%) (HCT) and respective alterations in blood serum 8-OHdG levels in between the time periods of radiotherapy. The stronger

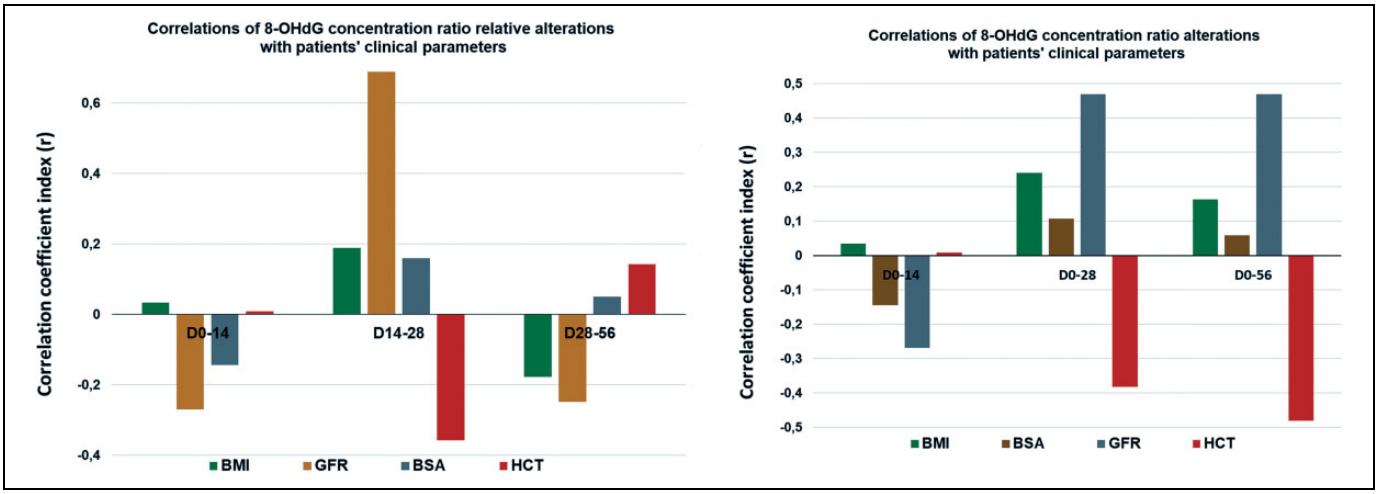

Figure 4. Pearson's correlation coefficient indexes (r) between the patients' clinical characteristics and parameters (BMI, BSA, GFR, HCT) and respective alterations in blood serum 8-OHdG levels in between the time periods of radiotherapy. No statistically significant correlations ($p > 0.05$) were found.

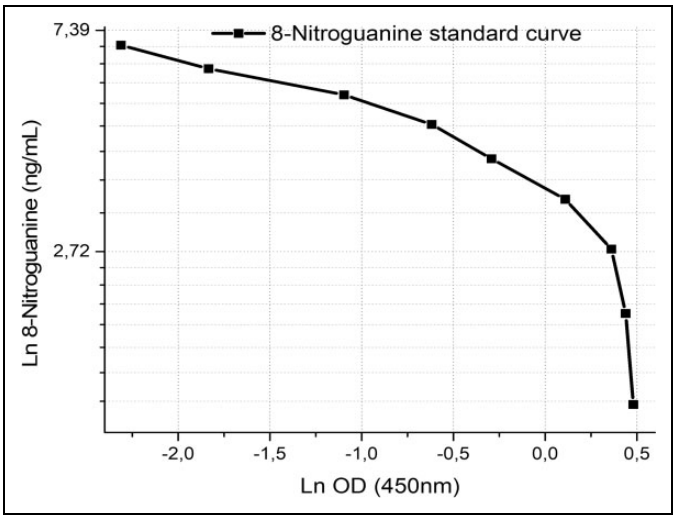

Figure 5. 8-NG standard curve fitted by logarithmic model. The standard curve was obtained by plotting the 8-NG concentration (ng/mL) against OD (450 nm) values.

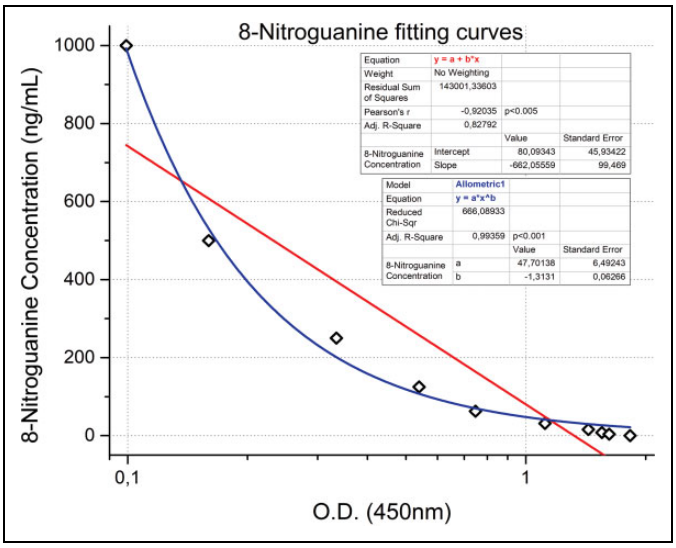

Figure 6. 8-NG fitting curves were determined according to the linear and allometric mathematic models. The blue line corresponds to the allometric and the red line to the linear.

correlations were found between alterations in serum 8-OHdG levels and GFR among D14-D28 of radiotherapy (r = 0.69), and between alterations in serum 8-OHdG levels and HCT among D0-D56 of radiotherapy (r = −0.48).

Plasma 8-NG Levels in Patients Undergoing Radiotherapy

In order to quantify the 8-NG concentration (ng/mL) in the blood serum samples of patients who underwent radiation therapy, the standard curve of 8-NG was firstly required. As **Figure 5** shows, the logarithmic model was followed, by plotting the 8-NG concentration (ng/mL) of the 9 standard samples against the corresponding absorbance values (OD). Furthermore, 2 fitting curves of plasma 8-NG concentration (ng/mL) resulted following the 8-NG standard curve. According to **Figure 6**, the two 8-NG fitting curves were developed using the equations of the linear and allometric models. Regarding the linear fitting curve, the utilized equation was y = a + b*x with residual sum of squares = 143001.336, Pearson's r = −0.92 ($p < 0.005$) and adjusted R-square = 0.828. The relevant equation for obtaining the allometric fitting curve was y = a*x^b with residual sum of squares = 666,089 and adjusted R-square = 0.994 ($p < 0.001$). Similar to 8-OHdG, plasma 8-NG concentration (ng/mL) was also altered in relation to radiation dose and time treatment. More specifically, as the polynomial fitting curve (95% prediction band and 95% confidence band) suggests, the levels of 8-NG were significantly increased within the 4 first weeks (Day0-Day28; 0-30 Gy) whereas no further changes in 8-NG concentration (ng/mL) were

recorded in the next 4 weeks (Day28-Day56; 30-60 Gy), resulting in a plateau (**Figure 7**) ($p < 0.001$).

As it is shown in **Figure 8**, no statistically significant correlations ($p > 0.05$) were found between the Pearson's correlation coefficient indexes (r) of patients' clinical characteristics and parameters (BMI, BSA, GFR, HCT) and respective alterations in blood serum 8-NG levels in between the time periods of radiotherapy. The strongest correlations were found between alterations in serum 8-NG levels and GFR among days 0-14 of radiotherapy ($r = -0.48$), and between alterations in serum 8-NG levels and HCT among days 14-28 of radiotherapy ($r = -0.69$). Altogether, the alterations in plasma 8-OHdG and 8-NG concentration (ng/mL) during radiotherapy were evaluated by following the Lognormal and polynomial regression model, respectively. Different best fit models (curves) were applied in each case due to the different dose-response alterations of 8-OHdG and 8-NG serum levels. The dose-response curves of 8-OHdG and 8-NG demonstrate the way of alteration of 8-OHdG and 8-NG levels in relation to radiation dose and treatment time.

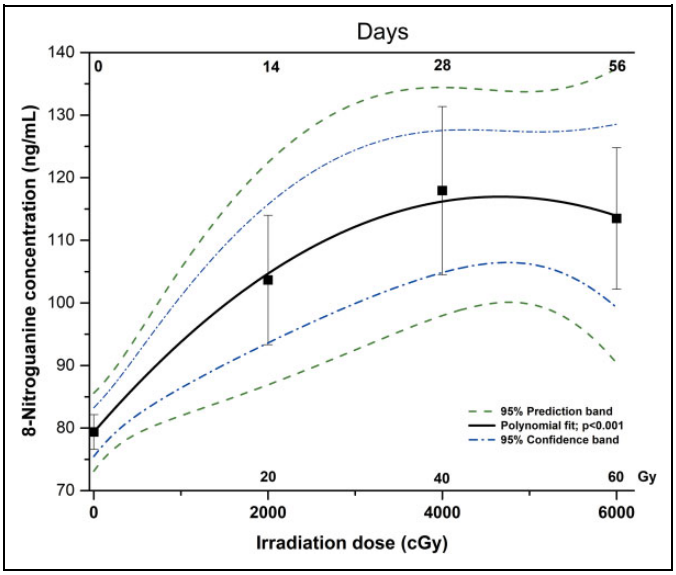

Figure 7. Alterations in plasma 8-NG levels correlated with the radiation dose and time period of radiotherapy. The radiation dose-response curve was made according to polynomial regression (95% prediction band and 95% confidence band; $p < 0.001$) by plotting 8-NG concentration (ng/mL) against the range of radiation dose which was delivered to breast cancer patients (0, 20, 40 and 60 Gy) within 60 days of radiation therapy.

Discussion

Blood serum samples, from irradiated cancer patients, were used in order to verify the presence of the 2 biomarkers of oxidative DNA damage, 8-OHdG and 8-NG, and explore potential correlations of their altered concentrations (ng/mL) with the administered radiation doses (Gy). More precisely, we have studied and showed the increase of 8-OHdG and 8-NG serum concentrations due to irradiation of normal tissues as a consequence of adjuvant regional radiotherapy of operated patients for breast cancer. As far as it concerns the increase of 8-OHdG and 8-NG concentrations in blood of tumoured irradiated patients there are very few clinical studies. Erhola et al.[20] recorded elevated 8-OHdG levels in the urine of lung cancer patients upon receiving radiotherapy and chemotherapy. According to their findings, the maximal urinary excretion of 8-OHdG/creatinine was recorded after total cumulative doses of 10 and 30 Gy.[20] Bergman et al.[21] also observed a rapid increase of 8-OHdG levels in the urine of irradiated patients with hematological malignancies. To our knowledge, there are no clinical studies concerning the detection of increased 8-NG levels in irradiated patients who bear cancer.

With reference to the dose-dependent relationship of 8-OHdG and irradiation, a growing body of evidence supports a positive correlation of the increased 8-OHdG levels with radiation exposure. The relationship between 8-OHdG and radiation dose has been investigated in different groups of occupational radiation workers as well as cancer patients who undergo radiation treatment. There are several examples

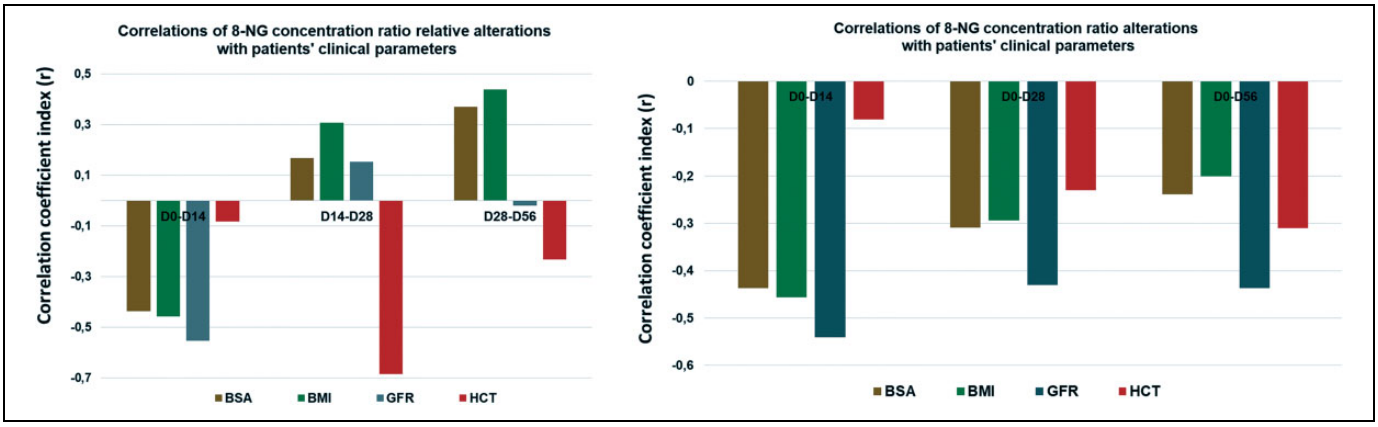

Figure 8. Pearson's correlation coefficient indexes (r) between the patients' clinical characteristics and parameters (BMI, BSA, GFR, HCT) and respective alterations in serum 8-NG levels in between the time periods of radiotherapy. No statistically significant correlations ($p > 0.05$) were found.

suggesting that 8-OHdG levels are positively correlated with radiation including pilots exposed to cosmic radiation and radiographers, whose 8-OHdG levels were higher than those of unexposed individuals. By contrast, there is another point of view regarding prolonged irradiation which primarily concerns cancer patients receiving radiotherapy; 8-OHdG levels not only can be decreased but also can be found lower than those of healthy subjects due to DNA repair mechanisms.[22,23]

Gao and his colleagues[23] demonstrated that serum 8-OHdG levels were significantly elevated in interventional radiologists compared with other categories of radiation workers, highlighting that the extend of oxidative DNA damage is proportional to the radiation dose. In other words, increasing radiation doses result in a more severe oxidative DNA damage, thereby indicating a distinct dose-response relationship. As our findings indicate, blood serum 8-OHdG levels were significantly increased within the first 2 weeks of radiotherapy with radiation dose being ranged from 10 to 30 Gy. During the time interval where patients received radiation dose from 30 to 40 Gy, blood serum 8-OHdG levels were decreased and then maintained constant until the end of the radiation regimen where radiation dose ranged from 45 to 60 Gy (**Figure 3**). It is noteworthy that later studies suggest the existence of a threshold as irradiation over 3 Gy leads to a high percentage of cell death, decreasing this way 8-OHdG levels. With respect to cancer patients who underwent radiotherapy, it has been shown that serum 8-OHdG levels were lower than those who did not received any treatment. Further studies implied the reduction of urinary 8-OHdG levels upon radiotherapy as well as absence of a linear correlation of 8-OHdG with the accumulated radiation dose.[23] There are different ways for explaining the declined 8-OHdG levels upon radiation exposure. First of all, DNA repair systems negate DNA damage induced by radiation and consequently such lesions cannot be accumulated. Secondly, due to the fact that DNA repair is a time-consuming procedure, repair may be carried out months after radiation exposure. Thirdly, since 8-OHdG constitutes a biomarker of carcinogenesis, cancer patients express higher levels of 8-OHdG than healthy people. Nonetheless, 8-OHdG levels can be decreased due to radiation treatment which is received by most of cancer patients.[23] Except radiation-induced oxidative stress, immune response causes the generation of ROS and RNS as well, reflecting that irradiation effects conform with those of inflammatory reactions and thereby generating a number of different ROS and RNS.[24] Nevertheless, RNS are mostly produced under inflammatory conditions in inflammatory and epithelial cells; activation of iNOS leads to an abundant formation of NO which in turn is converted into various RNS, including $ONOO^-$.[25,13,14] However, inflammation and the concomitant immune response, impelled by ionizing irradiation, depend on several factors including the radiation type, dose, intensity/fractionation and total cumulative dose.[26] Recent studies have demonstrated an association between NO pathway and immune response induced by irradiation while both of them are regulated in a radiation-dose-dependent manner.[27-30] More precisely, high-doses of radiation, predominantly used in cancer treatment, trigger an immune response comprising secretion of cytokines and chemokines which interact with endothelial cells and other cell types of the immune system.[31] In vivo studies have shown that high-dose irradiation stimulates the activation of macrophages as well as iNOS production which is followed by increased levels of NO and O_2^- while a series of pro-inflammatory cytokines are also excreted including IL-1β, IL-6 and TNF-α.[32-34] Further in vivo studies also indicated a dose-response relationship between NO and radiation as the expression of iNOS and subsequent production of NO were significantly increased in mice which were irradiated up to 50 Gy.[28] Meanwhile, low-dose irradiation (single dose $\leq$1.0 Gy) induce the anti-inflammatory effects of macrophages and inhibits NO pathway.[35] According to ex vivo studies, administration of low-dose (0.5 Gy) Xray decreased the excretion of pro-inflammatory cytokine IL-1β in contrast to the enhanced production of anti-inflammatory cytokine TGF-β.[36] It is noteworthy that IL-1β, TNF-α, TGF-β1 και IL-6 pro-inflammatory cytokines are crucially involved in dermatitis and increased levels of these cytokines constitute markers of dermal toxicity in patients receiving radiotherapy. Furthermore, TGI-β1 probably plays a major role as its activation triggers a sequence of cellular events that lead to fibrosis induced by exposure to radiation.[37] Taking into consideration that NO pathway is modulated in a radiation-dose-dependent manner and 8-NG is generated by $ONOO^-$, a dose-response relationship is likely to exist between 8-NG levels and radiation. So far, there are hardly any findings concerning the detection of 8-NG in biologic fluids of cancer patients who received radiation treatment. According to our results, the nitrative DNA lesion, 8-NG, was detected in the blood serum of cancer patients who were irradiated for 60 days. Moreover, blood serum 8-NG concentration (ng/mL) was altered in accordance with radiation doses and treatment time, similarly to 8-OHdG. More specifically, blood serum 8-NG levels were remarkably increased within the first 3 weeks where patients were irradiated up to 30 Gy while its concentration (ng/mL) remained stable until the end of radiotherapy (Figure 7). Consequently, the alterations in 8-NG levels were positively correlated with radiation doses (0-30 Gy) for the first 3 weeks, indicating a dose-response relationship as well as potential involvement of DNA repair mechanisms preventing any further increases, similar to 8-OHdG. In addition to the above, we can put forward the hypothesis that the production of 8-NG depends on radiation dose since NO pathway, though which is formed, is modulated in a radiation-dose-dependent-manner.

It is worthy to mention that alterations of 8-NG plasma concentration rather represent the DNA damage induced due to the irradiation mediated inflammatory oxidative processes, while the alterations of 8-OHdG plasma concentration mostly represent the DNA damage induced due to the direct or indirect oxidative effects of irradiation. Therefore, they concern to distinct oxidative processes. As a consequence, the dose-dependent curves of the 2 oxidative DNA damage markers do not represent quantitative differences but qualitative.

It is notable that among all the clinical parameters included in this study, HCT and GFR showed a strong trend of correlation with the respective alterations of 8-OHdG and 8-NG levels in serum (Figures 4 and 8). The negative correlation between the respective alterations in either 8-OHdG or 8-NG levels and HCT indicates a decreasing tendency of HCT due to radiation toxicity. Prior studies have shown that exposure to ionizing radiation leads to a decreased HCT due to high radiation-sensitivity of bone marrow and hematopoietic cells, rendering ionizing radiation capable of destroying hematopoietic stem cells as well as mature blood cells.[38] Furthermore, it is worthy to mention the strong trend of correlation between the respective alterations in 8-NG levels and GFR corresponding to the kinetics of 8-NG levels as illustrated by the corresponding dose-response curve. Moreover, a strong trend of correlation between GFR and the kinetics of 8-OHdG levels was also displayed, in conformation with the relevant dose-response curve. BMI and BSA were also correlated with the altered levels of 8-OHdG and 8-NG but without any statistical significance.

Due to lack of clinical studies related to oxidative DNA damage markers and radiotherapy, it is not increasingly clear the relationship of 8-OHdG and 8-NG levels with therapeutic efficacy and/or radiation-induced side effects. So far, there are some studies supporting a potential correlation between urinary DNA damage biomarkers and response to treatment, although these studies have been of too short duration to prove this point. Crohns et al.[39] conducted a study in which lung cancer patients were followed up for 6 years in order to evaluate the association between adverse events and responses to radiotherapy with 8-OHdG. According to their findings, no significant association was observed between 8-OHdG and adverse events during radiotherapy, response to radiation treatment or overall survival.[39] With regard to 8-NG, there are no yet published clinical data that demonstrate relation of the increased 8-NG levels with either therapeutic efficacy or radiation-induced side effects.

The increase of 8-OHdG levels in urine has been studied stringently in cancer patients as far as concerns its prognostic significance but almost at all as predictive biomarker. Recent findings demonstrate a correlation of 8-OHdG levels with the development of carcinogenesis and prognosis of cancer patients. For instance, increased urinary 8-OHdG levels have been found in patients with colorectal cancer and patients with tumor metastasis in contrast to healthy individuals and patients without tumor metastasis. Moreover, low 8-OHdG levels are associated with poor prognosis in breast cancer patients whereas increased 8-OHdG expression is related to poorer overall survival and progression-free survival in patients with serous ovarian carcinoma. Therefore, 8-OHdG may serve as a useful biomarker of carcinogenesis providing the possibility of early warning, detection and risk estimation.[40-44] Nevertheless, there are studies supporting 8-OHdG as predictor of developing drug resistance in cancer patients and thus assisting in the choice of chemotherapy. As observed in patients with epithelial ovarian carcinoma, the elevated serum 8-OHdG levels reflected the early development in platinum resistance. Therefore, the utility of 8-OHdG in predicting chemoresistance may have a beneficial impact on the decision of the primary treatment mode.[45]

Conclusion

A very few studies support a clear dose-response relationship between either 8-OHdG or 8-NG levels and radiation therapy conditions. Our study primarily indicated the production of 8-OHdG as well as 8-NG upon radiation treatment of normal tissues and subsequently a significant positive correlation of their concentration levels with radiation dose and exposure time. However, based on biochemical and molecular mechanisms, it is possible that the dose-dependent relationship of either 8-OHdG or 8-NG with radiation may be related to the therapeutic efficacy and side-effects of radiotherapy. Further investigations need to be carried out in that field.

Acknowledgments

The authors are grateful to ATHENA Institute of Biomedical Sciences and ENERGONBIO TECHNOLOGIES S.A., Greece, for supporting this work and publication.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Sofia Sagredou https://orcid.org/0000-0001-9281-7947

References

1. Bese N, Kiel K, El-Gueddari B, Campbell O, Awuah B, Vikram B. Radiotherapy for breast cancer in countries with limited resources: program implementation and evidence-based recommendations. *Breast J*. 2006;12(suppl1):S96-S102.
2. Masuda Y, Kamiya K. Molecular nature of radiation injury and DNA repair disorders associated with radiosensitivity. *Int J Lab Hematol*. 2012;95(3):239-245.
3. Borrego-Soto G, Ortiz-López R, Rojas-Martínez A. Ionizing radiation-induced DNA injury and damage detection in patients with breast cancer. *Genetic Mol Biol*. 2015;38(4):420-432.
4. Patel RR, Arthur DW. The emergence of advanced brachytherapy techniques for common malignancies. *Hematol Oncol Clin North Am*. 2006;20(1):97-118.
5. Hall EJ, Giaccia AJ. *Radiobiology for the Radiologist*. 6th ed. Lippincott Williams & Wilkins; 2006.
6. Azzam E, Jay-Gerin J, Pain D. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. *Cancer Lett*. 2012;327(1-2):48-60.
7. Platzman R. The physical and chemical basis of mechanisms in radiation biology. In: Claus W, (ed). *Radiation Biology and*

Medicine: Selected Reviews in the Life Sciences. Reading: Addison-Wesley; 1958:15-72.
8. O'Neill P, Wardman P. Radiation chemistry comes before radiation biology. *Int J Radiat Biol*. 2009;85(1):9-25.
9. Valerie K, Yacoub A, Hagan MP, Curiel DT, Fisher PB, Grant S, Dent P. Radiation-induced cell signaling: inside-out and outside-in. *Mol Cancer Ther*. 2007;6(3):789-801.
10. Kasai H. Analysis of a form of oxidative DNA damage, 8-hydroxy-2V-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res*. 1997;387(3): 147-163.
11. Jay-Gerin JP, Ferradini C. Are there protective enzymatic pathways to regulate high local nitric oxide (•NO) concentrations in cells under stress conditions? *Biochimie*. 2000;82(2):161-166.
12. Frongillo Y, Goulet T, Fraser MJ, Cobut V, Patau JP, Jay-Gerin JP. Monte Carlo simulation of fast electron and proton tracks in liquid water. II. Nonhomogeneous chemistry. *Radiat Phys Chem*. 1998;51(3):245-254.
13. Inoue S, Kawanishi S. Oxidative DNA damage induced by simultaneous generation of nitric oxide and superoxide. *FEBS Lett*. 1995;371(1):86-88.
14. Yermilov V, Rubio J, Becchi M, Friesen MD, Pignatelli B, Ohshima H. Formation of 8-nitroguanine by the reaction of guanine with peroxynitrite in vitro. *Carcinogenesis*. 1995;16(9): 2045-2050.
15. Yermilov V, Rubio J, Ohshima H. Formation of 8-nitroguanine in DNA treated with peroxynitrite in vitro and its rapid removal from DNA by depurination. *FEBS Lett*. 1995;376(3):207-210.
16. Loeb LA, Preston BD. Mutagenesis by apurinic/apyrimidinic sites. *Annu Rev Genet*. 1986;20:201-230.
17. Spitz DR, Azzam EI, Li JJ, Gius D. On: a unifying concept in stress response biology. *Cancer Metastasis Rev*. 2004;23(3-4): 311-322.
18. Petkau A. Role of superoxide dismutase in modification of radiation injury. *Br J Cancer Suppl*. 1987;8:87-95.
19. Martin MT, Vulin A, Hendry JH. Human epidermal stem cells: role in adverse skin reactions and carcinogenesis from radiation. *Mutat Res*. 2016;770(Pt B):349-368.
20. Erhola M, Toyokuni S, Okada K, et al. Biomarker evidence of DNA oxidation in lung cancer patients: association of urinary 8-hydroxy-2'-deoxyguanosine excretion with radiotherapy, chemotherapy, and response to treatment. *FEBS Lett*. 1997;409(2): 287-291.
21. Bergman V, Leanderson P, Starkhammar H, Tagesson C. Urinary excretion of 8-hydroxydeoxyguanosine and malondialdehyde after high dose radiochemotherapy preceding stem cell transplantation. *Free Radic Biol Med*. 2004;36(3):300-306.
22. Salehi A, Ebrahimpour K, Forouharmajd F, Zarean M. The relationship between collective effective doses of radiation and urinary concentration of 8-Dihydroxy-2'-Deoxyguanosine among radiography staff. *Int J Radiat Res*. 2020;18(3):587-592.
23. Gao Y, Wang P, Wang Z, et al. Serum 8-Hydroxy-2′-deoxyguanosine level as a potential biomarker of oxidative DNA damage induced by ionizing radiation in human peripheral blood. *Dose-Response*. 2019;17(1):1559325818820649.
24. Bubici C, Papa S, Dean K, Franzoso G. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. *Oncogene*. 2006;25(51): 6731-6748.
25. Hu CW, Chang YJ, Chen JL, Hsu YW, Chao MR. Sensitive detection of 8-nitroguanine in DNA by chemical derivatization coupled with online solid-phase extraction LC-MS/MS. *Molecules*, 2018;23(3):605.
26. McKelvey KJ, Hudson AL, Back M, Eade T, Diakos CI. Radiation, inflammation and the immune response in cancer. *Mammalian Genome*. 2018;29(11-12):843-865.
27. Yakovlev VA. Role of nitric oxide in the radiation-induced bystander effect. *Redox Biol*. 2015;6:396-400.
28. Gorbunov NV, Pogue-Geile KL, Epperly MW, Bigbee WL, Draviam R. Activation of the nitric oxide synthase 2 pathway in the response of bone marrow stromal cells to high doses of ionizing radiation. *Radiat Res*. 2000;154(1):73-86.
29. Hildebrandt G, Seed MP, Freemantle CN, Alam CA, Colville-Nash PR, Trott KR. Effects of low dose ionizing radiation on murine chronic granulomatous tissue. *Strahlentherapie und Onkologie*. 1998;174(11):580-588.
30. Hildebrandt G, Seed MP, Freemantle CN, Alam CA, Colville-Nash PR, Trott KR. Mechanisms of the anti-inflammatory activity of low-dose radiation therapy. *Int J Radiat Biol*. 1998;74(3): 367-378.
31. Schröder S, Kriesen S, Paape D, Hildebrandt G, Manda K. Modulation of inflammatory reactions by low-dose ionizing radiation: cytokine release of murine endothelial cells is dependent on culture conditions. *J Immunol Res*. 2018; 2018(45):1-13.
32. Ibuki Y, Goto R. Ionizing radiation-induced macrophage activation: augmentation of nitric oxide production and its significance. *Cell Mol Biol (Noisy-le-grand)*. 2004;50(Online Pub): OL617-OL626.
33. Ibuki Y, Goto R. Enhancement of NO production from resident peritoneal macrophages by in vitro gamma irradiation and its relationship to reactive oxygen intermediates. *Free Radic Biol Med*. 1997;22(6):1029-1035.
34. Hosoi Y, Miyachi H, Matsumoto Y, et al. Induction of interleukin-1beta and interleukin-6 mRNA by low doses of ionizing radiation in macrophages. *Int J Cancer*. 2001;96(5): 270-276.
35. Gough MJ, Young K, Crittenden M. The impact of the myeloid response to radiation therapy. *Clin Dev Immunol*. 2013;2013: 281958.
36. Wunderlich R, Ernst A, Rodel F, et al. Low and moderate doses of ionizing radiation up to 2 Gy modulate transmigration and chemotaxis of activated macrophages, provoke an anti-inflammatory cytokine milieu, but do not impact upon viability and phagocytic function. *Clin Exp Immunol*. 2015;179(1): 50-61.
37. Chen M, Chen W, Lai C, Hung C, Liu K, Cheng Y. Predictive factors of radiation-induced skin toxicity in breast cancer patients. *BMC Cancer*. 2010;10(1):508.
38. Akeem S, Lukman O, Eltahir K, Fatai O, Abiola B, Khadijat O. Bone marrow and peripheral blood cells toxicity of a single 2.0

Gy Cobalt^{60} ionizing radiation: an animal model. *Ethiop J Health Sci*. 2019;29(2):195-202.

39. Crohns M, Saarelainen S, Erhola M, Alho H, Kellokumpu-Lehtinen P. Impact of radiotherapy and chemotherapy on biomarkers of oxidative DNA damage in lung cancer patients. *Clin Biochem*. 2009;42(10-11):1082-1090.
40. Kuo H, Chou S, Hu T, Wu F, Chen D. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and genetic polymorphisms in breast cancer patients. *Mutat Res/Genetic Toxicol Environ Mutag*. 2007; 631(1):62-68.
41. Karihtala P, Soini Y, Vaskivuo L, Bloigu R, Puistola U. DNA adduct 8-hydroxydeoxyguanosine, a novel putative marker of prognostic significance in ovarian carcinoma. *Int J Gynecol Cancer*. 2009;19(6):1047-1051.
42. Qing X, Shi D, Lv X, Wang B, Chen S, Shao Z. Prognostic significance of 8-hydroxy-2′-deoxyguanosine in solid tumors: a meta-analysis. *BMC Cancer*. 2019;19(1):997.
43. Guo C, Li X, Wang R, et al. Association between oxidative DNA damage and risk of colorectal cancer: sensitive determination of urinary 8-Hydroxy-2'-deoxyguanosine by UPLC-MS/MS analysis. *Sci Rep*. 2016;6:32581.
44. Xu X, Wang Y, Guo W, et al. The significance of the alteration of 8-OHdG in serous ovarian carcinoma. *J Ovarian Res*. 2013;6(1): 74.
45. Pylväs-Eerola M, Karihtala P, Puistola U. Preoperative serum 8-hydroxydeoxyguanosine is associated with chemoresistance and is a powerful prognostic factor in endometrioid-type epithelial ovarian cancer. *BMC Cancer*. 2015;15:493.