X-irradiation induces acute and early term inflammatory responses in atherosclerosis-prone ApoE$^{-/-}$ mice and in endothelial cells

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Abstract. Thoracic radiotherapy is an effective treatment for many types of cancer; however it is also associated with an increased risk of developing cardiovascular disease (CVD), appearing mainly ≥10 years after radiation exposure. The present study investigated acute and early term physiological and molecular changes in the cardiovascular system after ionizing radiation exposure. Female and male ApoE$^{-/-}$ mice received a single exposure of low or high dose X-ray thoracic irradiation (0.1 and 10 Gy). The level of cholesterol and triglycerides, as well as a large panel of inflammatory markers, were analyzed in serum samples obtained at 24 h and 1 month after irradiation. The secretion of inflammatory markers was further verified *in vitro* in coronary artery and microvascular endothelial cell lines after exposure to low and high dose of ionizing radiation (0.1 and 5 Gy). Local thoracic irradiation of ApoE$^{-/-}$ mice increased serum growth differentiation factor-15 (GDF-15) and C-X-C motif chemokine ligand 10 (CXCL10) levels in both female and male mice 24 h after high dose irradiation, which were also secreted from coronary artery and microvascular endothelial cells *in vitro*. Sex-specific responses were observed for triglyceride and cholesterol levels, and some of the assessed inflammatory markers as detailed below. Male ApoE$^{-/-}$ mice demonstrated elevated intercellular adhesion molecule-1 and P-selectin at 24 h, and adiponectin and plasminogen activator inhibitor-1 at 1 month after irradiation, while female ApoE$^{-/-}$ mice exhibited decreased monocyte chemoattractant protein-1 and urokinase-type plasminogen activator receptor at 24 h, and basic fibroblast growth factor 1 month after irradiation. The inflammatory responses were mainly significant following high dose irradiation, but certain markers showed significant changes after low dose exposure. The present study revealed that acute/early inflammatory responses occurred after low and high dose thoracic irradiation. However, further research is required to elucidate early asymptomatic changes in the cardiovascular system post thoracic X-irradiation and to investigate whether GDF-15 and CXCL10 could be considered as potential biomarkers for the early detection of CVD risk in thoracic radiotherapy-treated patients.

Introduction

Adjuvant radiotherapy is an effective treatment for thoracic malignancies and has resulted in a significant improvement in the chances of cancer patient's survival over the past decades (1-4). However, while high-energy radiotherapy treatment successfully kills cancer cells, unavoidable radiation exposure to the heart and large arteries occurs during treatment, resulting in radiation-induced secondary cardiovascular disease (CVD) in cancer survivors (5-16). Indeed, epidemiological, clinical and experimental data have established the link between radiation exposure at high and medium doses that are received during radiotherapy (>0.5 Gy) and the risk for CVD (6,9,10,17-20). In addition, recent data suggest that radiation-induced CVD occurs at much lower doses than previously thought (<0.5 Gy) (7,10,19,21-24). However, the underlying cellular and molecular mechanisms of radiation-induced CVD are not fully understood, possibly resulting in improper radiation protection. Most of the clinical and experimental data focus on identifying the late cardiovascular response after radiation exposure, although physiological changes and cellular and molecular damage in the cardiovascular system may happen directly after radiation exposure (7,18,25-32). Moreover, there are currently no validated biomarkers that predict the risk to develop CVD after radiotherapy. Therefore, experimental animal studies of acute and early cardiovascular response after thoracic

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irradiation may improve our knowledge on early asymptomatic changes in the cardiovascular system after ionizing exposure. This may help in identifying biomarkers indicating developing cardiovascular complications after radiotherapy, which may help in screening patients at risk for developing CVD, thus countermeasures and early medical intervention might be applied to prevent further cardiac toxicity.

Atherosclerosis, a progressive inflammatory disease of the arterial wall, is the main underlying cause of major CVD. Multiple inflammatory markers have been correlated with the pathogenesis of atherosclerosis (33-36). Radiation exposure is also known to trigger the release of several inflammatory and adhesion molecules, involved in the pathogenesis of atherosclerosis (18,26,37-42). Here, we explored a large panel of inflammatory markers in the serum of atherosclerosis-prone ApoE$^{-/-}$ mice at 24 h (acute response) and 1 month (early term response) of thoracic X-ray irradiation. The inflammatory markers were further verified *in vitro* in coronary artery and microvascular endothelial cell lines exposed to low and high dose X-rays. Serum triglyceride and cholesterol levels, which are known to be strongly associated with atherogenesis (43,44), were also evaluated at 24 h and 1 month post-irradiation. We identified two inflammatory markers, growth differentiation factor-15 (GDF-15) and C-X-C motif chemokine ligand 10 (CXCL10), which were significantly elevated 24 h after irradiation in both female and male mice, which were found to be also increased in irradiated coronary artery and microvascular endothelial cells. Interestingly, we observed gender-specific responses in triglyceride and cholesterol levels 1 month post-irradiation, and in the assessed inflammatory markers at 24 h and 1 month post-irradiation.

Materials and methods

Animals. Animal experiments were approved by the Ethical Committee Animal Studies of the Faculty of Medicine and Health Sciences, Ghent University (ECD 17/60), and were performed in compliance with the Belgian laboratory animal legislation and the European Communities Council Directive of 22 September 2010 (2010/63/EU). Apolipoprotein E-deficient ApoE$^{-/-}$) mice were purchased from Charles River Laboratories, and animals were housed at the animal facility of UGent. Pups were weaned at the age of three weeks and housed in temperature-controlled, individually ventilated cages, with a 12 h light-dark cycle. They received a standardized mouse chow diet (3.7% fat) and water *ad libitum*. At the age of 10-12 weeks, ApoE$^{-/-}$ mice were randomly allocated to receive irradiation or sham treatment. In total 72 female and male ApoE$^{-/-}$ mice were used (6 mice per group, except for the control group at 1 month, which were 3 for male and 9 for female mice).

Mouse irradiation. The Small Animal Radiation Research Platform (SARRP; Xstrahl®; in collaboration with INFINITY lab, UGent) was used to irradiate ApoE$^{-/-}$ mice. Mice were anesthetized with isoflurane (5% induction and 2% maintenance) and subjected to a full body CT-scan (50 kV, 1.5 mA, 360 projections >360°, 1 mm aluminium filter) prior to irradiation. CT images were analyzed by using Muriplan software (Xstrahl®) to determine the coordinates of the isocenter for subsequent irradiation. Thoracic irradiation was performed in a ventro-dorsal direction, with 220-kV X-rays, operating at 13 mA and filtered with 0.15 mm of copper, operating with a dose rate of 3.4 Gy/min. The field size, created by the collimator of 10x10 mm, was encompassing both carotid arteries, the aortic arch and apical portion of the heart. The doses that were delivered to the heart region are 0 Gy (sham treatment; also referred to as 'controls'), 0.1 and 10 Gy.

Cell culture. Telomerase Immortalized human Microvascular Endothelial cells (TIME) from the American Type Cell Culture (ATCC, France; ATCC® CRL-4025™) (https://www.lgcstandards-atcc.org/Products/All/CRL-4025) was used. In addition, TICAE cells, which are primary human coronary artery endothelial cells from the European Collection of Authenticated Cell Cultures (ECACC; HCAECs cat. no. 300-05a) that were transduced with retroviruses bearing the est2 gene, a yeast homologue of the human TERT protein (45,46), were used. TIME and TICAE cells are from a male human donor. MesoEndo Cell Growth Medium (Sigma-Aldrich; Merck KGaA) was used for TIME and TICAE cells. The passage number that was used in the experiments is 38 for TIME cells and 33 for TICAE cells. Cells were grown at 37˚C in a humidified incubator supplemented with 5% CO_2 and were split with a 0.05% trypsin supplemented with 0.02% ethylenediaminetetraacetic acid (EDTA) every 3-4 days. Moxi Z Mini Automated Cell Counter (Orflo Technologies) was used to count the cells. For the cytokine detection experiment, TIME and TICAE cells were seeded in 6-well plate at a density of $2.5x10^5$ per well in 6 biological replicates. Three days later, cells reached 100% confluence. The medium was changed before irradiation, and at 24 and 72 h after irradiation the supernatant was collected.

Cell irradiation. Both TIME and TICAE cells were irradiated at 100% confluence. Single X-rays doses [0 Gy (also referred to as 'control'), 0.1 and 5 Gy] were applied to the cells using a vertical X-ray beam using a Xstrahl RX generator (320 kV, Filtration: 3.8 mm Al and 1 mm Cu, tube current: 12 mA) (Camberley), at a dose rate of 0.5 Gy/min. X-irradiation was performed in accordance to ISO 4037 and under ISO 17025 accreditation of the Laboratory for Nuclear Calibrations (LNK) of the Belgian Nuclear Research Centre (SCK•CEN). Cells were moved to the irradiation facility using a mobile incubator.

Blood sampling. The blood collection from each mouse was done at two different time points: 1) One week before irradiation and 2) a specific time period after radiation exposure (24 h or 1 month). The mice were anesthetized intraperitoneally with xylazine (10 mg/kg) and ketamine (80 mg/kg) using a 30G needle, and anesthetic depth was assessed using the pedal withdrawal reflex. Retro-orbital puncture was performed to take a 100 μl blood sample with a Brand micropipette one week before irradiation, and at the specific time points after irradiation, where the eye was pulled out to collect at least 500 μl blood. Immediately after the second blood sampling, anesthetized mice were euthanized due to exsanguination via transcardial perfusion. To obtain serum, the collected blood was allowed to clot in eppendorf tubes for 1.5 h at room temperature and was subsequently centrifuged at 1,500 g for

15 min at 4˚C. The supernatant was removed and centrifuged again at the same conditions to obtain pure serum. The latter was collected in eppendorf tubes and stored at -80˚C.

Triglyceride quantification. The Triglyceride Quantification assay kit (Abcam, ab65336) was used according to the manufacturer's instructions. Briefly, a 50 μl triglyceride standard curve was prepared and 1 μl serum was added to 49 μl assay buffer and all samples were performed in duplicate. Subsequently, 2 μl of lipase, which converts the triglycerides to free fatty acids and glycerol, was added to each well and incubated for 20 min at room temperature. Then, 50 μl reaction mix consisting of 46 μl triglyceride assay buffer, 2 μl triglyceride probe and 2 μl triglyceride enzyme mix, was added to each well and incubated at room temperature for 60 min protected from light. The glycerol molecules are oxidized to generate hydrogen peroxide (H_2O_2), which reacts with the probe to generate fluorescence that was measured at Ex/Em 535/587 nm with a microplate reader (Victor3, 1420 multilabel counter, PerkinElmer).

Cholesterol quantification. The Cholesterol Assay kit (Abcam, ab65390) was used according to the manufacturer's instructions. A 50 μl cholesterol standard curve was prepared. The serum samples were diluted 1/400 in assay buffer and 50 μl of this diluted serum was used. Then, 50 μl of a cholesterol reaction mix, consisting of 44 μl assay buffer, 2 μl cholesterol probe, 2 μl enzyme mix, and 2 μl cholesterol esterase was added to each well for an incubation period of 60 min at 37˚C. The cholesterol esterase hydrolyzes cholesteryl esters to free cholesterol. Subsequently, cholesterol oxidase specifically recognizes free cholesterol and produce H_2O_2 that reacts with the probe to generate fluorescence (Ex/Em=538/587 nm) that was measured with a microplate reader (Victor3, 1420 multilabel counter, PerkinElmer).

Inflammatory cytokine detection. Simultaneous detection of multiple inflammatory markers in mice serum samples (Adiponectin, CRP, CXCL10, Endoglin, FGF-basic, GDF-15, ICAM-1, IL-6, IL-1β, MCP-1, P-selectin, PAI-1, PCSK9, and uPAR) or in cell supernatant samples (GDF-15, CXCL10, ICAM-1, MCP-1, uPAR, PAI-1, P-selectin, FGF-basic and IL-6) was performed using multiplex bead assay (Luminex® MAGPIX Assay, R&D Systems) following manufacturer's instructions. Luminex® MAGPIX technology is based on the use of a mixture of magnetic microspheres that is added to the sample in a pre-coated plate with analyte-specific capture antibodies, followed by adding specific biotinylated detection antibodies and phycoerythrin that binds to the biotinylated antibodies. Magnetic beads are captured and held in a monolayer by a magnet, while the beads are illuminated by two spectrally distinct light-emitting diodes (LEDs), one to identify the analyte and the other to determine amount of analyte based on the magnitude of the phycoerythrin-derived signal. Signals are captured and imaged with a charge-coupled device (CDD) camera (47-50). Briefly, standards, serum or supernatant samples, and magnetic microparticles cocktail were incubated into cytokine-specific antibodies in a pre-coated 96-well plate. After 2-h incubation in the dark, the plate was washed, and a biotinylated detection antibody cocktail was added. After applying a second wash to remove the unbound biotinylated antibodies, a streptavidin-phycoerythrin conjugate was added to each well to bind to the biotinylated antibodies. Further, a final wash was performed and the microparticles were detected using the Luminex® MAGPIX Analyzer. Inflammatory markers concentration in the supernatant of TIME and TICAE cells was normalized to cell count performed using IncuCyte ZOOM™ phase contrast imaging with x10 magnification.

Statistical analysis. Analysis of cholesterol, triglyceride and supernatant inflammatory cytokine data were performed using Kruskal-Wallis test followed by Dunn's test, and the P-value was adjusted using Benjamini-Hochberg procedure to control the False Discovery Rate. Data are presented as mean ± standard error of the mean. GraphPad Prism 5.01 (GraphPad Software Inc.) was used for these statistical analysis. For *in vivo* serum inflammatory cytokines, the fold change in inflammatory marker levels before and after irradiation was first calculated, followed by statistical analysis. Statistical analysis was done with a Kruskal-Wallis test followed by Dunn's test and the P-value was adjusted using Benjamini-Hochberg procedure, using *ggsignif* R package. The results are considered significant if the P-value is <0.05. Data are presented as boxplot, *ggplot2* R package, showing the median with the lower and upper hinges that correspond to the first and third quartiles (the 25 and 75th percentiles) and upper/lower whisker extends from the hinges to the largest/smallest value no further than 1.5 * IQR from the corresponding upper and lower hinge, respectively. Data beyond the end of the whiskers are called 'outlying' points and are plotted individually. *In vivo* data are represented separately in female ApoE$^{-/-}$ mice and male ApoE$^{-/-}$ mice, or as the sum of female and male data, which are referred to as combined data.

Results

Thoracic irradiation induces early term alterations in triglyceride and cholesterol levels in ApoE$^{-/-}$ mice

At 24 h post-thoracic irradiation. Thoracic irradiation of female ApoE$^{-/-}$ mice with 10 Gy induced a significant decrease in triglyceride and cholesterol levels at 24 h after irradiation, compared to age-matched controls (Fig. 1A and B), (summarized in Table SI). In male ApoE$^{-/-}$ mice, thoracic irradiation induced a significant increase in triglyceride level only at 0.1 Gy, but no significant changes in cholesterol level was observed at 24 h after irradiation (Fig. 1A and B). Female and male ApoE$^{-/-}$ mice combined data showed no significant changes in triglyceride and cholesterol levels at 24 h after irradiation (Fig. 1A and B).

At 1 month post thoracic irradiation. At 1 month after irradiation, a significant increase in triglyceride levels in female ApoE$^{-/-}$ mice was observed at 10 Gy, whereas total cholesterol levels were significantly decreased at 0.1 and 10 Gy, compared to age-matched controls (Fig. 1C and D). In male ApoE$^{-/-}$ mice, a significant decrease in triglyceride level was observed in a dose-dependent manner at 1 month after irradiation, but no significant changes in cholesterol level was observed (Fig. 1C and D). Female and male ApoE$^{-/-}$ mice combined data

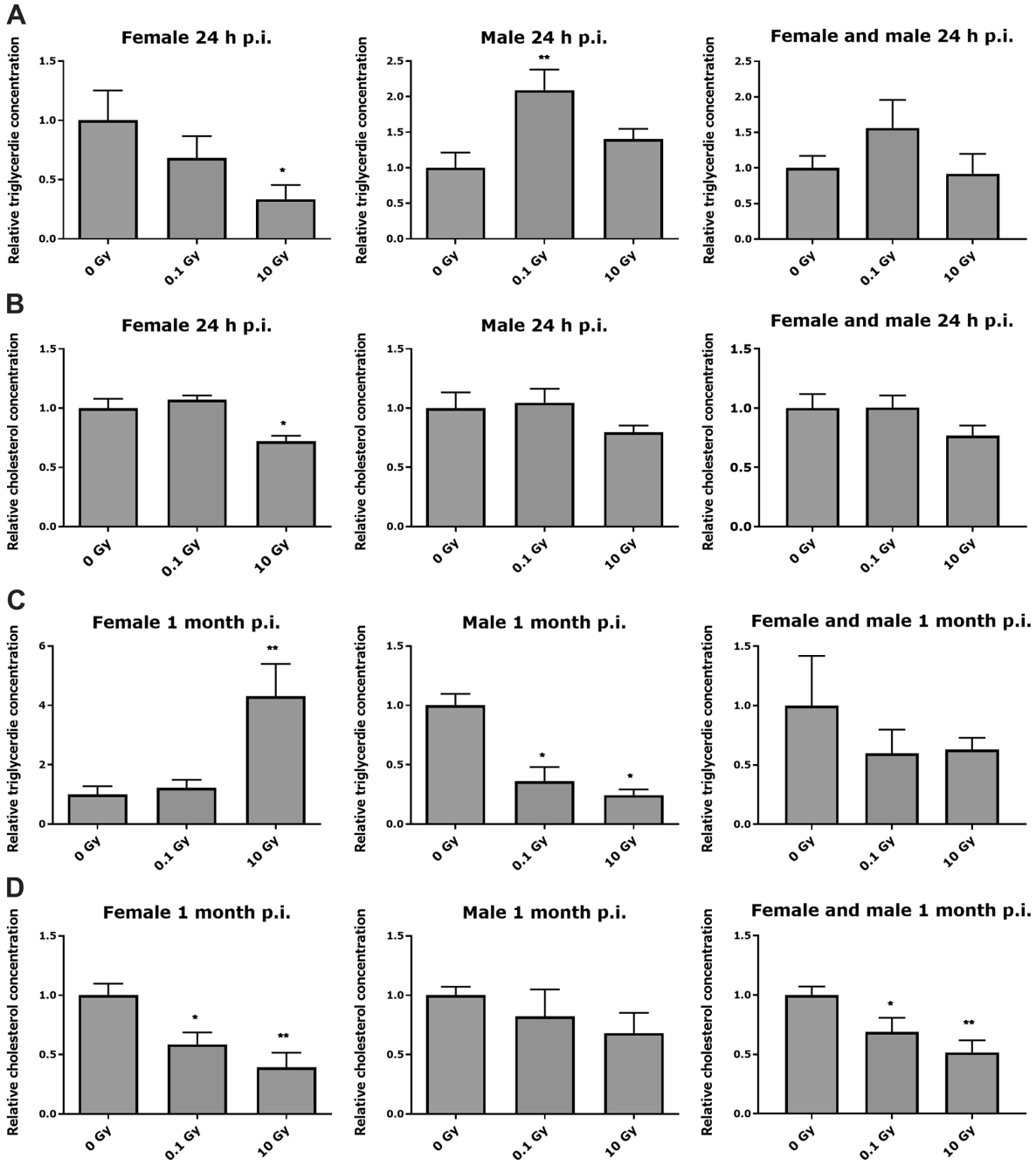

Figure 1. Triglyceride and total cholesterol concentration in serum of female (left panels), male (middle panels) and combined female and male ApoE$^{-/-}$ mice (right panels). (A) Triglyceride level at 24 h, (B) total cholesterol at 24 h, (C) triglyceride level at 1 month and (D) total cholesterol at 1 month after irradiation. Values represent average ± SEM of 6 mice per group, except for the 1 month time point in the 0 Gy male group, which had 3 mice. *P<0.05 and **P<0.01 vs. age-matched 0 Gy controls. p.i., post irradiation.

showed no significant changes in triglyceride levels, while a significant dose-dependent decrease in cholesterol level at 1 month after irradiation was observed (Fig. 1C and D).

Thoracic irradiation induces acute and early term systemic inflammatory response in ApoE$^{-/-}$ mice. To assess the inflammatory response, fourteen different cytokines, which are involved in the pathogenesis of atherosclerosis (Adiponectin, CRP, CXCL10, Endoglin, FGF-basic, GDF-15, IGMA-1, IL-6, IL-1β, MCP-1, P-selectin, PAI-1, PCSK9, and uPAR), have been assessed in serum samples of female and male ApoE$^{-/-}$ mice at 24 h and 1 month after local thoracic X-ray irradiation. Fold changes between the baseline cytokines levels and at 24 h or at 1 month post irradiation cytokine levels were analyzed (summarized in Table SII).

At 24 h post thoracic irradiation. At 24 h post thoracic irradiation, a significant increase in GDF-15 at 0.1 and 10 Gy in female ApoE$^{-/-}$ mice and a significant increase at 10 Gy in male ApoE$^{-/-}$ mice were observed (Fig. 2A). Female and male ApoE$^{-/-}$ mice combined data showed a significant increase in GDF-15 at 10 Gy. A significant increase in CXCL10 was also observed at 10 Gy in female and male ApoE$^{-/-}$ mice, as well as in the combined female and male data (Fig. 2B). In addition,

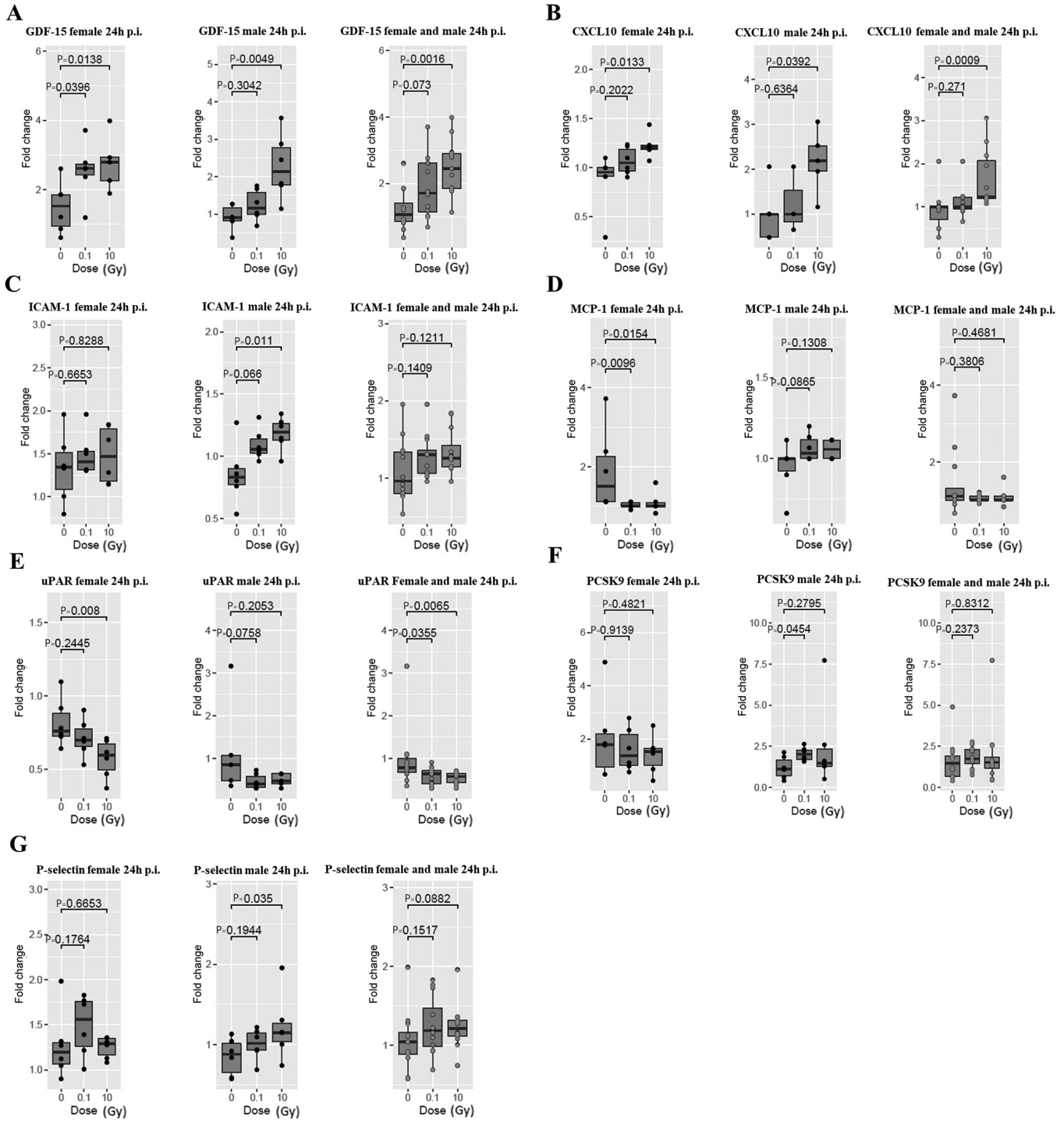

Figure 2. Systemic inflammatory response in female (left panels), male (middle panels) and combined female and male ApoE$^{-/-}$ mice (right panels) at 24 h post thoracic irradiation. (A) GDF-15, (B) CXCL10, (C) ICAM-1, (D) MCP-1, (E) uPAR, (F) PCSK9 and (G) P-selectin cytokines were analysed. The remaining are listed in Fig. S1. Data are presented as the fold change of the post-irradiation cytokine level relative to the baseline level. Statistical analysis was performed using a Kruskal-Wallis test, with the P-value being adjusted using the Benjamini-Hochberg method. Data are presented as a boxplot showing the median value of 6 mice per group. p.i., post irradiation; GDF-15, growth differentiation factor-15; CXCL10, C-X-C motif chemokine ligand 10; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; uPAR, urokinase-type plasminogen activator receptor; PCSK9, proprotein convertase subtilisin/kexin type 9.

a significant increase in ICAM-1 was observed at 10 Gy only in male ApoE$^{-/-}$ mice (Fig. 2C). Whereas, thoracic irradiation induced a significant decrease in MCP-1 at 0.1 and 10 Gy only in female ApoE$^{-/-}$ mice (Fig. 2D). Moreover, a significant decrease in female uPAR at 10 Gy was observed, and female and male uPAR combined data showed a significant decrease at 0.1 and 10 Gy (Fig. 2E). Next to that, in male ApoE$^{-/-}$ mice, there was a significant increase in PCSK9 at 0.1 Gy (Fig. 2F), and an increase in P-selectin at 10 Gy (Fig. 2G). Nonetheless, no significant changes were observed in adiponectin, PAI-1, CRP, endoglin, FGF-basic, IL-1β and IL-6 at 24 h post thoracic irradiation (Fig. S1).

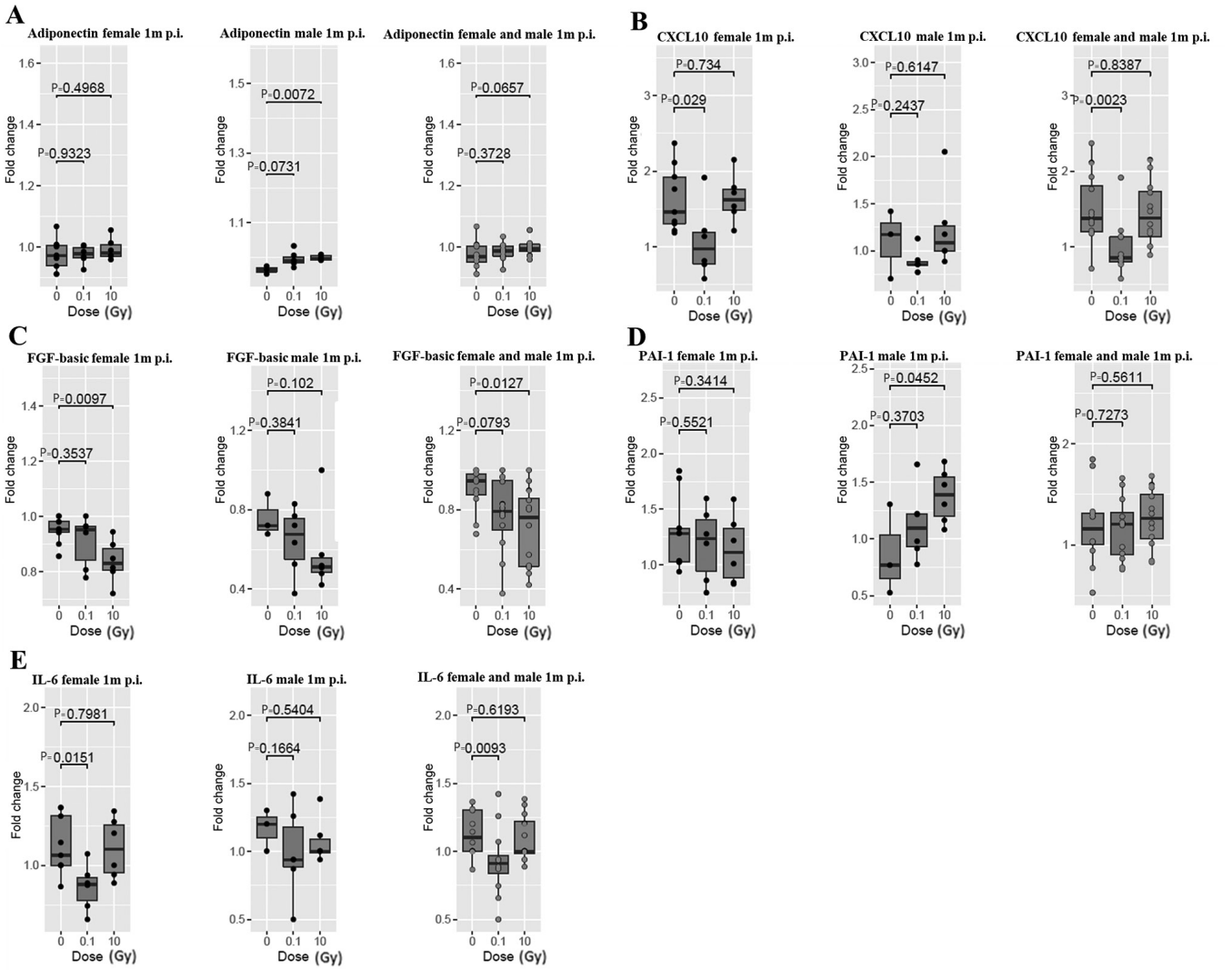

Figure 3. Systemic inflammatory response in female (left panels), male (middle panels) and combined female and male ApoE$^{-/-}$ mice (right panels) at 1 month post thoracic irradiation. (A) Adiponectin, (B) CXCL10, (C) FGF-basic, (D) PAI-1 and (E) IL-6 cytokines were assessed. The remaining are listed in Fig. S2. Data are presented as the fold change of the post-irradiation cytokine level relative to the baseline inflammatory cytokine level. Data are considered significant when P<0.05. Data are presented as boxplots showing the median of 6 mice per group, except for the male 0 Gy group, which had 3 mice. p.i., post irradiation; m, month; CXCL10, C-X-C motif chemokine ligand 10; FGF-basic, basic fibroblast growth factor; PAI-1, plasminogen activator inhibitor-1.

At 1 month post thoracic irradiation. The systemic inflammatory response at 1 month was different from the acute response at 24 h post thoracic irradiation. Adiponectin was significantly increased at 10 Gy post-irradiation only in male ApoE$^{-/-}$ mice (Fig. 3A). CXCL10 and IL-6 were significantly decreased at 0.1 Gy in female ApoE$^{-/-}$ mice, and in combined female and male data (Fig. 3B and E). FGF-basic level was significantly decreased at 10 Gy in female mice, and in the female and male mice combined data (Fig. 3C). Furthermore, PAI-1 showed a significant increase at 10 Gy in male ApoE$^{-/-}$ mice (Fig. 3D). No significant changes were observed in Endoglin, P-selectin, GDF-15, IL-1β, ICAM-1, MCP-1, PCSK-9, and uPAR at 1 month post thoracic irradiation (Fig. S2).

X-irradiation induces inflammatory response in coronary artery and microvascular endothelial cells. To verify whether the observed inflammatory responses in serum cytokines at 24 h and 1 month after irradiation is linked to endothelial cell responses, two endothelial cell lines (TICAE and TIME cells, originating from a male donor) were irradiated at low dose (0.1 Gy) or high dose (5 Gy), with supernatant being collected at 24 h or 72 h after irradiation. The 72 h time point was chosen since it is not possible to keep cells in culture for 1 month post-irradiation without passaging them. In addition, the high dose was chosen to be 5 Gy *in vitro*, since we previously reported that 5 Gy induced significant apoptotic cell death from 4 h after X-irradiation in TICAE and TIME cells, which increased with time (51), and also considering that we are irradiating a monolayer of endothelial cells (not tissue like the *in vivo* situation). The cytokines tested were GDF-15, CXCL10, ICAM-1, MCP-1, uPAR, IL-6, PAI-1, P-selectin, and FGF-basic (summarized in Table SIII).

At 24 h post endothelial irradiation. At 24 h after irradiation, GDF-15 and CXCL10 increased significantly at 5 Gy in TICAE and TIME cells (Fig. 4A, B, J and K) which correspond to the increase in these markers in female and male mice at 24 h post irradiation (Fig. 2A and B). A significant increase in

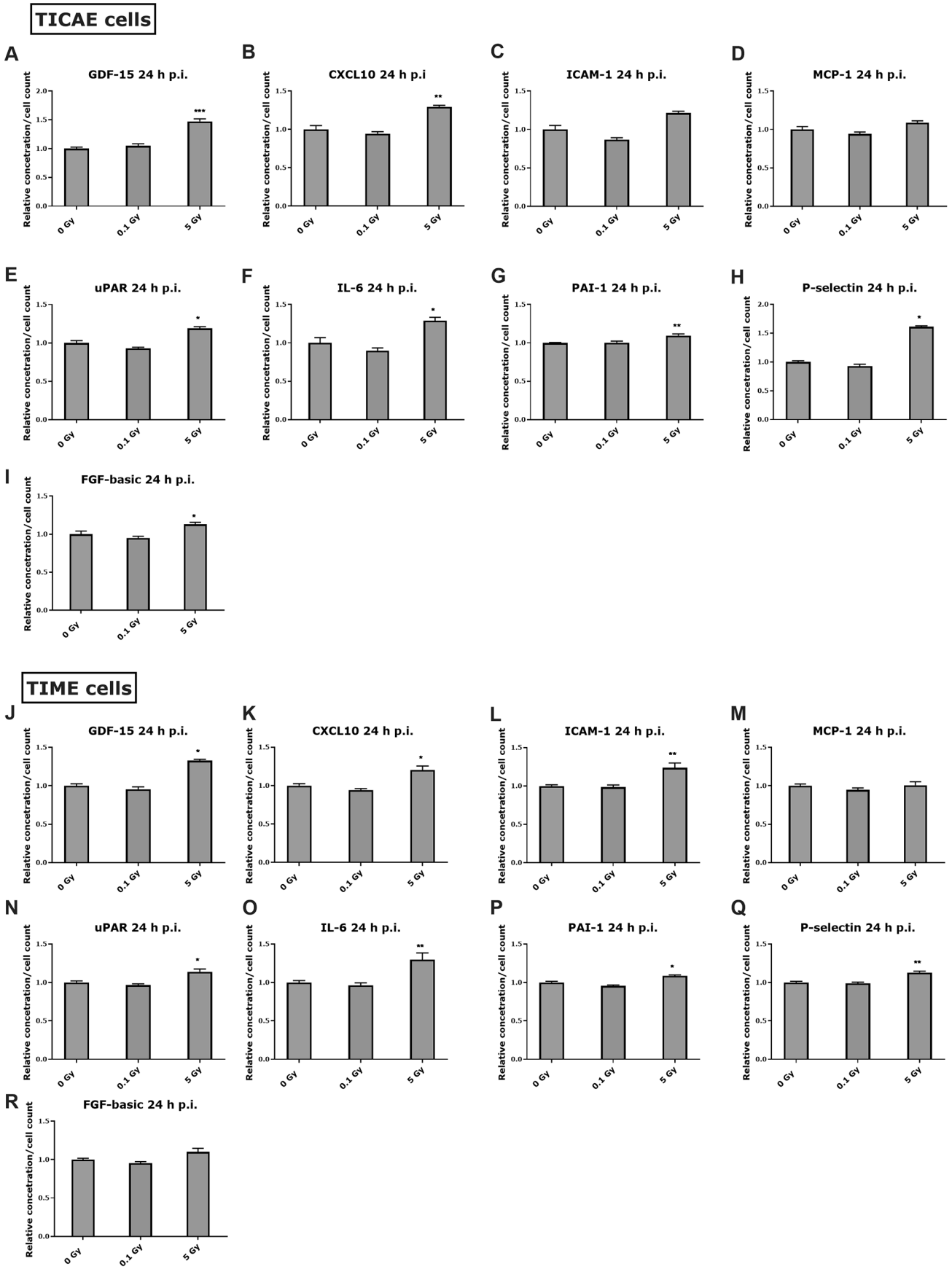

Figure 4. Inflammatory response in endothelial cells at 24 h after irradiation. The response of various inflammatory markers at 24 h after 0.1 and 5 Gy irradiation is represented in (A-I) TICAE cells and (J-R) TIME cells, normalized to cell count. The Kruskal-Wallis test was used to analyse the data and the P-value was adjusted using the Benjamini-Hochberg method. Values represent the average ± SEM of 6 biological replicates. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 Gy. p.i., post irradiation; GDF-15, growth differentiation factor-15; CXCL10, C-X-C motif chemokine ligand 10; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; uPAR, urokinase-type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; FGF-basic, basic fibroblast growth factor.

ICAM-1 was observed at 5 Gy in TIME cells (Fig. 4L), which may correlate with the increase in serum ICAM-1 in male mice at 10 Gy for the 24 h time point (Fig. 2C). Additionally, MCP-1 level was not changed in response to radiation in TICAE and TIME cells at 24 h post irradiation (Fig. 4D and M), and this was the case in the irradiated male mice at the same time point (Fig. 2D). Moreover, a significant increase in P-selectin was observed at 5 Gy in TICAE and TIME cells at 24 h post irradiation (Fig. 4H and Q), which corresponds to the observed trend of increase in male mice data (Fig. 2G). The levels of uPAR, IL-6 and PAI-1 were significantly increased in TICAE and TIME cells, and FGF-basic increased in TICAE cells at 5 Gy after 24 h of irradiation (Fig. 4E-G, I, N, O and P), while this was not the case in the irradiated female and male mice at the same time point.

At 72 h post endothelial irradiation. At 72 h after irradiation, GDF-15 was still significantly elevated in TICAE and TIME cells at 5 Gy (Fig. 5A and J). CXCL10, ICAM-1, MCP-1, uPAR, IL-6 and FGF-basic were also significantly elevated at 5 Gy in the irradiated TICAE cells (Fig. 5B-E, F and I), while ICAM-1 and MCP-1 significantly decreased in a dose-dependent manner in the irradiated TIME cells at 72 h post irradiation (Fig. 5L and M). Moreover, there was a significant increase in P-selectin at 5 Gy in TICAE cells, and significant increase in PAI-1 at 5 Gy in TICAE and TIME cells (Fig. 5G, H and P).

Discussion

Thoracic cancer radiotherapy significantly increases the risk for developing CVD (6,9,10,52,53). The underlying pathophysiology is complex and the mechanisms are not fully understood (6,7,9,17-21). Previous *in vivo* studies have identified late cardiovascular responses to ionizing radiation exposure, which was mainly manifested by pro-inflammatory responses and accelerated formation of atherosclerotic lesions/plaques 2-15 months after irradiation (17,18,26-29,54). Most of these pre-clinical studies were performed in a single gender, with limited data available related to possible gender differences. Here, we investigated acute (24 h) and early term (1 month) cardiovascular responses to ionizing radiation exposure, by assessing triglyceride and total cholesterol levels, and exploring a large panel of inflammatory markers in serum of female and male ApoE$^{-/-}$ mice that received low or high doses of local thoracic X-ray irradiation. We report, for the first time, that local thoracic irradiation of ApoE$^{-/-}$ mice increases serum GDF-15 and CXCL10 in both female and male mice 24 h after high dose irradiation (10 Gy). GDF-15 and CXCL10 levels were also significantly elevated at 24 h in irradiated coronary artery and microvascular endothelial (TICAE and TIME) cells *in vitro* after high dose of X-ray irradiation. In addition, we report gender-specific responses in the tested triglyceride and cholesterol levels at 1 month after irradiation, and in the assessed inflammatory markers at 24 h and 1 month post-irradiation. Below we discuss these findings in more detail.

Increased cholesterol and triglyceride levels in atherosclerosis have been shown in several clinical studies (55), and it was suggested that triglyceride level can be used as independent risk factor biomarker for coronary artery disease (56). Increased cholesterol and triglyceride levels may contribute to a large number of biological actions and consequences, including inflicting endothelial cell injury, increasing adhesion molecule expression, increasing leukocytes recruitment, as well as the formation of foam cells, therefore contributing to the atherosclerotic process (43,44). We observed different responses in cholesterol and triglyceride levels in the irradiated female and male ApoE$^{-/-}$ mice, especially at 1 month after exposure. Although triglyceride levels were increased in 10 Gy-irradiated female mice, levels dose-dependently decreased in male mice, demonstrating that triglyceride levels display gender specific responses. In contrast, cholesterol levels dose-dependently decreased in female mice, and showed a decreasing trend in male mice, resulting in a significant dose-dependent decrease when combining female and male responses 1 month post-irradiation. A similar observation for the decrease in total cholesterol and the increase in triglyceride levels in female mice was reported in female breast cancer patients after 50-60 Gy of radiotherapy which were given over 5 weeks (57). Previous studies performed 2-5 months after irradiation of ApoE$^{-/-}$ mice, revealed variable responses in cholesterol and triglyceride levels depending mainly on the time point after exposure, gender used in the experiment, and on radiation doses/quality used (17,18,27,29). Cholesterol and triglyceride changes can perhaps be secondary to the inflammatory or oxidative stress responses after radiation exposure, though further investigations are needed to unveil the involved mechanisms.

Our results further demonstrate that local thoracic irradiation induced an increase in serum GDF-15, which was significant at low and high doses (0.1 and 10 Gy) in female mice and at high dose in male mice 24 h after irradiation. *In vitro*, GDF-15 was secreted from irradiated TICAE and TIME cells at 24 h after high dose (5 Gy) irradiation. An increased GDF-15 gene expression was previously observed in human aortic endothelial cells at 4-24 h after 4 Gy (58), and in carotid arteries of male ApoE$^{-/-}$ mice at 1 week after 14 Gy of local neck X-ray irradiation (59). GDF-15 is a member of the transforming growth factor β superfamily that increases its expression under inflammatory conditions (60,61). Elevated GDF-15 serum levels have been associated with an increased risk for a range of CVD, including atherosclerosis, and currently being evaluated as a biomarker in CVD (62-65). Previous studies revealed that GDF-15 may contribute to the initiation and the progression of atherosclerotic lesions by regulating apoptosis and IL-6-dependent inflammatory responses (66), promoting migration of macrophage, and by contributing to plaque instability (67). In line with this, our *in vitro* data showed an increased IL-6 level in TICAE and TIME cells 24 h after 5 Gy irradiation. GDF-15 may play an important role in ionizing radiation-induced endothelial cell senescence through an oxidative stress-mediated p16 pathway (58). Another inflammatory marker of potential interest is CXCL10, also known as Interferon-γ-inducible protein 10 (IP-10), which acts as a chemoattractant cytokine. CXCL10 was shown to promote atherosclerosis by recruitment and retention of activated T lymphocytes to vascular wall lesions during the atherogenesis process (68,69), and also is being investigated as a potential biomarker for CVD (70-73). In our study, the serum level of CXCL10 was significantly elevated in female and male ApoE$^{-/-}$ mice 24 h after high dose thoracic irradiation, and

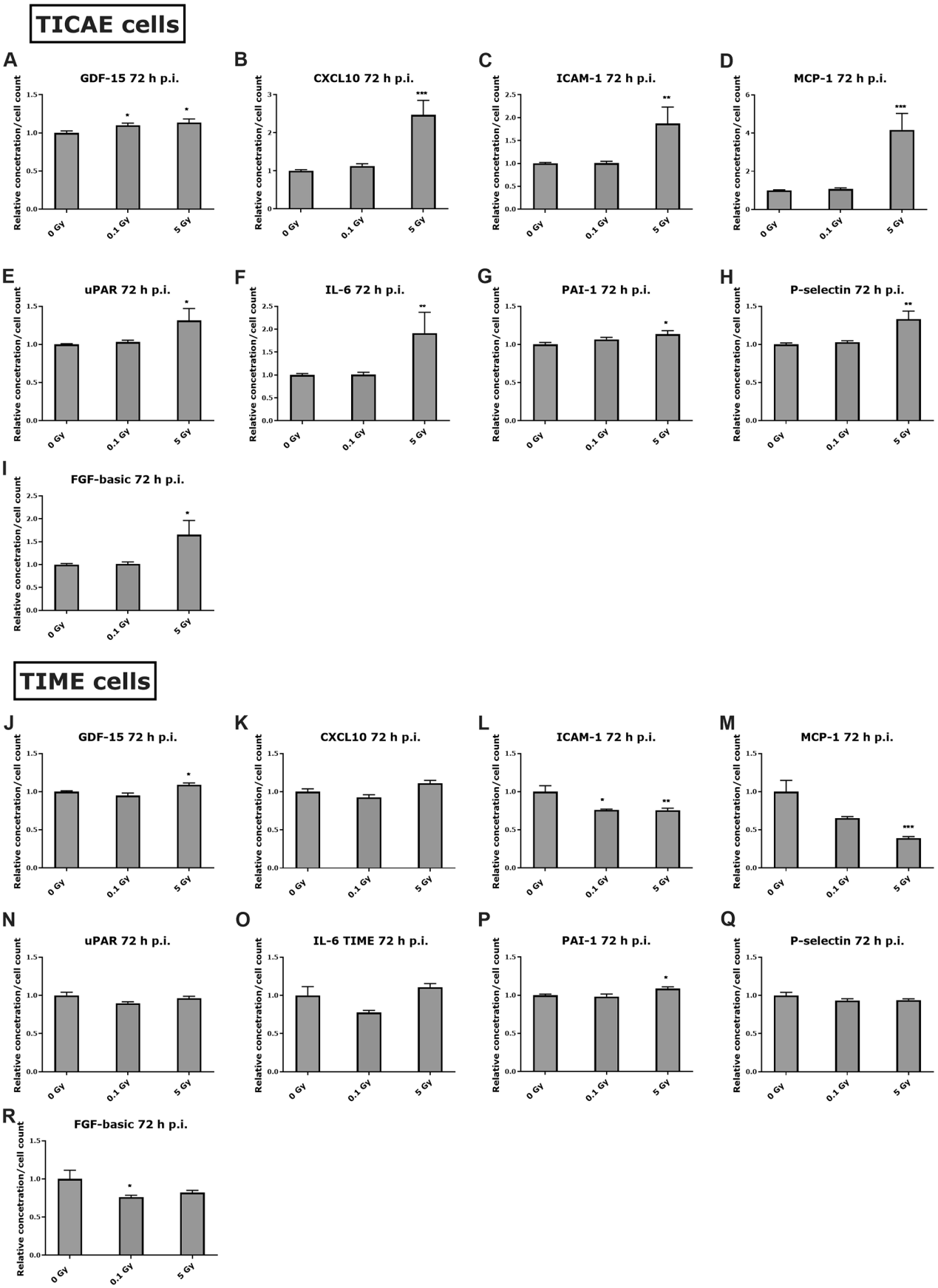

Figure 5. Inflammatory response in endothelial cells at 72 h after irradiation. The response of various inflammatory markers at 72 h after 0.1 and 5 Gy irradiation is presented in (A-I) TICAE cells and (J-R) TIME cells. Data were normalized to cell count. The Kruskal-Wallis test was used to analyse the data and the P-value was adjusted using the Benjamini-Hochberg method. Values represent the average ± SEM of 6 biological replicates. *P<0.05, **P<0.01 and ***P<0.001 vs. 0 Gy. p.i., post irradiation; GDF-15, growth differentiation factor-15; CXCL10, C-X-C motif chemokine ligand 10; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; uPAR, urokinase-type plasminogen activator receptor; PAI-1, plasminogen activator inhibitor-1; FGF-basic, basic fibroblast growth factor.

was found to be secreted from both TICAE and TIME cells 24 h post-irradiation. In line with our *in vitro* data, CXCL10 gene expression was previously reported to be upregulated in human coronary artery endothelial cells after 10 Gy of fractionated X-irradiation (74), and in human umbilical vascular endothelial cells following exposure to 20 Gy of acute gamma irradiation (75).

One of the earliest responses to endothelial cell injury, which is considered one potential initiating event of the atherosclerotic process, is upregulation of adhesion molecules including ICAM-1 and P-selectin, leading to leukocyte adherence to the endothelium (30,76,77). Multiple *in vitro* and *in vivo* studies have reported post-irradiation increases in ICAM-1 and P-selectin levels, thereby promoting leucocyte adhesion to the endothelium (26,78-83). However, most of the *in vivo* studies were performed in endothelial cells of irradiated arteries weeks to months after radiation exposure. Here, we report an acute dose-dependent increase in serum ICAM-1 level 24 h after thoracic irradiation in male ApoE$^{-/-}$ mice, and this increase was stabilized 1 month later. Previous observations have shown that local irradiation of carotid arteries in female and male ApoE$^{-/-}$ mice did not induce ICAM-1 changes at 1 and 22 weeks after 14 Gy X-ray exposure (17,18), indicating that the early 24 h response reported here may be transient in nature. We further found a corresponding ICAM-1 increase in the irradiated TIME cells at 24 h for the 5 Gy dose, which is in line with previous *in vitro* studies performed at the same time window (39,84). Moreover, we showed that the serum level of P-selectin was increased 24 h after irradiation, again only in male ApoE$^{-/-}$ mice. This elevated P-selectin level was also observed in irradiated TICAE and TIME cells, which confirms previous *in vitro* findings (79). The inflammatory responses observed at high irradiation dose (5 Gy) in TICAE and TIME cells could be an injury response, since an increased DNA damage and persistent cell death were previously observed in these cells after 5 Gy X-irradiation (51).

Our data further demonstrate a significant decrease in atheroprotective basic fibroblast growth factor (FGF-basic), in female ApoE$^{-/-}$ mice at 10 Gy, and a decreasing trend in male ApoE$^{-/-}$ mice 1 month after irradiation, resulting is a significant dose-dependent decrease in the combined female and male data. A previous study performed in non-Hodgkin lymphoma cancer patients showed a significant decrease in FGF-2 serum level after doses ranging between 6 and 52 Gy of radiotherapy (85), and another study performed in patients with different tumour histotypes, also reported that FGF-2 serum level decreased after radiotherapy (86). FGF-basic was observed to have a protective effect against irradiation (87), since it inhibited radiation-induced apoptosis of endothelial cells under both *in vitro* and *in vivo* conditions (88,89), and was also reported to decrease VCAM-1 expression and macrophage presence in atherosclerotic plaques from rabbits fed with a high cholesterol diet (90).

In contrast to the proatherogenic inflammatory response after thoracic irradiation, our results showed an acute decrease in the proatherogenic cytokine MCP-1, only observed in female mice 24 h after irradiation. MCP-1 is involved in atherosclerosis initiation and progression by recruiting monocytes and contributing to macrophage infiltration into the subendothelial cell layer (91,92). Interestingly, it has been shown that post-irradiation MCP-1-mediated chemoattraction is regulated by the proatherogenic uPAR expression (93,94). In line with this observation, our results showed a decrease in uPAR serum level only in female ApoE$^{-/-}$ mice 24 h after 10 Gy exposure. It is worth mentioning that the observed alterations in inflammatory markers, as discussed, are limited to multiplex bead assay assessment, and further validation using other approaches, such as western blotting or RT-qPCR, are required.

The gender difference in response to radiation exposure could be explained by hormone differences between male and female. Several groups have reported that estrogen is protective against vascular dysfunction and atherosclerotic lesions in mice (95-98). Estrogen is known to exert protective and beneficial effects in the cardiovascular system by improving vascular function, increasing NO production and by inhibiting proliferation and migration of vascular smooth muscle cells (99-101). It was also shown that atherosclerotic lesions were significantly less extensive in female ApoE$^{-/-}$ mice than in male mice (101). Indeed, in our experiment, we observed an increase in the pro-inflammatory markers ICAM-1, P-selectin in male mice while a decrease in the pro-inflammatory MCP-1 and uPAR levels in female mice. Though, further investigations are required to unveil mechanisms behind the observed gender specific response after irradiation in ApoE$^{-/-}$ mice, by scrutinizing the effect of sex steroid hormones on cytokine response *in vitro*, or by using knock-down/out techniques *in vivo*.

Taken together, our results reveal acute and early term inflammatory responses after X-ray exposure of ApoE$^{-/-}$ mice and of coronary artery and microvascular endothelial cells. Future research is needed to fully grasp the scope of early changes in the cardiovascular system after thoracic irradiation, and to determine whether GDF-15 and CXCL10 could be used as potential biomarkers for early detection of cardiovascular risks in thoracic radiotherapy-treated patients, thus identifying patients who may benefit from early medical intervention.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

RR conducted *ex-vivo* and *in vitro* experiments, guided and supervised the *in vivo* irradiation and blood collection experiments, and wrote the manuscript. MC and EC conducted *in vivo* irradiation and blood sampling, as well as the cholesterol and

triglyceride experiments. MM analysed the *in vivo* experiments. RR and AA confirm the authenticity of all the raw data. ED, SB, AA and LL designed the experiments and supervised the study. All authors, except MC, contributed equally in reviewing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Mice were treated according to the European Ethics Committee guidelines and the study protocol was approved by the Animal Experiment Ethical Committee of the Faculty of Medicine and Health Sciences, Ghent University, Belgium (approval no. ECD 17/60).

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

References

1. Kong FM, Zhao L and Hayman JA: The role of radiation therapy in thoracic tumors. Hematol Oncol Clin North Am 20: 363-400, 2006.
2. Baskar R, Lee KA, Yeo R and Yeoh KW: Cancer and radiation therapy: Current advances and future directions. Int J Med Sci 9: 193-199, 2012.
3. Delaney G, Jacob S, Featherstone C and Barton M: The role of radiotherapy in cancer treatment: Estimating optimal utilization from a review of evidence-based clinical guidelines. Cancer 104: 1129-1137, 2005.
4. Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, Stein KD, Alteri R and Jemal A: Cancer treatment and survivorship statistics, 2016. CA Cancer J Clin 66: 271-289, 2016.
5. Darby S, McGale P, Peto R, Granath F, Hall P and Ekbom A: Mortality from cardiovascular disease more than 10 years after radiotherapy for breast cancer: Nationwide cohort study of 90 000 Swedish women. BMJ 326: 256-257, 2003.
6. Darby SC, Ewertz M, McGale P, Bennet AM, Blom-Goldman U, Brønnum D, Correa C, Cutter D, Gagliardi G, Gigante B, *et al*: Risk of ischemic heart disease in women after radiotherapy for breast cancer. N Engl J Med 368: 987-998, 2013.
7. Baselet B, Rombouts C, Benotmane AM, Baatout S and Aerts A: Cardiovascular diseases related to ionizing radiation: The risk of low-dose exposure (review). Int J Mol Med 38: 1623-1641, 2016.
8. Yusuf SW, Sami S and Daher IN: Radiation-induced heart disease: A clinical update. Cardiol Res Pract 2011: 317659, 2011.
9. Authors on behalf of ICRP, Stewart FA, Akleyev AV, Hauer-Jensen M, Hendry JH, Kleiman NJ, Macvittie TJ, Aleman BM, Edgar AB, Mabuchi K, *et al*: ICRP publication 118: ICRP statement on tissue reactions and early and late effects of radiation in normal tissues and organs-threshold doses for tissue reactions in a radiation protection context. Ann ICRP 41: 1-322, 2012.
10. Little MP: Radiation and circulatory disease. Mutat Res 770: 299-318, 2016.
11. Eftekhari M, Anbiaei R, Zamani H, Fallahi B, Beiki D, Ameri A, Emami-Ardekani A, Fard-Esfahani A, Gholamrezanezhad A, Seid Ratki KR and Roknabadi AM: Radiation-induced myocardial perfusion abnormalities in breast cancer patients following external beam radiation therapy. Asia Ocean J Nucl Med Biol 3: 3-9, 2015.
12. Kole TP, Aghayere O, Kwah J, Yorke ED and Goodman KA: Comparison of heart and coronary artery doses associated with intensity-modulated radiotherapy versus three-dimensional conformal radiotherapy for distal esophageal cancer. Int J Radiat Oncol Biol Phys 83: 1580-1586, 2012.
13. Tillman GF, Pawlicki T, Koong AC and Goodman KA: Preoperative versus postoperative radiotherapy for locally advanced gastroesophageal junction and proximal gastric cancers: A comparison of normal tissue radiation doses. Dis Esophagus 21: 437-444, 2008.
14. Hong JC, Rahimy E, Gross CP, Shafman T, Hu X, Yu JB, Ross R, Finkelstein SE, Dosoretz A, Park HS, *et al*: Radiation dose and cardiac risk in breast cancer treatment: An analysis of modern radiation therapy including community settings. Pract Radiat Oncol 8: e79-e86, 2018.
15. Al-Kindi SG and Oliveira GH: Incidence and trends of cardiovascular mortality after common cancers in young adults: Analysis of surveillance, epidemiology and end-results program. World J Cardiol 8: 368-374, 2016.
16. Drost L, Yee C, Lam H, Zhang L, Wronski M, McCann C, Lee J, Vesprini D, Leung E and Chow E: A systematic review of heart dose in breast radiotherapy. Clin Breast Cancer 18: e819-e824, 2018.
17. Hoving S, Heeneman S, Gijbels MJ, te Poele JA, Russell NS, Daemen MJ and Stewart FA: Single-dose and fractionated irradiation promote initiation and progression of atherosclerosis and induce an inflammatory plaque phenotype in ApoE(-/-) mice. Int J Radiat Oncol Biol Phys 71: 848-857, 2008.
18. Stewart FA, Heeneman S, Te Poele J, Kruse J, Russell NS, Gijbels M and Daemen M: Ionizing radiation accelerates the development of atherosclerotic lesions in ApoE$^{-/-}$ mice and predisposes to an inflammatory plaque phenotype prone to hemorrhage. Am J Pathol 168: 649-658, 2006.
19. Kreuzer M, Auvinen A, Cardis E, Hall J, Jourdain JR, Laurier D, Little MP, Peters A, Raj K, Russell NS, *et al*: Low-dose ionising radiation and cardiovascular diseases-strategies for molecular epidemiological studies in Europe. Mutat Res Rev Mutat Res 764: 90-100, 2015.
20. Shimizu Y, Kodama K, Nishi N, Kasagi F, Suyama A, Soda M, Grant EJ, Sugiyama H, Sakata R, Moriwaki H, *et al*: Radiation exposure and circulatory disease risk: Hiroshima and Nagasaki atomic bomb survivor data, 1950-2003. BMJ 340: b5349, 2010.
21. Yamada M, Naito K, Kasagi F, Masunari N and Suzuki G: Prevalence of atherosclerosis in relation to atomic bomb radiation exposure: An RERF adult health study. Int J Radiat Biol 81: 821-826, 2005.
22. Azizova TV, Grigoryeva ES, Haylock RG, Pikulina MV and Moseeva MB: Ischaemic heart disease incidence and mortality in an extended cohort of Mayak workers first employed in 1948-1982. Br J Radiol 88: 20150169, 2015.
23. Kashcheev VV, Chekin SY, Karpenko SV, Maksioutov MA, Menyaylo AN, Tumanov KA, Kochergina EV, Kashcheeva PV, Gorsky AI, Shchukina NV, *et al*: Radiation risk of cardiovascular diseases in the cohort of Russian emergency workers of the chernobyl accident. Health Phys 113: 23-29, 2017.
24. Ramadan R, Vromans E, Anang DC, Decrock E, Mysara M, Monsieurs P, Baatout S, Leybaert L and Aerts A: Single and fractionated ionizing radiation induce alterations in endothelial connexin expression and channel function. Sci Rep 9: 4643, 2019.
25. Mathias D, Mitchel RE, Barclay M, Wyatt H, Bugden M, Priest ND, Whitman SC, Scholz M, Hildebrandt G, Kamprad M and Glasow A: Low-dose irradiation affects expression of inflammatory markers in the heart of ApoE$^{-/-}$ mice. PLoS One 10: e0119661, 2015.
26. Sievert W, Trott KR, Azimzadeh O, Tapio S, Zitzelsberger H and Multhoff G: Late proliferating and inflammatory effects on murine microvascular heart and lung endothelial cells after irradiation. Radiother Oncol 117: 376-381, 2015.
27. Mitchel RE, Hasu M, Bugden M, Wyatt H, Little MP, Gola A, Hildebrandt G, Priest ND and Whitman SC: Low-dose radiation exposure and atherosclerosis in ApoE(-)/(-) mice. Radiat Res 175: 665-676, 2011.
28. Mancuso M, Pasquali E, Braga-Tanaka I III, Tanaka S, Pannicelli A, Giardullo P, Pazzaglia S, Tapio S, Atkinson MJ and Saran A: Acceleration of atherogenesis in ApoE$^{-/-}$ mice exposed to acute or low-dose-rate ionizing radiation. Oncotarget 6: 31263-31271, 2015.
29. Kumarathasan P, Vincent R, Blais E, Saravanamuthu A, Gupta P, Wyatt H, Mitchel R, Hannan M, Trivedi A and Whitman S: Cardiovascular changes in atherosclerotic ApoE-deficient mice exposed to Co60 (γ) radiation. PLoS One 8: e65486, 2013.
30. Massberg S, Brand K, Gruner S, Page S, Müller E, Müller I, Bergmeier W, Richter T, Lorenz M, Konrad I, *et al*: A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med 196: 887-896, 2002.

31. Heidenreich PA, Hancock SL, Lee BK, Mariscal CS and Schnittger I: Asymptomatic cardiac disease following mediastinal irradiation. J Am Coll Cardiol 42: 743-749, 2003.
32. Yusuf SW, Venkatesulu BP, Mahadevan LS and Krishnan S: Radiation-induced cardiovascular disease: A clinical perspective. Front Cardiovasc Med 4: 66, 2017.
33. Corrado E, Rizzo M, Coppola G, Fattouch K, Novo G, Marturana I, Ferrara F and Novo S: An update on the role of markers of inflammation in atherosclerosis. J Atheroscler Thromb 17: 1-11, 2010.
34. Soeki T and Sata M: Inflammatory biomarkers and atherosclerosis. Int Heart J 57: 134-139, 2016.
35. Libby P: Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 32: 2045-2051, 2012.
36. Murabito JM, Keyes MJ, Guo CY, Keaney JF Jr, Vasan RS, D'Agostino RB Sr and Benjamin EJ: Cross-sectional relations of multiple inflammatory biomarkers to peripheral arterial disease: The Framingham offspring study. Atherosclerosis 203: 509-514, 2009.
37. Halle M, Gabrielsen A, Paulsson-Berne G, Gahm C, Agardh HE, Farnebo F and Tornvall P: Sustained inflammation due to nuclear factor-kappa B activation in irradiated human arteries. J Am Coll Cardiol 55: 1227-1236, 2010.
38. Kiyohara H, Ishizaki Y, Suzuki Y, Katoh H, Hamada N, Ohno T, Takahashi T, Kobayashi Y and Nakano T: Radiation-induced ICAM-1 expression via TGF-β1 pathway on human umbilical vein endothelial cells; comparison between X-ray and carbon-ion beam irradiation. J Radiat Res 52: 287-292, 2011.
39. Hallahan D, Kuchibhotla J and Wyble C: Cell adhesion molecules mediate radiation-induced leukocyte adhesion to the vascular endothelium. Cancer Res 56: 5150-5155, 1996.
40. Di Maggio FM, Minafra L, Forte GI, Cammarata FP, Lio D, Messa C, Gilardi MC and Bravatà V: Portrait of inflammatory response to ionizing radiation treatment. J Inflamm (Lond) 12: 14, 2015.
41. Baluna RG, Eng TY and Thomas CR: Adhesion molecules in radiotherapy. Radiat Res 166: 819-831, 2006.
42. Min X, Lu M, Tu S, Wang X, Zhou C, Wang S, Pang S, Qian J, Ge Y, Guo Y, *et al*: Serum cytokine profile in relation to the severity of coronary artery disease. Biomed Res Int 2017: 4013685, 2017.
43. Peng J, Luo F, Ruan G, Peng R and Li X: Hypertriglyceridemia and atherosclerosis. Lipids Health Dis 16: 233, 2017.
44. Sloop GD: A critical analysis of the role of cholesterol in atherogenesis. Atherosclerosis 142: 265-268, 1999.
45. Lowe D and Raj K: Premature aging induced by radiation exhibits pro-atherosclerotic effects mediated by epigenetic activation of CD44 expression. Aging Cell 13: 900-910, 2014.
46. Lowe D, Horvath S and Raj K: Epigenetic clock analyses of cellular senescence and ageing. Oncotarget 7: 8524-8531, 2016.
47. Leligdowicz A, Conroy AL, Hawkes M, Zhong K, Lebovic G, Matthay MA and Kain KC: Validation of two multiplex platforms to quantify circulating markers of inflammation and endothelial injury in severe infection. PLoS One 12: e0175130, 2017.
48. Vitkova V, Panek M, Janec P, Šibíková M, Vobruba V, Haluzík M, Živný J and Janota J: Endothelial microvesicles and soluble markers of endothelial injury in critically Ill newborns. Mediators Inflamm 2018: 1975056, 2018.
49. Bahlas S, Damiati L, Dandachi N, Sait H, Alsefri M and Pushparaj PN: Rapid immunoprofiling of cytokines, chemokines and growth factors in patients with active rheumatoid arthritis using luminex multiple analyte profiling technology for precision medicine. Clin Exp Rheumatol 37: 112-119, 2019.
50. Reslova N, Michna V, Kasny M, Mikel P and Kralik P: xMAP technology: Applications in detection of pathogens. Front Microbiol 8: 55, 2017.
51. Ramadan R, Vromans E, Anang DC, Goetschalckx I, Hoorelbeke D, Decrock E, Baatout S, Leybaert L and Aerts A: Connexin43 hemichannel targeting with TAT-Gap19 alleviates radiation-induced endothelial cell damage. Front Pharmacol 11: 212, 2020.
52. Darby SC, Cutter DJ, Boerma M, Constine LS, Fajardo LF, Kodama K, Mabuchi K, Marks LB, Mettler FA, Pierce LJ, *et al*: Radiation-related heart disease: Current knowledge and future prospects. Int J Radiat Oncol Biol Phys 76: 656-665, 2010.
53. Aleman BM, Moser EC, Nuver J, Suter TM, Maraldo MV, Specht L, Vrieling C and Darby SC: Cardiovascular disease after cancer therapy. EJC Suppl 12: 18-28, 2014.
54. Monceau V, Meziani L, Strup-Perrot C, Morel E, Schmidt M, Haagen J, Escoubet B, Dörr W and Vozenin MC: Enhanced sensitivity to low dose irradiation of ApoE$^{-/-}$ mice mediated by early pro-inflammatory profile and delayed activation of the TGFβ1 cascade involved in fibrogenesis. PLoS One 8: e57052, 2013.
55. Brunner D, Altman S, Loebl K, Schwartz S and Levin S: Serum cholesterol and triglycerides in patients suffering from ischemic heart disease and in healthy subjects. Atherosclerosis 28: 197-204, 1977.
56. Sarwar N, Danesh J, Eiriksdottir G, Sigurdsson G, Wareham N, Bingham S, Boekholdt SM, Khaw KT and Gudnason V: Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies. Circulation 115: 450-458, 2007.
57. Ozmen HK, Erdemci B, Askin S and Sezen O: Carnitine and adiponectin levels in breast cancer after radiotherapy. Open Med (Wars) 12: 189-194, 2017.
58. Park H, Kim CH, Jeong JH, Park M and Kim KS: GDF15 contributes to radiation-induced senescence through the ROS-mediated p16 pathway in human endothelial cells. Oncotarget 7: 9634-9644, 2016.
59. Hoving S, Heeneman S, Gijbels MJ, Te Poele JA, Visser N, Cleutjens J, Russell NS, Daemen MJ and Stewart FA: Irradiation induces different inflammatory and thrombotic responses in carotid arteries of wildtype C57BL/6J and atherosclerosis-prone ApoE(-/-) mice. Radiother Oncol 105: 365-370, 2012.
60. Bootcov MR, Bauskin AR, Valenzuela SM, Moore AG, Bansal M, He XY, Zhang HP, Donnellan M, Mahler S, Pryor K, *et al*: MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily. Proc Natl Acad Sci USA 94: 11514-11519, 1997.
61. Hsiao EC, Koniaris LG, Zimmers-Koniaris T, Sebald SM, Huynh TV and Lee SJ: Characterization of growth-differentiation factor 15, a transforming growth factor beta superfamily member induced following liver injury. Mol Cell Biol 20: 3742-3751, 2000.
62. Wollert KC and Kempf T: Growth differentiation factor 15 in heart failure: An update. Curr Heart Fail Rep 9: 337-345, 2012.
63. Chen J, Luo F, Fang Z and Zhang W: GDF-15 levels and atherosclerosis. Int J Cardiol 257: 36, 2018.
64. Kempf T and Wollert KC: Growth differentiation factor-15: A new biomarker in cardiovascular disease. Herz 34: 594-599, 2009.
65. Xu X, Li Z and Gao W: Growth differentiation factor 15 in cardiovascular diseases: From bench to bedside. Biomarkers 16: 466-475, 2011.
66. Bonaterra GA, Zugel S, Thogersen J, Walter SA, Haberkorn U, Strelau J and Kinscherf R: Growth differentiation factor-15 deficiency inhibits atherosclerosis progression by regulating interleukin-6-dependent inflammatory response to vascular injury. J Am Heart Assoc 1: e002550, 2012.
67. de Jager SC, Bermudez B, Bot I, Koenen RR, Bot M, Kavelaars A, de Waard V, Heijnen CJ, Muriana FJ, Weber C, *et al*: Growth differentiation factor 15 deficiency protects against atherosclerosis by attenuating CCR2-mediated macrophage chemotaxis. J Exp Med 208: 217-225, 2011.
68. Heller EA, Liu E, Tager AM, Yuan Q, Lin AY, Ahluwalia N, Jones K, Koehn SL, Lok VM, Aikawa E, *et al*: Chemokine CXCL10 promotes atherogenesis by modulating the local balance of effector and regulatory T cells. Circulation 113: 2301-2312, 2006.
69. Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P and Luster AD: Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. J Clin Invest 104: 1041-1050, 1999.
70. van den Borne P, Quax PH, Hoefer IE and Pasterkamp G: The multifaceted functions of CXCL10 in cardiovascular disease. Biomed Res Int 2014: 893106, 2014.
71. Ardigo D, Assimes TL, Fortmann SP, Go AS, Hlatky M, Hytopoulos E, Iribarren C, Tsao PS, Tabibiazar R and Quertermous T; ADVANCE Investigators: Circulating chemokines accurately identify individuals with clinically significant atherosclerotic heart disease. Physiol Genomics 31: 402-409, 2007.
72. Orn S, Breland UM, Mollnes TE, Manhenke C, Dickstein K, Aukrust P and Ueland T: The chemokine network in relation to infarct size and left ventricular remodeling following acute myocardial infarction. Am J Cardiol 104: 1179-1183, 2009.

73. Tavakolian Ferdousie V, Mohammadi M, Hassanshahi G, Khorramdelazad H, Khanamani Falahati-Pour S, Mirzaei M, Allah Tavakoli M, Kamiab Z, Ahmadi Z, Vazirinejad R, *et al*: Serum CXCL10 and CXCL12 chemokine levels are associated with the severity of coronary artery disease and coronary artery occlusion. Int J Cardiol 233: 23-28, 2017.
74. Palayoor ST, John-Aryankalayil M, Makinde AY, Falduto MT, Magnuson SR and Coleman CN: Differential expression of stress and immune response pathway transcripts and miRNAs in normal human endothelial cells subjected to fractionated or single-dose radiation. Mol Cancer Res 12: 1002-1015, 2014.
75. Heinonen M, Milliat F, Benadjaoud MA, François A, Buard V, Tarlet G, d'Alché-Buc F and Guipaud O: Temporal clustering analysis of endothelial cell gene expression following exposure to a conventional radiotherapy dose fraction using Gaussian process clustering. PLoS One 13: e0204960, 2018.
76. Libby P, Ridker PM and Maseri A: Inflammation and atherosclerosis. Circulation 105: 1135-1143, 2002.
77. Lusis AJ: Atherosclerosis. Nature 407: 233-241, 2000.
78. Hallahan DE and Virudachalam S: Accumulation of P-selectin in the lumen of irradiated blood vessels. Radiat Res 152: 6-13, 1999.
79. Tu J, Hu Z and Chen Z: Endothelial gene expression and molecular changes in response to radiosurgery in in vitro and in vivo models of cerebral arteriovenous malformations. Biomed Res Int 2013: 408253, 2013.
80. Hallahan DE and Virudachalam S: Ionizing radiation mediates expression of cell adhesion molecules in distinct histological patterns within the lung. Cancer Res 57: 2096-2099, 1997.
81. Gaugler MH, Squiban C, van der Meeren A, Bertho JM, Vandamme M and Mouthon MA: Late and persistent up-regulation of intercellular adhesion molecule-1 (ICAM-1) expression by ionizing radiation in human endothelial cells in vitro. Int J Radiat Biol 72: 201-209, 1997.
82. Haubner F, Leyh M, Ohmann E, Pohl F, Prantl L and Gassner HG: Effects of external radiation in a co-culture model of endothelial cells and adipose-derived stem cells. Radiat Oncol 8: 66, 2013.
83. Azimzadeh O, Sievert W, Sarioglu H, Merl-Pham J, Yentrapalli R, Bakshi MV, Janik D, Ueffing M, Atkinson MJ, Multhoff G and Tapio S: Integrative proteomics and targeted transcriptomics analyses in cardiac endothelial cells unravel mechanisms of long-term radiation-induced vascular dysfunction. J Proteome Res 14: 1203-1219, 2015.
84. Cervelli T, Panetta D, Navarra T, Andreassi MG, Basta G, Galli A, Salvadori PA, Picano E and Del Turco S: Effects of single and fractionated low-dose irradiation on vascular endothelial cells. Atherosclerosis 235: 510-518, 2014.
85. Ria R, Cirulli T, Giannini T, Bambace S, Serio G, Portaluri M, Ribatti D, Vacca A and Dammacco F: Serum levels of angiogenic cytokines decrease after radiotherapy in non-Hodgkin lymphomas. Clin Exp Med 8: 141-145, 2008.
86. Ria R, Portaluri M, Russo F, Cirulli T, Di Pietro G, Bambace S, Cucci F, Romano T, Vacca A and Dammacco F: Serum levels of angiogenic cytokines decrease after antineoplastic radiotherapy. Cancer Lett 216: 103-107, 2004.
87. Yang X, Liaw L, Prudovsky I, Brooks PC, Vary C, Oxburgh L and Friesel R: Fibroblast growth factor signaling in the vasculature. Curr Atheroscler Rep 17: 509, 2015.
88. Fuks Z, Persaud RS, Alfieri A, McLoughlin M, Ehleiter D, Schwartz JL, Seddon AP, Cordon-Cardo C and Haimovitz-Friedman A: Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death in vitro and in vivo. Cancer Res 54: 2582-2590, 1994.
89. Zhang S, Qiu X, Zhang Y, Fu K, Zhao X, Wu J, Hu Y, Zhu W and Guo H: Basic fibroblast growth factor ameliorates endothelial dysfunction in radiation-induced bladder injury. Biomed Res Int 2015: 967680, 2015.
90. Six I, Mouquet F, Corseaux D, Bordet R, Letourneau T, Vallet B, Dosquet CC, Dupuis B, Jude B, Bertrand ME, *et al*: Protective effects of basic fibroblast growth factor in early atherosclerosis. Growth Factors 22: 157-167, 2004.
91. Aiello RJ, Bourassa PA, Lindsey S, Weng W, Natoli E, Rollins BJ and Milos PM: Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 19: 1518-1525, 1999.
92. Harrington JR: The role of MCP-1 in atherosclerosis. Stem Cells 18: 65-66, 2000.
93. Nalla AK, Gogineni VR, Gupta R, Dinh DH and Rao JS: Suppression of uPA and uPAR blocks radiation-induced MCP-1 mediated recruitment of endothelial cells in meningioma. Cell Signal 23: 1299-1310, 2011.
94. Farris SD, Hu JH, Krishnan R, Emery I, Chu T, Du L, Kremen M, Dichek HL, Gold E, Ramsey SA and Dichek DA: Mechanisms of urokinase plasminogen activator (uPA)-mediated atherosclerosis: Role of the uPA receptor and S100A8/A9 proteins. J Biol Chem 286: 22665-22677, 2011.
95. Kimura M, Sudhir K, Jones M, Simpson E, Jefferis AM and Chin-Dusting JP: Impaired acetylcholine-induced release of nitric oxide in the aorta of male aromatase-knockout mice: Regulation of nitric oxide production by endogenous sex hormones in males. Circ Res 93: 1267-1271, 2003.
96. Adams MR, Golden DL, Register TC, Anthony MS, Hodgin JB, Maeda N and Williams JK: The atheroprotective effect of dietary soy isoflavones in apolipoprotein $E^{-/-}$ mice requires the presence of estrogen receptor-alpha. Arterioscler Thromb Vasc Biol 22: 1859-1864, 2002.
97. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgin J, Shaul PW, Thoren P, Smithies O, *et al*: Abnormal vascular function and hypertension in mice deficient in estrogen receptor beta. Science 295: 505-508, 2002.
98. Hodgin JB, Krege JH, Reddick RL, Korach KS, Smithies O and Maeda N: Estrogen receptor alpha is a major mediator of 17beta-estradiol's atheroprotective effects on lesion size in $Apoe^{-/-}$ mice. J Clin Invest 107: 333-340, 2001.
99. Hurtado R, Celani M and Geber S: Effect of short-term estrogen therapy on endothelial function: A double-blinded, randomized, controlled trial. Climacteric 19: 448-451, 2016.
100. Zheng S, Chen X, Hong S, Long L, Xu Y, Simoncini T and Fu X: 17β-Estradiol inhibits vascular smooth muscle cell migration via up-regulation of striatin protein. Gynecol Endocrinol 31: 618-624, 2015.
101. Zhang G, Li C, Zhu N, Chen Y, Yu Q, Liu E and Wang R: Sex differences in the formation of atherosclerosis lesion in $apoE^{-/-}$mice and the effect of 17β-estrodiol on protein S-nitrosylation. Biomed Pharmacother 99: 1014-1021, 2018.