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Correlation between DNA double strand breaks and cell death in peripheral blood lymphocytes from breast cancer patients.

Ángela Solana-Peña[1], Monica Pujol-Canadell[1], Juan-Sebastián López[1], Miquel Macià[2,3,4], Evelyn Martínez Pérez[2,3], Isabel Linares[2,3,4], Milica Stefanovic[2,3], Héctor Pérez-Montero[2,3], Javier González-Viguera[2,3], Marina Arangüena Peñacoba[2,3], Montse Ventura[2,3], Ferran Guedea[2,3,4], Nadina Erill[5], Víctor González-Rumayor[6], Gemma Armengol[1], Joan Francesc Barquinero[1*]

[1] Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain

[2] Radiobiology and Cancer, Institut d'Investigació Biomédica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat, Barcelona, Spain

[3] Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat, Barcelona, Spain

[4] Clinical Sciences Department. Facultat de Medicina i Ciències de la Salut. University of Barcelona, 08908 Hospitalet de Llobregat, Barcelona, Spain

[5] Atrys Health, Provenza 392, 08025 Barcelona, Spain

[6] Atrys Health, Principe de Vergara 132, 28002 Madrid, Spain

* Corresponding author: Joan Francesc Barquinero Estruch, Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain. email: Francesc.Barquinero@uab.cat; Telephone number: +34935811317

ORCID NUMBERS IN ALPHABETICAL ORDER

Ángela Solana-Peña, 0000-0001-5275-5278

Mònica Pujol-Canadell, 0000-0002-7403-9683

Juan-Sebastián López, 0000-0002-9384-7504

Miquel Macià, 0000-0002-7328-4767

Evelyn Martínez Pérez, 0000-0002-3463-5333

Isabel Linares 0000-0002-6205-3859

Milica Stefanovic, 0000-0001-9293-4385

Héctor Pérez-Montero 0000-0002-9756-5088

Javier González-Viguera, 0000-0001-9750-4667

Marina Arangüena Peñacoba, 0009-0000-6093-6845

Montse Ventura, 0000-0002-3366-8360

Ferran Guedea, 0000 0001 8798 8696

Nadina Erill, 0000-0003-3530-2669

Víctor González-Rumayor, 0000-0002-3205-1502

Gemma Armengol, 0000-0003-2345-1106

Joan Francesc Barquinero, 0000-0003-0084-5268

Notes on contributors

Ángela Solana-Peña, Ph.D. student, Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Mònica Pujol-Canadell, Post-Doctoral fellow, Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Juan-Sebastián López, Ph.D., Biologist, Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Miquel Macià, MD, MSc, Radiation Oncologist, Assistant Professor Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona - Spain ; Clinical Sciences Department. Facultat de Medicina i Ciències de la Salut. University of Barcelona (UB), L'Hospitalet de Llobregat, Barcelona, Spain.

Evelyn Martínez Pérez, MD, Ph.D., MSc, Radiation Oncologist, Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain.

Isabel Linares, MD, Ph.D., Radiation Oncologist, Associate Professor Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona - Spain ; Clinical Sciences Department. Facultat de Medicina i Ciències de la Salut. University of Barcelona (UB), L'Hospitalet de Llobregat, Barcelona, Spain.

Milica Stefanovic, MD, Ph.D., Radiation Oncologist, Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain.

Héctor Pérez-Montero MD, Ph.D., MSc, Radiation Oncologist, Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain.

Javier González-Viguera, MD, MSc, Radiation Oncologist, Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain.

Marina Arangüena Peñacoba, MD, MSc, Radiation Oncologist, Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain.

Montse Ventura, MSc, Project Manager, Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge (ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona - Spain

Ferran Guedea, MD, Ph.D., MSc, Radiation Oncologist, Full Professor, Radiobiology and Cancer, Institut d'Investigació Biomèdica de Bellvitge

(ONCOBELL-IDIBELL), Hospital Duran i Reynals, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona - Spain ; Radiation Oncology Department. Institut Català d'Oncologia, Gran Via de l'Hospitalet, 199-203. 08908 Hospitalet de Llobregat. Barcelona – Spain; Clinical Sciences Department. Facultat de Medicina i Ciències de la Salut. University of Barcelona (UB), L'Hospitalet de Llobregat, Barcelona, Spain

Nadina Erill, Ph.D., Biologist, R&D coordinator, Atrys Health, SA. Provença 392. 08025 Barcelona

Victor González-Rumayor, Ph.D., Geneticist, Research & Data Intelligence Director, Atrys Health. Principe de Vergara 132, 28002 Madrid.

Gemma Armengol, Ph.D., Biologist, Associate Professor, Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Joan Francesc Barquinero, Ph.D., Biologist, Full Professor, Unit of Biological Anthropology, Department of Animal Biology, Plant Biology and Ecology, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Abstract:

Purpose: Radiotherapy affects both cancer and healthy cells, potentially causing side effects. Factors like total dose administered or patient individual characteristics, such as genetic variability, influence radiotoxicity. Several biomarkers have been proposed to predict radiotoxicity, especially those based on apoptosis or DNA damage, like γ-H2AX, which correlates with DNA breaks. Our aim is to analyze how apoptosis and γ-H2AX correlate to each other and to link these results with selected SNPs associated with apoptosis.

Material and methods: Blood samples from 60 breast cancer patients in remission were collected. Mononucleated cells were isolated and γ-H2AX kinetics was evaluated at 1, 2, 4 and 24 h after 2 Gy irradiation. Apoptosis in CD8+ lymphocytes was assessed at 24 and 48 h after 8 Gy irradiation. SNP genotyping was performed for 27 apoptosis-related SNPs.

Results: Individual basal levels of γ-H2AX and apoptosis correlated positively with those observed at different post-irradiation times. High interindividual variability was observed in both γ-H2AX and apoptosis under all tested conditions. When both biomarkers were analyzed together, a negative correlation was observed between basal and residual γ-H2AX levels and apoptosis at 48 h post-irradiation. SNP analysis identified two apoptosis-associated SNPs located at *TP53* and *FAS* genes.

Conclusions: These findings support previous studies done in cancer patients showing a negative correlation between apoptosis and γ-H2AX. Overall, our results seem to indicate that individuals with less efficiency in removing damaged cells, probably due to genetic polymorphisms, presented more basal and residual levels of DNA damage.

Keywords: Radiosensitivity; polymorphisms; radioinduced apoptosis; radioinduced γ-H2AX; DNA damage response.

1. Introduction

Radiation therapy (RT) is a common and effective treatment for several types of cancer thanks to the ability of high-energy radiation to kill cancer cells or inhibit their growth. According to the American Cancer Society, more than 50% of patients with cancer receive RT (American Cancer Society 2024). Although it can be administered concomitantly with other treatments, such as chemotherapy or immunotherapy, RT is applied as monotherapy in about 15% of patients with solid cancer (Deloch et al. 2016). However, RT not only affects cancer cells but also healthy tissues or organs, which can develop adverse side effects, like erythema, ulcers and fibrosis (Majeed and Gupta 2023). Currently, technologic advances in RT, like stereotactic RT or intensity modulated RT, have improved dose conformation, delivering the maximum dose to the tumour and diminishing the exposure of healthy tissue and hence avoiding healthy tissue damage (Yarnold 2019; LaRiviere and Vapiwala 2022; Majeed and Gupta 2023). Despite this, it is accepted that between 5 and 10% of patients treated with RT will present adverse tissue reactions (Barnett et al. 2009; Greve et al. 2012). In accordance with the Hsu hypothesis (Hsu 1983), it is assumed that variation of healthy tissue reactions follows a Gaussian distribution, in which individuals affected by rare mendelian diseases that confer radiosensitivity, such as ataxia telangiectasia, Nijmegen syndrome, Fanconi's anaemia and Bloom syndrome, represent the most sensitive cases. However, the incidence of these diseases does not explain the assumed percentage of 5 to 10%. Several factors can influence the observed variation, some of them are related to treatment itself (total dose administrated, fractionation dose, irradiated volume and irradiated body part), additional treatments (concomitant chemotherapy and/or surgery), or patient individual characteristics (age, sex and co-morbid conditions) (Bentzen and Overgaard 1994; Constine et al. 2021). But the characteristics mentioned above only explain about 20% of the variation and the remaining variation is likely due to genetic factors (Turesson et al. 1996; Andreassen et al. 2002). Due to the increasing number of cancer patients and therefore, of patients who will receive RT in the future, there is an interest in the search of biomarkers capable of predicting the toxicity resulting from RT. Currently, the most studied biomarkers are those based on DNA damage and on cell death in response to radiation, as well as germline genetic variants or radio-induced changes in gene expression (Greve et al. 2012;

Gomolka et al. 2020; Naderi et al. 2023; Kolnohuz et al. 2024). In this sense, one of the earliest DNA damage detection events is the phosphorylation of the histone H2AX (γ-H2AX) at the DNA sites with double-strand breaks (DSB) (Adams et al. 2012; González JE et al. 2012). The analysis of γ-H2AX has been suggested as biomarker of radiotoxicity in several studies where ex vivo lymphocytes were irradiated at doses ranging from 0.5 to 2 Gy. Some of these studies have described that the DSB repair capacity is diminished in cancer patients with RT toxicity (Lobachevsky et al. 2016; Schuler et al. 2014). Other studies have proposed the residual post-irradiation γ-H2AX foci as a radiosensitivity predictor (Adams et al. 2012; Goutham et al. 2012; Li et al. 2013; Van Oorschot et al. 2016).

Another group of well-established DNA damage biomarkers are based on cytogenetic techniques (Vinnikov et al. 2020). Some studies have seen an increase of radio-induced chromosomal aberrations in patients with toxicity in non-tumoral tissue after RT (Matsubara et al. 1988; Greulich-Bode et al. 2012; Fahrig et al. 2018; Dunst et al. 1995). Moreover, by using the G2 assay, several authors have found that the lower ability to arrest cell cycle damaged cells is related to radiosensitivity in breast, head and neck, colorectal and prostate cancer, as well as in some types of paediatric cancer (Barber et al. 2000; Andreassen et al. 2002; Brzozowska et al. 2012) Furthermore, evaluating chromosomal aberrations by premature chromosome condensation techniques, some studies have shown an increase in extra chromosomal fragments in patients that suffer RT side effects (Kondrashova et al. 1997; Borgmann et al. 2002, 2008). Finally, some previous works have suggested that micronuclei, which are formed when a broken chromosome fragment remains outside the nucleus, are a good marker to detect patients with increased radiosensitivity (Widel et al. 2003; Lee et al. 2003).

The ability of cells to enter in programmed cell death or apoptosis is another possible biomarker of radiation sensitivity (Biechonski et al. 2018). It has been proposed that *ex vivo* radiation-induced apoptosis of cytotoxic T lymphocytes (CD8+), known as RILA assay, may help to predict late toxicity induced by RT. Some studies show that patients with side effects after RT present lower levels of apoptosis than patients without them (Ozsahin et al. 2005; Schnarr et al. 2009;

Chua MLK et al. 2014; Azria et al. 2015). Variability in apoptosis levels depends on several factors, like the influence of genetic polymorphisms and their interactive network (Cavalcante et al. 2019; Mališić et al. 2022).

Genetic variations are assessed in radiogenomic studies with the aim of proposing them as biomarkers capable of predicting which individuals may develop adverse effects after RT. There are different types of studies, such as those in which differences in gene expression are studied (Dai et al. 2021) but also those in which different single nucleotide polymorphisms (SNP) are the target of study (Alsbeih et al. 2014). In the latter, the reported genes participate in different pathways that may affect the response to radiation, including apoptosis (e.g. *TP53*), cell proliferation (e.g. CDKs), cell cycle regulation (e.g. *ATM* or *CHK2*) or DNA repair (e.g. *XRCC4*). Additionally, genes associated with the response to oxidative stress, as well as those related to inflammatory and pro-fibrotic cytokines, have also been related to RT (Andreassen et al. 2006; Barnett et al. 2012; Lumniczky et al. 2021). However, these findings have not been replicated in another cohort (Tam et al. 2023) .

Notably, most of the above-mentioned studies have analyzed the radiosensitivity biomarkers individually. Few of them have tested the correlation between markers and particularly with genetic variants. Considering all the evidence mentioned above, the objective of the present work is to study how apoptosis and a DNA damage biomarker, such as γ-H2AX, correlate to each other in a group of patients who had been treated for breast cancer and to link these results with selected SNPs associated with apoptosis. This study is part of a larger study in which a cohort of patients is being followed to determine factors, either treatment-related or intrinsic to the patients, that may affect the appearance of adverse late effects after RT.

2. Materials and methods

2.1 Patients

The study comprised 60 females, aged between 43 and 73 years, who had been treated for breast cancer and who were recruited for the study between 3 and 15 years after therapy. All of them were in complete remission. Patients were recruited in the Radiation Oncology Department at Institut Català d'Oncologia,

Barcelona, Spain. The recruitment period was from November 2020 to March 2023. All patients received RT, 40 of them with a boost treatment and the mean dose received was 57.31 ± 8.9 Gy. Out of these patients, 33 were also treated with chemotherapy, 50 with hormone therapy and 7 with monoclonal anti-HER2 antibody. This study was approved by the Research Ethical Committee of the Bellvitge Hospital and Institut Català d'Oncologia (PRED-RAD, reference PR044/20). All patients signed a written informed consent before blood collection and research methods followed Good Clinical Practice (GCP) guidelines and were performed in accordance with the Declaration of Helsinki. Clinical data needed in this study was only available by clinicians. Anonymized information was available from September-2024 to November-2021-4 for the other authors for research purposes.

2.2 Peripheral blood samples obtention

During a follow-up medical appointment, a peripheral blood sample of 27 ml was obtained by phlebotomy. Out of these ml, 18 ml of blood were collected in sodium heparin tubes (Vacutest, Arzergrande, Italy), 9 ml of blood were placed in EDTA tubes, both were transported to the Universitat Autònoma de Barcelona. Lymphocyte isolation was performed, from the sodium heparin tubes, using lymphosep solution (Biowest, Nuaillé, France) following the manufacturer's protocol. Once isolated, lymphocytes were diluted in 10.5 ml of RPMI 1640 (Biowest) supplemented with 15% of fetal bovine serum (FBS) (Life Technologies, Madrid, Spain). Then, the lymphocyte suspension was divided into seven different tubes, each containing 1.5 ml at a final lymphocyte concentration of 35,000 cells/ml. These tubes were incubated overnight at 37ºC. Five of these tubes were used for the γ-H2AX assay and two for the apoptosis assay. DNA extraction was carried out from the EDTA tubes.

2.3 Irradiation conditions

For the γ-H2AX assay, four tubes were irradiated at 2 Gy and one tube was non-irradiated. For the apoptosis assay, one tube was irradiated at 8 Gy and another tube was non-irradiated. Lymphocyte suspensions were irradiated with a ^{137}Cs-source (IBL437C, CIS Bio international, Gif-Sur-Yvette, France) located in a separated building in the Universitat Autònoma de Barcelona Irradiations were

done in air at room temperature, and dose rate ranged from 4.27 to 4.05 Gy/min due to the source decay. After irradiation, lymphocytes were placed on ice to halt DNA repair during transportation to the laboratory for 10 min.

2.4 γ-H2AX immunofluorescence staining and flow cytometry analysis

For each patient, we evaluated the background level of γ-H2AX, and the kinetics of its appearance and repair after 1, 2, 4 and 24 h of incubation at 37ºC. In a previous study no differences in the background levels of γ-H2AX were observed in the non-irradiated lymphocytes between 0.5 and 24 h incubation (López et al 2022). For this reason, the non-irradiated sample was processed at the same time as the 1 hour post irradiated sample. To detect the presence of γ-H2AX, first, cells were fixed in 2% paraformaldehyde (Sigma-Aldrich Quimica, Madrid, Spain) for 5 min at 4ºC and then cells were centrifuged at 600 g for 5 min. After that, the supernatant was removed, and 70% ethanol was added at least for 2 h at -20ºC. Then, cells were centrifuged at 600 g for 5 min, next the supernatant was removed, and cells were resuspended with 1xPBS and centrifuged again at 600 g for 5 min. Afterwards, 1xPBS-0.5% Tritonx100 (Sigma-Aldrich) was added for 10 min for cell permeabilization. Then, cells were washed with 1xPBS. A blocking treatment was conducted for 30 min with 1xPBS-0.1% Tween20 (Sigma-Aldrich) and 2% of FBS. Next, cells were centrifuged and after supernatant removal, they were incubated overnight at 4ºC with the mouse monoclonal anti-γ-H2AX (Ser139) antibody (Abcam, Cambridge, UK) diluted 1:500 in 1XPBS-0.1% Tween20 and 2% FBS. After this first incubation, cells were washed twice with 1xPBS-0.1% Tween20 solution and a second incubation was done using a secondary anti-mouse antibody, labelled with FITC (Invitrogen, Thermo-Fisher, Eugene, OR, USA) diluted 1:400 in 1XPBS-0.1% Tween20 and 2% FBS. DNA staining was done with a solution of 1 µl of propidium iodide (PI) (1 mg/ml) and 1 µl of RNase (1 mg/ml) in 1 ml of 1xPBS.

Analysis of γ-H2AX was done using the Cytoflex flow cytometer (Beckman Coulter, Indianapolis, IN, USA). This cytometer has two active lasers of 488 nm and 638 nm. To measure the γ-H2AX intensity of fluorescence in suspension, FITC and PI were excited with the 488 nm laser to detect γ-H2AX and DNA content respectively. For each sample the fluorescence intensity of approximately 10.000 events was recorded. The values of γ-H2AX fluorescence at different

post-irradiation time points and in non-irradiated samples were measured using FlowJo vX.0.7 software (FlowJo, Ashland, OR, USA). Considering the raw data was in logarithmic scale, the geometric Mean Fluorescence Intensity of FITC (MFI-FITC) was used for comparisons (Wanotayan et al. 2022). Apoptotic cells, which typically display elevated γ-H2AX fluorescence, were excluded from the analysis through a gating strategy based on cell size and morphology, and the DNA content.

2.5 Radio-induced apoptosis assay with Annexin V/ PI

The ability of CD8+ lymphocytes from each patient to enter apoptosis was evaluated 24 and 48 h after 8 Gy irradiation. At the same time, non-irradiated samples were evaluated to determine the basal apoptosis level. To perform the apoptosis assay, the Annexin-V-FLUOS-Staining Kit (Roche, Barcelona, Spain) was used according to manufacturer's instructions. Apoptosis was evaluated in the CD8+ lymphocyte population. For this reason, the APC anti-human CD8+ mouse antibody, SK1 clone, (Biolegend, San Diego, CA, USA) was added to the kit mix. Viable, Early Apoptotic (EA) and Late Apoptotic (LA) cells were scored by the Cytoflex flow cytometer. The CD8+ lymphocytes and Annexin V positive cells were identified using the 638 nm laser and the PI positive cells using the 488 nm laser. For each sample approximately 50.000 events were counted. Results were then analyzed using the FlowJo vX.0.7 software.

To evaluate the radio-induced cell death, each irradiated sample was normalized by subtracting its own non-irradiated control. Live cells were defined as cells negative for Annexin V and PI. EA cells were defined as positive cells for Annexin V and negative for PI and LA cells were defined as cells positive for both Annexin V and PI. For comparisons, total radiation-induced apoptosis (EA+LA) was also considered.

2.6 DNA extraction, quantification and SNP genotyping

DNA was extracted from blood samples using the QIAamp DNA Blood Midi Kit (QIAGEN, Hilden, Germany) and quantified using the Qubit dsDNA BR Assay Kit

(Invitrogen) following manufacturer's instructions for both kits. All DNA samples were diluted to a final concentration between 30 and 50 ng/ul. DNA was transferred to an Open Array Plate (Thermo-Fisher) with 96 wells. A total of 27 SNPs in genes related to apoptosis selected from the literature were evaluated. Studied SNPs are located in the following genes: *ADRB2, BAX, BCL2, BIRC5, CASP8, FAS, FASLG, GSTM1, GSTP1, iASPP, LGALS3, MCL1, MDM2, PDCD5, PDE4B, RASSF1, TNF* and *TP53*. Specific studied positions can be seen in Supplementary material 1.

2.7 Statistical analysis

Data were analyzed by GraphPad Prism version 8.0.1 (GraphPad Software, Boston, MA, USA) setting as statistically significant a p value < 0.05. First, normality tests (Shapiro-Wilk test and Kolmogorov-Smirnov test) were performed to γ-H2AX analysis values. After that, the Wilcoxon test between the different time points and the Man-Whitney test between means of the slopes of the γ-H2AX disappearance were performed. Apoptosis assay was analyzed with t-test between two groups corresponding to 24 h and 48 h post-irradiation. Correlation of individual values, either for γ-H2AX results or apoptosis rate, was tested also with Spearman test.

In the genotyping assay, potential prognostic variables, in this case SNPs genotype and covariates, such as clinical parameters, were evaluated to know their implication in cell death. First, two different apoptosis groups were established using the median of the total radiation-induced apoptosis observed at 48 h post-irradiation as the cut-off. Genetic variants were assessed for association with apoptosis using R 4.2.1 software (R core team 2024) and R package SNPassoc 2.1.0 (González JR et al. 2007). First, Hardy-Weinberg equilibrium was explored for the 27 SNPs using the Chi-square test. Then, logistic regression was used to test for association under five genetic models for each SNP (codominant, dominant, recessive, overdominant and log-additive). Moreover, some covariates were added to analyze their implication in the model. These covariates were: qualitative, such tumour and nodule types, stage of the tumour; binary, as presence of metastasis, smoking habit, dyslipidemia,

chemotherapy, hormone therapy and monoclonal antibody therapy; and quantitative, using continuous numerical values, such as age, total RT dose and boost dose. Bonferroni correction was applied to adjust for multiple testing.

3. Results

3.1 γ-H2AX assay

Descriptive statistics of the results of γ-H2AX analysis are shown in Table 1. There was a wide range of the observed values for the MFI-FITC; in the non-irradiated samples and the irradiated samples evaluated at different post-irradiation times. To figure out if some values were significant outliers, the ROUT test was applied, using as a Q value the recommended 1%. Out of the 300 measurements, five were considered as outliers (two in the non-irradiated samples, one in the analysis performed 2 h post-irradiation and two in the 4 h post-irradiation time). Once these outlier values were removed, normality was evaluated using the Kolmogorov-Smirnov test. Only the MFI-FITC values 24 h post-irradiation agrees with the normal distribution. For this reason, further comparisons were conducted using non-parametric tests.

The MFI-FITC was significantly different between each post-irradiation time point, except between 1 and 2 h post-irradiation (Fig 1). The basal or non-irradiated (NI) value of γ-H2AX intensity median level was always significantly lower than those observed after irradiation, even after 24 h of repair. Individual MFI-FITC values observed in the non-irradiated samples were positively correlated to those reported after 1, 2, 4 and 24 h post-irradiation. The R values were 0.38 ($p = 0.003$), 0.44 ($p = 0.001$), 0.56 ($p < 0.0001$) and 0.57 ($p < 0.0001$), respectively. Despite showing this positive correlation between the different post-irradiation points, the interindividual results show a big variability, with CVs higher than 29% even after outlier correction. When the DNA repair kinetics of γ-H2AX disappearance from 1 to 24 h were compared between the two groups with different apoptosis levels, the mean slopes were not statistically different ($p= 0.358$).

3.2 Apoptosis assay

For each individual, the EA and LA percentages from CD8+ lymphocytes observed after 24 and 48h non-irradiation and post-irradiation at 8 Gy (Table 2) were considered jointly after subtracting the respective values observed in the non-irradiated samples; to avoid outliers, negative results were removed. As can be seen in Fig 2, the percentage of radio-induced apoptosis at 48 h post-irradiation was statistically higher than that observed at 24 h.

To study deeply the reason for these differences, EA and LA at each time point were studied separately. In the irradiated samples and for both post-irradiation times 24 and 48 h, the percentages of EA and LA cells showed differences (Fig 3). After 24 h the percentage of EA cells was higher than that after 48 h (11.24 ± 7.18 vs. 8.60± 7.18, $p = 0.04$); and inversely after 48 h the percentage of LA cells was higher than after 24 h (43.84 ± 16.28 vs. 25.66 ± 14.92, $p = 0.0001$).

For both 24 and 48 h post-irradiation times, a positive correlation was noticed between the individual values of EA and LA in non-irradiated samples and the EA and LA values after 8 Gy irradiation ($p < 0.05$). Similarly to the γ-H2AX results, total radiation-induced apoptosis shows a high individual variability, as indicated by the CVs, which are 54% at 24 h and 41% at 48 h, respectively.

3.3 Correlation between γ-H2AX and apoptosis

The potential correlation between the apoptotic rate at 24 and 48 h and the γ-H2AX results were evaluated. After 8 Gy irradiation and after 24 h of incubation, individual level of apoptotic cells (considering together EA and LA) did not show any correlation with the observed MFI-FITC values at any post-irradiation time. However, after 48 h post-irradiation the apoptosis values showed a negative correlation with the basal values of MFI-FITC ($R = -0.31$, $p = 0.023$) and with radiation-induced MFI-FITC observed 24 h post-irradiation ($R = -0.41$, $p = 0.002$) (Fig 4).

3.4 SNP genotyping

The genotype distribution of all SNPs followed the Hardy-Weinberg equilibrium, except for *TNF* rs1799724 (p = 0.03). Out of the 27 SNPs evaluated, SNPassoc analysis found differences between the two apoptosis groups, established with the results obtained at 48 h, where the negative correlation with γ-H2AX level was significant. These differences were in: *TP53* rs1625895 (C/C vs C/T, OR = 6.88, 95% confidence interval (CI) [1.71-27.75], p = 0.003) in codominant model, *FAS* rs1800682 (G/G vs G/A+A/A, OR = 0.18, 95% CI [0.05-0.63], p = 0.004) in dominant model, *BCL2L11* rs724710 (C/C vs C/T+T/T, OR = 0.32, 95% CI [0.11-0.94], p = 0.034) in dominant model and *MCL1* rs9803935 (G/G+G/T vs T/T OR = 0.27, 95%CI [0.07-0.96], p = 0.034) in recessive model (Table 3). After using Bonferroni correction and for α = 0.05, none of them were statistically significant. Due to the limited number of patients forming our cohort, α = 0.1 was also considered. With α = 0.1, two statistically significant genes were identified: TP53 rs1625895, corresponding to a C>T change, where the T allele was more prevalent in patients with high levels of apoptosis under the codominant model; and FAS rs1800682, corresponding to an A>G change. Most patients showed the AG genotype, as expected since it is the most common in the population, and the A allele was more frequent in the group with low apoptosis levels under the dominant model. None of the covariates, such as age or the total dose received during the treatment, for example, was statistically significant in our analysis.

4. Discussion

Ionizing radiation is able to produce several types of DNA damage and all of them activate specific DNA repair mechanisms (Nikjoo et al. 2024). DSBs, which occur in smaller amounts compared to other DNA damage types, are considered the ones with a more biological impact. They are related to rearrangements of genomic material (inducing chromosomal aberrations) and to cell death (Reisz et al. 2014; Cheng et al. 2022). The analysis of γ-H2AX allows the evaluation of DSB induction as well as their repair kinetics. The γ-H2AX assay is widely used to study factors that can influence the DNA damage response and has been proposed to be used in clinical studies to identify radiosensitive patients (Dickey et al. 2009; Barroso and Aguilera 2021). In the present study, DSB were measured by detecting γ-H2AX, which is a common procedure in biological

dosimetry studies (López et al. 2022). However, non-specific signals cannot be entirely ruled out, as immunostaining inherently carries the risk of variable background signals. Notwithstanding this limitation, the highest value of γ-H2AX intensity was observed at 1 h post-irradiation, which agrees with other studies proving that the maximum levels of γ-H2AX occur between 0.5 and 1 h (Rothkamm and Horn 2009). Since constitutive levels of γ-H2AX can be considered to show the capacity to solve endogenous DNA damage (Tanaka et al. 2006), it could be hypothesized that the individual differences are related to the DSB repair capacity (Berthel et al. 2019). Despite the methodological limitations of the present study indicated above, the positive correlation observed between the individual basal values and the ones obtained at each post-irradiation time seems to indicate that those individuals less efficient in resolving endogenous DSBs are also those that show lower efficiency in resolving radiation-induced DSBs. Interestingly, in a previous study evaluating 94 healthy individuals, age, alcohol consumption and ethnicity were found to influence baseline γ-H2AX levels, whereas ethnicity and alcohol influenced repair kinetics (Sharma et al. 2015). In our study, all women belonged to the same ethnic group and alcohol consumption was not reported. When considering age, no correlation was seen with either initial γ-H2AX levels or repair kinetics. The variability in the γ-H2AX levels obtained in each evaluated condition highlights a limitation of the assay as a biomarker of individual radiosensitivity.

After exposure to ionizing radiation, in case of severe or irreparable DNA and to prevent cells with mutations from progressing or producing genomic instability, cells can trigger cell death (Biechonski et al. 2018). Currently, many forms of programmed cell death are described: intrinsic or extrinsic apoptosis, necrosis, ferroptosis, piroptosis and mitotic catastrophe, among others (Jiao et al. 2022). In G0 lymphocytes, ionizing radiation mainly promotes intrinsic apoptotic pathway (Maier et al. 2016). Radiation induced damage in the intracellular structures like DNA activates the intrinsic pathway releasing cytochrome C from mitochondria. In addition, ionizing radiation can also promote the extrinsic apoptosis, which is started by death receptors present in the cell membrane. In G0 cells, another conceivable way to produce apoptosis would be due to the membrane stress. In this case, radiation induces reactive oxygen species that oxidate cell membrane lipids triggering this cell death pathway. The present study confirms that

apoptosis can be better evaluated after 48 h post-irradiation rather than after 24 h as was previously reported (Foro et al. 2014; Azria et al. 2015), which may be because the EA cells at 24 h become LA cells at 48 h, increasing the percentage and in consequence the statistical power. However, an important aspect when considering the individual level of total radiation-induced apoptosis is the high variability observed, which is greater at 24 h than at 48 h. This variability also limits the use of apoptosis as an individual biomarker of radiosensitivity.

When the two biomarkers of radiation sensitivity, γ-H2AX and apoptosis, were correlated, the results indicated a significant negative correlation between the percentage of apoptotic cells, induced after 8 Gy and evaluated 48 h post-irradiation and the γ-H2AX values observed in the non-irradiated samples and in the samples irradiated at 2 Gy and analyzed 24 h later. Nevertheless, due to the high variability in the individual values of these two biomarkers, the observed negative correlation should be interpreted with caution. In a previous study with 16 breast cancer patients, Chua et al. (Chua MLK et al. 2014) found a negative, although non-significant, correlation between the mean number of γ-H2AX/53BP1 observed 24 h post-irradiation at 4 Gy and the mean apoptosis rate 48 h post-irradiation at 8 Gy. Later the same group (Chua KLM et al. 2018), evaluating 172 nasopharynx cancer patients, also saw a significant inverse association between residual DSB and percentage of apoptosis. These authors suggested that the negative correlation was due to a subgroup of patients with some intrinsic genomic or epigenomic factors that result in a blunting of the DNA damage response. Other authors have also observed this negative correlation, evaluating breast cancer patients before the start and at 1 and 2 weeks of RT (Marková et al. 2015). Although our results cannot be considered conclusive, they seem to support the existence of a negative correlation between apoptosis and background and residual levels of γ-H2AX. The negative correlation by using “ex vivo” irradiated lymphocytes suggests that those individuals that are more efficient at removing damaged cells through apoptosis are those that show lower levels of background or residual levels of DNA damage. In fact, the observed negative correlation appeared only in non-irradiated samples and after 24 h post-irradiation. This suggests that apoptosis is likely the main factor contributing to the negative correlation. No correlation was found when γ-H2AX was measured at 1, 2, and 4 h after irradiation. This result further supports that DNA repair is not

playing a major role in this observation. In addition, we compared DNA repair kinetics between the two groups with different levels of apoptosis. When we analyzed the mean slopes of γ-H2AX disappearance curves in these two groups, we did not observe any statistically significant differences. This suggests that their DNA repair capacities were similar.

To assess whether the variability observed in apoptosis rates was related to genetic polymorphisms, we performed a SNP analysis. The two SNPs differentially represented in the two groups were detected after Bonferroni correction at $\alpha = 0.1$. This α was selected because of the limited patient cohort size and the considerable variability in the apoptosis results. These two SNPs are related to different types of apoptosis. The first SNP was rs1625895 in the gene coding for TP53, which is activated in the presence of DNA damage, and it also engages in the intrinsic apoptosis pathway. This transcription factor induces the production of pro-apoptotic proteins of the Bcl-2 family, which allow the release of cytochrome C and the subsequent activation of Caspase-9, starting the cell death process itself (Yang et al. 2017). In both groups of patients, the most common genotype was CC, as described in the general population (Ensembl Genome Browser 2023). The positive association of this SNP with the radio-induced apoptosis rate followed a codominant model and CT genotype was more present in patients that showed elevated percentages of radio-induced apoptotic cells. Accordingly, the T allele has been observed in cells having a greater ability to enter apoptosis (Voropaeva et al. 2015).

The second SNP that was differentially represented in the two groups was rs1800896 in the gene coding for FAS, which is related to the extrinsic pathway of apoptosis. It is known that radiation causes an increase in the production of ligands, such as FasL and TNF-alfa, which bind to their cell membrane receptors to form the Death Inducing Cell Signalling Complex. This complex activates the Caspase-8, which triggers apoptosis (Jiao et al. 2022). For this SNP, our patients mostly presented the AG genotype, which agrees with what is expected from the general population (Ensembl Genome Browser 2023). In our study, this SNP showed a negative association with the radio-induced apoptosis rate in a dominant genetic model. Previous studies have reported that the G allele is associated with a lower apoptosis level but also with a higher risk to develop

breast cancer (Ji et al. 2009; Wang et al. 2016). Contrary to expectations, our results show that GG genotype is more present to the group with higher radio-induced apoptosis. A possible explanation for this contradictory result could be that this SNP is also associated with an increase in risk to develop breast cancer. Since our cohort is only composed of breast cancer patients, this can mask the statistical output.

Based on this result, we checked whether those patients with the higher apoptosis rate who presented the CT genotype in rs1625895 are the same as those with GG and AG genotypes for the rs1800682. Out of 13 patients with CT for rs1625895, 10 patients have the GG (5 patients) or AG (5 patients) genotype of rs1800682. This indicates that despite having genotypes (GG or AG) related to low levels of extrinsic apoptosis, the CT genotype, related to higher levels of intrinsic apoptosis, seems to have greater weight. These results support the idea that the intrinsic pathway, due to DNA damage, appears to play a more key role in radiation-induced apoptosis than the extrinsic pathway (Maier et al. 2016).

In conclusion, the results here presented seem to indicate that breast cancer patients with less efficiency in removing damaged cells have more background and residual levels of DNA damage, and that this variability could be explained by individual genetic variations. This negative correlation should be validated in studies including non-cancer patients and cancer patients evaluated before RT treatment. In addition, further studies will be carried out to test whether these and other biomarkers are associated with adverse RT effects in these patients.

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Declaration of interest statement

No potential conflict of interest was reported by the authors.

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Authors contribution

Conceptualization: Monica Pujol-Canadell, Miquel Macià, Evelyn Martínez Pérez, Ferran Guedea, Victor González-Rumayor, Gemma Armengol, Joan Francesc Barquinero

Data curation: Ángela Solana-Peña, Juan-Sebastián López

Formal analysis: Ángela Solana-Peña, Monica Pujol-Canadell, Gemma Armengol, Joan Francesc Barquinero

Funding Acquisition: Ferran Guedea, Victor González-Rumayor, Joan Francesc Barquinero

Investigation: Ángela Solana-Peña, Monica Pujol-Canadell, Juan-Sebastián López

Methodology: Monica Pujol-Canadell

Project administration: Monica Pujol-Canadell, Gemma Armengol, Joan Francesc Barquinero

Resources: Miquel Macià, Evelyn Martínez Pérez, Isabel Linares, Milica Stefanovic, Héctor Pérez-Montero, Javier González-Viguera, Marina Arangüena Peñacoba, Ferran Guedea.

Software: Ángela Solana-Peña

Supervision: Gemma Armengol, Joan Francesc Barquinero

Visualization: Ángela Solana-Peña, Monica Pujol-Canadell, Montse Ventura, Ferran Guedea, Nadina Erill, Victor González-Rumayor, Gemma Armengol, Joan Francesc Barquinero,

Writing- original draft: Ángela Solana-Peña, Joan Francesc Barquinero

Writing- review and editing: Ángela Solana-Peña, Monica Pujol-Canadell, Gemma Armengol, Joan Francesc Barquinero

Data Availability Statement

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878 **Tables**

879 **Table 1. Descriptive statistics of the geometric Mean Fluorescence Intensity**
880 **of FITC (MFI-FITC) values after γ-H2AX assay.**

a)	Time after irradiation				
	NI	1 h	2 h	4 h	24 h
	N = 60	N = 60	N = 60	N = 60	N = 60
Maximum	195470	321368	330946	324173	214257
Minimum	48474	63915	58295	68420	42324
Median	72820	138462	129690	118745	107953
SD	29524	53029	57117	48682	37367
Mean	81411	149122	145154	131840	108569
SEM	3812	6846	7374	6285	4824
CV	36.27%	35.56%	39.35%	36.92%	34.42%

b)	Time after irradiation				
	NI	1 h	2 h	4 h	24 h
	N = 58	N = 60	N = 59	N = 58	N = 60
Maximum	136750	321368	304116	234972	214257
Minimum	48474	63915	58295	68420	42324
Median	72494	138462	128748	117529	107953
SD	22672	53029	52090	37808	37367
Mean	77865	149122	142005	126119	108569
SEM	2977	6846	6781	4964	4824
CV	29.12%	35.56%	36.68%	29.98%	34.42%

881

882 a) Crude data; b) ROUT correction; NI, non-irradiated; N, number of patients; SD,
883 standard deviation; SEM, standard error of the mean; CV, coefficient of variation.

884

885

886 **Table 2. Descriptive statistics of the percentages of apoptotic cells in non-irradiated and irradiated samples.**

	N.I.				8Gy			
	24 h		48 h		24 h		48 h	
	EA	LA	EA	LA	EA	LA	EA	LA
	N=58	N=58	N=58	N=58	N=60	N=60	N=60	N=60
Maximum	26.20	31.69	29. 00	58.50	49. 00	92.10	35.20	91.70
Minimum	0.25	0.44	1.40	1.07	0.04	3.47	0.04	2.20
Median	9.81	7.40	10.35	15.60	20.50	31.95	18.75	64.30
SD	6.73	7.82	6.98	15. 00	10.21	21.91	9.02	22.63
Mean	10.80	10.61	11.11	19.94	21.42	36.98	17.69	59.19
SEM	0.88	1.05	0.92	1.90	1.32	2.83	1.16	2.92
CV	62.36	73.67	62.79	73.81	47.69	59.25	50.96	38.24

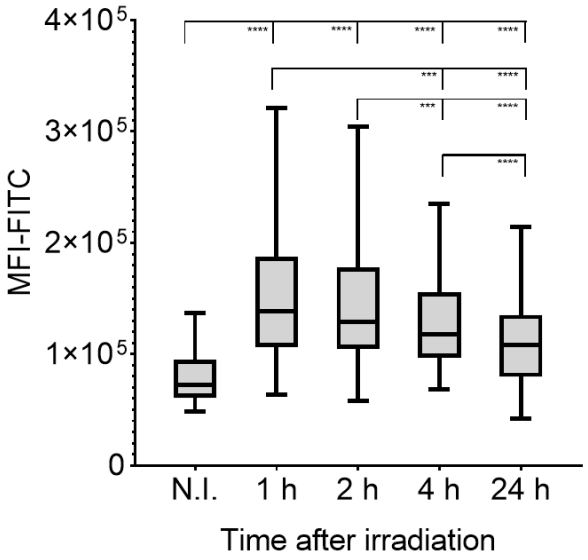
887

888 Early and late apoptotic rate (EA and LA) after 24- and 48-hours incubation at 37ºC for non-irradiated
889 samples (N.I.) and samples irradiated at 8 Gy; N, Number of patients; SD, standard deviation; SEM,
890 standard error of the mean; CV, coefficient of variation.

Table 3. Association of selected polymorphisms with apoptosis.

Polymorphism			Group of study			P value				
SNP	Gene	Genotype	Lower Apoptosis	Higher Apoptosis	Total	Codominant	Dominant	Recessive	Overdominant	Log AD
rs1625895	*TP53*	C/C	27	17	44	0.003*	-	-	-	-
		C/T	3	13	16					
		T/T	0	0	0					
rs1800682	*FAS*	G/G	4	14	18	0.016	0.004*	0.345	0.069	0.017
		A/G	18	11	29					
		A/A	8	5	13					
rs724710	*BCL2L11*	C/C	8	16	24	0.087	0.034	1	0.038	0.104
		C/T	19	11	30					
		T/T	3	3	6					
rs9803935	*MCL1*	G/G	8	7	15	0.06	0.766	0.034	0.038	0.272
		G/T	11	19	30					
		T/T	11	4	15					

SNP, Single Nucleotide Polymorphism; Log AD, Log-Additive *Statistically significant p value after Bonferroni correction (α = 0.1).



894

895 **Figure 1. Box-plot with mean geometric fluorescence intensity of FITC (MFI-**
896 **FITC)**. Values for each evaluated condition, non-irradiated (NI) and irradiated at
897 2 Gy, analyzed 1, 2, 4 and 24 h after irradiation. (****p < 0.0001, ***p < 0.001).

898

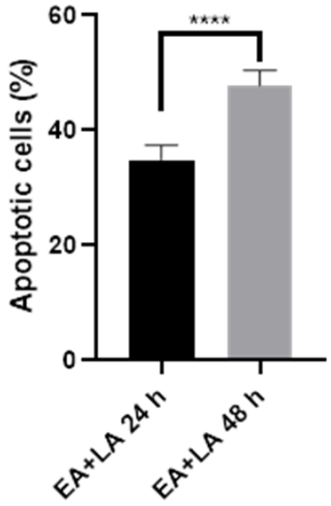
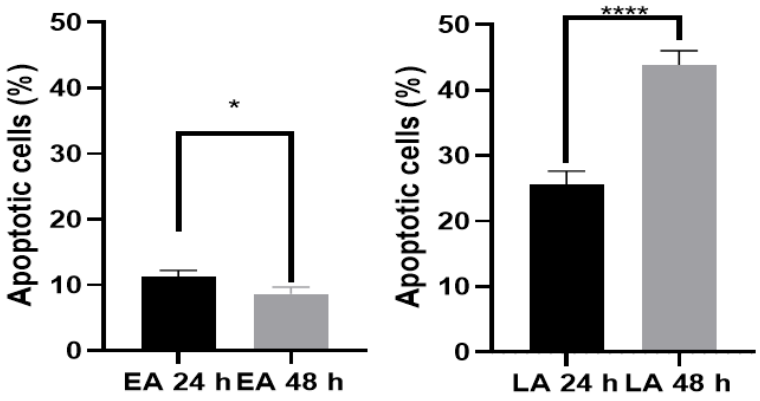


Figure 2. Percentage of total radiation-induced apoptotic cells (Early and Late-apoptosis, EA+LA). Evaluation after 24 h and 48 h post-irradiation at 8 Gy (**** $p < 0.0001$). Mean values and ± SEM values were used



905

906 **Figure 3. Radio-induced apoptosis measurement sample after 8 Gy.** Figures
907 show early (EA) and late apoptotic cells (LA) analyzed at 24 and 48 h after
908 irradiation (* $p<0.05$, **** $p<0.0001$). Mean and ± SEM values were used.

909

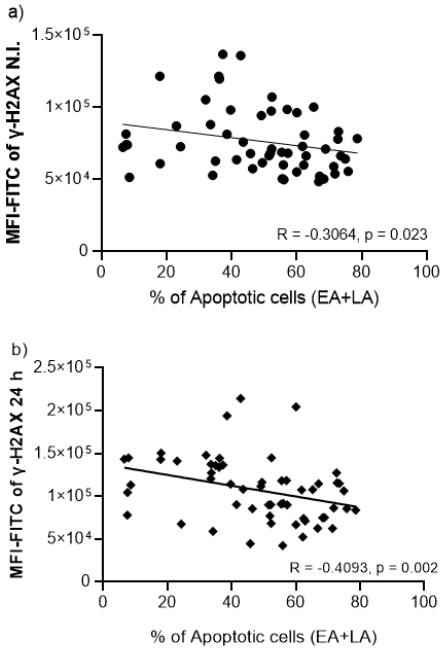


Figure 4. Correlation between the percentage of apoptotic cells and the geometric Means Fluorescence Intensity of FITC (MFI-FITC) of γ-H2AX. a) Apoptosis was measured after 48 h post-irradiation and MFI-FITC was measured in non-irradiated (N.I.) cells; and b) Apoptosis was measured after 48 h post-irradiation and MFI-FITC was measured after 24 h post-irradiation at 2 Gy.