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IONIZING RADIATION OR MITOMYCIN INDUCED-MICRONUCLEI IN LYMPHOCYTES OF *BRCA1* OR *BRCA2* MUTATION CARRIERS

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ABSTRACT

BRCA1 and *BRCA2* genes are essential in preserving the integrity of genome, and it is not unambiguously clear whether the heterozygosity status may affect BRCA1 or BRCA2 functions. This may have implications for the clinical management of *BRCA1* and *BRCA2* mutation carriers both in breast cancer (BC) screening modality and in cancer treatment based on DNA-damaging or DNA-repair-inhibiting drugs. We investigated whether lymphocytes carrying *BRCA1* or *BRCA2* mutations displayed an increased sensitivity to radiation or mitomycin C *in vitro* treatments.

Peripheral blood from 21 *BRCA1* mutation carriers (12 with BC and 9 healthy), 24 *BRCA2* carriers (13 with BC and 11 healthy), 15 familial BC patients without detected mutation in *BRCA1* or *BRCA2*, and 16 controls without familial history of cancer (5 with BC and 11 healthy) were irradiated or treated with mitomycin C (MMC). Chromosomal damage was measured using the cytokinesis-block micronucleus assay. We evaluated micronuclei (MN) and nucleoplasmic bridges (NPB).

The *BRCA2* mutation carriers and familial BC patients without detected mutation in *BRCA1* or *BRCA2* showed less basal NPB than *BRCA1* carriers and controls. The $BRCA1^{+/-}$ or $BRCA2^{+/-}$ lymphocytes did not have increased frequencies of MN or NPB after irradiation. In contrast, $BRCA2^{+/-}$ lymphocytes presented higher levels of MN after MMC exposure than *BRCA1* carriers and controls.

The monoallelic *BRCA1* or *BRCA2* pathogenic mutations seem not to be associated with an enhanced radiosensitivity. The mutation of one *BRCA2* allele conferred an increased sensitivity to MMC, presumably because of the role of this gene in the repair of MMC-induced DNA damage. This finding indicates that the MMC-induced MN analysis could be useful in identifying functional deficiencies of *BRCA2* or genes related to *BRCA2*. Since MMC can be used as an anti-cancer drug, these data may be relevant for the management and follow-up of *BRCA2* mutation carriers.

Keywords: *in vitro* radiation, mitomycin c, micronuclei, *BRCA1* and *BRCA2*.

INTRODUCTION

Heterozygous germline mutations in *BRCA1* or *BRCA2* tumour suppressor genes (*BRCA1/2*) confer susceptibility to breast (BC) and ovarian cancers (OC) with high penetrance, and account for approximately 20% of BC cases associated with family history [reviewed in ref. 1].

BRCA1/2 genes are important in the maintenance of genome stability. The main role of *BRCA2* appears to involve regulation of the *RAD51* function in DNA repair by homologous recombination. *BRCA1* is an E3 ubiquitin ligase that has a broader role upstream of *BRCA2*, participating in DNA repair, transcriptional regulation, cell cycle progression and meiotic sex chromosome inactivation [reviewed in refs. 2,3]. *BRCA1*$^{-/-}$ or *BRCA2* $^{-/-}$ cells from chicken DT40 knockout cell lines, knockout mice and tumour-derived human cell lines exhibit spontaneous chromosomal instability and are hypersensitive to DNA-damaging agents such as ionising radiation (IR) and DNA cross-linking agents [2,3].

In contrast, whether some functions of the BRCA1/2 proteins are diminished or deregulated while still heterozygous or only after the loss of both functional alleles is not yet well established [4,5]. The heterozygous status (*BRCA1*$^{+/-}$ or *BRCA2*$^{+/-}$) might contribute to the impairment of genomic stability and increase the risk of cancer promoting mutations, contributing to the loss of the remaining wild-type *BRCA1/2* allele [4-6]. Since BRCA1 and BRCA2 proteins are involved in DNA repair, *BRCA1*$^{+/-}$ or *BRCA2*$^{+/-}$ cells of mutation carriers may also have enhanced sensitivity to DNA-damaging agents, such as ionising radiation (IR) or mitomycin C (MMC); this phenotype may increase toxicity and the carcinogenic risk of chemotherapy, radiotherapy and mammography screening in *BRCA1* or *BRCA2* mutation carriers [4,5]. If the functions of the BRCA1/2 proteins are affected by heterozygosity status, two mechanisms may explain this phenotype: haploinsufficiency or reduction in gene dosage, and/or dominant negative mutations. Several studies have claimed the existence of phenotypes associated with *BRCA1/2* haploinsufficiency, whereas others have raised the possibility that BRCA1 or BRCA2 mutated proteins may block the function of the remaining wild-type *BRCA1/2* allele [reviewed in ref. 4].

Several studies in blood lymphocytes to know whether the heterozygosity for *BRCA1/2* mutations confers detectable sensitivity to genotoxic agents have been performed with contradictory results [reviewed in ref. 7]. Here, we have studied chromosomal sensitivity to ionising radiation or MMC treatments of peripheral blood lymphocytes from *BRCA1* or *BRCA2* germ-line mutation carriers with or without BC, familial BC patients without detected mutation in *BRCA1/2*, and controls with or without BC and no familial history of cancer. The chromosomal damage was measured by quantification of micronuclei (MN) and nucleoplasmic bridges (NPBs) using the cytokinesis-block micronucleus assay (CBMN). The *in vitro* proliferation capacity of the treated lymphocytes was also evaluated by the nuclear division index (NDI).

The MN in dividing cells are the result of fragments or whole acentric chromosomes/chromatids that lag behind in anaphase and are not included in the daughter nuclei in telophase [8]. These fragments can be originated from non-repaired or misrepaired DNA double-strand breaks (DSBs) [8]. Misrepair of DSBs could also lead to the formation of asymmetrical chromosome rearrangements producing dicentrics or ring chromosomes and acentric fragments. The centromeres of the dicentric chromosomes, dicentric ring chromosomes or concatenated double rings can be pulled to opposite poles of the cells at anaphase resulting in the formation of a nucleoplasmic bridges (NPB) between the daughter nuclei [9]. Therefore, the presence of MN or NPB could indirectly reflect the cell DNA repair capacity [8]. In fact, increased frequencies of spontaneous and mutagen induced MN or NPBs have been observed in different cells

deficient in DNA repair mechanisms such as non-homologous DNA end-joining [10] or homologous recombination [11,12].

The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring MN in cultured human cells because scoring is specifically restricted to once-divided binucleated (BN) cells, which are the cells that can show MN [8]. The inhibition of cytokinesis by cytochalasin B allows to discriminate between cells that did not divide after treatment and cells that did divide, thus preventing the confounding effects caused by differences in cell division kinetics [8]. Because cells are blocked in the BN stage, it is also possible to measure nucleoplasmic bridges [9]. In addition to micronuclei in binucleated cells and NPB, the CBMN assay also allows for scoring of other critical events, such as cell division and cell cytotoxicity by scoring of proportion of mono-, bi- and multicucleated cells to calculate the nuclear division index (NDI) [8]. To date the CBMN assay is one of the best-validated methods for measuring chromosome damage in human lymphocytes [13].

MATERIALS AND METHODS

Subjects and study design

Heparinized blood samples were collected from 76 women between 2004 to 2007: 60 with a family history of BC and OC and 16 without a family history of cancer. The ethical committee of the hospital approved the study and all women participating signed an informed consent.

Women with a family history of cancer were recruited from those attending the Cancer Genetics Clinic of Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. Subjects were referred for genetic assessment because of familial BC/OC, early onset BC or previous identified *BRCA1/2* mutation in the family. The mutation analysis of the *BRCA1* and *BRCA2* genes was performed in women fulfilling one of the following criteria: families with one or more cases of BC (at least 1 case diagnosed before age 50) and 1 or more cases of OC; site-specific female BC families with 2 or more cases (at least 1 diagnosed at 50 years of age); families with at least 1 case of BC or OC in addition to at least 1 male case of BC.

The entire coding sequence of *BRCA1* and *BRCA2* was analyzed for sequence variants using denaturing high-performance liquid chromatography (DHPLC) and for large deletions and duplications using Multiplex Ligation-dependent Probe Amplification (MLPA) (MRC Holland). In those women referred due to a *BRCA1/2* mutation previously identified in the family, the analysis was performed by direct sequencing or MLPA.

The mutational status of the 60 individuals with family history was: 21 *BRCA1* mutation carriers (12 with BC and 9 healthy), 24 *BRCA2* mutation carriers (13 with BC and 11 healthy) and 15 females with familial BC/OC without detected mutation in *BRCA1/2* (14 with BC and 1 with OC). The *BRCA1/2* mutations are shown in Table 1.

As controls, blood samples were also collected from 16 women without family history of BC/OC: five with sporadic BC and 11 healthy women. The sporadic BC patients came from those attending the Medical Oncology Department and the healthy subjects were recruited from the laboratory staff of the Genetics Department of the Hospital.

Considering the disease status of the analysed subjects, 31 were healthy and 45 had BC/OC. Most of the BC/OC patients (n=31) had received different combinations of adjuvant chemotherapy: cyclophosphamide, methotrexate and 5-fluorouracil (CMF) (n=7); 5-fluorouracil, epirubicin and cyclophosphamide (FEC) (n=6); adriamycin and cyclophosphamide (n=3); FEC in a high-dose chemotherapy regimen (with autologous transplantation) (n=2); FEC, trastuzumab and taxanes (n=2); CMF, adriamycin, taxol and capecitabine (n=2). The rest of patients (n=9) received individually different regimens each: FEC, taxanes and carboplatin; cyclophosphamide, adriamycin and taxol; FAC (5-fluorouracil, adriamycin and cyclophosphamide); FEC and taxanes; FAC and taxanes; FEC and CMF; CMF, MMC and vinblastine; anthracyclines; cyclophosphamide. Four patients required neo-adjuvant chemotherapy (2 patients with CMF, 1 with FEC and 1 with epirubicin and doxorubicin). Three patients required chemotherapy because of BC recurrence (one patient was treated with CMF, one with CMF, MMC and vinblastine, and one with MMC and vinblastine).

Culture and treatment with mutagenic agents

Peripheral blood cultures were set up by adding 0.5 mL of whole blood to 4.5 mL of RPMI 1640 medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 15% heat-inactivated foetal calf serum (PAA Laboratories GmbH), 1% antibiotics (penicillin and streptomycin), 1% L-glutamine (provided by Gibco, Invitrogen Corporation, Paisley, United Kingdom) and 1% phytohaemagglutinin (PHA)

to stimulate the division of T-lymphocytes (Gibco). Six cultures were set up for each subject immediately after drawing blood: 2 that will be treated in the G_0 phase with 2 Gy of gamma irradiation (before incubation at 37ºC with PHA), 2 that will be treated in the G_1/S phase with 0.05 µg/ml of MMC (24h after the beginning of the culture with PHA) and 2 replicates without any treatment.

Irradiation of the two respective cultures was done just after re-suspending the blood with the supplemented RPMI 1640 medium. All irradiations were performed at room temperature with 2 Gy of gamma irradiation from a source of Co-60 (Theratron 780 radiotherapy unit, Theratronics Ltd., Canada) at a high dose rate (HDR) of 0.6-0.8 Gy/min. To minimize the dose gradient within the tubes, the cultures were irradiated at a large distance (80 cm from the cobalt source). A polymethyl methacrylate (PMMA) scattering slab was placed on the irradiated tubes to minimize the build-up effect and to guarantee uniform irradiation.

Immediately after irradiation the 6 cultures per individual were incubated at 37ºC. After 24h of incubation and stimulation with PHA, mitomycin-C (Sigma, St. Louis, Missouri, USA) was added to the 2 corresponding cultures to obtain a final concentration of 0.05 µg/mL and cultures were returned to the incubator.

After 44 h of incubation with PHA at 37ºC, cytochalasin-B (Cyt-B, Sigma, St. Louis, Missouri, USA) was added to the 6 cultures to a final concentration of 6 µg/mL to arrest cytokinesis. Seventy-two hours after incubation with PHA at 37ºC the cells were centrifuged (800 rpm for 8 min at room temperature), the supernatant was aspirated, and the cells were re-suspended in a hypotonic solution (0.075 M KCl) for 3 min at 4°C. The cells were re-centrifuged, and a 3:1 (v/v) methanol:acetic acid solution gently added. This fixation step was repeated twice and the cells were then re-suspended in a small volume of fixative solution and dropped onto clean slides. The air-dried slides were subsequently stained with 1 µg/mL of 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, Missouri, USA) and 2 µg/mL of propidium iodide (PI; Sigma) in an antifade solution (Vectashield; Vector Laboratories, Burlingame, CA, USA). The slides were stored at 4°C until assessed by microscopy. All slides were randomized and coded before being scored by the same observer to minimize variability. The slides were scored under the 100x objective of a Nikon Eclipse E400 fluorescence microscope (Nikon, Tokyo, Japan) equipped with a triple-band-pass filter to visualize the nuclei and MN, in bright blue (DAPI), and the cytoplasm, in red (PI). The presence of MN was confirmed using the filter to view only the DAPI stain.

To determine the presence of both micronuclei (MN) and nucleoplasmic bridges (NPB), a total of 1000 binucleated cells with well-preserved cytoplasm for each subject (500 per replicate) were blind scored on coded slides according to previously described criteria [8]. In addition, a total of 500 lymphocytes (250 cells per replicate) were examined to determine the proportion of cells with different numbers of nuclei (1-4 nuclei) in order to calculate the nuclear division index (NDI) [8].

Statistical methods

In the untreated cultures, the outcome variables analyzed were the basal values of MN, NPB and NDI. In the treated cultures, the cytogenetic endpoints evaluated were the number of radiation or mitomycin-C-induced MN (calculated by subtracting the basal yields from the yields obtained in the treated samples), the absolute values of NPB after the mutagenic treatments and, the NDI index values in untreated cultures less NDI values in treated cultures.

The analysis included 2 factors: carrier/non-carrier, with 4 levels (*BRCA1* carrier, *BRCA2* carrier, familial BC without detected mutations in *BRCA1/2*, and controls without familial history of BC/OC) and, disease with 2 levels (healthy women or cancer patients groups). Potential confounding factors of MN were also considered.

Age at time of experiments, smoking habit, and number of cigarettes were recorded for all subjects. Given that the blood samples were not obtained concurrently and that experimental conditions may have varied between groups, the days elapsed since the first blood culture were calculated (as a centered variable) for each analyzed subject. For cancer patients the age at cancer diagnosis and the years elapsed between the end of radiotherapy and chemotherapy and time of MN cultures were also recorded.

Basic descriptive statistics of outcome and continuous variables were computed for all subgroups. The means for between-group demographic and clinical variables were compared using the *t*-test. To quantify the potential association among continuous and outcome variables, correlation matrices analyses were performed using the Pearson correlation coefficient.

The between-group differences in basal and treatment-induced MN, NPB and NDI were analyzed using generalized linear models (GLZ). A logarithmic link function was applied to the basal level of MN and NPB to achieve a best fit. The continuous variables that were shown by the correlation analysis to have a possible effect were included in the analyses. In the GLZ analysis of NPB after mutagens treatment, the basal NPB values were considered as a covariable since the treatment-induced NPB values, obtained by subtracting the basal yields from yields obtained after treatment, encompassed negative values which would be difficult to adjust in the GLZ analyses. A backward selection method was used and potential confounding factors that did not reach statistical significance were not considered in the final model. *Post hoc* comparisons using the sequential Bonferroni correction for multiple comparisons were also carried out.

The figures illustrating the between-group differences in this study show the estimated marginal means, which correspond to the mean adjusted for the other terms in the model.

For all analyses, differences were regarded as statistically significant at $P < 0.05$. Statistical computations were performed using the SPSS v.15.0 software (SPSS, Chicago, IL, USA) and the STATISTICA v.8.0 statistical software package (StatSoft, Tulsa, OK, USA).

RESULTS

Demographic, clinical and experimental factors

The mean value of age, days elapsed since first experiment as well as the clinical characteristics of the cancer patient groups are presented in Table 2. The group of all healthy women was significantly younger than the overall group of cancer patients (P<0.001). The peripheral blood cultures for cancer patients were established earlier than the cultures for healthy women and this difference in time, measured as the days elapsed since the first blood culture, was statistically significant (P<0.05). The blood cultures of *BRCA2* mutation carriers (with and without BC) were set up significantly earlier than the cultures of control group (BC patients and healthy women without familial history of BC) (P<0.01). The cultures of familial BC patients negative for *BRCA1/2* mutations were established earlier than the cultures of the all other groups and this difference in time is statistically significant (P<0.001). The average of the years elapsed since last chemotherapy and radiotherapy sessions in the group of familial BC patients that had no detected mutation in *BRCA1/2* is lower, although not statistically significant, than the average of the other BC patient groups.

Basal levels of MN, NPB and NDI

Table 3 shows the data (Mean ± SD) for the 3 endpoints evaluated in the untreated lymphocytes from all groups. The analysis of correlation matrices revealed higher basal MN levels when less time had elapsed since the last session of either chemotherapy or radiotherapy in BC patients. The decline in MN yield with time after cancer treatment showed a reasonably good fit to an inverse relationship (r= 0.799, P<0,001 and r=0.725, P<0.001 for chemotherapy and radiotherapy, respectively) (Fig. 1A). The mean lifetime of mature human T lymphocytes varies from 1.5-10 years [reviewed in ref. 14]. Therefore, the data showed in the Figure 1A are probably reflective of dying off of T lymphocytes with MN that were formed during the time of chemotherapy/radiotherapy. Given that the years elapsed since chemotherapy and radiotherapy were highly correlated, only the variable of chemotherapy was included in the subsequent analyses because it had a higher correlation coefficient with the basal MN level than the radiotherapy variable. The chemotherapy-years variable was inverse transformed in order to achieve a linear distribution. This allowed the assignment of a 0 value to the untreated healthy individuals, thereby including them in the analysis.

The results of the GLZ final model indicated that both cancer status (P=0.014) and the years since last sessions of chemotherapy (P<0.001) had a significant effect on the basal level of MN. Thus, the cancer patients showed higher basal MN frequencies than the healthy subjects irrespective of carrier status (20.16 versus 13.73 as adjusted means for cancer and healthy groups, respectively; P= 0.007) (Fig. 1B). The significant effect of the time since chemotherapy on basal MN indicates that the frequency of MN is higher when less time had elapsed since cancer therapy.

The GLZ analysis of basal NPB showed significant effects of cancer (P=0.027) and carrier status (P=0.018). The NPB in cancer patients were significantly higher than in healthy subjects (1.76 versus 0.81 as adjusted means for cancer and healthy groups, respectively; P= 0.025) (Fig. 1C). The carrier status affected the NPB basal level, since the levels of NPB in untreated cultures in both *BRCA2* carriers (adjusted mean, 0.80) and familial-BC group without detected mutation in *BRCA1/2* (0.63) were lower than the levels found in *BRCA1* carriers (1.81) and in non-carrier controls (2.22) (Fig. 2); nevertheless, the differences between the estimated marginal means, evaluated using the Bonferroni post-hoc test, did not attain statistical significance.

In terms of NDI levels in the cultures without any treatment, the analysis of the GLZ final models indicated no differences between healthy and cancer groups or

between mutation carriers and non-carriers. However, a slight non-significant increase (*P*= 0.062) in the basal NDI was observed in the group of *BRCA2* carriers (adjusted mean, 1.85) compared to that in the *BRCA1* (1.79), *BRCA1*/2 negative (1.75), and non-carrier control (1.77) groups. The final analysis model also showed that both subject age and time elapsed since chemotherapy or radiotherapy significantly affected the proliferation index values. Thus, lower NDI values appeared in older individuals (*P*= 0.002), and in those with less time elapsed since cancer treatment (*P*=0.006).

Effect of the radiation treatment in the G_0 phase

Table 4 shows the mean values of MN and NDI induced by radiation and the absolute values of NPB obtained after radiation treatment. The final model of the GLZ analysis did not indicate any effect of cancer or carrier status on MN and NPB after radiation with 2 Gy. Only days elapsed since first blood culture significantly affected both measures. The values of radioinduced MN were higher at the time of final experiments than those at the time of first experiments (*P*=0.002), and the frequency of NPB also increased with experimentation time (*P*<0.001).

To evaluate whether *in vitro* treatment with IR produced alterations in the capacity of cells to divide, we used the NDI value obtained by subtracting the values obtained after treatment from those obtained in the untreated cultures. The GLZ evaluation to assess the delay in the proliferation capacity after IR did not show any significant effect of carrier status, disease status or any confounding factor.

Effect of the mitomycin-C (MMC) treatment in the G_1/S phase

Table 5 shows the means of MN and NDI induced by MMC treatment and the values of NPB obtained after MMC treatment. The GLZ final models showed that carrier status had a significant effect on MMC-induced MN (*P*<0.001). The lymphocytes of *BRCA2* mutation carriers (irrespective to their disease status) presented higher levels of MN after exposure to MMC (adjusted mean, 135.48) than those of *BRCA1* carriers (96.39), and non-carrier controls (88.45) (*P*<0.001) (Fig. 3). The MMC-induced MN of *BRCA2* carriers was also higher (135.48) than that of familial cancer patients without detected mutation in *BRCA1/2* (111.63), but this difference was not statistically significant (Fig. 3). Furthermore, the frequency of MMC-induced MN increased with the continuous variable of days elapsed since the first blood extraction (*P*<0.001). The NPB obtained after MMC treatment was not significantly affected by carrier and cancer status or by any covariable.

The evaluation of between-group differences after adjusting the values of MMC-induced NDI for the different confounding factors did not show any significant effect of carrier or disease status on cell proliferation capacity after adding MMC to the cultures. The NDI yielded by MMC was only significantly correlated to the years elapsed since treatment for cancer (*P*=0.010). This correlation indicated a lower effect of MMC on cell proliferation with less time elapsed since the last cancer treatment. This apparent resistance to MMC in lymphocytes from subjects just treated with chemotherapy or radiotherapy could be explained by the fact that the cellular proliferating potential has been surpassed by the already lower cell proliferation, observed in the non-treated cultures, in subjects after cancer treatments and the high cytostatic effect of MMC (which is stronger than that of the radiation treatment; see NDI values in Tables 4 and 5).

DISCUSSION

Chromosomal sensitivity (CRS) to radiation and chemical mutagens, quantified by G_0 MN or G_2 chromosome aberrations assays, has been proposed as a marker for low-penetrance predisposition to several common cancers including breast cancer [7,15-17]. Several independent studies have shown enhanced *in vitro* sensitivity to the chromosome-damaging effects of ionizing radiation and other mutagens in a significant number of BC patients compared to normal healthy controls [18-22]. It has been also reported that the enhanced CRS to different mutagens is a heritable trait [19,21,23].

The studies that have investigated CRS to mutagens in blood lymphocytes from patients with germline mutations in *BRCA1* or *BRCA2* have yielded inconsistent results [18,24-35]. While it has been well documented that biallelic inactivation of *BRCA1* o *BRCA2* (*BRCA1*$^{-/-}$ or *BRCA2*$^{-/-}$) genes leads to repair deficiency, and hence to significantly enhanced CRS after treatment with different mutagens, the situation in heterozygous cells (*BRCA1*$^{+/-}$ or *BRCA2*$^{+/-}$) remains elusive.

In our study we assessed the chromosome sensitivity of *BRCA1*$^{+/-}$ or *BRCA2*$^{+/-}$ lymphocytes to ionising radiation or mitomycin-C treatment measuring MN and NPB with the MN G0 phase assay. Our results showed that, in both cancer and healthy women, heterozygosity for *BRCA1/2* mutations did not associated with an increased sensitivity to ionising radiation exposure in the G_0 phase. This result contrasts with the findings in lymphocytes heterozygote for *BRCA1*/2 mutations showing evidence of an enhanced MN radiation induction [7,18,32-35]. However, other studies have not reported a significant increase of MN after radiation in *BRCA1/2* mutated lymphocytes [18,24,30]. These inconsistent results may be due to small population sizes, heterogeneous reference groups and different techniques used for both irradiation and evaluation of MN [7,36].

We found that the frequency of radiation-induced MN as well as the MN induced by MMC increase with the days elapsed since first blood culture. This could not be related to a change in experimental conditions or to reported sources of MN variability such as storage time of the blood samples [37,38] and differences between scorers [39]. To reduce the inter-test variability, a direct comparison of cases with concurrent controls and/or automated counting by image analysis has been suggested [7,32].

The lack of increased radiosensitivity of *BRCA1*$^{+/-}$ or *BRCA2*$^{+/-}$ lymphocytes shown in our study may also suggest that the BRCA1/2 proteins do not play a role in the repair mechanisms acting on the radiation-induced DNA damage in the G_0/G_1 phase such as non-homologous DNA end-joining (NHEJ). NHEJ reseals DNA double-strand breaks (DSBs) efficiently throughout all cell cycle phases but especially in the G1 phase [40]. Homologous recombination (HR), the other mechanism that the cell employs to repair radiation induced DSBs, acts in the S and G_2 phase when a duplicated copy of DNA is available [41,42]. Despite some studies have described that *BRCA1*-deficient cells had significantly reduced NHEJ activity, it is not definitively clear whether *BRCA1* or *BRCA2* are required for efficient NHEJ of radiation-induced DNA breaks [43,44]. In contrast, it is well known the role of *BRCA1/2* in the functioning of HR pathway [2,3].

Given that HR is the most important mechanism for the repair of DSBs induced in the S and G_2 phases [41,42], impaired repair of radiation-induced DSBs may be expected at these phases in lymphocytes of *BRCA1/2* mutation carriers. When analysing the metaphases after irradiation at S phase Barwell et al. [26] found that the lymphocytes of *BRCA1/2* mutation carriers without cancer had increased chromosome breaks compared to age-matched unaffected controls. Blood cells of *BRCA1* mutation carriers irradiated with 8 Gy 24 hours after stimulation and cultured 6 days following irradiation exhibited a significantly higher level of chromosomal damage than those of

non-carrier controls [29]. However, using the G_2 assay, in which metaphases are analysed after irradiation at the G_2 phase of the cell cycle, some authors have described elevated levels of chromosomal aberrations in *BRCA1/2* lymphocytes [18,26-28,45], while other authors have not [24,25].

Knowing whether the normal cells of *BRCA1/2* mutation carriers exhibit a phenotype of *in vitro* enhanced radiosensitivity has implications for their cancer screening and treatment using modalities that involve irradiation. Epidemiological studies that have examined whether *BRCA1/2* mutation carriers have an increased risk of developing BC related to screening mammography have yield inconsistent results [reviewed in ref. 46]. Clinical studies of BC women with *BRCA1/2* mutations have not demonstrated increased acute or late toxicity after radiotherapy [47]. However, Broeks et al. [48] suggested that pathogenic alleles in *BRCA1/2*, *CHEK2* or *ATM* genes increase the risk of radiation-induced contralateral BC after radiotherapy for a first BC. It is clear that more and larger studies are warranted to determine the *in vitro* and *in vivo* radio-sensitivity phenotype of *BRCA1/2* normal cells.

In contrast to the lack of increased *in vitro* sensitivity to radiation observed in our study, *BRCA2* mutations confer enhanced mitomycin-C susceptibility on blood lymphocytes of both cancer and healthy women (Fig 3). Hence, the heterozygosity status for *BRCA2* mutations probably affects the DNA repair capacity for MMC-induced damage in the G_1/S phase of lymphocytes. To our knowledge, there are only 2 published studies on the effects of MMC on normal cells heterozygous for *BRCA1/2* . Shorrocks et al. [49] found no differences in MN response between *BRCA1* +/- and +/+ fibroblasts. Arnold et al. [6] measured by the Comet assay the repair of MMC-induced crosslinks and observed no clear differences in repair capacity between *BRCA2*+/- and *BRCA2* +/+ lymphoblastoid cell lines.

Although the repair of MMC-induced DNA damage in mammals has not been fully delineated, MMC induces several types of DNA damage that are repaired by multiple repair mechanisms such as nucleotide excision repair, HR and trans-lesion bypass repair [reviewed in ref. 50]. Under the experimental conditions used in our study, our results indicate a relevant role for *BRCA2* in the repair of MMC-induced lesions in the G_1/S phase but not for *BRCA1*. In this regard Venkitaraman [5] suggested that defects in DSBs repair in *BRCA1/2* mutated cells arise from distinct roles for BRCA1 or BRCA2 in HR. The major role of BRCA2 protein in DSB repair is the regulation of HR, acting as a mediator of interactions with RAD51 protein, while the functions of BRCA1 in DNA repair are not restricted to HR, performing varied functions in the cellular response to DNA breakage or replication arrest.

Our results with MMC may concur with those of some studies reporting high sensitivity to *in vitro* and *in vivo* MMC treatment of a pancreatic cancer cell line defective in *BRCA2* [51] or the response to a third-line MMC-based chemotherapy of a metastasic pancreatic adenocarcinoma in a *BRCA2* carrier patient [52]. Moreover, treatment of blood lymphocytes with MMC may be used to distinguish *BRCA2* mutation carriers and identify new *BRCA2* interacting genes.

The endpoint of nucleoplasmic bridges (NPB) is an indicator of DNA misrepair and/or telomere end-fusions [8]. With regard to this endpoint, the only significant finding we observed in our study was that *BRCA2* carriers, irrespective of their cancer status, and the familial BC patients without detected mutation in *BRCA1/2* had lower basal levels of NPB compared with those of *BRCA1* and non-carrier control groups (Fig 2). A similar trend was also observed by Beetstra et al. [53], who found that, in folate deficient medium cultures, the lymphocytes of *BRCA2* mutation carriers had a significantly lower frequency of NPB compared with controls. These authors suggested that the lower number of NPB may be due to an extended delay in cell division of *BRCA2* carriers. An extended cell division would allow more time for the bridges to

break during anaphase prior to cell harvesting. Beetstra et al. [53] based their hypothesis on data obtained by Daniels et al. [54], who reported that the period taken for cells to progress from anaphase onset to completion of cell division is significantly extended in cells with targeted gene disruption of *BRCA2* or reduced transcription of *BRCA2* by RNA interference when compared with controls. Likewise, Jonsdottir et al. [55] have demonstrated that *BRCA2* mutation carrier fibroblasts had delayed cytokinesis, being the mean cell division time 6 min longer compared with *BRCA2* wild type cells. However, in contrast to results from these two studies, Lekomtsev et al. [56] have recently indicated that *BRCA2* does not regulate cytokinesis in human cells. Thus, further research is required to verify a role of *BRCA2* in the regulation of cell division. Interestingly, in the familial BC patients without detected mutation in *BRCA1/2* a decrease in the basal NPB formation was also detected. It is tempting to speculate that *BRCA2* and other genes related to cell division might be associated with cancer susceptibility in familial BC patients negative for *BRCA1/2* mutations.

It should be mentioned that the group of familial BC patients who tested negative for *BRCA1/2* mutations did not present a different chromosomal sensitivity (CRS) to either radiation or MMC treatment compared with control non-carriers. These data do not support the findings of the studies carried out by Baeyens et al. [18,24]. They showed that the lymphocytes of non-*BRCA1/2* familial BC patients had a higher CRS to radiation, measured by G_0 MN and G_2 assays, than the control group. Further research will be needed to more precisely determine the CRS in the group of familial BC with no *BRCA1/2* mutations.

The nuclear division index (NDI), provides a measure of the proliferative status of the viable lymphocytes, indicating both the lymphocyte mitogenic response and the cytostatic effects of agents examined in the assay [8]. We did not find any significant difference in either basal or mutagen-induced levels of this proliferation index between *BRCA1/2* carriers and non carrier groups; this would indicate that the lymphocytes from heterozygous *BRCA1/2* mutation carriers have no important defects in the mitogenic response or cellular growth in culture after mutagenic treatments. However, it is interesting to note that, although differences were not statistically significant, the *BRCA2* carriers showed higher basal levels of NDI values than the other groups analysed. A similar finding has been reported by Beetstra et al. [53], who detected that the NDI of *BRCA2* mutation carriers in lymphocytes cultured with folate deficiency tended to be higher than in controls. Thus the *BRCA2* carriers may have a defect in cytokinesis that entails an increased sensitivity to the cytokinesis-blocking action of cytochalasin-B [53]. This may be attributed to an extended time for these cells to progress from anaphase onset to completed cell division, that allows the accumulation of binucleated cells in culture [54,55].

In conclusion, the *BRCA1* or *BRCA2* heterozygote status in peripheral blood lymphocytes is not related to an increased radiosensitivity. But the monoallelic *BRCA2* mutations are associated with a higher level of chromosomal damage induced by MMC, probably due to an impaired DNA repair capacity. This finding suggests that the MMC-induced MN analysis may be used in the identification of individuals with a deficiency in *BRCA2* or genes related to *BRCA2*. Given that MMC can be used as an anti-cancer drug, these data may be relevant for the management and follow-up of *BRCA2* mutation carriers.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

FIGURE CAPTIONS

FIG. 1 Effect of previous chemotherapy and radiotherapy of BC/OC patients on MN and NPB endpoints (A) Relationship between the basal values of MN of the cancer patients and the time passed since last session of chemotherapy. (B) Means adjusted for the other terms in the model and 95% confidence limits showing the differences of levels of MN in the non-treated cultures between cancer and healthy groups. (C) Means adjusted for the other terms in the model and 95% confidence limits illustrating the differences of basal levels of NPB in the non-treated cultures between cancer and healthy groups.

FIG. 2 Adjusted means and 95% confidence limits showing the differences of basal levels of NPB in the non-treated cultures between carrier and non-carrier groups.
BRCA negative: familial breast cancer patients without detected mutation in *BRCA1* or *BRCA2*. Control non carriers: healthy women and sporadic BC patients without history of familial cancer.

FIG. 3 Adjusted means and 95% confidence limits illustrating the differences of MN levels after MMC treatment between carrier and non-carrier groups.
BRCA negative: familial breast cancer patients without detected mutation in *BRCA1* or *BRCA2*. Control non carriers: healthy women and sporadic BC patients without history of familial cancer.

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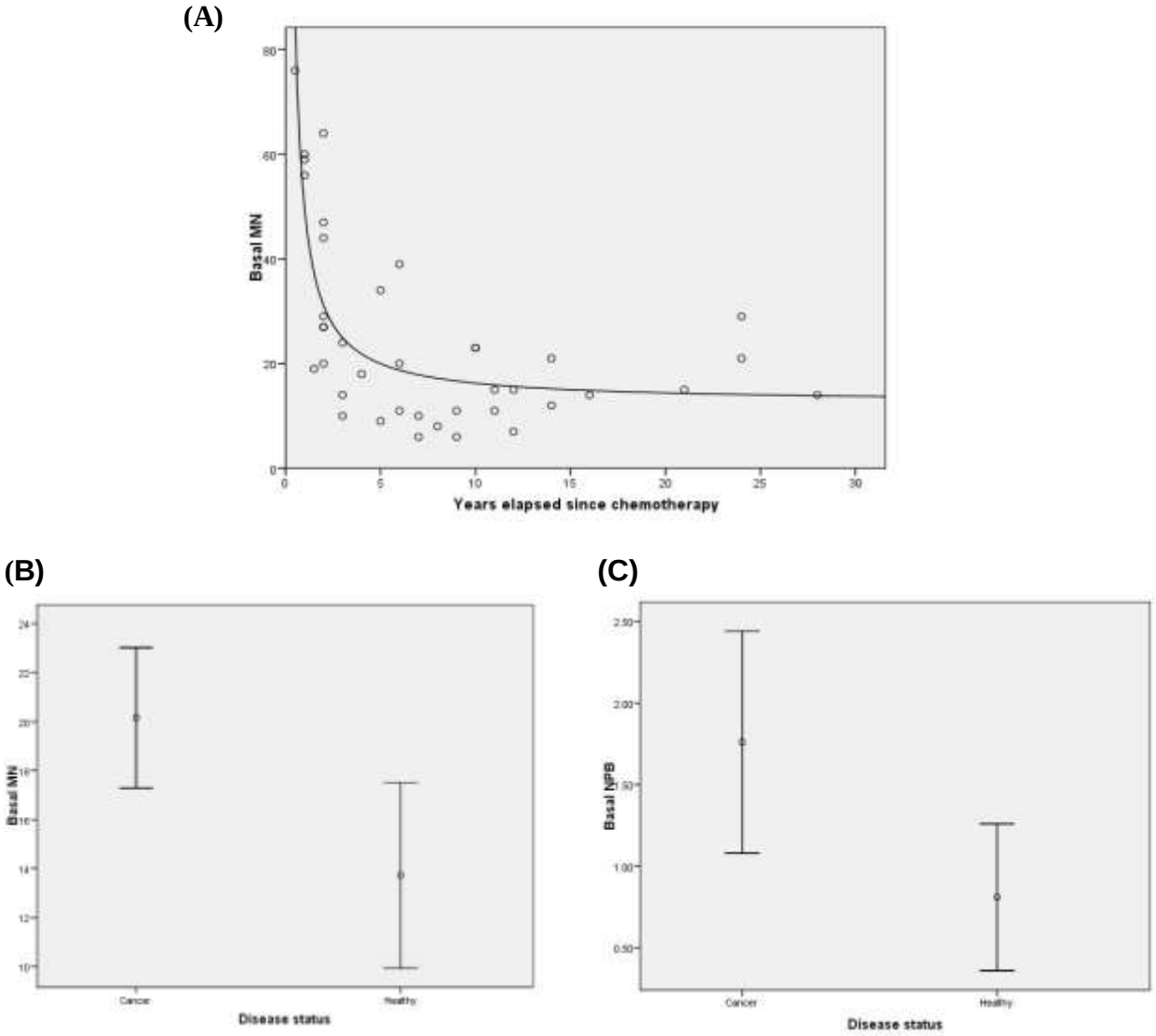

Fig 1

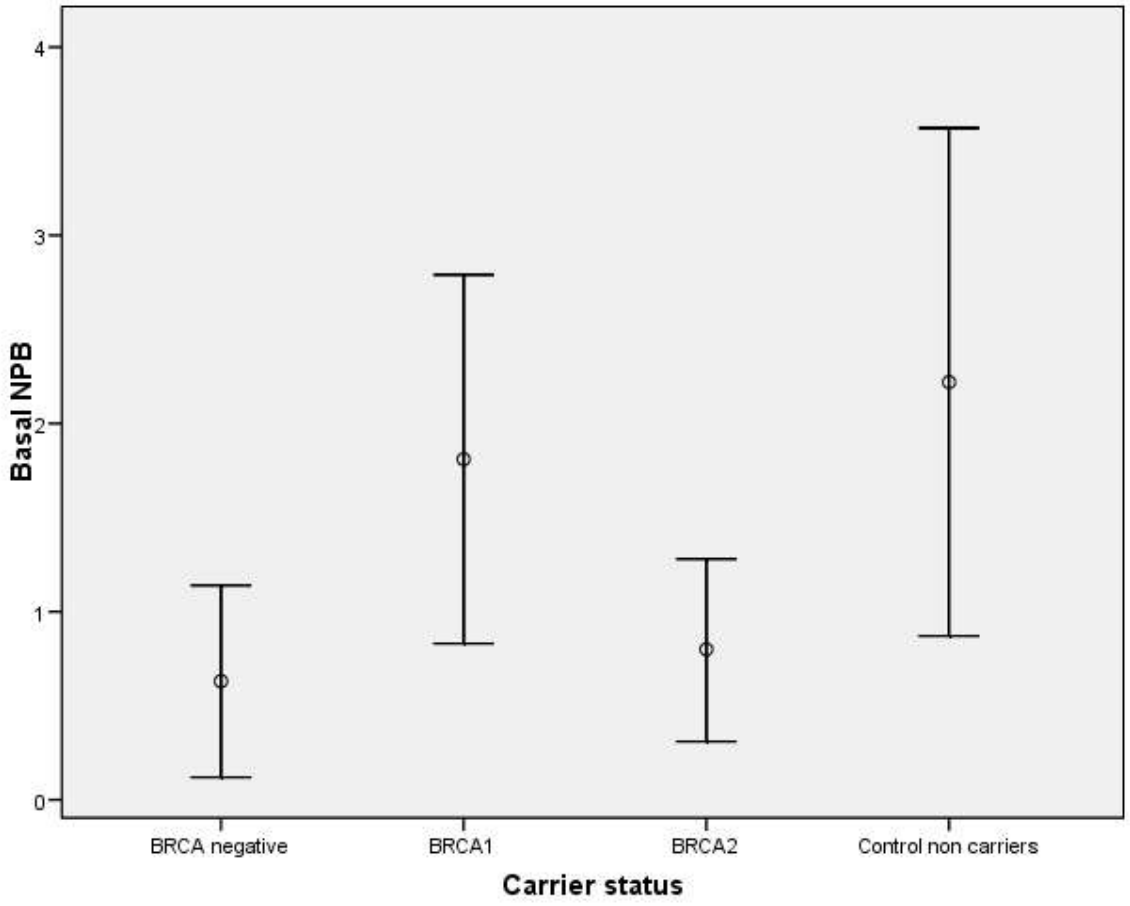
Basal NPB
4
3
2
1
0
BRCA negative
BRCA1
BRCA2
Control non carriers
Carrier status

Fig 2.

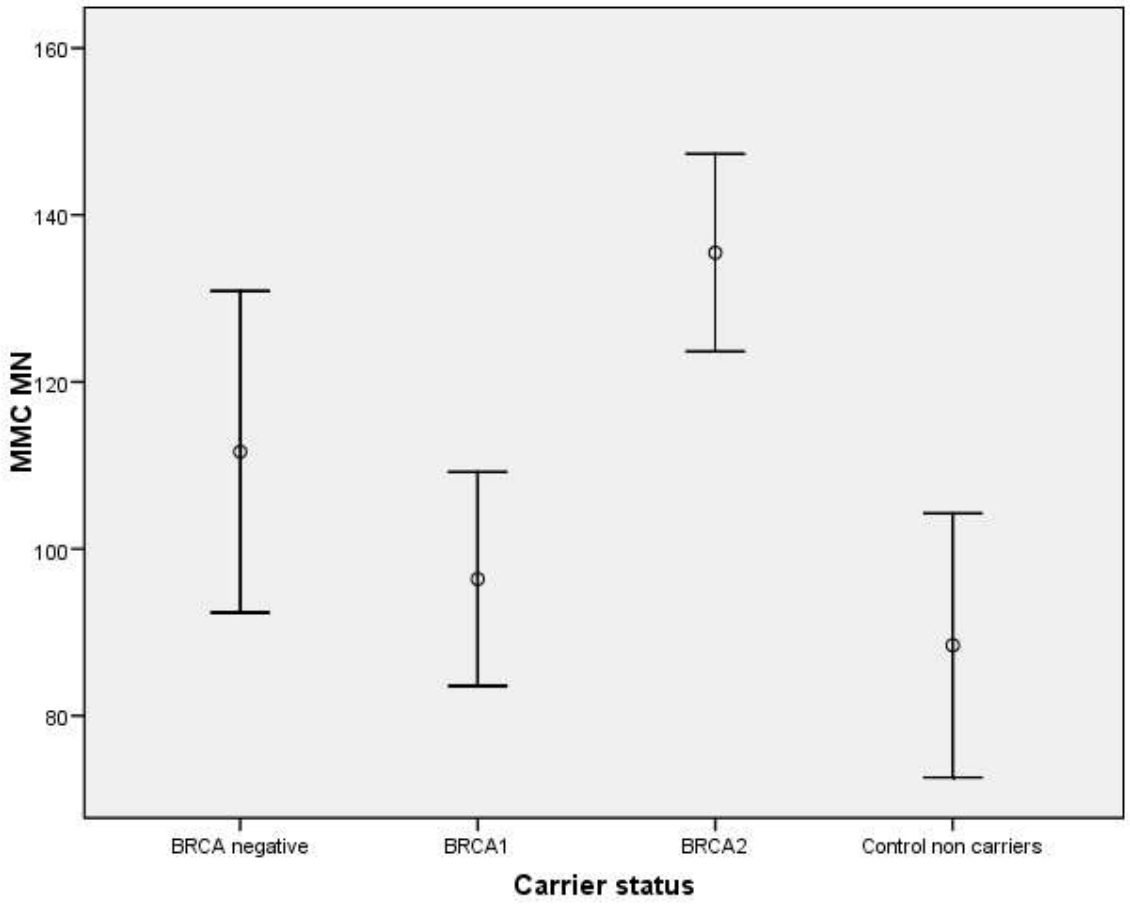

FIG. 3

Table 1. Description of *BRCA1* and *BRCA2* mutations amongst carriers with or without cancer

***BRCA1* carriers**		***BRCA2* carriers**	
with cancer	healthy	with cancer	healthy
185delAG[a]	185delAG[a]	373G>T[g]	373G>T[g]
330A>G	185delAG	del exons 10-12	886delGT
IVS5+1G>A	243delA[b]	1823_1825delA	1823_1825delA
954delC[c]	243delA[b]	3036_3039 del4	3036_3039del4
3478delTT	243delA	3374delA[h]	3036_3039del4
3598del11	del exons 10-12	3683T>G	3374delA[h]
3958del5ins4[d]	954delC[c]	6857delAA[i]	3492insT
3958del5ins4	3958del5ins4[d]	7636delTT	3492insT
5242C>A[e]	IVS22-2A>G[f]	8152_8154 delT	6857delAA[i]
5242C>A[e]	IVS22-2A>G[f]	8297_8300 insTT	9254delATCAT
5537delA		9254delATCAT	
del *BRCA1*		9254delATCAT	
		9514G>T	

Individuals sharing the same superscript letter are members of the same family

Table 2. Mean values and SD of subject age, age of cancer onset, years elapsed since radiotherapy and chemotherapy treatment and days since first experiment.

Group	Nº	Age	Days since first experiment	Cancer onset age	Chemotherapy years[a]	Radiotherapy years[a]
Healthy women	11	40.91 ± 9.03	777.73 ± 223.04			
BC non-familial or sporadic	5	52.6 ± 7.50	1051.8 ± 9.39	42.2 ± 8.17	10.0 ± 8.37	9.4 ± 7.09
Total group of control	16	44.56 ± 10.03	863.38 ± 224.50			
BRCA1 healthy	9	35.67 ± 10.25	822.89 ±285.12			
BRCA1 with cancer	12	48.5 ± 10.66	639.50 ± 334.85	36.83 ±7.71	8.09 ± 6.79	8.68 ± 7.67
Total group of *BRCA1* mutation carriers	21	43.0 ± 12.12	718.10 ± 320.68			
BRCA2 healthy	11	39.64 ± 11.42	654.18 ± 270.74			
BRCA2 with cancer	13	52.31 ± 9.56	620.15 ±110.49	38.69 ± 6.41	10.42 ± 8.32	8.83 ± 7.12
Total group of *BRCA2* mutation carriers	24	46.50 ± 12.10	635.75 ± 196.32			
Familial BC group without detected mutation in *BRCA1/2*	15	50.53 ± 13.45	324.20 ± 250.31	43.60 ± 12.44	4.42 ± 4.53	3.76 ± 5.66
Total healthy subjects	31	38.94 ± 10.17	747.0 ± 260.63			
Total cancer patients	45	50.73 ± 10.89	574.62 ± 316.73	40.22 ± 9.46	7.87 ± 7.07	7.53 ± 7.03

[a]years since last exposure. BC: breast cancer. *BRCA1/2*: *BRCA1* or *BRCA2* genes

Table 3. Mean values and SD of the different endpoints recorded between all analysed groups in the non-treated cultures.

Group	Nº	Basal MN	Basal BPN	Basal NDI
Normal non-carriers	11	14.09 ± 3.4	1.36 ± 1.91	1.84 ± 0.14
Sporadic cancer	5	12.6 ± 4.28	3.8 ± 1.92	1.71 ± 0.15
Total group of control	16	13.63 ± 3.61	2.13 ± 2.19	1.80 ± 0.15
BRCA1 healthy	9	8.89 ± 3.10	0.89 ± 1.36	1.89 ± 0.11
BRCA1 with cancer	12	27.75 ± 15.24	3.08 ± 3.29	1.68 ± 0.15
Total group of *BRCA1* carriers	21	19.67 ± 14.94	2.14 ± 2.82	1.77 ± 0.17
BRCA2 healthy	11	14.27 ± 8.10	0.82 ± 1.17	1.85 ± 0.15
BRCA2 with cancer	13	19.69 ± 14.76	0.85 ± 0.99	1.88 ± 0.15
Total group of *BRCA2* carriers	24	17.21 ± 12.24	0.83 ± 1.05	1.86 ± 0.15
Familial BC group without detected mutation in *BRCA1/2*	15	27.33 ± 21.56	0.93 ± 1.58	1.72 ± 0.16
Total healthy subjects	31	12.65 ± 5.85	1.03 ± 1.49	1.86 ± 0.13
Total cancer patients	45	23.6 ± 17.17	1.80 ± 2.35	1.75 ± 0.17

BC: breast cancer. *BRCA1/2*: *BRCA1* or *BRCA2* genes

Table 4. Mean ± SD values of MN, NBPs and NDI obtained in the blood cultures treated with 2 Gy of gamma-radiation.

Group	Nº	2 Gy MN[a]	2 Gy BPN[b]	2 Gy NDI[c]
Normal non-carriers	11	227.64 ± 25.47	18.18 ± 6.62	0.20 ± 0.13
Sporadic cancer	5	253.4 ± 23.73	42.4 ± 11.63	0.22 ± 0.10
Total group of control	16	235.69 ± 27.10	25.75 ± 14.13	0.20 ± 0.12
BRCA1 healthy	9	221.56 ± 39.81	26.56 ± 19.06	0.20 ± 0.08
BRCA1 with cancer	12	224.67 ± 47.03	18 ± 15.13	0.19 ± 0.07
Total group of *BRCA1* carriers	21	223.33 ± 43.05	21.67 ± 17.03	0.20 ± 0.07
BRCA2 healthy	11	245.91 ± 60.61	20.91 ± 14.47	0.22 ± 0.07
BRCA2 with cancer	13	213.23 ± 57.25	9.39 ± 6.32	0.23 ± 0.11
Total group of *BRCA2* carriers	24	228.21 ± 59.86	14.67 ± 12.1	0.22 ± 0.09
Familial BC group without detected mutation in *BRCA1/2*	15	184.0 ± 42.67	14 ± 7.33	0.15 ± 0.07
Total healthy subjects	31	232.36 ± 44.43	21.58 ± 13.9	0.21 ± 0.09
Total cancer patients	45	211.0 ± 50.81	16.9± 13.83	0.19 ± 0.09

BC: breast cancer. *BRCA1/2*: *BRCA1* or *BRCA2* genes.

[a]Calculated by subtracting the spontaneous yields from the yields obtained in the treated samples.

[b]Absolute values of NPB obtained in the cultures treated with radiation

[c]NDI index values in untreated cultures less NDI values in treated cultures.

Table 5. Mean ± SD values of MN, NBPs and NDI in the blood cultures treated with MMC

Group	Nº	MMC MN[a]	MMC BPN[b]	MMC NDI[c]
Normal non-carriers	11	108.36 ± 26.41	4.82 ± 3.49	0.29 ± 0.14
Sporadic cancer	5	105.60 ± 23.29	11 ± 3.81	0.28 ± 0.08
Total group of control	16	107.63 ± 24.87	6.75 ± 4.6	0.29 ± 0.12
BRCA1 healthy	9	105.78 ± 39.13	5.89 ± 4.29	0.26 ± 0.11
BRCA1 with cancer	12	97.5 ± 17.39	7.25 ± 5.15	0.28 ± 0.10
Total group of *BRCA1* carriers	21	101.05 ± 28.22	6.67 ± 4.74	0.27 ± 0.10
BRCA2 healthy	11	145.82 ± 60.49	6.64± 4.65	0.26 ± 0.09
BRCA2 with cancer	13	123.85 ± 43.99	5.31 ± 7.29	0.30 ± 0.12
Total group of *BRCA2* carriers	24	133.92 ± 52.20	5.92 ± 6.13	0.28 ± 0.11
Familial BC group without mutations in *BRCA1/2*	14	80.14 ± 26.16	3.14 ± 1.96	0.24 ± 0.09
Total healthy subjects	31	120.90 ± 47.06	5.77 ± 4.1	0.27 ± 0.11
Total cancer patients	44	100.73 ± 34.40	5.8 ± 5.5	0.27 ± 0.10

BC: breast cancer. *BRCA1/2*: *BRCA1* or *BRCA2* genes

[a]Calculated by subtracting the spontaneous yields from the yields obtained in the treated samples.

[b]Absolute values of NPB obtained in the cultures treated with MMC

[c]NDI index values in untreated cultures less NDI values in treated cultures.