DNA damage response in monozygotic twins discordant for smoking habits

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Previous studies in twins indicate that non-shared environment, beyond genetic factors, contributes substantially to individual variation in mutagen sensitivity; however, the role of specific causative factors (e.g. tobacco smoke, diet) was not elucidated. In this investigation, a population of 22 couples of monozygotic twins with discordant smoking habits was selected with the aim of evaluating the influence of tobacco smoke on individual response to DNA damage. The study design virtually eliminated the contribution of genetic heterogeneity to the intra-pair variation in DNA damage response, and thus any difference in the end-points investigated could directly be attributed to the non-shared environment experienced by co-twins, which included as main factor cigarette smoke exposure. Peripheral lymphocytes of study subjects were challenged *ex vivo* with γ-rays, and the induction, processing, fixation of DNA damage evaluated through multiple approaches. Folate status of study subjects was considered significant covariate since it is affected by smoking habits and can influence radiosensitivity. Similar responses were elicited by γ-rays in co-twins for all the end-points analysed, despite their discordant smoking habits. Folate status did not modify DNA damage response, even though a combined effect of smoking habits, low-plasma folic acid level, and ionising radiation was observed on apoptosis. A possible modulation of DNA damage response by duration and intensity of tobacco smoke exposure was suggested by Comet assay and micronucleus data, but the effect was quantitatively limited. Overall, the results obtained indicate that differences in smoking habits do not contribute to a large extent to inter-individual variability in the response to radiation-induced DNA damage observed in healthy human populations.

Introduction

DNA repair plays a fundamental role in the maintenance of genome integrity against the continuous threat of exogenous and endogenous insults, avoiding the accumulation of genetic damage, and its detrimental consequences on cell survival and function (1,2).

DNA repair capacity shows wide variation within the human species: beyond rare genetic instability syndromes, severely compromising DNA repair functions (3), remarkable differences in DNA damage response (DDR) are also observed in normal individuals (4,5).

In view of the wide polymorphism of genes implicated in DNA repair (6), it is conceivable that most individual variability in mutagen sensitivity observed in healthy subjects be determined by their genetic makeup (7). Indeed, previous studies in monozygotic (MZ) and dizygotic (DZ) twins demonstrated that heredity plays a major role as determinant of individual sensitivity to several genotoxic agents (8–15), even though a significant fraction of inter-individual variation was also attributed to the non-shared environment (10,15,16). In particular, the relative role of genetic and environmental influences on spontaneous and radiation-induced micronuclei (MN) levels has been evaluated in recent studies, which highlighted the complexity of this trait in peripheral blood lymphocytes, and the need for specifically designed investigations to define the role of specific environmental factors (14,15).

Among environmental factors possibly modulating DNA repair capacity, diet has so far received most consideration. Studies *in vitro* and *ex vivo,* in fact, demonstrated that the deficiency of selected micronutrients (17,18) or the dietary pattern (19) could significantly affect individual response to DNA damage. However, the contribution of other environmental or lifestyle factors still remains elusive.

Tobacco smoke could reasonably be anticipated to play a prominent role among environmental factors influencing individual sensitivity to mutagens. Smoking is still widely diffused, with an estimated worldwide one billion smokers consuming about six thousand billion cigarettes each year, and represents nowadays the main source of exposure to genotoxic carcinogens in human populations, far higher than most occupational circumstances or polluted environments. More than 60 carcinogens have been identified in cigarette smoke (20), exerting a role in cancer initiation and promotion (21,22), and it is conceivable that the challenge represented by repeated exposure to tobacco genotoxins may trigger cellular mechanisms implicated in DDR, ultimately modulating mutagen sensitivity. However, previous studies on the influence of tobacco smoke on DNA repair capacity in human populations have provided contrasting or equivocal results, from which no firm conclusion could be drawn (23–25). This inconsistent picture might be explained by bias due to confounders not adequately addressed in most investigations. Indeed, given the prevailing contribution of genetic factors to inter-individual variability in chromosomal stability, and the high human genetic heterogeneity, it can be anticipated that the relative role of genetic and environmental factors may be difficult to disentangle in conventional studies in human populations. In this respect, studies in MZ twins have greatly contributed to the understanding of environmental and lifestyle factors impacting human phenotypes (26). Among genetically identical individuals, in fact, phenotypic differences cannot be attributed to genetic heterogeneity: thus, monozygotic twins represent an ideal model to study the influence of environmental factors while controlling for genetic influences.

Based on the above considerations, in the present investigation a population of MZ twins with discordant smoking habits has been enrolled, with the aim of evaluating the modulating effects of smoking habits on DDR in individuals with identical genetic background. To this aim, peripheral blood mononuclear cells (PBMCs) from smoker and non-smoker co-twins were challenged with a single dose of ionising radiation and the individual response to DNA damage investigated. DDR includes a complex series of events aimed at signalling DNA damage, engaging DNA repair mechanisms, arresting cell-cycle progression and activating the apoptotic pathway (2). Herein, in particular, the efficiency in DNA damage signalling following the ionising radiation challenge was evaluated through the kinetics of phosphorylation of the histone H2AX, the kinetics of DNA damage repair by the Comet assay, the fixation of primary DNA damage in chromosome damage and the removal of damaged cells through necrosis and programmed cell death, by the comprehensive Cytome assay [cytokinesis-blocked micronucleus (CBMN) assay] (27). It is worth noting that the multiple approaches used allowed to monitor DDR both in unstimulated and mitogen-stimulated cells, thus overcoming possible bias due to differential cell growth, survival or elimination by apoptosis (28), which may represent an important drawback in population studies only based on the analysis of proliferating blood cells. Moreover, to overcome the limitations of most previous studies, where definition of smoking status was only based on qualitative criteria, in this work detailed information on smoking history of all study subjects was retrieved, and the results analysed taking into account intensity and duration of tobacco smoke exposure. Finally, data were evaluated also taking into account plasma folate and homocysteine levels of study subjects, as folate status—which is altered by smoking habits (29,30)—was shown to modulate chromosomal damage in smokers compared with non-smokers (31), and to influence ionising radiation sensitivity of lymphocytes *ex vivo* (32,33).

Materials and methods

Study population
Twenty-two pairs of monozygotic twins discordant for smoking habits were enrolled for a cross-sectional study conducted in accordance with the principles of Good Clinical Practice and approved by the Independent Ethics Committee of the University of Rome 'Tor Vergata'. Written informed consent was obtained from all subjects according to the Declaration of Helsinki. Information on demographic data, medical history, lifestyle, dietary habits, occupational and environmental exposures was collected by questionnaire. Saliva samples were taken from all subjects to verify the zygosity status. Testing for zygosity was performed using the AmpFiSTRs Identifier kit (Applied Biosystems, Darmstadt, Germany). All the analyses were carried out on coded samples.

Smoking history
Detailed information on smoking history of all the examined subjects was also collected. Smoking status was assigned as follows: *smokers*, inclusion criteria included an average daily tar intake of 60 mg, based on the number of cigarettes smoked per day multiplied by tar yield of the cigarette brand as declared on the pack. This cut-off was chosen to exclude irregular or occasional smokers; *non-smokers*, subjects who have never smoked or had stopped smoking more than 10 years previously. To avoid misclassifications based on the self-reported smoker or non-smoker status, urinary cotinine levels were measured in all study subjects using the Accutest NicAlert_test kit (Jant Pharmacal Corp., Encino, CA, USA) according to the manufacturer's instructions. Overall tobacco exposure during smokers' lifetime was quantified by the *pack year* parameter, which integrates the number of smoked cigarettes per day over the period of smoking. In particular, one *pack year* is defined as 20 manufactured cigarettes (one pack) smoked per day for 1 year, and the number of *pack years* for each smoker is obtained as follows: (number of *cigarettes* smoked per day × number of years smoked)/20.

Analysis of plasma folate, vitamin B12 and homocysteine
After lymphocytes separation, a plasma sample was taken and stored at −20°C for the determination of folic acid, vitamin B12 and homocysteine. Folic acid and vitamin B12 were simultaneously measured by a radioimmunoassay method (Quantaphase II, Bio-Rad). Plasma homocysteine was determined by a microparticle enzyme immunoassay method (Abbott Diagnostics). Both assays were carried out following the instructions of the manufacturers.

Processing of blood samples and γ-ray treatment
Blood samples from all donors were collected within 2 days, 1 week apart. Members of the same couple were sampled on the same day, and blood samples processes in parallel. From each donor, a blood sample was taken by venipuncture into tubes containing Ficoll (BD Vacutainer® CPT™). Manufacturer's protocol was followed for the separation of mononuclear cells from whole blood. After two washes in RPMI 1640, PBMCs were diluted at 10×10^6 cells/ml in freezing medium [RPMI 1640, 50% fetal bovine serum, 10% dimethyl-sulphoxide (DMSO)] and transferred in liquid nitrogen for long storage (up to 2 years). Isolated lymphocytes from the two individuals of the same couple were thawed and processed in parallel according to the experimental design; all the experiments were performed within 2 months. In details, cells were challenged in G_0 with 2 Gy γ-rays from a ^{137}Cs source at a dose rate of 0.8 Gy/min. During treatment, cells were maintained at 0°C to prevent DNA repair. Irradiated and control cells from each subject were partitioned into aliquots to evaluate the individual response to DNA damage by (i) the kinetic of phosphorylation of the histone H2AX; (ii) the kinetic of DNA damage repair by the Comet assay; (iii) the induction of apoptosis, necrosis and chromosome damage by the Cytome assay; (iv) the incidence of apoptosis by flow cytometry.

Analysis of the kinetics of histone H2AX phosphorylation
After irradiation cells were incubated at 37°C to allow DNA repair to occur. Aliquots were taken at four time intervals after irradiation (10 min, 1, 3 and 6 h) and cells harvested by cytospin centrifugation at 600 r.p.m for 5 min, fixed for 10 min in 4% formaldehyde and stored in phosphate-buffered saline (PBS) at 4°C. For immunofluorescence detection, lymphocytes were permeabilised with 0.5% Triton X-100, blocked with 3% foetal calf serum and stained with an antiphosphohistone H2AX antibody (Ser139, clone JBW301; Upstate Biotechnology, Charlottesville, VA, USA) (1:1000 in 3% goat serum/PBS) for 2 h in a humid chamber at room temperature. Samples were washed two or three times for 10 min in 0.02% Tween 20 in PBS, incubated with Alexa fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Eugene, OR, USA) (1:1000 in 3% goat serum/PBS) for 45 min at 37°C in a humid chamber. Nuclei were counterstained with 0.5 μl/ml DAPI (4′,6-diamidino-2-phenylindole) diluted in antifade solution (Vector). Slides were analysed at ×1000 magnification under a Leitz DM RB microscope. Based on the number of γ-H2AX foci, cells were classified in one of two classes: 0–9 signals or ≥10 signals. For the analysis of the kinetics of histone H2AX phosphorylation, the percentage of residual γ-H2AX foci at time *t* after irradiation (%RF) was calculated as follows = (γ-H2AX foci at time *t* after irradiation − γ-H2AX foci in control cells before irradiation)/(γ-H2AX foci immediately after irradiation − γ-H2AX foci in control cells before irradiation) × 100.

Analysis of the kinetics of DNA damage repair
The amount of basal and radiation-induced DNA damage was measured by Comet assay before and immediately after treatment, whereas residual DNA damage was measured after 15 and 30 min of incubation at 37°C. Samples were processed according to the alkaline comet assay protocol described in detail elsewhere (34). Briefly, cells were suspended in 0.7% (w/v) low-melting point agarose (Bio-Rad Laboratories, Hercules, CA, USA) and distributed onto a lower layer of 1% (w/v) normal-melting point agarose (Bio-Rad Laboratories) on microscope slides (Carlo Erba). The slides were immersed in a lysis solution (2.5 M NaCl, 100 mM Na₂ EDTA and 10 mM Tris, pH 10) containing 10% DMSO (Carlo Erba) and 1% Triton X-100 (Sigma), and incubated overnight at 4°C. At lysis completion, slides were placed in a horizontal electrophoresis tank with alkaline electrophoresis buffer (300 mM NaOH and 1 mM Na₂EDTA, pH 13) and left in the solution for 25 min at 4°C to allow the DNA to unwind and the alkali-labile sites to express. Electrophoresis was carried out at 4°C for 20 min, 20 V (1 V/cm), 300 mA. After electrophoresis, slides were immersed in 0.3 M sodium acetate in ethanol for 30 min. Slides were dehydrated in absolute ethanol at −20°C for 10 min and air-dried at room temperature. Immediately before scoring, slides were stained with 12 μg/ml ethidium bromide (Sigma) and analysed at ×250 magnification with a Leitz DM RB microscope under a 50W mercury lamp and an N2.1 filter block. One hundred cells were scored for each experimental point from two slides. DNA damage was quantified by the percentage of DNA in the tail (%DNA), calculated using an Image Analysis Software (IAS, Delta Sistemi, Rome, Italy). Nowadays, %DNA is the

parameter recommended to measure DNA damage in the Comet assay since it is most linearly related to the amount of single-stand breaks (35) and the easiest to intuitively understand (36,37).

For the analysis of DNA repair kinetics, the percentage of residual DNA damage at time *t* after irradiation (%RD DNA) was calculated as follows: (DNA damage at time *t* after irradiation − DNA damage in control cells before irradiation)/(DNA damage immediately after irradiation − DNA damage in control cells before irradiation) × 100 (38).

The Cytome assay

The Cytome assay (CBMN assay) was conducted following the protocol described by Fenech (39). PBMCs were incubated (1×10^6/ml) in RPMI 1640 medium (Gibco) supplemented with 20% fetal bovine serum (Hyclone), 2% phytohaemagglutinin (HA-15 PHA, Abbott check) and incubated at 37°C in a humidified atmosphere with 5% CO_2. Two cultures were set up for each experimental point. Forty-four hours after PHA stimulation, cytokinesis was blocked with 6 µg/ml cytochalasin B; 22 h later lymphocytes were harvested by cytospin centrifugation at 600 r.p.m for 5 min and fixed in absolute methanol at −20°C for 10 min. Cells were analysed in the comprehensive CBMN test following the criteria set up by Fenech (39). The frequencies of MN, buds, nucleoplasmic bridges (NPBs), apoptosis and necrosis were determined analysing 1000 binucleated lymphocytes with well-preserved cytoplasm. Two skilful cytogenetists contributed with 500 cells each from different areas of the same coded slide; results were merged adding data from both scorers. Data from the two scorers were highly significantly correlated ($P < 0.001$): Pearson correlation coefficients (*r*) between the two subsets of data were 0.569 and 0.839 for basal and radiation-induced MN, respectively. Cell proliferation was evaluated by determining a proliferation index (PI) in 200 cells. PI was calculated as follows: (number of binucleated cells + twice the number of multinucleated cells)/200 (40).

Analysis of apoptosis

Apoptosis was analysed by flow cytometry, using the Annexin-V-FLUOS Staining Kit (Roche). Briefly, at 0, 24 and 48 h following γ-ray treatment, 3×10^4 cells from irradiated and unirradiated cultures were washed in PBS and incubated in the dark for 15 min with a binding buffer containing 5 µl of annexin V-FITC and 5 µl of propidium iodide according to the manufacturer's instruction and then analysed by flow cytometry. The FACSCAN flow cytometer (Becton Dickinson) and Cell Quest software were used for data acquisition and analyses. Cells that were positively stained by annexin-V-FITC only (early apoptosis) and positive for both annexin-V-FITC and PI (late apoptosis/necrosis) were measured and overall lethality was calculated by the sum of both subpopulations.

Statistical analyses

Mean intra-pair differences were expressed as the mean of the smoker–non-smoker twin differences. The Wilcoxon signed-rank test was used to assess differences in DDR between smokers and non-smokers. Analysis of variance (ANOVA) was undertaken to estimate between-pairs and within-pairs means of squares and to evaluate the homogeneity of variances (F ratio). In order to quantify the similarity within pairs for responses to γ-irradiation for all the end-points analysed, intra-class correlation coefficient was computed as well.

The DNA repair capacity and the kinetics of repair were also evaluated in both groups by slope analysis. Random effect model was used to estimate beta coefficients and to test whether there are different time trends, for example, if the downtrend in the smoker group is steeper than in the non-smoker group.

In addition, twins were treated as individuals and association between study biomarkers and intensity of exposure (pack year) was evaluated using linear regression model adjusted by age, gender and body mass index (kg/m²).

For the comparison of serum nutrient in smokers and non-smokers, data were evaluated by the non-parametric two-tailed Mann–Whitney *U*-test. The correlation between biomarkers investigated was evaluated by Pearson correlation analysis.

An a-posteriori power analysis was carried out using G*Power (Version 3.1.3). For the main outcomes studied, we estimated the minimum differences between smoker and non-smoker twins (estimated intra-pair mean difference) that can be detected with type I error of 0.05 and 0.80 power, given the available sample size and the standard deviation of the observed intra-pair differences. Based on these estimates, we calculated the corresponding percent variations of observed intra-pair mean differences (observed intra-pair mean difference/observed mean value in non-smoker twins) × 100 and estimated intra-pair differences (intra-pair detectable difference/observed mean value in non-smoker twins) × 100.

Results

Characteristics of the study population

The study population consisted of 22 pairs of monozygotic twins (26 males and 18 females) with discordant smoking habits. Demographic characteristics, information on smoking habits, as well as plasma levels of folic acid, Vitamin B12 and homocysteine are summarised in Table I.

Kinetics of histone H2AX phosphorylation

The analysis of H2AX activation in smoker and non-smoker co-twins was performed in unstimulated lymphocytes. Comparably low background levels of γ-H2AX-positive cells (i.e. with ≥10 foci) were observed in the two groups (Figure 1 a and b), with an overall average percentage of positive γ-H2AX cells lower than 1% (mean ± standard deviation = 0.3 ± 0.7). The induction of γ-H2AX foci by irradiation was examined in cells harvested 10 min and, 1, 3 and 6 h after exposure to 2 Gy γ-rays. No significant differences could be observed comparing the average incidence of γ-H2AX-positive cells in twins discordant for smoking habits (Figure 1 a and b) at each sampling time. The kinetics of disappearance of γ-H2AX foci was also evaluated and no difference between the two groups could be observed (slope −32.75 vs. −33.31 in smoker and non-smoker co-twins, respectively; $P = 0.805$). Also the percentages of residual damage (%RF H2AX) 3 and 6 h after irradiation were not different between smoker and non-smoker twins (Table II), even if a significant decrease of γ-H2AX positive cells at the latest harvest time could be observed in both groups (Table II). In addition, data on H2AX histone phosphorylation were correlated with the intensity and duration of exposure to tobacco smoke, quantified by number of cigarettes/day and the *pack year* parameter, without disclosing any significant association (data not shown).

Kinetics of DNA damage repair

Basal and γ-ray-induced primary DNA damage were analysed by means of the alkaline comet assay. The percentages of DNA in the tail (%DNA) measured before and after irradiation (0, 15 and 30 min) are presented in Figure 1 c and d. Baseline levels of DNA damage were comparable in smokers and non-smokers (mean value ± standard deviation: 0.02 ± 0.0 for both groups). A 5-fold increase in average %DNA was observed immediately after irradiation, with no significant difference between groups.

Table I. Demographic characteristics, smoking habits and plasma levels of some nutrients in the study population

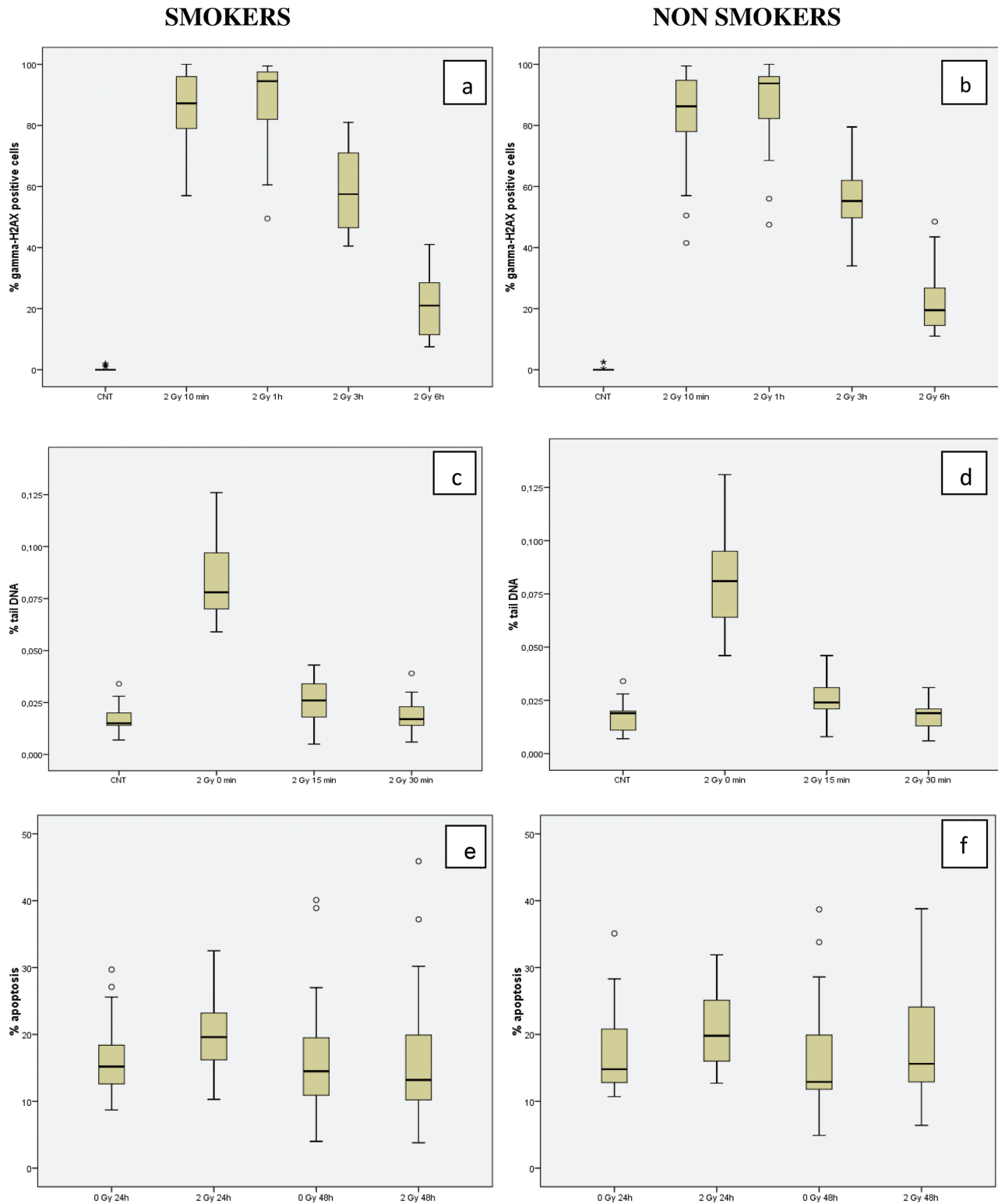|	Smokers	Non-smokers
Age (years)	31.5 ± 6.4 (23–46)[a]	31.5 ± 6.4 (23–46)
Gender		
Male	13	13
Female	9	9
Smoking habits		
n.cig/day	13.7 ± 5.5 (7–20)	—
Years of smoking	11.3 ± 6.6 (2–30)	—
Pack/year	7.9 ± 5.1 (1–23)	—
Plasma nutrients		
Folic acid (ng/ml)	4.8 ± 1.5 (2.5–9.7)	5.4 ± 2.7 (1.9–12.9)
Vitamin B12 (pg/ml)	567.1 ± 154.2 (283–988)	627.4 ± 308.4 (189–1469)
Homocysteine (µmol/l)	13.1 ± 9.1 (7.2–49.6)	11.1 ± 2.9 (5–15)

[a]Mean ± standard deviation (min–max in brackets).

SMOKERS

NON SMOKERS

Fig. 1. Analyses of the kinetics of signalling (**a**, **b**), repair of DNA damage (**c**, **d**), removal of damaged cells (**e**, **f**) in smoker and non-smoker co-twins before and after treatment with 2 Gy γ-rays. (a, b) Percentage of γ-H2AX positive cells observed in unstimulated lymphocytes. CNT: background levels; kinetics of foci appearance evaluated at 10 min, 1, 3 and 6 h after irradiation. (c, d) Percentage of tail fluorescence evaluated by the Comet assay in unstimulated lymphocytes. CNT: background levels; kinetics of DNA damage repair evaluated at 0 min (immediately), 15 and 30 min after irradiation. (e, f) Incidence of apoptotic cells observed by flow cytometry in stimulated lymphocytes at 24 and 48 h.

Also measurements of %DNA at 15 and 30 min after treatment did not show significant differences between smoker and non-smoker co-twins (Figure 1 c and d), highlighting a sharp decrease of induced DNA damage in both groups (Table II),

with fairly similar slopes in smokers and non-smokers (−0.032 for both groups; $P = 0.946$).

When data on %RD DNA were correlated with the intensity of exposure to tobacco smoke, a different profile of repair in light

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Table II. Kinetics of DNA damage signalling and repair in unstimulated lymphocytes after *in vitro* challenge with 2 Gy γ-rays

Variable – experimental condition	*N* pairs	Smoker MZ twins	Non-smoker MZ twins	
		Mean ± standard deviation	Mean ± standard deviation	*P*[*]
% RD H2AX – 3 h	21	0.70 ± 0.20	0.65 ± 0.15	0.39
% RD H2AX – 6 h	20	0.23 ± 0.10	0.26 ± 0.11	0.52
% RD tDNA – 15 min	21	0.14 ± 0.18	0.13 ± 0.14	0.89
% RD tDNA – 30 min	21	0.02 ± 0.10	0.002 ± 0.10	0.39

The residual percentage of γ-H2AX positive cells (%RF H2AX) and the residual percentage of DNA in the comet tail (%RD DNA), corrected for background values, are reported.
*Wilcoxon signed-rank test.

smokers (<12 cigarettes/day) compared with heavy smokers (>12 cigarettes/day) was observed. As shown in Figure 2, 15 min after irradiation the percentage of RD was significantly higher in light smokers than in paired twins ($P = 0.023$); in heavy smokers an opposite result was observed, %RD DNA being significantly lower compared with non-smoker co-twins ($P = 0.027$). No significant differences were detected 30 min after irradiation.

Analysis of apoptosis by flow cytometry

The induction of apoptosis was evaluated by flow cytometry at 24 and 48 h of culture, before and after radiation treatment. The results are reported in Figure 1 e and f. Data show comparable incidences of apoptotic cells in lymphocytes of smokers and non-smokers, in unirradiated cells as well as in irradiated cells 24 and 48 h after irradiation (Wilcoxon signed-rank test, $P > 0.20$ for all experimental conditions).

The Cytome assay

Data on baseline and radiation-induced binucleated cells with MN, buds and NPBs, as well as data on cell proliferation rates (PI), apoptosis and necrosis in cytokinesis-blocked

lymphocytes of the study subjects are summarised in Table III. Treatment with γ-rays induced a significant increase in the frequency of MN, buds and NPBs in all study subjects, with no remarkable differences between smoker and non-smoker co-twins. Also, cell proliferation rates and the percentages of necrotic and apoptotic cells were fairly similar in the smoker and non-smoker groups, both in unirradiated cells, as well as after irradiation with γ-rays. An analysis of the correlation between study biomarkers and the intensity and duration of exposure to tobacco smoke was performed applying a linear regression model after adjustment for age, gender and body mass index. A significant inverse relationship between the number of radiation-induced MN and the number of *pack years* ($\beta = -5.648$, $P = 0.017$; Figure 3) was observed.

Analysis of plasma micronutrients

Levels of folic acid, vitamin B12 and homocysteine were determined in plasma samples of all study subjects. Mean values of these micronutrients, which are reported to modulate cellular response to irradiation, in smokers and in non-smokers are shown in Table I. Lower mean levels of folic acid and vitamin B12 were observed in smokers compared with non-smokers, but the difference did not attain the statistical significance (paired T test $P = 0.851$). A significant inverse relationship between folate and homocysteine was observed (correlation coefficient: -0.335; $P = 0.026$).

A correlation analysis among study biomarkers, plasma nutrients and smoking habits showed statistically significant associations between low levels of folate/vit B12 and high incidence of apoptosis only in smokers ($P < 0.05$); this relationship was stronger after 48 h of culture and treatment with γ-rays as shown in Figure 4 (correlation coefficient folate–apoptosis: -0.508; $P = 0.02$; correlation coefficient vit B12–apoptosis: -0.597; $P = 0.004$). A significant positive relationship between homocysteine levels and apoptotic response was also observed (correlation coefficient: 0.553;
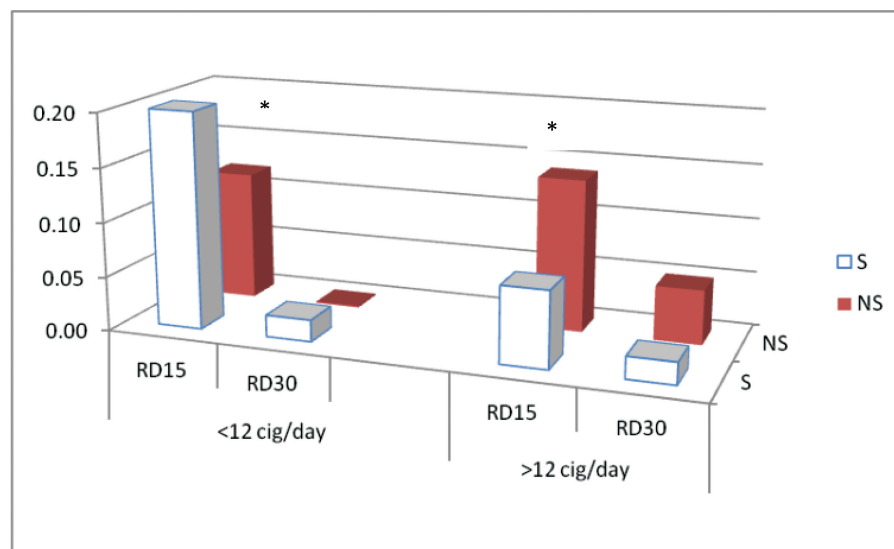
Fig. 2. Percentage residual DNA damage evaluated by the Comet assay in paired twins discordant for smoking habits; smokers stratified by smoking intensity (mean cigarettes/day). RD15: residual damage detected 15 min after irradiation; RD30: residual damage detected 30 min after irradiation. Paired T test between smokers and non-smokers *: $P < 0.05$.

Table III. Frequencies of MN, buds, NPBs, proliferation index (PI), necrotic and apoptotic cells observed in stimulated lymphocytes of smokers and non-smokers

	MN, ‰	Buds, ‰	NPB, ‰	PI	Necrotic cells, %	Apoptotic cells, %
0 Gy						
Smokers	11.4±4.4	8.7±3.7	4.9±3.5	0.31±0.07	33.2±13.3	3.4±1.7
Non-smokers	11.1±6.7	9.0±4.7	3.8±2.3	0.33±0.08	34.7±9.8	3.7±1.4
2 Gy						
Smokers	210.1±59.1	34.7±12.9	29.3±10.8	0.31±0.07	32.7±9.8	3.3±1.5
Non-smokers	200.9±41.4	33.2±10.4	29.1±10.1	0.31±0.07	31.1±7.5	3.6±1.3

$P = 0.009$), as expected in view of the inverse association between homocysteine and folate levels. No significant correlations were observed among these plasma nutrients and the other study biomarkers.

ANOVA: within-pairs and between-pairs comparison
The ANOVA has been performed to compare the similarities between and within twin pairs, with the aim of estimating smoke effect on the response to DNA damage, independently of the genetic background. The results obtained, reported in Table IV, show that within-pairs variance was always lower than between-pairs variance for all parameters (F values higher than 1), suggesting a weak smoke effect, and consistent with the existence of distinct genetic influence, unknown environmental factors, as well as lifestyle habits on the endpoints considered. Intra-class correlation coefficients estimated to evaluate the level of similarities between the two members of each twin pair(Table IV) show that MZ twins, regardless of their smoking habits, were remarkably similar in most damage and repair measurements (correlation

coefficients from 0.39 to 0.86, $P < 0.05$). Representations of intra-class correlations, showing individual differences within the pairs and linear regression lines, are provided as plots in supplementary Figure S1, available at *Mutagenesis* Online.

Assessment of the study power
For a set of representative measures of study parameters, that is the % tail DNA 15 min after treatment, the incidence of γ-H2AX positive foci 3 h after irradiation, the incidence of apoptosis after 48 h and the incidence of micronucleated cytokinesis-blocked cells, the minimum intra-pair differences that could be significantly ($\alpha = 0.05$) detected by this sample size with 80% power were calculated (supplementary Table I, available at *Mutagenesis* Online). Overall, the assessment of study power indicated that the minimum statistically significant intra-pair differences detectable with this study design correspond to modest percentage variations from the mean values observed in non-smoker twins (range 15–26%; full data in supplementary Table I, available at *Mutagenesis* Online). For example, for the
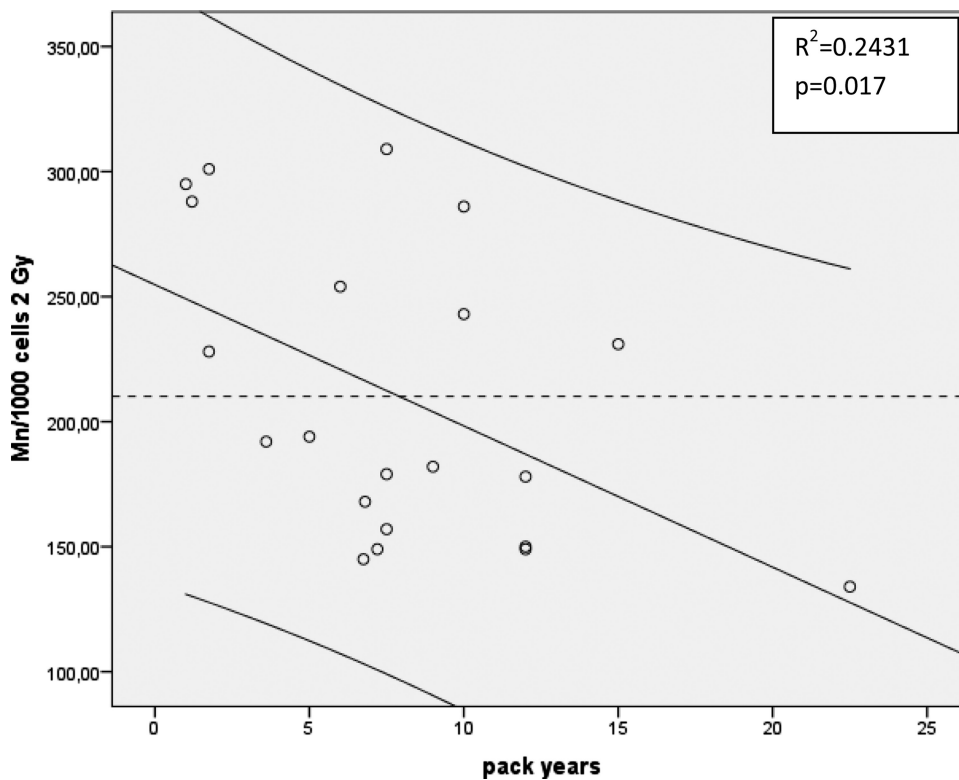

Fig. 3. Relationship between γ-ray induced MN in lymphocytes of smokers and cumulative tobacco consumption. Cumulative tobacco consumption expressed as *pack years* (number of packs/day per number of years of smoking). Dot line represents average MN frequency in non-smokers. 95% CIs are shown.
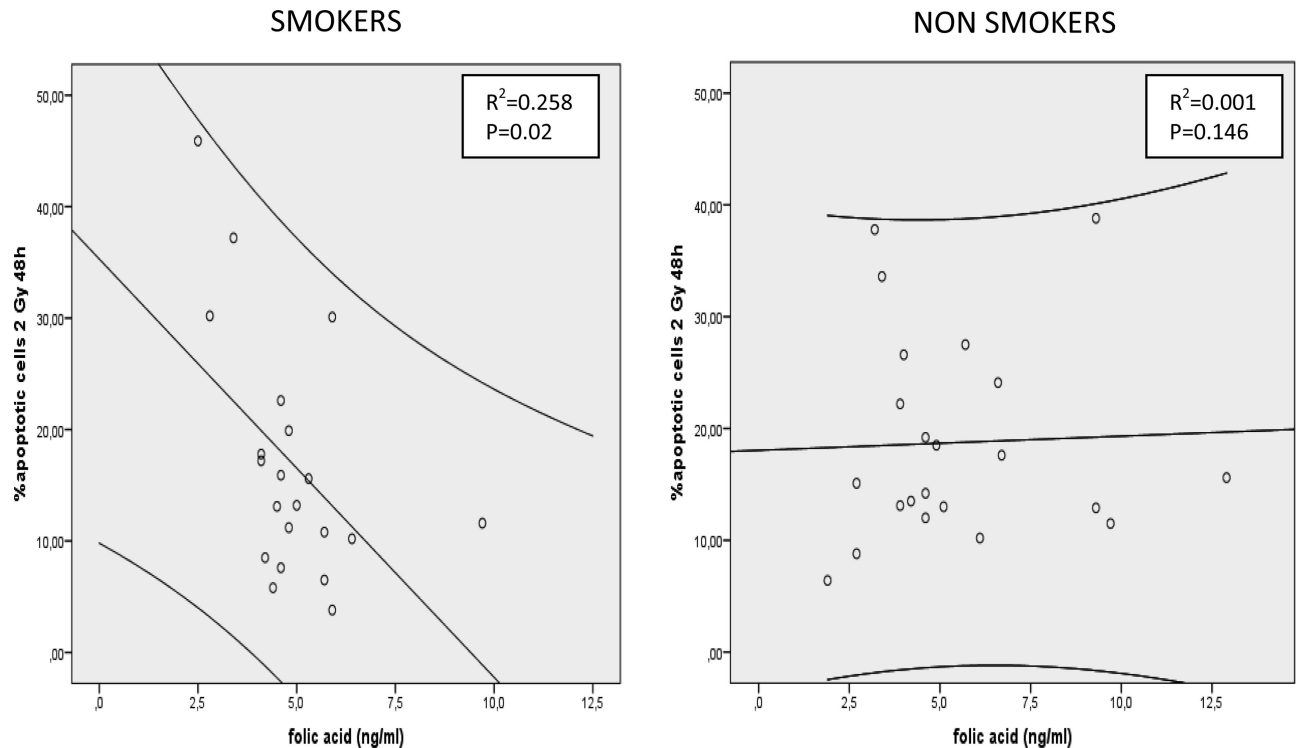
SMOKERS

NON SMOKERS

Fig. 4. Relationships between the frequency of apoptosis observed 48 h after irradiation and plasma levels of folic acid in smokers and non-smokers. 95% CI are shown.

Table IV. ANOVAs and intra-class correlations of the main parameters studied

Variable/experimental condition	Pairs (N)	ANOVA		intra-class correlation
		F^a	P^b	Coefficient (95% CI)
Comet 2 Gy% RD 15 min	21	3.80	<0.01	0.58 (0.30–0.87)
Comet% RD 30 min	21	2.77	0.01	0.47 (0.13–0.81)
H2AX foci 2 Gy%RD 3h	21	1.93	0.07	0.32 (0.00–0.71)
H2AX foci % RD 6 h	20	1.29	0.29	0.13 (0.00–0.57)
% apoptosis 0 Gy 24 h	21	13.16	<0.01	0.86 (0.75–0.97)
% apoptosis 2 Gy 24 h	22	10.12	<0.01	0.82 (0.68–0.96)
% apoptosis 0 Gy 48 h	21	4.32	<0.01	0.63 (0.37–0.89)
% apoptosis 2 Gy 48 h	21	7.24	<0.01	0.76 (0.58–0.94)
MN‰ 0 Gy	20	2.25	0.04	0.39 (0.02–0.77)
MN‰ 2 Gy	21	2.39	0.03	0.41 (0.05–0.77)

$^a F$ = variance between pairs/variance within pairs.
^{b}Probability for F values.
95% CI: 95% confidence interval.

frequency of radiation-induced MN, it was estimated that any real difference between smokers and non-smokers higher than 18% would have been detected as significant (at $P < 0.05$) in 80% of cases in this study. In practice, this corresponds to a difference of 37 MN per thousand cells, taking as reference the group of non-smokers where the overall incidence of radiation-induced MN was 200.9‰.

Discussion

In this study, the hypothesis that smoking habits can modulate individual response to DNA damage was evaluated. To this aim, blood lymphocytes of current and never smokers were challenged *ex vivo* with ionising radiation, and the induction, processing and fixation of DNA damage monitored with an array of laboratory methods. The study was performed in a population of MZ twins to minimise inter-individual differences due to genetic heterogeneity and simultaneously maximise differences due to environmental factors. Given the design of the study, based on intra-pair comparisons, any difference in the phenotype investigated could directly be attributed to the non-shared environment experienced by co-twins, which included as main factor cigarette smoke exposure.

To the best of our knowledge, this is the first study in which the induction and processing *ex vivo* of radiation damage, from primary DNA lesions to chromosome alterations, has been investigated in smoker and non-smoker co-twins. Overall, the main conclusion drawn from this investigation is that similar responses to γ-irradiation are elicited in co-twins with discordant smoking habits, and consequently that smoking is unlikely to contribute to a large extent to the fraction of variation in the sensitivity to ionising radiation attributed to non-shared environment. Overall, fairly similar results were obtained in smoker and non-smoker twins for all the end-points analysed, which intended to probe individual response along different steps of the DDR pathway, viz. DNA damage signalling, repair, fixation and elimination by apoptosis.

Regarding the signalling of DNA damage, despite *in vitro* experiments showed the induction of H2AX phosphorylation by cigarette smoke in human cell lines (41,42), no association with smoking habits was observed in this work, confirming the foreseeable discrepancies among cell types, as well as between *in vitro* and *in vivo* studies. No association with smoking habits was observed with the Comet and the CBMN assays either, in agreement with previous observation from this laboratory (34,43) and by others (16,23,24,44–46), even

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though opposite results have also been published (47,48). As to the discrepancies among studies on genetic damage in smokers, these can rely on different study protocols adopted, for example G_2 and G_0 protocols for cytogenetic assays (11) or on different degrees of tobacco smoke exposure of the study subjects. Indeed, a meta-analysis of previous studies indicated that only heavy smokers (>30 cigarettes/day) experience a detectable increase in cytogenetic damage in their peripheral lymphocytes (49).

Actually, when data were evaluated as function of smoke intensity and duration, a faster repair of DNA damage and a lower incidence of radiation-induced MN were observed in subjects with lower daily or cumulative exposure to tobacco smoke. These findings are reminiscent of similar results obtained in previous studies performed in our laboratory (34,43) and also in agreement with some literature data (16,23,24,46). The opposite trends observed among heavy and light smokers compared with non-smokers could underlie a subtle modification of DNA repair capacity related to the intensity and duration of tobacco exposure and could explain why a significant difference is not appreciated when all smokers are contrasted to non-smokers. The hypothesis that tobacco smoke exposure may lead to the accumulation of epigenetic modifications, resulting in an altered response to radiation, is biologically plausible as altered locus-specific DNA methylation, also targeting tumor-suppressor genes, has been associated with tobacco smoke exposure (50). Tobacco smoke could in principle affect mutagen sensitivity also interfering directly with components of the DNA repair machinery, through the oxidation and inactivation of key proteins (51), or the activation of other components, as recently reported for ATM (ataxia telangectasia mutated), a key player in the DDR (52). In any case, the effect resulting from these putative modification are quantitatively limited, and as stated above no significant difference in radiation sensitivity related to smoking habits *per se* was observed. These indications are in line with the conclusion of a previous study by Berasati *et al.* (23), in which it was shown that even though smoke may induce the upregulation of anti-oxidative defense mechanisms, impacting on pathways involved in DNA damage repair, this effect is quantitatively marginal, unlikely to be appreciated in light and moderate smokers. In this respect, it is worth noting that heavy smokers (i.e. subject smoking ≥ 20 cigarettes/day) were under-represented in the study population enrolled in this work (4 out of 21 smoking subjects).

In the present study, care was taken to identify and account for possible bias related to tobacco smoke which could indirectly affect individual sensitivity to radiation. In particular, it was considered of importance to assess and take into account the folate status (i.e. plasma levels of folic acid and vitamin B12) of the study subject, as folate status is negatively affected by smoke (29,30) and known to modulate baseline levels of DNA damage in blood cells *in vivo* (18) and radiation sensitivity *in vitro* (32,33). Indeed, even though the high content of folic acid in culture medium may overcome inter-individual differences in folate status when blood cells are cultured *in vitro* along several replication cycles, it is plausible that differences in the intracellular milieu related to folate levels can affect DDR at early times following stimulation, when critical repair processes occur. Interestingly, a combined effect of smoking habits, folic acid levels and ionising radiation could be observed herein on the incidence of apoptosis. This end-point was significantly affected by low levels of folic acid only

in smokers, and mostly after treatment with γ-rays. Folic acid deficiency is associated with the same extent of DNA damage produced by low doses of ionising radiation (between 5 and 20 cGy) (53) and may act synergically with γ-rays in DNA damage induction (54). Thus, it is plausible that when the effects of radiation and low folate add to the higher baseline exposure to genotoxic agents experienced by smokers, this would result in a higher incidence of highly damaged cells, then removed through apoptosis.

A crucial point in this, as well in other population studies, is the statistical power of the study. In this investigation, the response elicited by radiation was compared independently in each couple of twins, considering the whole study as 21 repetitions of the same trial on different twin couples. This resulted in a gain of statistical power, which allowed to rule out statistically significant variations associated with smoking habits, greater than 26% for the main end-points considered. This difference can be considered of limited biological significance, in view of the large inter-individual and inter-experimental variation commonly observed for the end-points analysed (55).

Finally, it has to be pointed out that this study, which only involved MZ twins, was not suitable, nor intended, to provide a quantitative measurement of the influence of genetic determinants on the end-point studied. To this aim a classical twin study, based on the biometric analysis of the results in MZ and DZ twins or siblings, should be conducted. Indeed previous investigations using such study design clearly highlighted the influence of genetic background on several of the end-points evaluated in this study (12–15). For baseline and radiation-induced micronucleus induction, in particular, the influence of both environmental and genetic factors were recently disclosed (15), highlighting as multiple factors contribute to modulate this complex trait. Even though a quantitative estimation of the heritability of DDR was not possible with this study design, it is however noteworthy that for all the end-points a significant influence of genetic makeup was indicated by the comparison of within-twin and between-twin differences by ANOVA.

In conclusion, the comprehensive multi-biomarker experimental design applied in this study on MZ twins with discordant smoking habits can be considered a powerful approach to evaluate the effects of smoking habits on individual mutagen sensitivity, minimising the interference of genetic susceptibility factors. The overall lack of straight differences in DDR between smokers and non-smokers allows to conclude that the exposure to tobacco smoke is not likely to be a major modifier of the processing and fixation of DNA damage induced by ionising radiation in human blood cells. A word of caution, however, is needed with respect to the general relevance of these findings, due the variety of mechanisms involved DDR and the variety of primary DNA lesions, which do not allow to anticipate that the same results obtained with ionising radiation would be obtained with other physical or chemical mutagens.

Supplementary data

Supplementary Table I and Figure S1 are available at *Mutagenesis* Online.

Funding

Italia S.p.A. n. Q78, years 2008–2010]. The financial support provided to the Istituto Superiore di Sanità (ISS) by British American Tobacco (BAT) Italia S.p.A. to perform this work was preliminary approved by the Scientific Committee of ISS. As stipulated in article 5 of the above-mentioned contract, in view of its role of public health authority ISS retains full and unique responsibility for the interpretation and diffusion of any result with health relevance.

Conflict of interest statement: The authors declare no conflict of interest.

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