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Biomarkers of genotoxicity measured in human lymphocytes exposed to benzo[a]pyrene: Aneugenic effect, and involvement multiple primary DNA lesions

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Abstract

Benzo-a-pyrene (B[*a*]P) is a polycyclic aromatic hydrocarbon classified as carcinogenic to human. Its metabolic activation leads to production of metabolites forming adducts with DNA, being at origin of B[*a*]P-induced DNA damages, mutagenesis and carcinogenesis. Human blood lymphocytes cultures established from 25 subjects aged 20 to 30 years old, living in Montréal (Québec, Canada) were exposed to B[*a*]P (0.4, 4, 20 and 40 μM) for 24 h, then washed and cultivated without B[*a*]P for an additional 24 h. B[*a*]P-DNA adducts, DNA single-strand breaks (SSBs), sister chromatid exchanges (SCEs), chromosomal aberrations (CAs) and micronuclei (MN) were analysed. Fluorescent *in situ* hybridization (FISH) analysis of MN using a pancentromeric probe was also done to assess MN content. Significantly increased formation of B[*a*]P-DNA adducts, CAs, MN and SCEs were observed starting at [B[*a*]P]=0.4 μM ($p<0.01$), with a significant decrease of B[a]P-DNA adducts and MN after exposure to 20 μM B[*a*]P. For DNA SSBs, no significant increase was observed in all conditions. Besides, FISH analysis showed that B[*a*]P-induced MN mostly contain centromeres (77.1% vs 68.5% for the control, $p<0.01$), and specifically three or more centromeres ($p<0.01$). This study suggests an aneugenic effect of B[*a*]P in human lymphocytes. Finally, statistical analysis by multiple linear regression showed that two variables (B[*a*]P exposure level and B[*a*]P-DNA adducts) significantly explained 53% of observed variability in SCE test, while the percentage of cell presenting a high frequency of SCE (% HFC) was the only variable significantly explaining the observed variability in CA and MN test (34% and 12%, respectively). These observations indicate that DNA breaks, in addition to B[*a*]P-DNA adducts, contributed to SCEs formation.

Introduction

Benzo[*a*]pyrene (B[*a*]P) is a polycyclic aromatic hydrocarbon (PAH) produced during the incomplete combustion of organic matter and is ubiquitously present in our environment. PAHs are common contaminants of ambient air through cigarette smoke and air pollution, as well as a food contaminant. Occupational exposure to PAHs occurs in coal gasification and aluminium production industries, as well as during use of coal tar, asphalt and mixtures of coal-tar pitch [1]. Currently, 15 PAHs are classified as carcinogenic, probably or possibly carcinogenic to human by the International Agency for Research on Cancer (groups 1, 2A and 2B) [1], and B[*a*]P is a prototypical member of this class of carcinogenic PAHs.

Bioactivation of B[*a*]P leads to production of r7,t8-dihydroxy-t-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE), which is considered as the ultimate carcinogenic species. BPDE can bind to DNA, forming adducts that will be removed mainly by nucleotide excision repair (NER) [2]. The major stable adduct of B[*a*]P is the BPdG adduct (r7,t8,t9-trihydroxy-c-10-(N^2-deoxyguanosyl)-7,8,9,10-tetrahydrobenzo[*a*]pyrene). In addition, B[*a*]P quinone metabolites can form stable and depurinating adducts with DNA. It can be redox-cycling, thus generating reactive oxygen species (ROS), which will oxidize DNA bases generating 8-oxo-7,8-dihydro-2`-deoxyguanosine (8-OH-dG) [3]. These adducts are at the origin of B[*a*]P-induced DNA damage, mutagenesis and carcinogenesis [4,5], and their levels are increased in occupationally exposed individuals presenting raised levels of cytogenetic biomarkers such as sister chromatid exchanges (SCEs), chromosome aberrations (CAs) and micronuclei (MN) [6-10]. As for DNA damage measured with the Comet assay, some studies report a significant increase in PAH exposed workers [11,12], while other studies did not report such an increase [13,14]. In order to

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evaluate DNA damage, biological monitoring is commonly performed on human lymphocytes, used as surrogate tissue, as they are considered to reflect damage present in target tissues [15].

In vitro exposure of human lymphocytes to B[*a*]P permitted the characterization of B[*a*]P-DNA adducts by ^{32}P-postlabeling [16,17], however failed in measuring DNA single-strand breaks with the Comet assay, following a short exposure period to B[*a*]P (1 or 4 h; 0.5–10 µM B[*a*]P) [18,19]. As for early biomarkers of genotoxicity, a significant production of SCEs was observed in lymphocytes treated with up to 250 µM B[*a*]P for a 48h exposure period [20-22]. Also, a significant production of MN was found in lymphocytes treated with up to 15 µM B[*a*]P for long exposure periods (72 and 64 h) [23,24], while for CAs significant increases were observed in lymphocytes treated with B[*a*]P for shorter periods (24 or 48 h) [20,25]. Evidences of B[*a*]P metabolism in human lymphocytes first came from quantification of BPDE and quinones [16], then from gene expression analyses showing presence of proteins involved in phase I and phase II metabolism of B[*a*]P (CYP1A1 and GSTP1), and in its induction (AhR, ARNT) [26]. Together with the fact that CYP1A1 enzyme is induced in benzanthracene treated lymphocytes [27], these studies suggest that human lymphocyte culture is a metabolically competent cell system and, therefore is a good model for *in vitro* studies of B[*a*]P-induced genotoxicity.

MN and CA tests can be used to identify the properties a particular compound. MN originate mainly from chromosome fragments or whole chromosome lagging at anaphase during cell division reflecting, respectively, clastogenic and aneugenic events [28]. As for CAs, chromatid- and chromosome-type aberrations reflect clastogenic events [29]. Following evaluation of the MN test by the GUM (a German speaking section of the European Environmental Mutagen Society) and the ECVAM (European Centre for the Validation of Alternative Methods) [30,31], B[*a*]P has been classified as a clastogen. However, reported MN studies on PAH or B[*a*]P did not evaluate content of MN using fluorescent *in situ* hybridization (FISH) with pancentromeric probe, which could distinguish between clastogenic and aneugenic events [32,33].

In this study, we evaluated the genotoxicity of B[*a*]P on human lymphocytes exposed to low B[*a*]P concentrations (0.4-40 µM) with a panel of selected biomarkers. Our goals were to evaluate the effect of B[*a*]P exposure on the levels of B[*a*]P-DNA adducts, DNA single-strand breaks (DNA SSBs) and cytogenetic biomarkers, and to confirm the clastogenic effect of B[*a*]P on human lymphocytes. We measured B[*a*]P-DNA adducts and DNA single strand breaks (SSBs) as primary DNA lesions. Cytogenetic effects of B[*a*]P were evaluated with SCE test, CA test, and MN assay, while MN content was assessed with a FISH-coupled MN assay. Finally, we studied correlations between the biomarkers, and examined sex differences, as they are only rarely assessed in occupational and environmental studies. To our knowledge, very few reports have assessed such a large number of early biomarkers following B[*a*]P exposure of human lymphocytes.

Materials and methods

Subjects

Twenty five subjects (13 women and 12 men), aged 20 to 30 years old (mean: 26.6 years), living in Montréal (Québec, Canada) were carefully selected to ensure minimal exposure to PAHs and other known genotoxic agents. PAH intake through the diet was not documented and blood collection of all subjects was performed during an entire year. All subjects were healthy non-smoking students at Université de Montréal, with no radiography or illicit drug use in the last three months, taking no medication on a regular basis (except for hormones such as contraceptives and thyroid replacement hormone therapy), never diagnosed with a genomic instability syndrome and with no chemo- or radio-therapy history. This research was approved by the research ethics board of Université de Montréal (reference numbers CERFM-68(05)#75 and CERFM-94(08)4#308). All subjects provided informed consent to participate in this study.

Subjects were abstaining from alcohol for 24 hours prior to a 30 ml blood draw. As there was a large number of biomarker analyses and the amount of blood was limited, all assays were not done on every subject. For B[*a*]P-DNA adducts measurements, 24 subjects were involved (11 males, 13 females). Ten subjects participated in DNA single-strand break measurement by EM-ISEL assay (5 males, 5 females). Also, 16 subjects were involved in SCEs test (8 males, 8 females), while 15 subjects had adequate chromosome preparations to perform the chromosome aberration test (7 males, 8 females). Finally, for micronuclei assay, 20 subjects participated (10 males, 10 females).

Chemicals

A benzo[*a*]pyrene (B[*a*]P) stock solution was prepared by dissolving the powder (CAS number: 50-32-8, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) in sterile 100% DMSO (cell culture grade, Sigma-Aldrich Canada Ltd) to reach a final concentration of 12.5 mg/ml. Daughter stock solutions of 1.25 and 0.125 mg/ml were prepared from initial B[*a*]P stock solution. All B[*a*]P stock solutions were sterile filtered and kept in aluminium-wrapped microtubes to prevent light degradation [34].

Cell cultures

For cell viability assays and B[*a*]P-DNA adduct measurement, lymphocyte/monocyte cell fraction were first isolated using Histopaque®-1077 (Sigma-Aldrich Canada Ltd) following the manufacturer's instructions. Cultures containing 3-5x10^6 cells (1.5x10^6 cells/ml) were established. Cells were cultivated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% phytohemagglutinin, 2 mM glutaMAX™-I and 0.1% gentamycin (all from Invitrogen Canada Inc., Burlington, ON, Canada). For DNA SSBs, CA test, MN assay and SCE test, independent peripheral blood cultures were established in parallel using standard cytogenetic procedures [35]. A 0.35 ml aliquot of blood was added to 5 ml supplemented RPMI medium. Cultures were incubated at 37°C for 24 h before B[*a*]P exposure. B[*a*]P was then added to cultures to reach final concentrations of 0.4–4–20 and 40 µM. Sterile DMSO (0.08% - final concentration) was used as a negative control. After a 24h exposure period, cells were washed twice with Hank's balanced salt solution (Invitrogen Canada Inc.) to remove the test compound, and then reincubated in fresh supplemented RPMI medium for 24 h, before harvest. This 24h period without B[*a*]P was required for the SCE test, since 5-bromo-2'-deoxyuridine (BrdU) was added to cultures, thus obtaining the dynamic DNA labeling permitting SCE visualization. Therefore, to be able to compare results obtained with all biomarkers, this period was respected for all tests performed.

BPDE-DNA Chemiluninescence Immunoassay (CIA) for measurement of BPdG

After culture, cells were centrifuged and pellets were frozen at -80°C until DNA preparation. DNA was isolated using Flexigene DNA kit for blood cells (Qiagen Inc., Mississauga, ON, Canada). DNA adducts were measured using a chemiluminescence immunoassay

(CIA), as described by Divi *et al.* [36]. The antibody used in the CIA is a BPDE-DNA antiserum (rabbit # 31, bleed # 08/16/78), at a 1: 8,000,000 dilution. Standard BPDE-DNA was serially diluted in calf-thymus DNA so that each well contained an equal quantity of DNA but varying amounts of BPdG adducts (0-640 adducts/10^9 nucleotides). These standards were prepared in triplicate for each microtiter plate and the lower limit of detection was 40 adducts/10^8 nucleotides. To determine the reproducibility of the experimental setting, four samples were assayed three times on different microtiter plates, resulting in an inter-assay variation of 1.16-fold (16%). One culture per treatment and per subject was prepared for B[*a*]P-DNA adducts measurements, resulting in each sample DNA being measured in one experimental well, as there was a limited amount of cells available for culture.

DNA SSB measurements with the Electron Microscopy *In Situ* End-labeling (EM-ISEL) assay

Colcemid (0.1 mg/ml – Invitrogen Canada Inc.) was added to cultures 2 h prior harvesting cells in metaphase. Cells were centrifuged, resuspended in 0.075 M KCl, incubated for 8 minutes at 37°C, centrifuged and fixed three times in Carnoy I. Finally, metaphase cells were spread on ice-cold precleaned microscope slides and air-dried overnight at room temperature. DNA SSBs were detected using the EM-ISEL assay, as described in Depault *et al.* [37]. One culture per treatment and per subject, was analysed with EM-ISEL assay, due to limitation in blood volume. Twenty nuclei were observed and photographed with a Philips EM208 transmission microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 36000x of magnification. Labeling density was evaluated by counting the number of immunogold particles (IGPs) per μm^2 of chromatin using the public domain software Scion Image for Windows, version 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

Sister chromatid exchanges (SCE) test

BrdU (10μM – Sigma-Altrich Canada Ltd) was added to cultures for the last 24 h of culture. Colcemid (0.1 mg/ml – Invitrogen Canada Inc.) was added to cultures 2 h prior harvesting cells in metaphase. Cells were centrifuged, resuspended in 0.075 M KCl, incubated for 8 minutes at 37°C, centrifuged and fixed three times in Carnoy I. Finally, metaphases were spread on ice-cold precleaned microscope slides. Fluorescence-Photolysis-Giemsa (FPG) technique was then performed to visualize sister chromatids and SCEs [38]. Two cultures per treatment and per subject were analyzed. For each culture, 25 M2 metaphases were analysed at 1000x of magnification on a Leica Aristoplan microscope (Leica Microsystems Canada Inc.), for SCE number. Selected metaphases contained 46 chromosomes, in accordance with OECD guideline 479 [39]. All microscopic analyses were done by a single observer. High frequency SCE cells (HFC) were evaluated following a modification of the method described by Kosmider *et al.* [40]. The number of SCE corresponding to the 95th percentile of SCE distribution of the negative control condition ([B[*a*]P]=0 μM) was determined for each subject. The percentage of cells having more SCEs than the 95th percentile was calculated for all subjects and all conditions.

Micronuclei (MN) assay

Cytokinesis-block MN assay was done following Fenech [28], with minor changes. 6 μM (final) cytochalasin-B was added to the cultures for the last 24 h of culture, before cell harvest. Cells were centrifuged, resuspended in 0.075 M KCl (Sigma-Aldrich Canada Ltd), incubated for 5 minutes at room temperature, centrifuged and fixed twice in Carnoy I (methanol: glacial acetic acid – 3:1 v/v, Fisher Scientific Company, Ottawa, ON, Canada). Finally, cells were spread on precleaned microscope slides (Fisher Scientific Company) and Giemsa staining was realized with freshly filtered 2% Giemsa diluted in Gurr's pH 6.8 buffer (both from Invitrogen Canada Inc.). Two cultures per treatment and per subject were analyzed. For each culture, 1000 binucleated (BN) cells with well preserved cytoplasm were analysed at 1000x magnification on a Leica Laborlux microscope (Leica Microsystems Canada Inc., Richmond Hill, ON, Canada). BN cells were selected and MN were analysed following the Fenech criteria [28]. Also, nuclear division index (NDI) was determined for all BaP conditions, by scoring the number of cells containing one (N1), two (N2), three (N3) or four (N4) nuclei on 1000 cells, as described in Fenech [28]. All slides were analysed by the same observer.

Chromosome Aberration (CA) test

For the CA test, colcemid (0.1 mg/ml – Invitrogen Canada Inc.) was added to cultures 2 h prior harvesting to arrest cells in metaphase. Cells were centrifuged, resuspended in 0.075 M KCl, incubated for 8 minutes at 37°C, and then fixed three times in Carnoy I. Finally, metaphases were spread on ice-cold precleaned microscope slides, and Giemsa staining was realized with freshly filtered 3% Giemsa diluted in Gurr's pH 6.8 buffer. Two cultures per treatment and per subject were analyzed. For each culture, 100 well spread metaphases were analysed at 1000x magnification on Leica Laborlux and Aristoplan microscopes (Leica Microsystems Canada Inc.). Chromosome number, gaps, breaks and complex aberrations were recorded. Cells with 46 ± 2 chromosomes were chosen for CA analysis, in accordance with OECD guideline 473 for the *in vitro* mammalian chromosome aberration test [41]. Cells with chromosome losses and gains were also recorded (43 ≤ chromosomes ≥ 49, triploid and tetraploid cells, as well as endoreduplications). Also, mitotic index (MI) was determined for all BaP conditions, by scoring the number of metaphases and nuclei on 1000 cells. All microscopic analyses were done by two observers, one duplicate culture per observer, for all subjects.

FISH-coupled MN assay

A pancentromeric probe (Cambio, Cambridge, UK) was used to assess the centromeric (Cen) content of MN for both the negative control and the [B[*a*]P]=20 μM conditions. For each subject, a slide for both conditions was stained with Giemsa; all MN present in BN cells were preselected, and their positions carefully recorded on a Leica Aristoplan dual photonic/fluorescence microscope (Leica Microsystems Canada Inc.) before hybridization. After destaining with Carnoy I, slides were treated with RNase A and pepsin (both from Sigma-Aldrich Canada Inc.) to remove endogenous RNA and cytoplasmic proteins. This step ensured an optimal penetration of the probe during hybridization, and thus an optimal visualization of Cen signals in MN. Hybridization was performed following manufacturer's instructions and slides were counterstained with DAPI (Roche Diagnostics, Laval, QC, Canada) before microscopic analysis. Positions of preselected MN were retrieved and number of Cen signals were recorded, for both the negative control and the [B[*a*]P]=20 μM condition. Only MN where the FISH signals were clearly visible and homogeneously dispersed within the BN cell wereanalysed.

Statistical analysis

For DNA SSBs (n=20 per subject and treatment) and SCEs (n=50 per subject and treatment), raw data were analysed on an individual level by one-way ANOVA followed by multiple comparison *post-hoc* test (Bonferroni for equal variances or Tamhane's T2 for unequal

variances). To perform statistical analysis at the group level, DNA SSB data were expressed as means normalized to the negative control, while SCE data were expressed as mean SCEs per cell, and as percentage HFC. For MN, tested indicators were: frequency of MN in BN cells, and number of MN per micronucleated BN cells (MN per MNBN). For CAs, tested indicators were: frequency of cells presenting one or more CA (CA cells), frequency of CA and percentage of cells with chromosome losses and gains. Dose-response relationships of B[*a*]P-DNA adducts and DNA SSBs were analysed with the non-parametric Wilcoxon signed-Rank test. For CA, MN, SCEs and % HFC, repeated measures ANOVA were performed. Inclusion or exclusion of gaps during statistical analysis did not change the results.

For analysis of MN content by FISH, MN were classified for the two conditions (negative control and [B[*a*]P]=20 μM) in the following categories, as suggested by Iarmarcovai *et al.* [33]. First, MN containing no Cen signal (C- MN), and containing 1 or more Cen signals (C+ MN) were enumerated. Second, C+ MN were divided in two new categories according to the number of Cen signals present: $C_{1\text{-}2}$+ MN (1-2 Cen signals) and C_{3+}+ MN (3 or more Cen signals). The two-proportion *z*-test for independent samples was used to test if proportion of C+ MN and of C_{3+}+ MN were higher following B[*a*]P exposure, when compared to negative control, for the group, as well as women and men separately.

Sex differences were investigated using two approaches. First, women and men samples were compared for every dose and indicators with the bilateral Student's *t*-test for independent samples (B[*a*]P-DNA adducts, DNA SSBs, CA frequency and MN frequency) or as a part of the repeated measures ANOVA (SCEs and % HFC). Second, dose-response relationships for all indicators were analyzed for women and men separately and were then compared to each other.

To study the relationships existing between the different biomarkers, Pearson's correlation, as well as partial correlation (adjusted on B[*a*]P exposure and sex), were performed. Also, multiple linear regression, testing for collinearity, was conducted to explain the variability observed in our biomarkers. Samples in which B[*a*]P-DNA adducts were non detected were assigned a value corresponding to half the limit of detection (LOD/2). Statistic analyses were done using SPSS 17.0 software for Windows (SPSS inc. Chicago, IL, USA), and *p*-values less than 0.05 were considered statistically significant.

Results

Preliminary experiments were done to determine lymphocyte viability (using Trypan blue exclusion assay) following 24 h exposure to 0–0.4–4–20 and 40 μM B[*a*]P. These experiments, performed on 4 subjects (2 females and 2 males) showed that more than 80% cells were viable after B[*a*]P exposure (data not shown). Also, analysis of NDI showed a significant decrease when cells were exposed to 40 μM B[*a*]P, compared to negative control (Table 1), suggesting the presence of a cytostatic effect. However, with an observed NDI of 1.60 ± 0.05 at B[*a*]P=40 μM, it is well above the lower threshold value of 1.3 cited by Fenech [28], indicating that viability of exposed cells was adequate, and did not compromise results of our genotoxicity testing. This was further confirmed by analysis of the mitotic index (Table 1), as no significant decreases were observed following B[*a*]P exposure. Together, these results reveal a trend towards a reduction in viability of human lymphocytes with increasing B[*a*]P exposure.

B[*a*]P-DNA adduct

Individual results for B[*a*]P-DNA adduct measurements by CIA are presented in Table 5, while group mean values are in Table 2. Interindividual variation was present when measuring the spontaneous ([B[*a*]P]=0 μM) B[*a*]P-DNA adduct level, as adducts were non detected in 76% of the unexposed samples (Table 5). Comparison at group level showed a significant increase in B[*a*]P-DNA adducts following B[*a*]P exposure, starting at [B[*a*]P]=0.4 μM ($p<0.001$ for the group –Table 2). Maximal B[*a*]P-DNA adduct level was observed at [B[*a*]P]=4 μM, followed by a significant decrease at [B[*a*]P]=20 μM, for both group ($p<0.05$), and women ($p<0.01$). Direct comparison of women and men results in all B[*a*]P-tested conditions showed no significant differences, despite the 1.6 fold difference at B[*a*]P 20μM condition (Table 2).

Table 1. Cell cycle, nuclear division index (NDI) and mitotic index (MI) obtained in human lymphocytes *in vitro* exposed to benzo[*a*]pyrene.

B[*a*]P (μM)	% N1*	% N2 †	% N3 + N4‡	NDI§	MI (%)§
0	39.5	52.9	7.6	1.71 ± 0.03	3.5 ± 0.4
0.4	37.6	54.3	8.1	1.73 ± 0.02	3.3 ± 0.8
4	41.0	53.4	5.5	1.67 ± 0.01	3.2 ± 0.7
20	38.9	54.7	6.5	1.70 ± 0.01	3.2 ± 0.7
40	49.4	43.8	6.8	1.60 ± 0.05**	3.3 ± 0.4

* % N1: percentage of mononucleated cells

† % N2: percentage of binucleated cells

‡ % N3 + N4: percentage of trinucleated and quadrinucleated cells

§Results are presented as means ± SEM, for the group.

p-values are presented for negative control vs B[*a*]P exposure.

** $p<0.05$

DNA single-strand breaks (DNA SSBs)

Statistical analysis at an individual level by one-way ANOVA showed significantly increased IGPs for 4/10 subjects at [B[*a*]P]=0.4 μM, when compared to negative control ($p<0.001$ – Table 6). At [B[*a*]P]=4 μM, differences between individuals were especially present, as 5/10 subjects presented a significant IGPs increase, while 2/10 subjects presented a significant decrease at this concentration (Supplementary Table 6 – comparison with negative control condition; $p<0.001$). The remaining three subjects presented no significant increases or decreases in IGPs. Group statistical analysis was performed after normalisation of the means, taking the negative control as reference (Table 2), and show a non-significant IGPs increase in all B[*a*]P conditions tested. Direct comparison of women and men results in all B[*a*]P-tested conditions showed no significant differences (Table 2).

Sister chromatid exchanges (SCEs)

Group analysis shows that significant increases in mean SCEs per cell and % HFC were observed following B[*a*]P exposure, starting at [B[*a*]P]=0.4 μM ($p<0.05$–Table 2). These increases were linear: individual mean SCEs per cell (data not shown) and individual % HFC (Table 7) presented a very good correlation with B[*a*]P exposure (Pearson's $r=0.64$ and $r=0.66$; $p=1.5 \times 10^{-8}$ and $p=2.9 \times 10^{-9}$, respectively – Table 4). However, men and women behaved differently (see Table 2), as women showed a non-significant increase in mean SCEs per cell at [B[*a*]P]=0.4 μM, and a significant increase at [B[*a*]P]=4 μM ($p<0.05$), while men presented highly significant mean SCE increases at both concentrations ($p<0.001$). When analyzing % HFC, the same situation was observed (Table 2): men had significantly increased % HFC at [B[*a*]P]=0.4 and 4 μM ($p<0.05$ and $p<0.001$, respectively), while women presented significant % HFC increases starting at [B[*a*]P]=4 μM ($p<0.05$).

Chromosome Aberration (CA) analysis

B[*a*]P exposure caused mainly gaps and chromatid breaks, but

Table 2. Biomarkers of genotoxicity (B[*a*]P-DNA adducts, DNA SSBs, mean SCEs per cell, percentage of HFC, MN frequency, MN per MNBN, CA frequency, percentage of cells containing CA and percentage of cells presenting chromosome gains /losses) measured in human lymphocytes drawn from healthy subjects, *in vitro* exposed to benzo[*a*]pyrene. Results expressed as means ± SEM.

	B[*a*]P concentration (µM)				
Biomarker	**0**	**0.4**	**4**	**20**	**40**
B[a]P-DNA adducts (per 10^8 nucleotides) measured with the BPDE-DNA CIA assay					
Group (n=24)	136 ± 54*	6935 ± 891‡‡	14661 ± 1330‡‡	10760 ± 1951‡‡	na
Women (n=15)	115 ± 62*	5792 ± 800††	13079 ± 1526††	8324 ± 1596††	na
Men (n=11)	160 ± 95*	8286 ± 1654††	16383 ± 2185††	13700 ± 3732††	na
DNA SSBs (normalised mean) measured with the EM-ISEL assay					
Group (n=10)	1.00	1.37 ± 0.17	1.68 ± 0.34	1.28 ± 0.35	1.72 ± 0.19
Women (n=5)	1.00	1.43 ± 0.21	1.54 ± 0.61	1.29 ± 0.28	1.61 ± 0.45
Men (n=5)	1.00	1.32 ± 0.30	1.82 ± 0.37	1.26 ± 0.73	1.84 ± 0.01
Mean SCEs per cell determined during the SCE test					
Group (n=16)	7.6 ± 0.12	11.1 ± 0.21‡‡	14.6 ± 0.22‡‡	16.2 ± 0.22‡‡	na
Women (n=8)	7.5 ± 0.15	10.7 ± 0.36	13.9 ± 0.31**	16.0 ± 0.34‡‡	na
Men (n=8)	7.8 ± 0.18	11.4 ± 0.22‡‡	15.3 ± 0.31‡‡	16.4 ± 0.30‡‡	na
HFC (%) determined during the SCE test					
Group (n=16)	5.1 ± 1.75	25.9 ± 16.11**	57.8 ± 19.56‡‡	66.9 ± 16.66‡‡	na
Women (n=8)	5.8 ± 1.98	25.3 ± 18.39	56.6 ± 24.43**	68.6 ± 20.22‡‡	na
Men (n=8)	4.5 ± 1.41	26.5 ± 14.73**	58.9 ± 14.83‡‡	65.4 ± 14.11‡‡	na
MN frequency (‰) determined during the MN assay					
Group (n=20)	9.9 ± 1.2	13.6 ± 1.5††	17.8 ± 1.8‡‡	20.0 ± 1.9‡‡	17.9 ± 1.5‡‡
Women (n=10)	10.4 ± 1.8	12.8 ± 2.3¶	18.8 ± 2.9††	21.1 ± 3.0††	18.7 ±2.5††
Men (n=10)	9.4 ± 1.6	14.4 ± 2.0**	16.9 ± 2.2††	18.9 ± 2.6††	17.0 ± 1.6††
MN per MNBN † determined during the MN assay					
Group (n=20)	1.061 ± 0.022	1.086 ± 0.020	1.135 ± 0.025††	1.100 ± 0.022**	1.083 ± 0.014
Women (n=10)	1.084 ± 0.040	1.076 ± 0.029	1.147 ± 0.033¶	1.086 ± 0.020	1.092 ± 0.021
Men (n=10)	1.037 ± 0.018	1.097 ± 0.029¶	1.124 ± 0.037**	1.114 ± 0.040**	1.074 ± 0.020
CA frequency (%) determined during the CA test					
Group (n=15)	1.6 ± 0.3	3.6 ± 0.5††	4.4 ± 0.8††	7.5 ± 1.0††	6.9 ± 1.6
Women (n=8)	2.1 ± 0.5	3.8 ± 0.5¶	5.4 ± 1.4**	7.9 ± 1.6**	8.0 ± 3.5
Men(n=7)	1.1 ± 0.3	3.4 ± 0.8**	3.2 ± 0.8**	7.1 ± 1.4**	5.8 ± 0.8
CA cells (%) ‡ determined during the CA test					
Group (n=15)	1.5 ± 0.2	3.3 ± 0.3††	3.6 ± 0.5††	6.6 ± 0.9††	5.8 ± 1.4†
Women (n=8)	1.8 ± 0.3	3.5 ± 0.3**	4.1 ± 0.7**	7.2 ± 1.5**	7.0 ± 3.0
Men(n=7)	1.1 ± 0.3	3.0 ± 0.5**	2.9 ± 0.7**	5.9 ± 1.1**	4.5 ± 0.5
Chromosome losses/gains (%)§ determined during the CA test					
Group (n=15)	0.21 ± 0.06	0.57 ± 0.13**	0.57 ± 0.09**	0.87 ± 0.14‡‡	0.25 ± 0.02

na: not available – chromosome preparation not available

*non detected were assigned a value corresponding to half the limit of detection.

† Mean number of MN per micronucleated cell (containing one or more MN)

‡Percentage of cells presenting one or more CAs

§ Cells with 43≤ chromosomes ≥49

¶p<0.1 ** p<0.05 †† p<0.01 ‡‡ p<0.001

chromosome breaks and complex aberrations were also recorded. Significant increases in CA cells and CA frequency (with and without gaps) were observed, starting at [B[*a*]P]=0.4 µM (group –p<0.01; Table 2). Maximal percentage of CA cells and CA frequency were observed at [B[*a*]P]=20 µM, followed by a slight decrease at [B[*a*]P]=40 µM. The CA cell decrease was moderate (non-significant) when compared to [B[*a*]P]=20 µM (p<0.1). We also observed a significant increase in cells presenting chromosome losses and gains, starting at [B[*a*]P]=0.4 µM (group: p<0.05 – Table 2). Direct comparison of women and men samples in all B[*a*]P-tested conditions (CA cells and CA frequency), showed no significant sex differences. On the other hand, dose-response relationship for CA frequency (Table 2) was different for the two sexes. Women behaved differently at [B[*a*]P]=0.4 µM, as they showed a moderate non-significant (p<0.1) increase in CA frequency, while men showed a significant increase (p<0.05).

Micronuclei (MN) analysis

An interindividual variation was present among the subjects with spontaneous ([B[*a*]P]=0 µM) MN frequencies per 1000 BN cells ranging from 3.5 to 21 for women, and from 1 to 17 for men (not shown), with a mean MN frequency of 9.9 ± 1.2 for the group (Table 2). While women had an increased spontaneous MN frequency when compared to men, it was non-significant. Following B[*a*]P exposure, significant increases in MN frequency was observed, starting at [B[*a*]P]=0.4 µM (p<0.01 – Table 2). Maximal MN frequency was observed at [B[*a*]P]=20 µM, followed by a decrease at [B[*a*]P]=40 µM. This decrease was significant only for men, when compared to [B[*a*]P]=20 µM (p<0.05), while the group showed only a moderate non-significant decrease (p<0.1). The number of MN per MNBN also increased following B[*a*]P exposure, being significant and maximal at [B[*a*]P]=4 µM (p<0.01 – Table 2). It

Table 3. FISH analysis of micronuclei (MN), using a pancentromeric probe, for [B[*a*]P]=0μM (negative control) and [B[*a*]P]=20μM (exposed condition).

	B[*a*]P (μM)	MN analyzed	C- MN (%)*	C+ MN (%)†	C_{1-2}+ MN‡	C_{3+}+ MN(%)§
Group	0	235	74 (31.5)	161 (68.5)	54 (33.5)	107 (66.5)
	20	477	109 (22.9)	368 (77.1) ††	80 (21.7)	288 (78.3) ††
Women	0	138	39 (28.3)	99 (71.7)	37 (37.4)	62 (62.6)
	20	236	52 (22.0)	184 (78.0) †	44 (23.9)	140 (76.1) ††
Men	0	97	35 (36.1)	62 (63.9)	17 (27.4)	45 (72.6)
	20	241	57 (23.7)	184 (76.3) ††	36 (19.6)	148 (80.4) ¶

The number (and percentage) of C-, C+, C_{1-2}+ and C_{3+}+ MN are presented for the group, as well as women and men separately.

Percentages of C+ and C_{3+}+ MN present in the [B[*a*]P] = 20 μM condition were compared to the negative control condition using the two-proportion *z*-test.

*C- MN: MN containing no centromere

†C+ MN: MN containing one or more centromeres

‡C_{1-2}+ MN: MN containing one or two centromeres

§C_{3+}+ MN: MN containing 3 or more centromeres

p-values are presented for negative control vs B[*a*]P exposure

¶p<0.1 ** p<0.05 †† p<0.01 ‡‡ p<0.001

Table 4. Pearson's correlation coefficients and partial correlation coefficients (controlling for exposure and sex) existing between the biomarkers studied in human lymphocytes *in vitro* exposed to benzo[*a*]pyrene. Significant correlations are in bold.

Biomarker		% HFC	Adducts (LOD/2)*	Mean SCEs per cell	Normalised SSBs	MN frequency	MN per MNBN	CA frequency
B[*a*]P exposure †	*r*= *p*= *n*=	**0.664** **2.9 x10^{-9}** **62**	**0.311** **0.003** **91**	**0.641** **1.5 x10^{-8}** **62**	0.165 0.309 39	**0.279** **0.008** **88**	-0.009 0.930 88	**0.518** **1.6 x10^{-5}** **61**
% HFC‡	*r*= *p*= *n*=		**0.393** **0.002** **57**	**0.752** **1.9 x10^{-12}** **61**	0.022 0.912 26	0.233 0.160 37	0.345 0.034 37	0.367 0.020 39
Adducts (LOD/2)‡	*r*= *p*= *n*=			**0.344** **0.008** **57**	0.188 0.281 34	0.165 0.170 70	0.119 0.321 70	0.106 0.432 56
Mean SCEs per cell‡	*r*= *p*= *n*=				0.84 0.679 26	0.214 0.198 37	0.131 0.433 37	-0.032 0.844 39
Normalised SSBs‡	*r*= *p*= *n*=					0.160 0.415 27	0.099 0.616 27	0.180 0.307 33
MN frequency‡	*r*= *p*= *n*=						**0.473** **3.2 x10^{-6}** **87**	0.074 0.604 51
MN per MNBN‡	*r*= *p*= *n*=							0.351 0.011 51

*non detected were assigned a value corresponding to half the limit of detection.

† Pearson's correlation coefficients

‡ Partial correlation coefficients

slowly decreased at higher B[*a*]P concentrations, being significant for the group at [B[*a*]P]=40 μM, when compared to [B[*a*]P]=4 μM (p<0.05). Direct comparison of women and men samples in all B[*a*]P-tested conditions and for all MN assay indicators showed no significant differences. However, dose-response relationship for MN frequency was different for the two sexes. Women behaved differently at [B[*a*]P]=0.4 μM, as they showed a moderate non-significant increase (p<0.1 – Table 2) in MN frequency, while men showed a significant increase (p<0.05).

FISH-coupled MN analysis

Classification of FISH results in four categories (C-, C+, C_{1-2}+ and C_{3+}+ MN) shows that B[*a*]P-induced MN were mostly C+ MN (p<0.01 – Table 3). Also, subdividision of C+ MN into two categories (C_{1-2}+ and C_{3+}+ MN) revealed a proportional increase of MN containing three or more centromeric signals (C_{3+}+ MN), following B[*a*]P exposure (p<0.01). Sex analysis suggests that women and men behave differently: in women, B[*a*]P-induced C_{3+}+MN were significantly increased (p<0.01), while in men those C_{3+}+ MN were non-significantly increased (p<0.1). However, direct comparison of the proportions of C+ and C_{3+}+ MN showed no significant differences between the sexes.

Correlations between biomarkers

Since in our study most biomarkers (B[*a*]P-DNA adducts, mean SCEs per cell, % HFC, MN and CA frequencies) were significantly correlated with B[*a*]P exposure (see Pearson's correlation coefficients, Table 4), partial correlation coefficients were calculated, controlling for B[*a*]P exposure and sex. B[*a*]P-DNA adducts showed significant partial correlations with both SCEs indicators (mean SCEs per cell and % HFC – p=0.008 and p=0.002, respectively). Interestingly, % HFC also

Table 5. B[*a*]P-DNA adducts measured with the BPDE-DNA CIA in human lymphocytes drawn from 24 subjects, *in vitro* exposed to benzo[*a*]pyrene. Results are expressed as adducts per 10^8 nucleotides.

Identifier	B[*a*]P concentrations (µM)			
	0	0.4	4	20
F1*	nd	2316	17291	16851
F2	nd	5030	7093	1761
F3	nd	6757	13114	12934
F4	nd	7443	23105	4166
F5	nd	11501	12740	40383
F6	nd	3132	13907	7419
F7	nd	3279	5049	2054
F8	nd	3782	19307	12581
F11	nd	10171	13357	12764
F12	nd	4666	15154	17458
F13	83	3319	9159	3811
F14/	11	8402	7677	2143
F15	19	5500	na	3957
M1†	98	15699	14045	5894
M2	nd	9200	19615	9203
M3	nd	2511	13727	24574
M4	nd	13077	31323	38033
M5/	nd	6315	25796	32880
M6	nd	6458	18357	13357
M7	nd	17542	13978	8481
M8	55	1099	1535	674
M9	nd	1490	12131	3637
M10	nd	5434	11095	2226
M11	nd	2434	4830	5682

nd: non detected

na: not available –not enough DNA in sample

* F: Female subject

†M: Male subject

showed a significant partial correlation with CA frequency (p=0.020) and the number of MN per MNBN (p=0.034). Finally, MN per MNBN and CA frequency showed a significant partial correlation with each other (p=0.011).

Multiple linear regression analysis was performed to identify the explanatory variables affecting our early biomarkers of genotoxicity. B[*a*]P exposure was the most important variable affecting % HFC, as it explained 44% of its variability (r^2=0.441; p=1.2 x10^{-4}). Addition of B[*a*]P-DNA adducts to the regression explained 53% of observed variability in % HFC (r^2=0.528; p=8.5 x10^{-5}). As for mean SCEs per cell, B[*a*]P exposure explained 41% of its variability (r^2=0.411; p=2.4 x10^{-4}). About CA and MN frequencies, the only significant explanatory variable was % HFC, as it explained 34% of CA frequency's variability (r^2=0.335; p=0.001), and 12% of MN frequency's variability (r^2=0.124; p=0.028).

Discussion

Early biomarkers of genotoxicity show different dose-response curves

In our cohort, 76% of unexposed lymphocyte samples had an undetectable level of B[*a*]P-DNA adducts, as measured by CIA (Table 5). This is in the upper range of reported values for control or exposed populations (13–77%), when adducts are measured with techniques having similar limits of detection [42-44]. This high rate of undetectable samples may be explained, in part, by application of strict selection criteria in our study, which minimizes exposure to PAH. On the other hand, as the blood samples were obtained from our subjects throughout the year, and as the dietary intake of PAH was not controlled, the unexposed samples containing detectable levels of B[*a*]P-DNA adducts may reflect charbroiled meat consumption or increased air pollution occurring in winter, as observed in Mexico city [45-47].

Following B[*a*]P exposure, B[*a*]P-DNA adducts measurements showed a progressive increase over the control values for the first two B[*a*]P concentrations (Table 2), and at [B[*a*]P]=20 µM, B[*a*]P-DNA adducts significantly decreased (p<0.05). This dose-response curve has an inverted U-shape also observed with CAs and MN. Indeed, significant induction of CAs and MN were observed at all B[*a*]P conditions tested (Table 2). As for the B[*a*]P-DNA adducts, a significant decrease in MN frequency (p<0.05), and a moderate (non-significant – p<0.1) decrease in CA frequency were observed after exposure to [B[*a*]P]=40 µM, when compared to [B[*a*]P]=20 µM. Similar observations were reported in the literature, for both *in vitro* studies and population studies. Indeed, less DNA adducts were observed in human lung fibroblasts exposed to 1 µM B[*a*]P, when compared to 0.25 µM [48]. Also, higher exposed

Table 6. DNA single-strand breaks quantified in human lymphocytes drawn from 10 subjects, *in vitro* exposed to benzo[*a*]pyrene. Results expressed as mean Immunogold particles (IGP) per µm² of chromatin ± SEM, and as means normalised to the negative control ([B[*a*]P]=0 µM).

Identifier	Indicator	B[*a*]P concentrations (µM)				
		0	0.4	4	20	40
F1*	IGP/ µm²	93 ± 3.7	164 ± 8.1‡‡	366 ± 12.0‡‡	na	191 ± 5.4‡‡
	Normalised mean	1.00	1.76	3.92		2.05
F4/	IGP/ µm²	681 ± 31.6	634 ± 26.7	466 ± 35.0‡‡	502 ± 47.5††	na
	Normalised mean	1.00	0.93	0.68	0.74	
F5/	IGP/ µm²	233 ± 6.7	438 ± 19.8‡‡	358 ± 11.9‡‡	362 ± 8.0‡‡	na
	Normalised mean	1.00	1.88	1.54	1.56	
F11	IGP/ µm²	312 ± 17.1	291 ± 13.7	260 ± 15.4	na	361 ± 13.0
	Normalised mean	1.00	0.93	0.83		1.16
F12	IGP/ µm²	52 ± 1.6	85 ± 3.2‡‡	39 ± 1.2‡‡	81 ± 3.0‡‡	na
	Normalised mean	1.00	1.64	0.75	1.57	
M1†	IGP/ µm²	278 ± 13.0	306 ± 10.2	576 ± 16.9‡‡	na	508 ± 41.9‡‡
	Normalised mean	1.00	1.10	2.07		1.83
M2	IGP/ µm²	560 ± 32.2	565 ± 23.2	538 ± 16.3	323 ± 23.9‡‡	na
	Normalised mean	1.00	1.01	0.96	0.58	
M3	IGP/ µm²	75 ± 7.2	86 ± 4.9	100 ± 5.4	37 ± 1.6‡‡	na
	Normalised mean	1.00	1.14	1.33	0.49	
M4	IGP/ µm²	276 ± 13.8	231 ± 12.69	451 ± 15.8‡‡	577 ± 19.3‡‡	na
	Normalised mean	1.00	0.83	1.63	2.72	
M11	IGP/ µm²	165 ± 11.4	415 ± 24.9‡‡	514 ± 24.5‡‡	na	307 ± 17.8‡‡
	Normalised mean	1.00	2.51	3.11		1.85

na: not available – chromosome preparation not available

* F: Female subject

† M: Male subject

p-values are presented for negative control vs B[*a*]P exposure; One-way ANOVA followed by Bonferroni or Tamhane's T2 *post-hoc* test.

†† p<0.01 ‡‡ p<0.001

Table 7. Percentage of High Frequency SCE Cells (HFC) in human lymphocytes drawn from 16 subjects, *in vitro* exposed to benzo[*a*]pyrene. The 95th percentile of the SCE distribution was determined individually, for each subject, after analysis of their own unexposed cultures. Fifty metaphases per condition (two cultures per condition) were analyzed for all subjects.

Identifier	95th percentile SCE distribution (negative control)	HFC cells (%)			
		B[*a*]P (μM)			
		0	0.4	4	20
F1*	13.9 SCE	4	10	8	44
F2	12 SCE	6	50	82	98
F6	11 SCE	6	8	62	86
F7	13 SCE	10	36	75	na
F11	13 SCE	6	16	32	44
F12	14 SCE	6	22	64	64
F13	13.5 SCE	4	8	66	68
F14	14.5 SCE	4	52	64	76
M1†	20.5 SCE	4	2	28	38
M2	14.9 SCE	4	10	60	62
M3	12.5 SCE	4	26	58	62
M4	11.9 SCE	4	20	64	64
M5	12.5 SCE	4	36	80	82
M8	13 SCE	8	36	70	84
M11	13.9 SCE	4	40	58	70
M12	10.9 SCE	4	42	52	60

na: not available – chromosome preparation not available

* F: Female subject

† M: Male subject

workers of a coke oven plant presented less MN than medium exposed workers [49]. Furthermore, significantly less bulky DNA adducts and less MN were observed in individuals living in the Ostrava region of Czech republic, when compared to individuals living in Prague, despite higher B[*a*]P air pollution in the Ostrava region [42,50].

An explanation for this phenomenon is that this decrease could be related to the saturation of metabolic activation enzymes, as suggested by Slikker *et al.* [51]. A saturation of CYP1A1 enzyme was observed in rat hepatocytes [52], and in breast cancer MCF-7 and HepG2 cells [53], at high benzo[*a*]pyrene concentrations. In addition, phase II metabolism induction occurs when a sufficient amount of ROS is produced, thereby stabilizing Nrf2 and activating transcription of aldo-ketoreductases (AKRs), glutathione-s-transferases (GSTs) and other conjugating enzymes [54]. Acting together, those could result in production of less CA- or MN-inducing lesions following high B[*a*]P exposure, such as DNA adducts.

Our results suggest that a third factor, an increased DNA repair rate, could be involved. This is supported by our observation of a linear dose-response curve for mean SCEs per cell and % HFC (Table 2). Those SCEs are generated during homologous recombination (HR) repair, which is engaged in response to stalled replication forks. SCEs are a consequence of an incorrect resolution of Holliday junctions during HR [55]. These fork arrests result from the presence of DNA double-strand breaks (DSBs), DNA interstrand crosslinks, modified DNA bases or DNA adducts [56]. In human blood lymphocytes treated with B[*a*]P, the major BPDE diastereoisomer is (+)-*anti*-BPDE, representing 85% of all BPDE diastereoisomers, followed by (+)-*syn*-BPDE [16]. Adduction of (+)-*anti*-BPDE to guanine is mainly in *trans* (95%) in bronchoalveolar cells, while (+)-*syn*-BPDE generates *cis* and *trans* dG adducts [57]. These adducts are mainly repaired by NER [2] and repair efficiency is modulated by conformational changes caused by insertion of different BPDE diastereoisomer adducts in DNA [58]. Using an *in vitro* repair assay with human lymphoblastoid cell extracts, Custer *et al.* [59] showed that *trans*-(±)-*anti*-BPDE-dG adducts were the less efficiently repaired of all *trans* adducts. Given that these adducts would be the major ones in blood lymphocytes (as in bronchoalveolar cells), and as they are not efficiently repaired by NER, we can postulate that these adducts could still be present in DNA when replication forks progress. Presence of these unrepaired adducts would stall replication forks during the next cell cycle turn, therefore engaging HR repair to a greater extent, and leading to production of an increased number of SCEs. Conversely, if NER repair efficiency was increased, fewer DNA adducts would persist, HR repair would be less engaged and fewer SCEs would be observed. It is actually the case in smoking individuals having an increased NER repair efficiency, where decreased mean SCEs were observed [60].

As we report a significant decrease in B[*a*]P-DNA adduct level at [B[*a*]P]=20 μM, a decrease in SCE formation would be expected at this concentration. Clearly, this is not the case here (Table 2). This could result from the presence of other SCEs-inducing DNA lesions, such as DNA SSBs or 8-OH-dG, which are generated during redox cycling of B[*a*]P quinones [3]. Those damages were detected in HepG2 cells with the COMET assay, following a 4 h exposure to 2 μM B[*a*]P [61]. As for human lymphocytes, two studies failed in detecting significant DNA damages with the COMET assay after short-term (1 h and 4 h) B[*a*]P exposure [18,19], only they were using quiescent lymphocytes, unable to metabolize B[*a*]P [62]. Even if using cell cycling lymphocytes, our study did not succeed in demonstrating an increased formation of DNA SSBs with the EM-ISEL assay, following B[*a*]P exposure (Tables 2 and 6). This was also the case for Tao *et al.* [63], who did not observe significant DNA damages with the COMET assay in bronchoalveolar cells, 24 h post-B[*a*]P exposure. But when performed immediately or 12h after B[*a*]P exposure, the COMET assay did detect a significant DNA damage increase. These results suggest a fast repair of the damages detected by the COMET assay and, probably, by our EM-ISEL assay, explaining why no significant DNA SSB increase was observed in our setting.

B[*a*]P is a Clastogen and an Aneugen

In our study, exposure to B[*a*]P induced mainly formation of gaps and chromatid breaks. This is in line with the type of aberrations produced by BPDE in human lymphocytes [64], and classification of B[*a*]P as a S-dependent agent by Natarajan and Paliti [29]. Moreover, significant increase of all CA types in our study supports classification of B[*a*]P as a clastogen compound. This classification was realized after review of literature by the GUM group and based on reported significant induction of CA and MN by B[*a*]P, in various cell systems [30]. In the same way, our study also reports an increased MN frequency (Table 2). On the other hand, we observed significantly increased percentage of cells presenting chromosome losses and gains after B[*a*]P exposure, maximal at [B[*a*]P]=20 μM ($p<0.001$ – Table 2). More, using FISH-coupled MN assay, we found that exposure to [B[*a*]P]=20 μM induced most frequently formation of MN containing one or more centromeres (C+ MN – group: 77.1% compared to 68.5%; $p<0.01$ – Table 3). Percentage of C+ MN observed in our study in the control condition (68.5%) is in the range of values reported in literature, which shows a quite high variation: from 33% C+ MN in Decordier *et al.* [65], up to 77% in Iarmarcovai *et al.* [33]. Intermediate values, such as 45% C+ MN in the control subjects of Vl achodimitropoulos [66] or 65% in Cakmak Demircigil *et al.* (2011) [67] are also reported.

We created two new categories to further refine our analysis: C_{1-2}+ and C_{3+}+ MN. Those permitted distinction between events possibly involving only one chromosome or its two chromatids (C_{1-2}+ MN), and events necessarily involving 2 or more chromosomes (C_{3+}+ MN). This new categorization revealed that, in our study, C+ MN generated after B[*a*]P exposure contained 3 or more centromeres in a significantly increased proportion, compared to the negative control (group: 78.3% compared to 66.5%; p<0.01 – Table 3). This suggests that B[*a*]P also has an aneugenic effect on human lymphocytes, and this effect is observed similarly in men and in women (Table 2). As most MN produced by B[*a*]P contain three or more centromeres in our study, this could be related to an impairment at the centrosome level [68]. Indeed, Shinmura *et al.* [69] found that BPDE exposure of a p53-deficient lung cancer cell line induced an excessive centrosome duplication following a prolonged S-phase arrest, and subsequently, chromosome instability appearance. On the other hand, our results also support the findings of Matsuoka *et al.* [70] on a V79-MZ hamster cell line. They found that in B[*a*]P-treated cells (1.25 to 10 μM for 24 h), distribution of chromosome number ranged from a diploid to a tetraploid number. Further analyses showed presence of spindle disturbances (incomplete spindles, multipolar spindles and lagging chromosomes) immediately after B[*a*]P exposure, as well as appearance of hyperdiploidy in V79-MZ cells cultured up to five months after exposure [71]. Interestingly, these cells contain no detectable aryl hydrocarbon hydroxylase activity, suggesting a direct action of unmetabolized B[*a*]P on cellular components, probably on the mitotic spindle, as observed with classic aneugenic agents such as colchicine and vinscristine [72].

Correlations between B[*a*]P exposure, B[*a*]P-DNA adducts and early biomarkers of genotoxicity

In our study, early biomarker levels (B[*a*]P-DNA adducts, SCEs, % HFC, MN and CA frequencies) correlated significantly with B[*a*]P exposure (Table 4). Those correlations were not established in previous published *in vitro* studies using human lymphocytes. Nevertheless, correlations were analyzed in occupational studies (e.g. non experimental designs) with contradictory results. Indeed, mean SCEs, MN frequency and/ or CA frequency were associated with PAH exposure in coke oven workers and coal exposed workers in many studies [6,8,9,73], while no association was found in other reports [7,49]. The same contradictory situation was reported for DNA adducts and PAH exposure, as reviewed by Brandt and Watson [74].

Investigation of relations existing between early biomarkers measured in our study revealed that B[*a*]P-DNA adducts correlated significantly with the two SCE indicators (mean SCEs per cell and % HFC; p=0.008 and p=0.002, respectively – Table 4), when controlling for B[*a*]P exposure and sex. In literature, significant positive correlations between SCEs and B[*a*]P-induced or bulky DNA-adducts were reported only in one *in vitro* study conducted on human lymphocytes of two subjects [17], and in one cohort study exposed to environmental pollution in Upper Silesia (Poland) [75]. However, in coke oven workers, no significant correlations were observed between SCEs or % HFC, and bulky DNA-adducts [14,76].

In our study, multiple linear regression analysis showed that a large part of the variability present when analysing SCE indicators (mean SCEs per cell and % HFC) can be explained by B[*a*]P exposure and by B[*a*]P-DNA adducts. This analysis also showed that % HFC is the only variable significantly affecting CA and MN frequencies, providing a link between SCEs, CAs and MN. Furthermore, we report a significant correlation existing between CA frequency and MN per MNBN (r=0.351, p=0.011; Table 4), likely related to the role of chromosome breaks in MN formation [32]. On the other hand, in addition to DNA adducts, other DNA lesions caused by B[*a*]P exposure, such as DSBs and 8-OH-dG, are also known to lead to SCE formation [56]. While DSBs are responsible for CA and MN formation [77], importance of 8-OH-dG in MN formation should not be excluded, as MN and 8-OH-dG increases were significantly associated with PAH exposure in coke oven workers [9]. Also, as B[*a*]P quinones were detected in B[*a*]P-treated human lymphocytes [16], 8-OH-dG formation is almost certainly present in this cell type. In our study, because % HFC's variability is linked to B[*a*]P-DNA adducts, CA frequency and MN frequency (when excluding effect of B[*a*]P exposure and sex), this gives support to a role of B[*a*]P-DNA adducts, DNA DSBs and 8-OH-dG, as DNA primary lesions, in the production of cells containing a high number of SCEs. These factors could explain why % HFC is considered a more sensible genotoxic endpoint than mean SCEs per cells [78]. At last, heritability has a major role in MN and CA formation, explaining up to 70% of interindividual variation [79,80], while it explains only 30% of interindividual variation in SCEs [81]. Thus, it is not surprising that, in our study, an environmental factor such as B[*a*]P exposure, cannot significantly explain the observed variability in CA and MN frequencies. Only % HFC, which reflects action of both environmental and genetic factors, significantly explains a small part of CA and MN frequencies (34% and 12%, respectively).

Men could be more sensitive to genotoxic effects caused by low B[*a*]P exposure

In our cohort, sex differences were investigated by direct comparison of all early biomarker levels (B[*a*]P-DNA adducts, DNA SSBs, SCE, CA and MN), between men and women. Following B[*a*]P exposure of blood lymphocytes, statistical analysis did not reveal significant sex differences (bilateral Student's *t*-test for independent samples – Table 2), even if these levels were increased in women. On the other hand, when analyzing the dose-response curves observed in women and men separately, an increased sensitivity of men showed up, especially to our lower B[*a*]P concentrations. Taking into account B[*a*]P-DNA adducts, men presented more adducts than women in all B[*a*]P-exposure conditions, even though this difference did not reach statistical significance (Table 3). Also, only men presented a significant increase in the following biomarkers at [B[*a*]P]=0.4 μM: mean SCEs, % HFC, CA frequency and MN frequency (Table 2).

Sex differences in DNA damages have not been reported frequently and could be related to specific exposures. In individuals exposed to air pollution, a significantly increased bulky DNA adduct level in lymphocytes was reported only in males [82]. On the other hand, in Upper Silesia (Poland), significantly increased blood DNA adduct level, mean SCEs and MN frequency were found in girls, but not in boys, in response to a complex environmental exposure [75]. Sex differences in genotoxic response to B[*a*]P or PAHs could be associated with different metabolic capacities, specific for a particular cell type. In fact, women have a significantly lower baseline CYP1A1 activity in blood cells, and this activity is less inducible by 3-methylcholanthrene, when compared to men [83]. This could explain the lower B[*a*]P-DNA adducts level observed in women of our cohort. On the contrary, significantly more bulky DNA adducts are found in lung tissues taken from non-smoking females, when compared with non-smoking male tissues [84]. Furthermore, lung adenocarcinoma cell lines of female origin have significantly more B[*a*]P-induced DNA adducts, increased basal and induced CYP1A1 levels, and increased CYP1A1 activity [85]. Together,

these findings support the concept that sex differences could exist, and may be related to specific exposures and cell types.

In summary, our study shows that, when measuring early biomarkers (B[*a*]P-DNA adducts, MN and CAs), an inverted-U dose-response curve is present in human lymphocytes exposed to low B[*a*]P concentrations. This particular dose-response curve could be caused by the coordinate action of three pathways, namely a saturation in metabolic activation enzyme activity, the induction of phase II metabolic enzymes, and an increased DNA repair rate in high B[*a*]P exposure conditions, as observed in some population studies. On the other hand, as SCE test gave a linear dose-response curve, it suggests that some DNA lesions, such as unrepaired DNA adducts or 8-OH-dG, engaged HR repair to a greater extent at high B[*a*]P concentrations. As unrepaired adducts could persist, a kinetic study evaluating formation and repair rates of B[*a*]P-DNA adducts in human lymphocytes could be interesting, using similar and lower B[*a*]P concentrations. Also, our multiple regression analysis independently linked % HFC's variability to three other biomarkers: B[*a*]P-DNA adducts, CA frequency and MN frequency, when controlling for B[*a*]P exposure and sex. Since % HFC is the only variable significantly affecting CA and MN frequency, and since CA and MN are formed through a common DNA lesion, namely DNA DSBs, this suggests a role for DNA DSBs, and probably for 8-OH-dG, in the production of cells containing a high number of SCEs.

Additionally, clastogenic activity of B[*a*]P in normal (untransformed) human cells was confirmed by our results. And, most importantly, aneugenic activity of B[*a*]P on human lymphocytes was suggested by the presence of metaphases presenting chromosome losses and gains after B[*a*]P exposure. This was confirmed by FISH-coupled MN assay showing that most B[*a*]P-induced MN contained three or more centromeres, also suggesting an aneugenic effect. Use of increased proportion of C+ MN and/or C_{3+}+ MN as biomarkers of aneugenic activity must be validated. The work recently published by Speit *et al.* on formaldehyde [72] describes an interesting approach to *in vitro* characterization of potential aneugen compounds integrating FISH-coupled MN assay to more classical assays. This integrative approach could be interesting to investigate further the aneugenic activity of B[*a*]P on human cells. Our results, together with karyotypic changes [70], spindle disturbances [71] and centrosome duplication [69], provide additional evidences that B[*a*]P could play a role in the progression step of carcinogenesis. Involvement of B[*a*]P and other PAH during cancer progression needs to be confirmed in studies involving humans environmentally or occupationally exposed to PAH, particularly using FISH-coupled MN assay. This might be of particular importance given that exposure to B[*a*]P and other PAHs (smoking, environmental and occupational exposure) are chronic and may last for decades.

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References

1. Straif K, Baan R, Grosse Y, Secretan B, El Ghissassi F, et al. (2005) Carcinogenicity of polycyclic aromatic hydrocarbons. *Lancet Oncology* 6: 931-932.
2. Xue W, Warshawsky D (2005) Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. *Toxicol Appl Pharmacol* 206: 73-93. [Crossref]
3. Park JH, Troxel AB, Harvey RG, Penning TM (2006) Polycyclic aromatic hydrocarbon (PAH) o-quinones produced by the aldo-keto-reductases (AKRs) generate abasic sites, oxidized pyrimidines, and 8-oxo-dGuo via reactive oxygen species. *Chem Res Toxicol* 19: 719-728. [Crossref]
4. Luch A (2005) Nature and nurture - lessons from chemical carcinogenesis. *Nat Rev Cancer* 5: 113-125. [Crossref]
5. Shimada T (2006) Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. *Drug Metab Pharmacokinet* 21: 257-276. [Crossref]
6. Celik M, Donbak L, Unal F, Yüzbasioglu D, Aksoy H, et al. (2007) Cytogenetic damage in workers from a coal-fired power plant. *Mutat Res* 627: 158-163. [Crossref]
7. Siwińska E, Mielzyńska D, Kapka L. (2004) Association between urinary 1-hydroxypyrene and genotoxic effects in coke oven workers. *Occup Environ Med* 61: e10. [Crossref]
8. Donbak L, Rencuzogullari E, Yavuz A, Topaktas M (2005) The genotoxic risk of underground coal miners from Turkey. *Mutat Res* 588: 82-87. [Crossref]
9. Liu AL, Lu WQ, Wang ZZ, Chen WH, Lu WH, et al. (2006) Elevated levels of urinary 8-hydroxy-2 -deoxyguanosine, lymphocytic micronuclei, and serum glutathione S-transferase in workers exposed to coke oven emissions. *Environ Health Perspect* 114: 673-677. [Crossref]
10. Deng Q, Huang S, Zhang X, Zhang W, Feng J, et al. (2014) Plasma microRNA expression and micronuclei frequency in workers exposed to polycyclic aromatic hydrocarbons. *Environ Health Perspect* 122: 719-725. [Crossref]
11. Xiao C, Chen S, Li J, Hai T, Lu Q, et al. (2002) Association of HSP70 and genotoxic damage in lymphocytes of workers exposed to coke-oven emission. *Cell Stress Chaperones* 7: 396-402. [Crossref]
12. Yang X, Zheng J, Bai Y, Tian F, Yuan J, et al. (2007) Using lymphocyte and plasma Hsp70 as biomarkers for assessing coke oven exposure among steel workers. *Environ Health Perspect* 115: 1573-1577. [Crossref]
13. Carstensen U, Hou SM, Alexandrie AK, Hogstedt B, Tagesson C, et al. (1999) Influence of genetic polymorphisms of biotransformation enzymes on gene mutations, strand breaks of deoxyribonucleic acid, and micronuclei in mononuclear blood cells and urinary 8-hydroxydeoxyguanosine in potroom workers exposed to polyaromatic hydrocarbons. *Scand J Work Environ Health* 25: 351-360. [Crossref]
14. van Delft JH, Steenwinkel MS, van Asten JG, de Vogel N, Bruijntjes-Rozier TC, et al. (2001) Biological monitoring the exposure to polycyclic aromatic hydrocarbons of coke oven workers in relation to smoking and genetic polymorphisms for GSTM1 and GSTT1. *Ann Occup Hyg* 45: 395-408. [Crossref]
15. Cavallo D, Ursini CL, Rondinone B, Iavicoli S (2009) Evaluation of a suitable DNA damage biomarker for human biomonitoring of exposed workers. *Environ Mol Mutagen* 50: 781-790. [Crossref]
16. Okano P, Miller HN, Robinson RC, Gelboin HV (1979) Comparison of benzo(a)pyrene and (-)-trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene metabolism in human blood monocytes and lymphocytes. *Cancer Res* 39: 3184-3193. [Crossref]
17. Wiencke JK, McDowell ML, Bodell WJ (1990) Molecular dosimetry of DNA adducts and sister chromatid exchanges in human lymphocytes treated with benzo[a]pyrene. *Carcinogenesis* 11: 1497-1502. [Crossref]
18. Frenzilli G, Bosco E, Barale R (2000) Validation of single cell gel assay in human leukocytes with 18 reference compounds. *Mutat Res* 468: 93-108. [Crossref]
19. Kleinsasser NH, Wallner BC, Kastenbauer ER, Muenzenrieder RK, Harreus UA (2000) Comparing the genotoxic sensitivities of human peripheral blood lymphocytes and mucosa cells of the upper aerodigestive tract using the Comet assay. *Mutat Res* 467: 21-30. [Crossref]
20. Guven GS, Guven M, Onaran H, Ulutin T, Hacianefioglu S (2006) Individual sensitivity

to cytogenetic effects of benzo[alpha]pyrene in cultured human lymphocytes: Influence of glutathione S-transferase M1 genotype. *Genet Mol Biol* 29: 142-147.

21. Hatzi VI, Terzoudi GI, Stavropoulou C, Malik SI, Makropoulos V, et al. (2011) Lack of association between GSTT1 polymorphism and endogenous or benzo[a]pyrene-induced sister chromatid exchanges as analyzed in metaphase or G2-phase lymphocytes. *Mol Biol Rep* 38: 3959-3966. [Crossref]

22. Salama SA, Sierra-Torres CH, Oh HY, Hamada FA, Au WW (2001) Variant metabolizing gene alleles determine the genotoxicity of benzo[a]pyrene. *Environ Mol Mutagen* 37: 17-26. [Crossref]

23. Cho YH, Chung HW (2003) The effect of extremely low frequency electromagnetic fields (ELF-EMF) on the frequency of micronuclei and sister chromatid exchange in human lymphocytes induced by benzo[a]pyrene. *Toxicol Lett* 143: 37-44. [Crossref]

24. Warshawsky D, Livingston GK, Fonouni-Fard M, LaDow K (1995) Induction of micronuclei and sister chromatid exchanges by polycyclic and N-heterocyclic aromatic hydrocarbons in cultured human lymphocytes. *Environ Mol Mutagen* 26: 109-118. [Crossref]

25. Hastak K, Lubri N, Jakhi SD, More C, John A, et al. (1997) Effect of turmeric oil and turmeric oleoresin on cytogenetic damage in patients suffering from oral submucous fibrosis. *Cancer Lett* 116: 265-269. [Crossref]

26. Siest G, Jeannesson E, Marteau JB, Samara A, Marie B, et al. (2008) Transcription factor and drug-metabolizing enzyme gene expression in lymphocytes from healthy human subjects. *Drug Metab Dispos* 36: 182-189. [Crossref]

27. Lin P, Hu SW, Chang TH (2003) Correlation between gene expression of aryl hydrocarbon receptor (AhR), hydrocarbon receptor nuclear translocator (Arnt), cytochromes P4501A1 (CYP1A1) and 1B1 (CYP1B1), and inducibility of CYP1A1 and CYP1B1 in human lymphocytes. *Toxicol Sci* 71: 20-26. [Crossref]

28. Fenech M (2007) Cytokinesis-block micronucleus cytome assay. *Nat Protoc 2*: 1084-1104. [Crossref]

29. Natarajan AT, Palitti F (2008) DNA repair and chromosomal alterations. *Mutat Res* 657: 3-7. [Crossref]

30. Miller B, Potter-Locher F, Seelbach A, Stopper H, Utesch D, et al. (1998) Evaluation of the in vitro micronucleus test as an alternative to the in vitro chromosomal aberration assay: position of the GUM Working Group on the in vitro micronucleus test. Gesellschaft fur Umwelt-Mutations-forschung. *Mutat Res* 410: 81-116.

31. Corvi R, Albertini S, Hartung T, Hoffmann S, Maurici D, et al. (2008) ECVAM retrospective validation of in vitro micronucleus test (MNT). *Mutagenesis* 23: 271-283. [Crossref]

32. Norppa H, Falck GC (2003) What do human micronuclei contain? *Mutagenesis* 18: 221-233. [Crossref]

33. Iarmarcovai G, Bonassi S, Sari-Minodier I, Baciuchka-Palmaro M, Botta A, et al. (2007) Exposure to genotoxic agents, host factors, and lifestyle influence the number of centromeric signals in micronuclei: a pooled re-analysis. *Mutat Res* 615: 18-27. [Crossref]

34. Dabrowska D, Kot-Wasik A, Namiesnik J (2008) Stability studies of selected polycyclic aromatic hydrocarbons in different organic solvents and identification of their transformation products. *Pol J Environ Stud* 17: 17-24.

35. Lemieux N, Drouin R, Richer CL (1990) High-resolution dynamic and morphological G-bandings (GBG and GTG): a comparative study. *Hum Genet* 85: 261-266. [Crossref]

36. Divi RL, Beland FA, Fu PP, Von Tungeln LS, Schoket B, et al. (2002) Highly sensitive chemiluminescence immunoassay for benzo[a]pyrene-DNA adducts: validation by comparison with other methods, and use in human biomonitoring. *Carcinogenesis* 23: 2043-2049. [Crossref]

37. Depault F, Cojocaru M, Fortin F, Chakrabarti S, Lemieux N (2006) Genotoxic effects of chromium(VI) and cadmium(II) in human blood lymphocytes using the electron microscopy in situ end-labeling (EM-ISEL) assay. *Toxicol In Vitro* 20: 513-518. [Crossref]

38. Richer CL, Senécal-Quevillon M, Duquette P (1987) Analysis of low concentrations of 5-bromo-2-deoxyuridine on sister chromatid exchanges in human lymphocytes. *Genome* 29: 165-168. [Crossref]

39. OECD, Test No. 479: In vitro sister chromatid exchange assay in mammalian cells, OECD Publishing, Paris, 1986.

40. Kosmider B, Wyszynska K, Janik-Spiechowicz E, Osiecka R, Zyner E, et al. (2004) Evaluation of the genotoxicity of cis-bis(3-aminoflavone)dichloroplatinum(II) in comparison with cis-DDP. *Mutat Res* 558: 93-110. [Crossref]

41. OECD, Test No. 473: In vitro Mammalian Chromosome Aberration Test, OECD Publishing, Paris, 1997.

42. Rossner P, Jr Svecova V, Schmuczerova J, Milcova A, Tabashidze N, et al. (2013) Analysis of biomarkers in a Czech population exposed to heavy air pollution. Part I: bulky DNA adducts. *Mutagenesis 2*8: 89-95. [Crossref]

43. Poirier MC, Weston A, Schoket B, Shamkhani H, Pan CF, et al. (1998) Biomonitoring of United States Army soldiers serving in Kuwait in 1991. *Cancer Epidemiol Biomarkers Prev* 7: 545-551. [Crossref]

44. Pavanello S, Favretto D, Brugnone F, Mastrangelo G, Dal Pra G, et al. (1999) HPLC/fluorescence determination of anti-BPDE-DNA adducts in mononuclear white blood cells from PAH-exposed humans. *Carcinogenesis* 20: 431-435. [Crossref]

45. Kang DH, Rothman N, Poirier MC, Greenberg A, Hsu CH, et al. (1995) Interindividual differences in the concentration of 1-hydroxypyrene-glucuronide in urine and polycyclic aromatic hydrocarbon-DNA adducts in peripheral white blood cells after charbroiled beef consumption. *Carcinogenesis* 16: 1079-1085. [Crossref]

46. van Maanen JM, Moonen EJ, Maas LM, Kleinjans JC, van Schooten FJ (1994) Formation of aromatic DNA adducts in white blood cells in relation to urinary excretion of 1-hydroxypyrene during consumption of grilled meat. *Carcinogenesis* 15: 2263-2268. [Crossref]

47. García-Suástegui WA, Huerta-Chagoya A, Carrasco-Colín KL, Pratt MM, John K, et al. (2011) Seasonal variations in the levels of PAH-DNA adducts in young adults living in Mexico City. *Mutagenesis* 26: 385-391. [Crossref]

48. Binková B, Giguère Y, Rössner P Jr, Dostál M, Srám RJ (2000) The effect of dibenzo[a,1]pyrene and benzo[a]pyrene on human diploid lung fibroblasts: the induction of DNA adducts, expression of p53 and p21(WAF1) proteins and cell cycle distribution. *Mutat Res* 471: 57-70. [Crossref]

49. Van Hummelen P, Gennart JP, Buchet JP, Lauwerys R, Kirsch-Volders M (1993) Biological markers in PAH exposed workers and controls. *Mutat Res* 300: 231-239. [Crossref]

50. Rossner P Jr, Rossnerova A, Spatova M, Beskid O, Uhlirova K, et al. (2013) Analysis of biomarkers in a Czech population exposed to heavy air pollution. Part II: chromosomal aberrations and oxidative stress. *Mutagenesis* 28: 97-106. [Crossref]

51. Slikker W, Jr., Andersen ME, Bogdanffy MS, Bus JS, Cohen SD, et al. (2004) Dose-dependent transitions in mechanisms of toxicity. *Toxicol Appl Pharmacol* 201: 203-225. [Crossref]

52. Till M, Riebniger D, Schmitz HJ, Schrenk D (1999) Potency of various polycyclic aromatic hydrocarbons as inducers of CYP1A1 in rat hepatocyte cultures. *Chem Biol Interact* 117: 135-150. [Crossref]

53. Skupinska K, Misiewicz I, Kasprzycka-Guttman T (2007) A comparison of the concentration-effect relationships of PAHs on CYP1A induction in HepG2 and Mcf7 cells. *Arch Toxicol* 81: 183-200. [Crossref]

54. Jiang H, Gelhaus SL, Mangal D, Harvey RG, Blair IA, et al. (2007) Metabolism of benzo[a]pyrene in human bronchoalveolar H358 cells using liquid chromatography-mass spectrometry. *Chem Res Toxicol* 20: 1331-1341. [Crossref]

55. Hirano S, Yamamoto K, Ishiai M, Yamazoe M, Seki M, et al. (2005) Functional relationships of FANCC to homologous recombination, translesion synthesis, and BLM. *EMBO* J 24: 418-427. [Crossref]

56. Andreassen PR, Ho GP, D'Andrea AD (2006) DNA damage responses and their many interactions with the replication fork. *Carcinogenesis* 27: 883-892. [Crossref]

57. Ruan Q, Gelhaus SL, Penning TM, Harvey RG, Blair IA (2007) Aldo-keto reductase- and cytochrome P450-dependent formation of benzo[a]pyrene-derived DNA adducts in human bronchoalveolar cells. *Chem Res Toxicol* 20: 424-431. [Crossref]

58. Hess MT, Gunz D, Luneva N, Geacintov NE, Naegeli H (1997) Base pair conformation-dependent excision of benzo[a]pyrenediol epoxide-guanine adducts by human nucleotide excision repair enzymes. *Mol Cell Biol* 17: 7069-7076. [Crossref]

59. Custer L, Zajc B, Sayer JM, Cullinane C, Phillips DR, et al. (1999) Stereospecific differences in repair by human cell extracts of synthesized oligonucleotides containing trans-opened 7,8,9, 10-tetrahydrobenzo[a]pyrene 7,8-diol 9,10-epoxide N2-dG adduct stereoisomers located within the human K-ras codon 12 sequence. *Biochemistry (Mosc)* 38: 569-581. [Crossref]

60. Stierum RH, Hageman GJ, van Herwijnen MH, van der Veer MS, Kleinjans JC (1997) Age-related negative associations between parameters of cytogenetic damage and ex vivo (+/-)-anti-benzo(a)pyrenediolepoxide-induced unscheduled DNA synthesis in smoking humans. *Cancer EpidemiolBiomarkersPrev* 6: 943-948.

61. Tarantini A, Maitre A, Lefebvre E, Marques M, Marie C, et al. (2009) Relative contribution of DNA strand breaks and DNA adducts to the genotoxicity of benzo[a] pyrene as a pure compound and in complex mixtures. *Mutat Res* 671: 67-75. [Crossref]

62. Holz O, Krause T, Rüdiger HW (1991) Differences in DNA adduct formation between monocytes and lymphocytes after in vivo incubation with benzo[a]pyrene. *Carcinogenesis* 12: 2181-2183. [Crossref]

63. Tao GH, Yang LQ, Gong CM, Huang HY, Liu JD, et al. (2009) Effect of PARP-1 deficiency on DNA damage and repair in human bronchial epithelial cells exposed to Benzo(a)pyrene. *Mol Biol Rep* 36: 2413-2422. [Crossref]

64. Wu X, Lippman SM, Lee JJ, Zhu Y, Wei QV, et al. (2002) Chromosome instability in lymphocytes: a potential indicator of predisposition to oral premalignant lesions. *Cancer Res* 62: 2813-2818. [Crossref]

65. Decordier I, Dillen L, Cundari E, Kirsch-Volders M (2002) Elimination of micronucleated cells by apoptosis after treatment with inhibitors of microtubules. *Mutagenesis* 17: 337-344. [Crossref]

66. Vlachodimitropoulos D, Norppa H, Autio K, Catalán J, Hirvonen A, et al. (1997) GSTT1-dependent induction of centromere-negative and -positive micronuclei by 2:3,4-diepoxybutane in cultured human lymphocytes. *Mutagenesis* 12: 397-403. [Crossref]

67. Cakmak Demircigil G, Aykanat B, Fidan K, Gulleroglu K, Bayrakci US, et al. (2011) Micronucleus frequencies in peripheral blood lymphocytes of children with chronic kidney disease. *Mutagenesis* 26: 643-650. [Crossref]

68. Iarmarcovai G, Botta A, Orsière T (2006) Number of centromeric signals in micronuclei and mechanisms of aneuploidy. *Toxicol Lett* 166: 1-10. [Crossref]

69. Shinmura K, Iwaizumi M, Igarashi H, Nagura K, Yamada H, et al. (2008) Induction of centrosome amplification and chromosome instability in p53-deficient lung cancer cells exposed to benzo[a]pyrene diol epoxide (B[a]PDE). *J Pathol* 216: 365-374. [Crossref]

70. Matsuoka A, Ozaki M, Takeshita K, Sakamoto H, Glatt HR, et al. (1997) Aneuploidy induction by benzo[a]pyrene and polyploidy induction by 7,12-dimethylbenz[a] anthracene in Chinese hamster cell lines V79-MZ and V79. *Mutagenesis* 12: 365-372. [Crossref]

71. Matsuoka A, Matsuura K, Sakamoto H, Hayashi M, Sofuni T (1998) Spindle disturbances induced by benzo[a]pyrene and 7, 12-dimethylbenz[a]anthracene in a Chinese hamster cell line (V79-MZ) and the stability of the numerical chromosome aberrations that follow. *Mutat Res* 419: 1-12. [Crossref]

72. Speit G, Zeller J, Neuss S (2011) The in vivo or ex vivo origin of micronuclei measured in human biomonitoring studies. *Mutagenesis* 26: 107-110. [Crossref]

73. Kalina I, Brezáni P, Gajdosová D, Binková B, Salagovic J, et al. (1998) Cytogenetic monitoring in coke oven workers. *Mutat Res* 417: 9-17. [Crossref]

74. Brandt HC, Watson WP (2003) Monitoring human occupational and environmental exposures to polycyclic aromatic compounds. *Ann Occup Hyg* 47: 349-378. [Crossref]

75. Mielzynska D, Siwinska E, Kapka L, Szyfter K, Knudsen LE, et al. (2006) The influence of environmental exposure to complex mixtures including PAHs and lead on genotoxic effects in children living in Upper Silesia, Poland. *Mutagenesis* 21: 295-304. [Crossref]

76. Popp W, Vahrenholz C, Schell C, Grimmer G, Dettbarn G, et al. (1997) DNA single strand breakage, DNA adducts, and sister chromatid exchange in lymphocytes and phenanthrene and pyrene metabolites in urine of coke oven workers. *Occup Environ Med* 54: 176-183. [Crossref]

77. Delacôte F, Lopez BS (2008) Importance of the cell cycle phase for the choice of the appropriate DSB repair pathway, for genome stability maintenance: the trans-S double-strand break repair model. *Cell Cycle* 7: 33-38. [Crossref]

78. Bonassi S, Fontana V, Ceppi M, Barale R, Biggeri A (1999) Analysis of correlated data in human biomonitoring studies. The case of high sister chromatid exchange frequency cells. *Mutat Res* 438: 13-21. [Crossref]

79. Surowy H, Rinckleb A, Luedeke M, Stuber M, Wecker A, et al. (2011) Heritability of baseline and induced micronucleus frequencies. *Mutagenesis* 26: 111-117. [Crossref]

80. Tedeschi B, Cicchetti R, Argentin G, Caporossi D, Pittaluga M, et al. (2004) Aphidicolin and bleomycin induced chromosome damage as biomarker of mutagen sensitivity: a twin study. *Mutat Res* 546: 55-64. [Crossref]

81. Hirsch BA, Sentz KK, McGue M (1992) Genetic and environmental influences on baseline SCE. *Environ Mol Mutagen* 20: 2-11. [Crossref]

82. Georgiadis P, Topinka J, Stoikidou M, Kaila S, Gioka M, et al. (2001) Biomarkers of genotoxicity of air pollution (the AULIS project): bulky DNA adducts in subjects with moderate to low exposures to airborne polycyclic aromatic hydrocarbons and their relationship to environmental tobacco smoke and other parameters. *Carcinogenesis* 22: 1447-1457. [Crossref]

83. Smart J, Daly AK (2000) Variation in induced CYP1A1 levels: relationship to CYP1A Ah receptor and GSTM1 polymorphisms. *Pharmacogenetics* 10: 11-24. [Crossref]

84. Cheng YW, Hsieh LL, Lin PP, Chen CP, Chen CY, et al. (2001) Gender difference in DNA adduct levels among nonsmoking lung cancer patients. *Environ Mol Mutagen* 37: 304-310. [Crossref]

85. Uppstad H, Osnes GH, Cole KJ, Phillips DH, Haugen A, et al. (2011) Sex differences in susceptibility to PAHs is an intrinsic property of human lung adenocarcinoma cells. *Lung Cancer* 71: 264-270. [Crossref]