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Increased levels of chromosomal aberrations and DNA damage in a group of workers exposed to formaldehyde

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Abstract

Formaldehyde (FA) is a commonly used chemical in anatomy and pathology laboratories as a tissue preservative and fixative. Because of its sensitising properties, irritating effects and cancer implication, FA accounts probably for the most important chemical-exposure hazard concerning this professional group. Evidence for genotoxic effects and carcinogenic properties in humans is insufficient and conflicting, particularly in regard to the ability of inhaled FA to induce toxicity on other cells besides first contact tissues, such as buccal and nasal cells. To evaluate the effects of exposure to FA in human peripheral blood lymphocytes, a group of 84 anatomy pathology laboratory workers exposed occupationally to FA and 87 control subjects were tested for chromosomal aberrations (CAs) and DNA damage (comet assay). The level of exposure to FA in the workplace air was evaluated. The association between genotoxicity biomarkers and polymorphic genes of xenobiotic-metabolising and DNA repair enzymes were also assessed. The estimated mean level of FA exposure was 0.38 ± 0.03 ppm. All cytogenetic endpoints assessed by CAs test and comet assay % tail DNA (%TDNA) were significantly higher in FA-exposed workers compared with controls. Regarding the effect of susceptibility biomarkers, results suggest that polymorphisms in *CYP2E1* and *GSTP1* metabolic genes, as well as, *XRCC1* and *PARP1* polymorphic genes involved in DNA repair pathways are associated with higher genetic damage in FA-exposed subjects. Data obtained in this study show a potential health risk situation of anatomy pathology laboratory workers exposed to FA (0.38 ppm). Implementation of security and hygiene measures may be crucial to decrease risk. The obtained information may also provide new important data to be used by health care programs and by governmental agencies responsible for occupational health and safety.

Introduction

Formaldehyde (FA) is a building block for many chemical compounds with a wide range of industrial and medical uses. It is a high production volume chemical (HPV) worldwide, to which many people are exposed both occupationally and environmentally. The major route of exposure to FA is inhalation. Given its economic importance

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and wide spread use, occupational exposure to FA involves not only individuals employed in the direct manufacture of FA and products containing it but also those using the products (e.g. hairdressers, embalmers). Although environmental exposure typically occurs at much lower levels than occupational exposure, general population is exposed to FA through tobacco smoke, off-gassing from construction and home-furnishing products or combustion sources, such as automobiles and refineries. The highest level of human exposure to FA occurs in occupational settings, namely in anatomy pathology laboratories where it is commonly used as a fixative and tissue preservative. Indoor air analyses have consistently shown that the levels of airborne FA in anatomy laboratories exceed recommended exposure criteria (ranging from 0.30 to 2 ppm) (1,2). In the last decade, a large number of toxicological studies were published about FA. The genotoxicity is confirmed in a variety of experimental systems ranging from bacteria to rodents. Although these positive findings may provide a basis for extrapolation to humans, in human biomonitoring studies the genotoxic effects of FA have been inconsistent with both positive and negative outcomes (3,4). Biological evidence of toxicity on distant-site such as peripheral lymphocytes and bone marrow is still insufficient and conflicting (5–7). Some authors stated that as inhaled FA is rapidly metabolised, it would not be expected to enter the systemic circulation and for that reason genotoxic and carcinogenic effects (leukaemia) in animals and humans are limited to local effects, in the area of first contact (8,9).

Listed since 2004 by the International Agency for Research on Cancer as a human carcinogen (3), FA status was recently revised by the US government that reclassified this compound as known to be a *human carcinogen* (10). Both classifications are based on sufficient evidence of carcinogenicity from epidemiologic studies, supporting data on mechanisms of carcinogenesis and experimental evidence in animals. Numerous epidemiological studies demonstrated a causal relationship between occupational exposure to FA and cancer (11,12). Consistent findings of increased risks of nasopharyngeal carcinoma and leukaemia, particularly myeloid leukaemia, were found among workers with high measures of exposure to FA (exposure level or duration) (4).

Cytogenetic endpoints have long been applied in human biomonitoring studies for the detection of early biological effects of genotoxic agents. Among these biomarkers, chromosome aberrations (CAs) are the most widely used and best validated biomarker of early effects (13). The formation of structural CAs requires one or several DNA double-strand breaks (DSBs). Structural CAs may be induced by direct DNA breakage, replication of a damaged DNA template, inhibition of DNA synthesis or other mechanisms (14). CAs in lymphocytes revealed to be predictive of overall cancer risk in human populations (15). Thus, their frequency in lymphocytes is believed to represent a surrogate endpoint for more specific chromosome alterations in target tissues undergoing carcinogenesis. Despite the biological relevance and predictive value of CAs, only a few studies have addressed the frequency of this endpoint on FA-exposed subjects.

During the last years, the single cell gel electrophoresis assay or comet assay has been proven to be a very sensitive tool in human biomonitoring for the detection of DNA damage at the individual cell level (16). In its simplest form, with an alkaline treatment and electrophoresis at high pH, it detects DNA DSBs (single and double stranded) and also alkali-labile sites, particularly apurinic/apyrimidinic sites (17).

Genetic polymorphisms are considered to play a primary role in individual response to carcinogen-induced disease. Therefore, polymorphic genes involved in the metabolism of xenobiotics and in DNA repair pathways have been studied as susceptibility biomarkers in order to understand the possible modulator effect of these genetic determinants on genetic damage and to assess the individual risk of exposure to genotoxic chemicals.

The aim of this study was to evaluate cytogenetic alterations by CAs test and DNA damage by comet assay in peripheral blood lymphocytes (PBLs) of workers exposed to FA in anatomy pathology laboratories. Air sampling was also performed in order to determine FA-level of exposure of each worker. In addition, the effect of genetic polymorphisms of xenobiotic-metabolising enzymes (*CYP2E1*, *GSTM1*, *GSTT1*, *GSTP1*) and DNA repair enzymes (*XRCC3, XRCC1, PARP1, MUTYH*) on the endpoints studied was determined.

Materials and Methods

Subjects

Study population consisted of 84 workers exposed to FA from nine Hospital Anatomy Pathology laboratories, located in the North and centre of Portugal, working for at least 1 year, and 87 non-exposed control employees working in the same area in administrative offices and without occupational exposure history to FA. Exclusion criteria regarding health parameters previously defined included cancer/tumour history, radiation therapy or chemotherapy treatments, last year surgical intervention with anaesthesia and blood transfusions. Health conditions, general medical history, medication, diagnostic tests (X-rays, etc.) and relevant individual information such as age, smoking habits, alcohol consumption and dietary habits were assessed by means of questionnaires.

The survey on dietary habits was based on Bonassi *et al.* (18) and included the daily consumption of tea, coffee, fruits, vegetables (salad and soup), protein intake (fish and meat) and vitamin supplements. Subjects of the exposed group also gave information related to working practices, namely use of personal protective equipment, years of employment, specific symptoms related to FA exposure and chronic respiratory diseases such as asthma or others. All individuals who agreed to participate in the study were fully informed about the procedures and objectives of the work in progress, and prior to the study, each subject signed an informed consent form. Ethical approval for this study was obtained from the ethical board of the National Institute of Health.

Environmental monitoring

Air sampling was performed in the workers breathing zone for representative working periods during FA-related tasks. Other workplace sites considered relevant for the assessment were also sampled. Analysis of the samples allowed the calculation of the 8-h time-weighted average (8-h TWA) level of exposure to FA for each subject. Air sampling and FA analysis were performed according to the National Institute for Occupational Safety and Health method no. 3500 (19).

Biologic monitoring

Peripheral blood samples (10 ml) were collected between 10 and 11 am from each donor (non-fasting) and processed immediately for the different methodologies used in this study. All samples were coded and analysed under blind conditions. For one subject (exposed group), it was only possible to collect a limited volume of blood, used for chromosomal aberrations (CAs) test and genotype analysis.

CAs test

Duplicate lymphocyte cultures for CAs were established using 0.5 ml of heparinised whole blood as described in Roma-Torres *et al.* (20). CAs analysis was performed on a Nikon Eclipse E400 light microscope; slides were scored blindly by the same reader. One hundred metaphases with well-spread chromosomes were analysed for each

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individual, 50 from each duplicate culture, using 500× (to identify the metaphases) and 1250× magnification (to analyse and classify CAs). Gaps, chromosome-type aberrations (CSAs; e.g. chromosome-type breaks and dicentric and ring chromosomes) and chromatid-type aberrations (CTAs; e.g. chromatid-type breaks, symmetrical homologous figures, radial figures) were identified and classified according to Savage *et al.* (21). Gaps were not included in CAs parameters. The criteria for distinguishing chromatid-type breaks from gaps were the acentric piece displaced with respect to the chromosome axis and the size of the discontinuity exceeded the width of the chromatid. Acentric fragments were considered together with chromatid-type breaks. A dicentric with an acentric fragment was scored as one aberration. Numerical CAs were also scored and the number of aneuploid metaphases assessed (46 ± 1). In addition, known variables of chromosome fragility were also evaluated for each subject, namely the number of aberrant and multiaberrant cells according to Castella *et al.* (22) and Oostra *et al.* (23). Exchanges (figures, dicentric and ring chromosomes) were converted into the number of breaks necessary to form each figure, two breaks. A metaphase exhibiting one break (corresponding to a chromatid-break or a chromosome-break) was considered aberrant. A metaphase presenting exchanges, two or more fragments or breaks in different chromosomes were accounted as multiaberrant.

Comet assay

Peripheral blood mononuclear cells were isolated using BD Vacutainer™ CPT™ Cell Preparation Tubes with sodium heparin (Becton Dickinson), following manufacturer's instructions. Cell viability, determined by trypan blue exclusion, was higher than 85% in all cases. The alkaline comet assay was performed as described by Singh *et al.* (24) with minor modifications (5). Two gels were prepared for each donor and a 'blind' scorer examined 50 randomly selected cells from each gel (100 cells/donor) using a magnification of 400×. Microscopic analyses were performed on a Nikon Eclipse E400 Epi-fluorescence microscope (G2A filter, Nikon C-SH61). Image capture and analysis were performed with Comet Assay IV software (Perceptive Instruments). The percentage of DNA in the comet tail (%TDNA) was the DNA damage parameter evaluated.

Genotype analysis

Genomic DNA was obtained from heparinised whole blood samples (350 µl) using a commercially available kit (Qiagen EZ1 DNA Blood kit; Qiagen BioRobot EZ1 System), according to the manufacturer's instructions. DNA samples were stored at −20°C until analysis. The *CYP2E1* intronic polymorphism (rs6413432) was determined by PCR–restriction fragment length polymorphism (RFLP) as described by Lin *et al.* (25) with minor modifications (26). *GSTM1* and *GSTT1* genotyping for gene deletions were carried out through a multiplex PCR technique described elsewhere (25) with slight modifications described in Teixeira *et al.* (27). The *GSTP1* Ile105Val polymorphism (rs1695) was determined by PCR and RFLP according to the method of Harries *et al.* (28) with minor modifications (26). The *XRCC1* (rs1799782 and rs25487), *PARP1* (rs1136410) and *MUTYH* (rs3219489) gene polymorphisms were determined by real-time PCR using TaqMan® SNP Genotyping Assays from Applied Biosystems (ABI assays references: C_11463404_10, C_622564_10, C_15115368_1_, C_27504565_10, C_ 7482700_10, C_30590701_10, C_2547422_10, respectively) following Conde *et al.* (29) and Silva *et al.* (30). In order to assure uniformity in genomic DNA content (2.5 ng/µl) in all samples, DNA was quantified using the fluorimetric Quant-iT™ Picogreen® dsDNA Assay Kit (Invitrogen) and a Zenyth 3100 plate reader (Anthos Labtech Instruments), according to the manufacturer's recommendations. The PCR amplification was performed in a 7300 Real-Time PCR System thermal cycler (Applied Biosystems), with 96-well microplates. *XRCC3* (rs861539) polymorphism was determined by PCR–RFLP as described previously (30) with slight modifications. The genotype analysis of all genetic polymorphisms was not possible for some samples due to inconclusive results or technical reasons; therefore, the number of genotyped individuals will vary according to the polymorphism in question.

Statistical analysis

A general description of the study population was performed through univariate analysis. The distribution within the study groups of sociodemographic and lifestyle factors was evaluated with the Student's *t*-test for continuous variables and the Pearson's Chi-square test for categorical variables. The effect of exposure on the level of genotoxicity biomarkers was preliminarily assessed by Student's *t*-test. To achieve a better approximation to the normal distribution, a logarithmic transformation of the data was applied to percentage of DNA in the comet tail (% TDNA). As no improvement was achieved with transformation, the Mann–Whitney *U*-test was applied to all parameters obtained from CAs test: total-CAs, CSAs, CTAs, gaps, aneuploidies, aberrant cells and multiaberrant cells. Best-fitting multiple regression models were used to estimate the effect of the exposure. Linear regression was applied on the log-transformed %TDNA; negative binomial regression on non-transformed data was carried out for total-CAs, CTAs, CSAs, gaps, aberrants and aneuploidies; lastly, Poisson regression on non-transformed data was fitted for multiaberrants. All models included age, gender, smoking habits (subjects who stopped smoking since at least 2 years ago were considered non-smokers) and parameter-specific actual confounders. An ancillary regression analysis was carried out to assess the effect of FA exposure level, exposure duration and professional activity, only in the exposed population. Adjustment for age, gender, smoking habits and actual confounders was applied. A possible role as effect modifiers of genetic polymorphisms, as candidate biomarkers of susceptibility, on the alterations induced by the exposure was also tested. As the number of homozygous variant individuals was low (or inexistent) for most genes studied, these were merged with the group of heterozygous subjects, assuming a dominant model for their inheritance. Differences in genotype distributions were evaluated by the Pearson's Chi-square test. Mean ratio (MR) was used as the point estimate of effect, accompanied by its 95% confidence interval (CI). Associations between variables were analysed by Spearman's rank correlation. The level of statistical significance was set at 0.05. All analyses were performed using the IBM SPSS Statistics V. 20 software and STATA/SE 12.0 for Windows software.

Results

General characteristics of the study population are summarised in Table 1. In total, 172 subjects (84 exposed and 87 controls) were involved in the study. Both groups were similar in distribution of gender, age and smoking habits.

The mean TWA-level of workers' exposure to FA was 0.38 ± 0.03 ppm (range 0.08–1.39 ppm). The current Portuguese occupational exposure limit is 0.30 ppm (ceiling level), meaning the maximum safe FA concentration that should never be exceeded during any length of time in the workers' breathing zone. Germany and the American Conference of Governmental Industrial Hygienists also

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set for FA occupational exposure a maximum concentration limit of 0.30 ppm (TWA and ceiling, respectively). The peak emission of FA occurred mainly during two routine tasks, macroscopic examination of FA-preserved specimens (range 0.3–3.2 ppm) and disposal of specimens and waste solutions (range 0.3–2.8 ppm). These results show that the professionals studied are exposed to levels of FA higher than admissible air standards and guidelines, both national and international. In most FA-related tasks, workers were only using masks for biological hazard, not appropriate to protect from FA vapours. The primary reason given by workers for not using goggles and appropriate masks (if available) was interference in efficiency of activities performed, namely taking notes and handling material, and communication difficulties.

Univariate comparisons of genotoxicity biomarkers by study group are reported in Table 2. All CAs endpoints and the DNA damage parameter were significantly higher in the FA-exposed workers compared with control subjects.

To further evaluate the genotoxic effect of exposure and the possible influence of some known confounders on the frequencies of the endpoints studied, multivariate modelling was carried out. Fruit consumption was found to be a confounder for multiaberrant cells and %TDNA, and therefore, it was included on the analysis. The result on genotoxicity biomarkers, adjusted for gender, age, smoking habits and actual parameter-specific confounders (dietary habits), are reported in Tables 3 and 4.

The significant effect of exposure was confirmed with FA-exposed workers showing significantly higher MRs for all biomarkers compared with controls. No significant influence of gender was observed on the endpoints studied, with the exception of multiaberrant cells; a significant decrease was observed in males compared with females. Age was found to be a significant confounder but only for CSAs. CSAs frequency was significantly higher in subjects between ages 35 and 45. It was also elevated in individuals >45 years old, but with a near significance ($P = 0.06$). Regarding to smoking habits, a significant decrease was noted for aneuploidies among smokers. Furthermore, the significant association found between this variable and packs/year ($r = -0.270$, $P < 0.001$) confirmed the influence of smoking on aneuploidies frequency. Fruit consumption was found to significantly decrease multiaberrant cells frequency and DNA damage evaluated by comet assay (%TDNA). The associations between the genotoxicity indicators were evaluated and some were found to be significant. CTAs were significantly correlated with CSAs ($r = 0.343$, $P \leq 0.001$). A significant positive association was also found between gaps and CA-total ($r = 0.521$, $P \leq 0.001$), CSAs ($r = 0.291$, $P \leq 0.001$), CTAs ($r = 0.498$, $P \leq 0.001$), aberrant ($r = 0.511$, $P \leq 0.001$) and multiaberrant cells ($r = 0.315$, $P \leq 0.001$). Gaps were also correlated with %TDNA ($r = 0.273$, $P \leq 0.001$). Moreover, a weak but significant association was found between the comet assay parameter and CSAs ($r = 0.174$, $P \leq 0.001$).

Table 1. Characteristics of the study population

	Controls ($N = 87$)	Exposed ($N = 84$)	P-value
Gender			
Females	67 (77%)	65 (77%)	0.944[a]
Males	20 (23%)	19 (23%)	
Age (years)[b] (range)	38.9 ± 11.0 (20–61)	39.8 ± 9.5 (23–60)	0.563[c]
Years of employment[b]		12.0 ± 8.2	
FA-level of exposure[d] (ppm)		0.38 ± 0.03	
Smoking habits			
Non-smokers	65 (75%)	63 (75%)	0.947[a]
Smokers	22 (25%)	21 (25%)	
Years smoking[b]	21.7 ± 11.3	20.4 ± 11.0	0.704[c]
Cigarettes/day[b]	13.7 ± 6.9	11.0 ± 5.8	0.160[c]
Pack-years[b]	14.8 ± 11.5	11.2 ± 8.1	0.248[c]

[a]Chi-square test (bilateral).
[b]Mean ± SD.
[c]Student's t-test.
[d]Eight-hour TWA.

Table 2. Results of biomarkers of genotoxicity in the study groups

	Controls		Exposed		P-value
	N	Mean ± SE (range)	N	Mean ± SE (range)	
CA-total	87	2.09 ± 0.25 (0–13)	84	3.96 ± 0.34 (0–13)	<0.001[a]
CSAs, CA-chromosome type	87	0.48 ± 0.10 (0–4)	84	0.98 ± 0.14 (0–5)	0.004[a]
CTAs, CA-chromatid type	87	1.61 ± 0.19 (0–10)	84	3.00 ± 0.28 (0–12)	<0.001[a]
Gaps	87	3.49 ± 0.32 (0–14)	84	5.70 ± 0.31 (0–13)	<0.001[a]
Aneuploidies	87	2.13 ± 0.19 (0–6)	84	3.49 ± 0.19 (0–8)	<0.001[a]
Aberrant cells	87	1.90 ± 0.19 (0–9)	84	3.18 ± 0.28 (0–11)	0.001[a]
Multiaberrant cells	87	0.14 ± 0.04 (0–2)	84	0.55 ± 0.09 (0–3)	<0.001[a]
%TDNA, comet assay	87	7.50 ± 0.47 (0.86–24.40)	83	11.67 ± 0.72 (0.23–28.07)	<0.001[b]

SE, standard error.
[a]Mann–Whitney U-test.
[b]Student's t-test.

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Table 3. Effect of exposure, gender, age and smoking habits on the frequencies of CA-total, CSAs, CTAs and gaps with estimates of MRs

	N	CA-total		CSAs		CTAs		Gaps	
		MR	95% CI	MR	95% CI	MR	95% CI	MR	95% CI
Exposure									
Controls	87	1.00		1.00		1.00		1.00	
Exposed	84	1.91***	1.44–2.53	2.07**	1.27–3.38	1.86***	1.39–2.48	1.65***	1.34–2.03
Gender									
Females	132	1.00		1.00		1.00		1.00	
Males	39	0.85	0.60–1.21	0.53	0.27–1.05	0.98	0.68–1.42	1.03	0.80–1.33
Age									
<35 years	59	1.00		1.00		1.00		1.00	
35–45 years	63	1.11	0.80–1.55	1.89*	1.03–3.46	0.97	0.69–1.37	0.98	0.77–1.24
>45 years	49	1.20	0.84–1.70	1.84	0.98–3.46	1.08	0.76–1.54	0.96	0.75–1.25
Smoking habits									
Non-smokers	128	1.00		1.00		1.00		1.00	
Smokers	43	0.82	0.58–1.15	0.88	0.48–1.60	0.79	0.56–1.14	0.81	0.63–1.05

*$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$, significant difference with regard to the corresponding reference category (MR = 1.00).

Table 4. Effect of exposure, gender, age and smoking habits on the frequencies of aneuploidies, aberrant cells, multiaberrant cells and comet assay parameter with estimates of MR

		Aneuploidies		Aberrant cells		Multiaberrant cells		%TDNA		
	N	MR	95% CI	MR	95% CI	MR	95% CI	*N*	MR	95% CI
Exposure										
Controls	87	1.00		1.00		1.00		87	1.00	
Exposed	84	1.64***	1.36–1.98	1.66***	1.28–2.17	3.96***	2.09–7.48	83	1.50**	1.14–1.96
Gender										
Females	132	1.00		1.00		1.00		131	1.00	
Males	39	0.89	0.70–1.13	1.04	0.75–1.46	0.50*	0.17–0.96	39	1.05	0.75–1.47
Age										
<35 years	59	1.00		1.00		1.00		59	1.00	
35–45 years	63	0.89	0.71–1.11	1.12	0.81–1.54	0.81	0.42–1.55	63	1.22	0.88–1.67
>45 years	49	0.99	0.79–1.24	1.17	0.84–1.62	1.06	0.57–1.98	48	1.10	0.78–1.56
Smoking habits										
Non-smokers	128	1.00		1.00		1.00		128	1.00	
Smokers	43	0.67**	0.52–0.86	0.80	0.58–1.12	0.80	0.58–1.12	42	0.88	0.64–1.20
Fruit consumption (number of pieces/day)	129					0.74*	0.55–0.99	130	0.88*	0.78–0.99

CI, confidence interval.
*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, significant difference with regard to the corresponding reference category (MR = 1.00).

In the exposed population, the influence of FA-level of exposure corresponding to each exposed individual, time of exposure and professional activity on genotoxicity endpoints was also evaluated by multivariate regression; however, no significant results were found.

The genotypic frequencies of polymorphisms in genes involved in the metabolism and DNA repair pathways for sets of exposed workers, controls and whole population are presented in Table 5.

The distribution frequencies of the genotyped polymorphisms in the study population were in agreement with Hardy–Weinberg equilibrium. Further, the distribution in the study population of the metabolic and DNA repair polymorphisms are in accordance with previous studies carried out in Caucasians (31,32) and more specifically in Portuguese populations (33–35). No significant difference was found between the study groups regarding the frequency of the genetic polymorphisms assessed. The influence of genetic polymorphisms in metabolic and DNA repair enzymes on the level of genotoxicity outcomes is reported in Table 6 (only data with statistically significant results are shown). The wild-type homozygous genotypes were always the reference category.

In the exposed group, a significant increase in the %TDNA MR was observed in the *CYP2E1* intron (*T/T*) homozygous wild-type individuals, whereas for carriers of the *A* variant allele, the %TDNA MR was significantly decreased. CSAs MR was significantly higher in the *GSTP1* homozygous wild-type exposed workers compared with reference, whereas for subjects carrying the Val allele, the MR was significantly decreased. The *XRCC1* Arg194Trp genotype influenced %TDNA in both control and exposed groups. Significantly higher %TDNA MRs were observed in exposed individuals, the increase was more pronounced for heterozygous subjects. Among controls, however, heterozygotes showed a significant decrease in %TDNA MR. Nevertheless, it should be noted the low number of heterozygous subjects found in both exposed and control group. Lastly, multiaberrant cells MR was significantly higher in exposed wild-type homozygous for *PARP1* Val762Ala, whereas heterozygous subjects showed the opposite result.

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Table 5. Genotype frequency of genes involved in xenobiotic metabolism and in DNA repair in the study population

Gene	Genotype	All		Controls		Exposed		*P*-value
		N	%	*N*	%	*N*	%	
CYP2E1 rs6413432	*T/T*	132	82.0	68	79.1	64	85.3	0.552
	T/A	27	16.8	17	19.8	10	13.3	
	A/A	2	1.2	1	1.2	1	1.3	
GSTM1 deletion	*Present*	78	45.6	40	46.0	38	45.2	0.923
	Null	93	54.4	47	54.0	46	54.8	
GSTT1 deletion	*Present*	140	81.9	70	80.5	70	83.3	0.626
	Null	31	18.1	17	19.5	14	16.7	
GSTP1 rs1695	*Ile/Ile*	69	40.4	32	36.8	37	44.0	0.499
	Ile/Val	84	49.1	44	50.6	40	47.6	
	Val/Val	18	10.5	11	12.6	7	8.3	
XRCC1 rs1799782[a]	*Arg/Arg*	151	91.5	83	95.4	68	87.2	0.058
	Arg/Trp	14	8.5	4	4.6	10	12.8	
XRCC1 rs25487	*Gln/Gln*	63	38.2	32	36.8	31	39.7	0.917
	Gln/Arg	68	41.2	37	42.5	31	39.8	
	Arg/Arg	34	20.6	18	20.7	16	20.5	
PARP1 rs1136410[a]	*Val/Val*	141	85.5	75	87.2	66	83.5	0.505
	Val/Ala	24	14.5	11	12.8	13	16.5	
MUTYH rs3219489	*Gln/Gln*	81	54.0	45	53.6	36	54.5	0.992
	Gln/His	55	36.7	31	36.9	24	36.4	
	His/His	14	9.3	8	9.5	6	9.1	
XRCC3 rs861539	*Thr/Thr*	49	28.8	24	27.9	25	29.8	0.051
	Thr/Met	93	54.1	42	48.8	51	60.7	
	Met/Met	28	16.3	20	23.3	8	9.5	

[a]No variant homozygotes.

Table 6. Influence of biomarkers of susceptibility on genotoxicity parameters (only models showing significant effect are included)

	Controls			Exposed		
	N	MR	95% CI	*N*	MR	95% CI
CYP2E1 rs6413432						
%TDNA						
T/T	53	1.00		51	1.61*	1.20–2.16
T/A + A/A	15	0.84	0.54–1.30	7	0.42**	0.20–0.89
GSTP1 rs1695						
CAcs						
Ile/ Ile	32	1.00		37	5.43**	2.04–14.46
Ile/Val + Val/Val	55	1.79	1.14–7.94	47	0.26*	0.97–3.27
XRCC1 rs1799782						
%TDNA						
Arg/Arg	67	1.00		53	1.46**	1.10–1.93
Arg/Trp	2	0.19**	0.06–0.57	6	4.93*	1.33–18.32
PARP1 rs1136410						
Multiaberrant cells						
Val/Val	60	1.00		50	5.97***	2.34–15.25
Val/Ala	8	3.00	0.55–16.40	9	0.09*	0.01–0.95

CI, confidence interval.
$*P \le 0.05$, $**P \le 0.01$, $***P \le 0.001$, adjusted for gender, age and smoking habits and parameter-specific actual confounders (dietary habits).

Discussion

Biomarkers of genotoxicity

In this study, all cytogenetic parameters evaluated—total-CA, CSAs, CTAs, gaps and aneuploidies—were significantly elevated in anatomy pathology professionals exposed to FA (mean 0.38 ppm) compared with control subjects. FA-exposed individuals showed an increase of 91% in total-CAs frequency compared with controls (Table 3). Mean frequencies of both CAs types, CSAs and CTAs were also significantly higher in exposed workers (Table 3). Although there is a paucity of studies assessing CAs in FA occupationally exposed subjects, our findings are in agreement with most of the published literature. He *et al.* (36) found higher frequencies of CAs in PBLs of 13 anatomy students exposed to FA (mean level 2.37 ppm) during a 12-week anatomy class. Similarly, in a recent study involving FA-exposed personnel working in pathology departments (n = 21; mean level 0.72 ppm), total-CA and CTAs were significantly elevated compared with controls, but no significant differences were found

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for CSAs (37). A significant increase in CAs frequencies was also observed in industrial workers (38). In contrast, no significant differences were found in CAs frequencies between individuals working in different laboratories of a Cancer Research Institute, including an anatomical pathology laboratory (6); however, the results obtained may be attributed to the low level of FA exposure (range 0.01–0.05 ppm) and the reduced number of subjects evaluated (n = 36).

In this study, both CAs sub-types were statistically elevated in PBLs of FA-exposed workers. Although CSAs are thought to be formed by direct double-stranded breakage *in vivo* in G0/G1 lymphocytes (being duplicated during replication), CTAs formation requires DNA-replication and arises *in vitro* from other lesions pre-existing in DNA. However, CTAs that survive to an earlier division can be converted by duplication into secondary chromosome types and in next cell generation appear as CSAs (21,39). Indeed the significant positive association found in this study between CTAs and CSAs (r = 0.343, $P \le 0.001$) may confirm this association. Hence, our findings indicate that FA exposure is able to induce lesions in chromosomal DNA, which during repair or DNA synthesis generate DSBs and CAs formation. In addition, some stable forms of CTAs seem to survive division and pass in a modified form to next cell generation. Other variables were analysed to obtain additional information about the biological impact of our results. Therefore, we assessed the number of aberrant cells (metaphases with one CSAs or CTAs break) and multiaberrant cells (metaphases with CSAs or CTAs type exchanges or with more than two breaks/fragments). Multiaberrant cells frequency was significantly higher (4-fold) in FA-exposed workers than in control individuals, whereas aberrant cells frequency was significantly increased by 1.7-fold in the exposed group (Table 4). Accumulated data confirm that high CAs frequencies in lymphocytes of healthy individuals are predictive of cancer risk (15). Although the majority of CAs are lethal to the cell, others may lead to oncogenic transformation by several mechanisms, such as inactivation of a tumour suppressor gene or by generating novel fusion proteins capable of initiating carcinogenesis (39). A number of factors may collectively influence the association between CAs and cancer including exposure to genotoxic carcinogens and internal generation of genotoxic species (e.g. oxidative stress) (40). In fact, a significant association was found between cancer incidence and CAs (chromatid breaks) and aberrant cells frequency in a group of miners exposed to radon; the authors estimated that an increase of 1% in the frequency of CAs was associated with 64% increase incidence of cancer (41,42).

Our results also showed that FA-exposed workers had significantly higher frequencies of gaps (65% increase) than controls. Jakab *et al.* (37) and Schmid *et al.* (43) found similar results in PBLs of pathologists exposed to FA and *in vitro* experiments, respectively. The validity of scoring and analysing gaps in human biomonitoring studies has been the subject of much discussion, in spite of the positive results observed in several studies (44,45). One point of discussion is whether a gap represents a true double-stranded break in the DNA of a chromatid, a staining discontinuity or an error in chromosome condensation process ('folding defect') (46). Our findings revealed significant positive correlations between gaps and all structural CAs endpoints, showing an association with established CAs parameters, and confirming the sensitivity of this parameter for FA genotoxicity evaluation. Results from a recent comparative analysis of different types of CAs observed under light microscopy and by means of atomic force microscopy showed that most gaps are chromosome alterations and should be included in genotoxicity studies (47). This statement is further supported by evidence that DNA lesions such as DSBs may affect chromatin condensation (46) and by the observation that chromatin relaxation is a fundamental pathway in the DNA damage response (48). Also of note, in opposition to CAs, gaps are reparable and produce no further structural damage on transmission (46), so by being a reversible form of damage it may reflect relatively recent genotoxic exposure (49). Paz-y-Miño *et al.* (50) found an association between gaps and comet assay. We also found a significant positive association between gaps and the comet assay parameter (r = 0.273; $P \le 0.001$), which confirms the hypothesis above on the possible biological significance of gaps and reinforces the inclusion of this event as a genotoxicity parameter in biomonitoring studies.

In this study, a significant increase in aneuploid PBLs was found among FA-exposed workers compared with controls. There are limited studies evaluating the frequency of numerical CAs in subjects occupationally exposed to FA and the available data are conflicting (51,52). An earlier study reported an increase in aneuploid cells in a group of pathologists compared with controls, but it did not reach significance (53). Conversely, a significant decrease in aneuploidy was found among workers exposed to FA (0.72 ppm) in a pathology department, but the observed lower frequency of aneuploid cells may be attributed to the significant increase of apoptotic cells found in the FA-exposed workers investigated (37). In fact, *in vitro* experiments in cell lines showed that FA, at concentrations around 1 mM, enhanced apoptosis and decreased cell proliferation, whereas at lower doses (0.1 mM), it decreased apoptosis and increased cell proliferation (54). Although apoptosis induction was not evaluated in this study, this finding may explain the difference in aneuploidy outcome found in our group of workers, exposed to 0.38 ppm, when compared with Jakab *et al.* (37) study (0.72 ppm). Furthermore, in a recent study, FA showed to deregulate the expression of micro RNAs involved in apoptosis signalling (55). Other studies based on a different assay (e.g. fluorescence *in situ* hybridisation) have also yielded mixed results (56,57). Nevertheless, it should be noted that aneuploidy is a natural event occurring in healthy subjects. Indeed, different studies have showed that ~3% of human lymphocytes are aneuploid (58). It is possible that aneuploid cells are present in all tissue types and because of their presence in low percentages, they do not represent any significant pathological danger including oncogenic transformation. Furthermore, aneuploidy is a prominent phenotype of cancer, and it has been discussed whether aneuploidy is only a by-product of the oncogenic processes or it can induce tumourigenesis (58). Zhang *et al.* (51) reported leukaemia-specific chromosome changes (monosomy 7 and trisomy 8) in cultured peripheral blood myeloid progenitor cells of workers exposed to FA; this finding suggests that FA exposure may have an adverse impact through an aneugenic effect on the haematopoietic system. However, the mechanisms behind aneuploidy are difficult to dissect due to countless factors that may be involved. Therefore, considering the above mentioned, further studies on the FA potential aneugenic activity are needed before reaching any solid conclusion.

In this study, the levels of DNA damage measured as %TDNA were significantly higher in PBLs of FA-exposed professionals compared with controls. A similar result was obtained earlier in a smaller group of anatomy pathology workers with a higher mean level of exposure to FA (0.44 ppm) (5,59). This result agrees with Yu *et al.* (60) and Jiang *et al.* (61), who reported a significant increase in comet assay parameters, comet tail length and olive tail moment, in workers exposed to FA (mean level 0.83 ppm) from two plywood factories. In contrast, no significant differences in comet assay endpoints were found in PBLs of workers from fibreboard plants

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compared with controls (62); however, the results obtained in Aydin *et al*. (62) study may be attributed to the low level of exposure to FA (0.2 ppm).

Comet assay has also proved to be a sensitive biological indicator in the evaluation of FA genotoxic effects in several *in vitro* experiments using cell lines or in cells from FA-exposed rodent or humans (63,64). Interestingly, in most of these studies, FA showed a 'two-phase' dose-response relationship. At low doses, FA induced an increase in DNA migration, whereas at higher doses, a decrease in DNA migration was observed. The DNA damaging and crosslinking effects of FA may explain this finding. Hence, at low concentrations, it induces strand breaks, whereas at higher concentrations, crosslinking activity seems to become the dominant lesion (65). The excess of FA-induced lesions at high concentrations may overwhelm the cell repair capacity and result in the accumulation of cross-link lesions and the decrease in DNA migration, whereas the strand breaks observed at low concentrations are indicative of repair processes in progress or incomplete. Indeed, single-strand breaks (SSBs) are often intermediates during repair of other DNA lesions. A significant association was found in this study between CSAs and %TDNA, but the coefficient was relatively low. This association is not surprising as %TDNA detects DNA strand breaks, including DSBs.

In human biomonitoring studies, it is important to assess the influence of major potential confounding factors, such as gender, age and smoking habits in the biomarkers studied. In this study, significantly lower multiaberrant cells frequency was observed in males when compared with females. Although gender is not a known confounder for CAs, there are some studies that show significant increases in females compared with males (66). Regarding age, our data agree with previous studies reporting a positive association between age and CAs (67). The observed age-related effect is probably associated with the progressive increase in spontaneous chromosome instability and the loss of efficiency of DNA repair mechanisms, which may result in the accumulation of genetic lesions with increasing age (68). Cigarette smoking had a significant influence on aneuploidy in PBLs (decrease) confirmed by the significant correlation found with pack/year. Tobacco smoke contains a high number of mutagenic and carcinogenic substances including FA. Some authors have reported lower damage in PBLs of healthy smokers compared with never-smokers (69,70). Furthermore, smokers have showed an increase on baseline repair capacity (71), probably as an adaptation resulting from the increased demand for repair stimulated by the continuous damage caused by tobacco carcinogens. The genotoxicity of mainstream tobacco smoke and cigarette smoke condensate has been demonstrated *in vitro* and *in vivo* experiments, although human studies have produced mixed results (72). The significant decrease of aneuploid PBLs found in smokers might also be related with a higher apoptotic activity in these subjects induced by the increased levels of DNA damage resulting from tobacco smoking as reported in rats exposed to mainstream cigarette smoke (73). Nevertheless, it is important to note that the unbalanced number of smokers and non-smokers in this study limits the value of the data obtained and restricts possible conclusions, so larger studies are necessary to confirm this result. Fruit consumption was found to significantly decrease multiaberrant cells and DNA damage measured by comet assay parameter %TDNA. Fruits are rich in several phytochemicals and antioxidants such as vitamin C and flavonoids that inactivate reactive oxygen species (ROS) involved in the initiation or progression of several chronic diseases (74). Therefore, regular fruit consumption protects against the oxidative damage of DNA and thus might prevent mutation and cancer. In studies by Maffei *et al*. (75) and Yong *et al*. (76), fruit intake was associated with decreased frequency of cytogenetic alterations, chromosome translocation and micronuclei, respectively. Human supplementation trials investigating the antioxidant effect of fruit consumption measured by comet assay have, however, given ambiguous results, with positive (77) and negative associations (78). Our data suggest that regular consumption of fruit may actually protect against DNA damage; however, one shortcoming of this study was the lack of detailed information concerning the type of fruit consumed, which would have enabled further conclusions.

Biomarkers of susceptibility

In addition to this investigation, only a few studies were carried out on FA-exposed populations to understand the influence of genetic polymorphisms in xenobiotic-metabolising enzymes and DNA repair proteins on observed genetic damage. Our results showed that *CYP2E1* intronic polymorphism (rs6413432) significantly influenced the level of DNA damage induced by FA exposure. These results suggest a possible protective effect of the variant allele to DNA damage induced by FA inhalation. The CYP2E1 enzyme is responsible for the oxidation of various compounds producing ROS that can deplete glutathione (79). Glutathione is determinant for FA detoxification because it is the cofactor of FA-dehydrogenase, the key enzyme in the metabolic inactivation of FA. One explanation to the higher DNA damage found in wild-type individuals may be the activity of the encoded *CYP2E1* enzyme, probably more effective than the protein expressed by variant allele carriers. However, to our knowledge, no conclusive information is available so far on the effect of this polymorphism on the enzyme activity. An increase in intracellular ROS due to enzyme activity may result in a reduction of glutathione cell content, which in turn may lead to a decrease in FA detoxification and an increase in FA toxicity. Nevertheless, these results must be cautiously interpreted given the low number of heterozygous subjects found. Concerning *GSTP1*, our result agrees with Sram *et al*. (80) who found among policemen exposed to urban air pollution higher levels of chromosomal translocations associated with *GSTP1* lle/lle genotype compared with heterozygous genotype (lle/Val). Glutathione S-transferases (GSTs) detoxification enzymes catalyse the conjugation of reduced glutathione with different species of electrophilic compounds. There is some evidence that *GSTP1* Val allele encodes an enzyme with lower conjugating activity when compared with the *GSTP1* Ile allele phenotype. Also, the inhibition of *GSTP1* expression was found to induce cell death in human HFL-1 lung fibroblasts, including apoptosis (81). Therefore, the observed decrease in CSAs in Val allele carriers may be related to the maintenance of glutathione cell content and/or to an increase of apoptotic PBLs potentially induced by a less efficient antioxidant activity of *GSTP1* enzyme. More studies are needed to clarify *GSTP1* influence on modulating genotoxicity endpoints frequency in FA-exposed workers.

In this study, no significant influence of null GSTs genes was detected on the MRs of the endpoints studied. Similarly, the increase on CAs found in a group of pathologists by Santovito *et al*. (82) was not affected by GSTM1 or GSTT1 null genotypes. However, the result may have been influenced by the low number of individuals included in the study. Nevertheless, in a previous study, with a smaller population and different group of workers, we also did not find any association between GSTM1, GSTT1 and genotoxicity induced by FA exposure (5).

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DNA repair is a very important mechanism in the protection against multiple types of DNA damage, specifically those induced by endogenous and exogenous agents. Common polymorphisms in DNA repair genes may alter protein function and an individual's capacity to repair damaged DNA. A deficient repair capacity may lead to genetic instability and ultimately to cancer initiation. In this study, we found significant influences of *XRCC1* (rs1799782) and *PARP1* (rs1136410) on the effect of exposure to FA. *XRCC1* and *PARP1* are major genes involved in the base excision repair (BER) pathway cooperating in the repair of DNA SSBs (83). XRCC1 is an important protein for coordination of DNA damage repair, forming complexes with DNA polymerase β and other repair enzymes. Significant influence of exposure on %TDNA was observed for *XRCC1* Arg194Trp in both wild-type homozygous and heterozygous exposed individuals compared with reference controls. A protective influence of *XRCC1* Arg194Trp substitution on genetic damage has been observed in some studies (69,83). In this study, this effect was only obtained in heterozygous controls. However, the small number of heterozygotes found among exposed and control groups should be noted. PARP1 protein functions as a DNA damage sensor, activating the poly(ADP-ribosyl)ation of target proteins involved in BER and DSBs repair pathways and recruiting repair proteins to the sites of DNA damage (84). Our results suggest a probable protective effect of the heterozygous genotype with regard to the genotoxic effects induced by FA exposure. To our knowledge, this is the first study reporting the influence of *CYP2E1*, *XRCC1* and *PARP1* polymorphisms on genotoxicity biomarkers induced by FA exposure. These findings must be cautiously interpreted as further studies in larger populations are needed to confirm our results. The contribution of genetics to the variability of the expression and/or activity of enzymes is controversial. In many cases, genotype does not correlate well with phenotype, a phenomenon which is likely due to the fact that these enzymes are also induced to varying degrees by external factors (lifestyle factors, drugs, stress) (85).

The data from this study show that subjects working in anatomy pathology laboratories are regularly exposed to average levels of FA (0.38 ppm) near or higher than national and international limit values. Regarding the genotoxicity evaluation, both cytogenetic and DNA damage endpoints were significantly elevated in the PBLs of FA-exposed workers compared with control subjects. In a preliminary study of a small group (n = 35) of individuals also included in this study, we found higher levels of micronucleus formation and sister-chromatid exchange (86), CAs results confirms FA genotoxicity at a chromosome level (structure). Moreover, the significant increase of chromosome breakage measured by CAs and comet assay suggests a clastogenic mode of action for FA genotoxicity. On the other hand, it confirms FA ability to induce DNA damage on circulating PBLs resulting from concurrent (detected by comet assay) and past exposure (detected by CAs test). Further, the present results support the biological plausibility of inhaled FA to induce genotoxicity on circulating blood cells and potentially on other distant-site cells, reinforcing recent epidemiological data and FA classification as a human carcinogen. We also found significant influence of *CYP2E1*, *GSTP1*, *XRCC1* and *PARP1* polymorphic genes on the endpoints studied, indicating that polymorphisms in DNA repair and xenobiotic metabolising enzymes may affect an individual response to the genotoxic damage induced by occupational exposure to FA. Overall, our findings indicate a potential health risk situation associated to FA occupational exposure. Implementation of security and hygiene measures, such as periodic air sampling and medical surveillance, as well as good practice campaigns, may be crucial to decrease the risk.

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