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ORIGINAL ARTICLE

# Micronuclei and chromosome aberrations in subjects occupationally exposed to antineoplastic drugs: a multicentric approach

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**Abstract**
*Objectives* Recently published works showed that occupational exposure to antineoplastic drugs (ANPD) is still frequent in hospital settings, despite significant safety policy improvements. The aim of this study was to assess the current level of occupational exposure to ANPD and any potentially associated cytogenetic damages in hospital nurses routinely handling ANPD.
*Methods* Occupationally ANPD-exposed ($n = 71$) and ANPD-unexposed ($n = 77$; control) nurses were recruited on a voluntary basis from five hospitals in Northern and Central Italy. Evaluation of surface contamination and dermal exposure to ANPD was assessed by determining cyclophosphamide (CP) on selected surfaces (wipes) and on exposed nurses' clothes (pads). The concentration of unmetabolized CP—as a biomarker of internal dose—was measured in end-shift urine samples. Biomonitoring of genotoxic effects (i.e., biological effect monitoring) was conducted by analyzing micronuclei (MN) and chromosome aberrations (CA) in peripheral blood lymphocytes. Genetic polymorphisms for enzymes involved in metabolic detoxification (i.e., glutathione *S*-transferases) were analyzed as well.
*Results* We observed a significant increase in MN frequency (5.30 ± 2.99 and 3.29 ± 1.97; mean values ± standard deviation; $p < 0.0001$) in exposed nurses versus controls, as well as in CA detection (3.30 ± 2.05 and 1.84 ± 1.67; $p < 0.0001$), exposed subjects versus controls. Our results provide evidence that, despite safety controlled conditions, ANPD handling still represents a considerable genotoxic risk for occupationally exposed personnel.
*Conclusions* Because both MN and CA have been described as being predictive of group-increased cancer risk, our findings point to a need for improving specific safety procedures in handling and administering ANPD.

**Keywords** Antineoplastic drugs · Occupational exposure · Genotoxic hazard · Micronuclei · Chromosome aberrations · *GSTM1* and *GSTT1* polymorphisms

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## Introduction

Over the past few years, an increasing rate of neoplastic diseases has led to a parallel increase in the use of antineoplastic drugs (ANPD), disparate in nature as to

origin, chemical structure, and mechanism of cytotoxicity. Approximately a dozen ANPD has been classified as Group 1 (human carcinogens) by the International Agency for Research on Cancer, among which are busulfan, chlorambucil, cyclophosphamide (CP), etoposide, and tamoxifen (IARC 2012). Because of the increased use of such potentially mutagenic and carcinogenic ANPD, there is growing concern regarding occupational risks for people involved in handling those drugs, including manufacturers, clinical pharmacists, nurses, and physicians responsible for patients' care. Although health workers are exposed to much lower doses than cancer patients, low-dose exposure over a long period of time may have long-term effects on the workers' health. Moreover, such occupational exposure is typically and durably associated with the use of multiple drugs, such that no threshold dose can be clearly identified for their combined genotoxic and carcinogenic effects. This implies that unnecessary exposure to those compounds should be avoided or limited by the use of safety procedures (Connor and McDiarmid 2006; Kiffmeyer and Hadtstein 2007).

Inhalation and skin or mucosa adsorption are considered to be the main potential routes of exposure. Contact with skin or mucosae may occur accidentally at any stage in the handling of these substances (transport, preparation, administration, and disposal). The inhalation of powders and aerosols may occur via aerosolization of the product (e.g., on drawing the needle back through the perforable cap or opening vials). Other means of penetration into the body can be regarded as incidental events, such as eye contamination by spurts, or hand-to-mouth contact causing absorption of ANPD via the gastrointestinal tract (Turci et al. 2003; Turci and Minoia 2006). The chemical and physical properties of the drug, the quantity administered, the availability of personal protection devices and/or collective protective equipment, and the worker's skill all contribute to determining the level of ANPD contamination (Davis et al. 2011).

Environmental monitoring studies of occupational exposures carried out in hospital units have shown detectable levels of cytotoxic agents in the air (McDevitt et al. 1993; Sessink et al. 1994a), on surfaces (Connor et al. 2005; Larson et al. 2002; Minoia et al. 1998), gloves (Ziegler et al. 2002), and on different body sites (Fransman et al. 2004). Several biomonitoring methods for the detection of occupational exposure to ANPD have been developed and validated (Sorsa and Anderson 1996). The presence of those drugs in the urine of hospital personnel has been widely studied and documented (DeMeo et al. 1995; Ensslin et al. 1994; Sessink et al. 1994b). Moreover, an association between occupational exposure and increased urine mutagenicity was first reported in 1979 (Falck et al. 1979). Urine mutagenicity was later traced to skin contact (Labuhn et al. 1998; Sessink et al. 1995). This has led to improved safety instructions for handling of ANPD (ASHP 2006; NIOSH 2010; OSHA 2000) and a decreased risk of occupational contamination in hospitals.

Recently, hygienic guidance values have been proposed for hospital personnel preparing ANPD (Kiffmeyer et al. 2013; Schierl et al. 2009) and health care workers involved in drug administration and nursing (Hedmer and Wohlfart 2012). In Italy, guidelines for the prevention of this occupational exposure were published in 1999 (GURI 1999).

Apart from environmental monitoring and biomonitoring of urine samples, it is of particular interest to assess the degree of cytogenetic damage. Many anticancer agents are known to be genotoxic and have a potential for causing genetic alterations in target tissues (Keshava and Ong 1999). Biomonitoring of genotoxic hazards (i.e., biological effect monitoring) has been reported in several studies by the use of cytogenetic assays, such as analysis of chromosome aberrations (CA), micronuclei (MN), and sister chromatid exchanges (SCE) in peripheral blood lymphocytes (PBL) of exposed subjects. Since the early 80s, a lot of studies worldwide have been attempting to identify the possible cytogenetic consequences of occupational exposure to ANPD. However, those studies generated conflicting data on MN and CA frequencies in PBL from this type of workers (Turci et al. 2003), the discrepancy being probably traceable to the disparate safety procedures adopted in handling cytostatic drugs. In addition, most of the studies reckoned without the possible contribution of factors that may affect the nonspecific indicators of genetic damage (mostly, smoking habits).

The aim of the present study was to assess the association between ANPD exposure and genetic damage (i.e., MN and CA), taking into account the confounding effects of non-occupational exposures and the modulating effects of *GSTM1* or *GSTT1* gene polymorphisms. The epidemiological design of the study was cross-sectional (Moretti et al. 2011).

## Materials and methods

### Study subjects

For this multicentric approach, health care workers were recruited on a voluntary basis from five hospital departments in Northern and Central Italy (i.e., Bologna, Brescia, Padova, Parma, and Perugia). To reduce the potential impact of confounding factors, the study was carried out on healthy non-smoker female subjects. Exclusion criteria, together with male gender and active smoking, also included radiography, radiotherapy, or chemotherapy in the past 12 months. A total of 71 exposed subjects handling ANPD and 77 subjects working as nurses in the same hospitals—and

not occupationally exposed to ANPD (controls)—were recruited in the study. Participants were asked to fill in three standardized questionnaires to provide details regarding individual and occupational data. Questionnaire A was aimed at obtaining demographic/anthropometric data (age, height, and weight), previous and present diseases, lifestyle habits (diet, passive smoking, alcohol and medicine consumption, physical activity, and other leisure activities), non-occupational exposures to mutagenic and carcinogenic agents, and outdoor environment (residence close to intense traffic areas and/or factories). Questionnaires B and C were administered to exposed nurses only, and were aimed at investigating work experience and occupational exposures, years of service, cytostatics most frequently handled in the previous 12 months (Questionnaire B), names and quantities of ANPD handled during the last two work shifts (Questionnaire C) (Moretti et al. 2011). Exposed and non-exposed workers were informed about the aim and the experimental details of the study, and written informed consent was obtained from all participating subjects.

### Monitoring of exposure

Because CP was the most widely used ANPD in all of the five considered hospitals, according to Italian Guidelines (GURI 1999), exposure monitoring was carried out by considering the concentration of this drug as a marker for exposure to ANPD. Sampling was carried out only on days when the CP was actually included in the preparation or administration of drugs. Samples were collected as previously described (Moretti et al. 2011). Briefly, wipe and pad samples were used to evaluate contamination of surfaces and clothes of nurses exposed to ANPD, respectively. The standard sampling sites for wipe tests were located on the hood surface (preparation site) or the drip surface (administration site), while the pads were applied to clothes of the left forearm (non-dominant arm) of each subject during the working shift. The analysis was carried out by gas chromatography coupled with an ion-trap mass spectrometry (GC–MS/MS).

The concentration of unmetabolized CP as a biomarker of internal dose was also measured in end-shift urine samples from exposed and control subjects. One spot fresh urine sample collected from each subject at the end of the work shift was analyzed by liquid chromatography coupled with a triple-quadrupole mass spectrometer equipped with an electrospray source (LC–ESI–MS/MS) after urine sample purification and concentration using solid-phase extraction (SPE).

### Biological effect monitoring

End-shift blood samples were collected by venipuncture in heparinized vacuum tubes (Moretti et al. 2011). Coded tubes were shipped overnight to the laboratories in Perugia and Rome for subsequent processing. MN and CA analyses were performed at the University of Perugia and at ENEA-Casaccia, Rome, respectively. Blood sampling of exposed and non-exposed subjects was carried out during the same period.

For the cytokinesis-block micronucleus test, lymphocyte cultures were established by adding 0.3 ml whole blood to 4.7 ml RPMI-1640 medium supplemented with 20 % heat-inactivated fetal calf serum, 2 mM L-glutamine, 2 % phytohemagglutinin (PHA), and penicillin–streptomycin (100 IU/ml and 100 μg/ml, respectively). Whole-blood cultures were incubated for 72 h at 37 °C in a humidified atmosphere with 5 % $CO_2$ (Fenech 2000). To obtain binucleated cells, cytochalasin-B (final concentration 3 μg/ml) was added at 44 h of culture (Fenech et al. 1999). Cells were then collected by centrifugation, re-suspended in a pre-warmed hypotonic solution (75 mM KCl) for 15 min at 37 °C and fixed in acetic acid–methanol (1:5 v:v).

For cytogenetic analysis, air-dried preparations were stained with 4 % Giemsa, the slides were coded, and the entire analysis was carried out in a blinded fashion. A total of 2,000 binucleated lymphocytes (1,000 cells/scorer) with preserved cytoplasm were scored for each exposed or control subject. MN evaluation was based on the standard criteria (Fenech et al. 2003). For cell cycle analysis, 500 lymphocytes/subject were scored to evaluate the percentage of binucleated cells, and the nuclear division index (NDI) was then calculated (Eastmond and Tucker 1989).

For CA test, cell cultures were established by adding 0.5 ml whole blood to 4.5 ml RPMI-1640 medium supplemented with 10 % heat-inactivated fetal calf serum, 2 mM L-glutamine, 2 % PHA, and penicillin–streptomycin (100 IU/ml and 100 μg/ml, respectively). According to a standard protocol (IAEA 2001), lymphocytes proliferation was induced by PHA exposure for 48 h. Colcemid was added at a final concentration of 0.2 μg ml 90 min before harvesting, to arrest dividing cells at metaphase.

For CA analysis, air-dried metaphase spreads were stained according to the conventional unbanded Giemsa method. Similar to MN evaluation, CA slides were coded and the entire analysis was carried out blindly. An average 100 well-spread metaphases containing 46 (±1) centromeres were analyzed for each subject; CA were classified according to the International System of Cytogenetic Nomenclature (ISCN) (Savage and Holloway 1988). Total CA included chromosome-type (chromosome-type breaks, ring chromosomes, dicentrics) and chromatid-type (chromatid-type breaks and chromatid exchanges) aberrations (CsA and CtA, respectively). Gaps were not scored as aberrations.

### Genotype analysis

Blood samples were collected by venipuncture in lithium EDTA vacuum tubes (Moretti et al. 2011). Coded samples

were shipped to the University of Padova for genotyping. DNA from leukocytes was isolated with a Wizard® Genomic DNA purification kit (Promega, Italy). Lysis of red blood cells, followed by lysis of white blood cells and their nuclei, RNase digestion, salt precipitation of cellular proteins, and final genomic DNA concentration by isopropanol precipitation were performed according to the manufacturer's specifications.

A multiplex PCR method was used for the simultaneous detection of the allelic status (i.e., presence or absence) of *GSTM1* and *GSTT1*. In PCR assay, primer pairs for both *GSTM1* and *GSTT1* were included in the same amplification mixture; a third primer pair for β-globin was also included as an internal positive PCR control (Pavanello et al. 2002). Amplicons were resolved in an ethidium bromide-stained 2 % agarose gel. The presence or the absence of *GSTM1* and/or *GSTT1* genes was defined by the occurrence of the specific bands (i.e., 215 and 480 bp, respectively); a band at 285 bp (corresponding to β-globin) was used as an internal control to document successful PCR amplification (Hirvonen et al. 1996). The absence of the *GSTM1*- or *GSTT1*-specific fragment indicates the corresponding null genotype (*0/*0).

### Ethical approval

The study protocol was approved by institutional review boards appointed by all universities involved in the research project (Moretti et al. 2011).

### Statistical analysis

Statistical analysis was performed to ascertain any association between occupational exposure to ANPD and biomarkers of early biological effects (MN and CA) using the SPSS statistical package (SPSS Inc., IL, USA). Pearson's $\chi^2$ test was used to evaluate the differences in distributions for age and genetic polymorphisms between exposed and non-exposed subjects. The distribution of cytogenetic parameters was evaluated by the Kolmogorov–Smirnov test, which showed significant departures from the normal distribution; therefore, the presence of possible significant differences between exposed and non-exposed subjects was tested by nonparametric Mann–Whitney *U* test (significance level set at $p < 0.05$). Differences between subgroups were investigated through Kruskal–Wallis *H* test. For significant results ($p < 0.05$), post hoc analysis was performed by running separate Mann–Whitney *U* tests on the different combinations of related groups (multiple pairwise comparisons) with Bonferroni correction of the $\alpha$ in order to maintain the overall probability of a type I error at 0.05.

Significant results in the univariate analyses were included in a multiple linear regression model to examine the influence of exposure status, age, occupational assignment, job seniority, and personal protection as independent variables on the frequency of MN. Moreover, the effect size of exposure, in terms of cytogenetic damage, was measured by calculating the ratio of means (RoM) and the corresponding conventional confidence intervals at the 95 % (95 % CI) level, RoM being defined as the mean value in the exposed group divided by the mean value in the control group (Friedrich et al. 2011).

## Results

### Population characteristics

Characteristics of the studied population are summarized in Table 1. The two groups were age-matched, with no significantly different (Mann–Whitney *U* test) mean values. Exposed and control subjects were divided according to an age cutoff (i.e., 40 years) defined according to the mean value for age in the whole population (i.e., 39.54 years). Exposed and controls were equally distributed (Pearson's $\chi^2$ test) in the corresponding subgroups. The exposed nurses were gathered into two subgroups according to their job seniority: the exposed subjects were almost equally distributed in the subgroups, with 39 individuals exposed for less than 10 years and 32 individuals exposed for more than 10 years. Exposed and control subjects were also gathered according to *GSTM1* and *GSTT1* genotype profiles; similar frequency distributions (Pearson's $\chi^2$ test) were observed in the corresponding subgroups.

Although little is known on the impact of specific diets on chromosomal damage rates, cooking meat at high temperatures may result in the formation and ingestion of carcinogenic compounds such as heterocyclic amines and polycyclic aromatic hydrocarbons (PAH). To check for any such confounding factors, we included queries on cooking habits and food consumption (e.g., grilled or barbecued, baked or roasted) in the questionnaire. For each subject, exposure to total PAH was summarized using a cumulative exposure index. Subjects were then classified according to eating habits as having a low or a high PAH score. Moreover, alcohol units drunk per week were also considered.

### Surface and dermal contamination (wipes and pads analysis)

On the basis of data gathered from the questionnaires, exposed subjects handled a multiplicity of ANPD, very often in mixtures of two or more drugs. Table 2 summarizes the frequencies of ANPD handling reported as the percentage of subjects handling each drug at least on one occasion over a period of 6 months before environmental and biological monitoring.

The results of environmental monitoring are reported in Table 3. All wipe samples were positive, with CP levels

**Table 1** Main characteristics of the study population[a]

| | Exposed | Controls |
|---|---|---|
| Subjects[b] | 71 | 77 |
| City | | |
| Bologna[b] | 17 (23.9) | 19 (24.7) |
| Brescia[b] | 3 (4.2) | 3 (3.9) |
| Padova[b] | 16 (22.5) | 20 (26.0) |
| Parma[b] | 19 (26.8) | 18 (23.4) |
| Perugia[b] | 16 (22.5) | 17 (22.1) |
| Diet/alcohol | | |
| Low PAH score | 27 (38.0) | 38 (49.4) |
| High PAH score | 44 (62.0) | 39 (50.6) |
| ≤4 alcohol units/week | 65 (91.5) | 63 (81.8) |
| >4 alcohol units/week | 6 (8.5) | 14 (18.2) |
| Age (years)[c] | 39.06 ± 7.66 | 39.99 ± 8.65 |
| <40[b,d] | 38 (53.5) | 34 (44.2) |
| ≥40[b,d] | 33 (46.5) | 43 (55.8) |
| Job seniority (years)[c] | 9.20 ± 7.18 | – |
| <10[b] | 39 (54.9) | – |
| ≥10[b] | 32 (45.1) | – |
| *GSTM1* | | |
| Positive[b,e] | 36 (53.7) | 36 (46.8) |
| Null[b,e] | 31 (46.3) | 41 (53.2) |
| *GSTT1* | | |
| Positive[b,e] | 59 (88.1) | 67 (87.0) |
| Null[b,e] | 8 (11.9) | 10 (13.0) |

a According to the inclusion criteria, all subjects were females and non-smokers

b Data reported as the number of subjects (% between brackets)

c Age and job seniority in the specific task are expressed in years and reported as the group mean ± SD

d Cutoff defined according to the mean value (i.e., 39.54 years) of the observed age distribution in the whole population

e Four exposed subjects were not genotyped

ranging from 0.01 to 1,400 ng/cm$^2$ on working surfaces. All pads positioned on forearms of exposed subjects were also positive, with CP concentrations from 0.03 to 64 ng/cm$^2$. Even though the majority of tested surfaces showed low levels of CP, in eight samples the concentration of CP was between 100 and 500 ng/cm$^2$, in three wipes CP was between 500 and 1,000 ng/cm$^2$, and two wipes had a CP concentration higher than 1,000 ng/cm$^2$. However, although high CP values were mainly found in working places of nurses with an age >40 or a job seniority >10 years, differences did not reach the significance level, possibly owing to a large variability. CP in urine samples was always below the limit of detection (i.e., 0.04 µg/L) in exposed and control subjects, except for samples from two exposed nurses with CP levels of 0.08 and 0.12 µg/L, respectively.

## Micronuclei frequencies

MN frequencies in cytokinesis-blocked peripheral blood lymphocytes are reported in Table 4. The response to the mitogenic (PHA) stimulus in lymphocyte cultures was evaluated by determining the NDI. No significant inter-group variations were observed for this parameter (data not shown). In the CBMN test, cytochalasin-B added after 44 h of incubation (before the first mitotic wave for most cells) yielded about 60 % binucleated cells at 72 h of PHA stimulation (58.7 and 56.1 % for exposed and control subjects, respectively), in the range of 35–60 % binucleated cells, thus indicating optimal culture conditions (Fenech 2000).

A statistically significant increase in MN frequency was observed in nurses handling ANPD (exposed subjects) as compared to control (unexposed) subjects when the whole population was considered. When exposed and control subjects were compared with reference to the city, the level of significance was reached for Bologna, Padova, and Parma. Globally, exposed subjects always showed higher MN frequencies, as compared to controls, for both age subgroups (i.e., < or ≥40 years). MN frequencies were not influenced by eating habits, neither in exposed nor in control subjects. Age was not associated with any increase in the frequency of MN in the exposed subjects, whereas a statistically significant increase in MN with increasing age was observed in the reference group. Among the exposed subjects, no statistically significant differences were observed as to the occurrence of MN in relationship to job seniority.

The results of CBMN test with regard to genetic polymorphisms are also shown in Table 4. Regarding the *GSTM1* and *GSTT1* genotypes, no significant increase in MN values was observed among exposed or control individuals for null subjects, with significant results obtained only when exposed subjects were compared with corresponding controls.

## Chromosome aberrations frequencies

CA group mean values (±SD) for both specific (i.e., CsA and CtA) and total CA found in exposed and control subjects are summarized in Table 5. Statistical analysis showed a significantly higher level of total CA and CtA in exposed nurses compared to controls. When exposed and control subjects were compared with reference to the city, the level of significance was reached for Bologna (CtA), Padova (CA, CsA, and CtA), and Parma (Ca and CtA). No influence of age or diet was observed among either exposed or control subjects. Among exposed subjects, a statistically significant increase for CsA was observed in relationship to job seniority. Control subjects with the *GSTM1* null genotype showed a significant increase in total CA and CtA

**Table 2** Frequencies of ANPD handling

| ANPD | % | ANPD | % |
|---|---|---|---|
| Cyclophosphamide[1] | 83.9 | Gemcitabine[NL] | 56.5 |
| Etoposide[1] | 77.4 | Paclitaxel[NL] | 53.2 |
| Methotrexate[3] | 75.8 | Vinorelbine[NL] | 53.2 |
| Cisplatin[2A] | 71.0 | Oxaliplatin[NL] | 51.6 |
| Carboplatin[NL] | 64.5 | Irinotecan[NL] | 46.8 |
| Doxorubicin[2A] | 64.5 | Docetaxel[NL] | 41.9 |
| Ifosfamide[3] | 64.5 | Bleomicyn[2B] | 38.7 |
| Vincristine[3] | 61.3 | Topotecan[NL] | 38.7 |
| 5-Fluorouracil[3] | 59.7 | Cytarabine[NL] | 33.9 |
| Epirubicin[NL] | 58.1 | Daunorubicin[2B] | 33.9 |

Data reported as the percentage of subject who have handled each drug at least once over a period of 6 months (data obtained from questionnaires)

ANPD classified by the International Agency of Research as [1] carcinogenic to humans (Group 1), [2A] probably carcinogenic to humans (Group 2A), [2B] possibly carcinogenic to humans (Group 2B), [3] not classifiable as to its carcinogenicity to humans (Group 3), [NL] not listed by IARC (refs.: IARC 1976, 1981, 1987, 2000, 2012)

**Table 3** Concentrations of CP (ng/cm$^2$) detected on surfaces or clothes over the course of the study

| | *n* | Surfaces[a] (wipes) | Left forearms (pads) |
|---|---|---|---|
| Total | 71 | 112.9 ± 279.7 | 2.4 ± 8.7 |
| Age (years) | | | |
| <40 | 38 | 100.8 ± 244.8 | 1.2 ± 1.9 |
| ≥40 | 33 | 126.7 ± 318.9 | 3.6 ± 12.4 |
| Job seniority (years) | | | |
| <10 | 39 | 84.81 ± 256.9 | 1.0 ± 1.9 |
| ≥10 | 32 | 145.1 ± 305.2 | 3.9 ± 12.6 |

Data are reported as the group mean (±SD)

[a] Each sampled workplace was associated with an exposed subject

when compared to control individuals with a *GSTM1*-positive genotype.

### Regression analysis and effect size

Multiple regression analysis was performed on exposure status, age, job seniority, genetic polymorphism profiles, and environmental CP as independent variables. The analysis was first done on the whole population and thereafter on subjects stratified by occupational exposure (Table 6). This statistical approach indicated that variance in MN and CA frequency in the study population was mainly explained by the subjects' occupation, thus confirming a statistically significant positive association of cytogenetic damage with exposure to ANPD. As shown in Table 6, in the whole population, MN frequency tended to rise in association with age and a *GSTM1* null genotype. In exposed nurses, job seniority was found to be associated with increased frequency of total CA and CsA. In controls, the frequency of MN was influenced by age, whereas *GSTM1* polymorphism influenced CA and CsA counts.

By evaluating the effect size in terms of increased cytogenetic damage (i.e., RoM with 95 % CI) in the exposed subjects considering MN, total CA, and CtA, we observed increases (RoM) of 1.61 (95 % CI 1.34–1.94), 1.79 (95 % CI 1.39–2.31), and 2.10 (95 % CI 1.58–2.79) times over those of unexposed individuals. Among the exposed subjects, job seniority >10 years was associated with a 1.80-fold increase (95 % CI 1.07–3.04) for CsA. In contrast, among controls, age >40 years was associated with a 1.39-fold increase (95 % CI 1.04–1.84) for MN and *GSTM1* null genotype to 1.78 (95 % CI 1.13–2.80) times higher total CA. These effects were not observed in the exposed subjects probably because cytogenetic damage is mainly accounted to exposure.

## Discussion

In the present study, contamination of work sites by ANPD was assessed by determining CP concentrations in selected surfaces (wipes) and nurses' clothes (pads). All wipes were positive for the monitored model compound, with CP levels ranging from 0.01 to 1,400 ng/cm$^2$. Dermal exposure also occurred in hospital personnel handling ANPD, with all pads positive with CP concentrations from 0.03 to 64 µg/L. Two subjects had detectable urinary concentration of CP. Overall, environmental (surface/clothing) contamination levels were found to be similar to those reported for other Italian hospitals (Sottani et al. 2012). We also observed a significant induction of MN (group mean values ± standard deviation: 5.30 ± 2.99 and 3.29 ± 1.97, in exposed nurses and control subjects, respectively) and CA (3.30 ± 2.05 and 1.84 ± 1.67, in exposed and control subjects, respectively) associated with occupationally exposure to ANPD. However, in the exposed subjects, no relationships were observed between MN or CA frequencies and the extent of surface contamination assessed by means of wipes and pads.

These findings demonstrate that, even when the personnel is specifically trained, and drug handling occurred according to the current Italian Guidelines (GURI 1999), the risk of accidental contamination and thus exposure is still present in the administration process. Similar to our findings, positive results for both MN and CA induction were reported in other investigations on workers exposed to ANPD (Bouraoui et al. 2011; El-Ebiary et al. 2013; Kopjar et al. 2009); positive results have been reported also in studies evaluating only MN (Cornetta et al. 2008; Ladeira et al. 2014; Maluf and Erdtmann 2000b; Rekhadevi et al. 2007;

**Table 4** Frequency of MN per 1,000 binucleated lymphocytes in nurses exposed to ANPD and non-exposed subjects with respect to age and genetic polymorphisms (whole population), and job seniority (exposed subjects)

| | Exposed | | Controls | | RoM (95 % CI) |
|---|---|---|---|---|---|
| | *n* | Micronuclei | *n* | Micronuclei | |
| Total | 71 | 5.30 ± 2.99* | 77 | 3.29 ± 1.97 | 1.61 (1.34–1.94) |
| City | | | | | |
| Bologna | 17 | 4.47 ± 1.64# | 19 | 3.26 ± 2.51 | 1.37 (0.93–2.02) |
| Brescia | 3 | 4.17 ± 2.36 | 3 | 3.60 ± 3.21 | 1.16 (0.35–3.83) |
| Padova | 16 | 4.78 ± 1.88# | 20 | 2.63 ± 1.68 | 1.82 (1.29–2.55) |
| Parma | 19 | 7.21 ± 3.88# | 18 | 4.06 ± 2.24 | 1.78 (1.25–2.52) |
| Perugia | 16 | 4.66 ± 3.17 | 17 | 3.22 ± 1.35 | 1.45 (0.98–2.13) |
| Diet/alcohol | | | | | |
| Low PAH score | 27 | 5.11 ± 3.33# | 38 | 3.37 ± 1.92 | 1.52 (1.12–2.06) |
| High PAH score | 44 | 5.42 ± 2.79# | 39 | 3.21 ± 2.03 | 1.69 (1.31–2.17) |
| ≤4 alcohol units/week | 56 | 5.55 ± 3.07# | 63 | 3.17 ± 1.87 | 1.75 (1.43–2.15) |
| >4 alcohol units/week | 6 | 5.08 ± 2.75# | 14 | 3.82 ± 2.34 | 1.33 (0.78–2.28) |
| Age (years) | | | | | |
| <40 | 38 | 4.78 ± 2.54# | 34 | 2.70 ± 1.95 | 1.77 (1.32–2.38) |
| ≥40 | 33 | 5.91 ± 3.37# | 43 | 3.74 ± 1.87§ | 1.58 (1.24–2.02) |
| Job seniority (years) | | | | | |
| <10 | 39 | 5.15 ± 2.70 | – | – | – |
| ≥10 | 32 | 5.48 ± 3.34 | – | – | – |
| *GSTM1* | | | | | |
| Positive | 36 | 4.81 ± 2.48# | 36 | 3.05 ± 1.82 | 1.58 (1.22–2.04) |
| Null | 31 | 6.19 ± 3.41# | 41 | 3.45 ± 2.09 | 1.79 (1.37–2.35) |
| *GSTT1* | | | | | |
| Positive | 59 | 5.62 ± 3.08# | 67 | 3.19 ± 1.98 | 1.76 (1.44–2.16) |
| Null | 8 | 4.19 ± 2.07 | 10 | 3.90 ± 1.88 | 1.07 (0.68–1.69) |

Data reported as the group mean (±SD) of individual counts

*RoM* ratio of means, *95 % CI* confidence intervals at the 95 % level

Statistical significance (two-sided *p* value <0.05, nonparametric Mann–Whitney *U* test): * whole population, exposed versus controls; # subgroups, exposed versus corresponding non-exposed nurses (post hoc analysis, Mann–Whitney *U* test multiple pairwise comparisons with Bonferroni correction for positive Kruskal–Wallis *H* tests); § subgroups, <40 versus ≥40 years (post hoc analysis with Bonferroni correction)

Rombaldi et al. 2009), or CA (Burgaz et al. 2002; Jakab et al. 2001; Musak et al. 2009; Testa et al. 2007). However, negative findings have also been reported for MN (Cavallo et al. 2007; Hessel et al. 2001; Laffon et al. 2005; Maluf and Erdtmann 2000a). In a previous study by the group in Perugia (Villarini et al. 2012), no differences in MN frequency were observed between nurses with occupational exposure to ANPD and unexposed controls. The Perugia-based subjects in the present and the previous study were not the same; however, regarding the health care workers at the hospital of Perugia, the present results confirm the absence of an exposure-related excess of MN in exposed subjects compared to controls.

It has been reported that the number of MN in PBL increases with age in both males and females (Fenech and Bonassi 2011), whereas the relationship between aging and CA is much less clear (Bolognesi et al. 1997). In our study, MN but not CA frequencies tended to rise with age, although to a significant extent only in the control group, probably because in the exposed subjects the effect of exposure was predominant. No correlation was found between job seniority and the levels of cytogenetic damage; this finding is in accordance with data retrieved in the literature (Maluf and Erdtmann 2000a; Testa et al. 2007).

In a recently published paper (Buschini et al. 2013), we have reported on the extent of primary, oxidative, and "cryptic" DNA damage as evaluated by comet assay in circulating leukocytes from the same nurses (exposed and controls) evaluated herein for cytogenetic damage. In such earlier work, we did not observe any statistically significant difference between exposed nurses and control subjects when primary DNA damage was evaluated in leukocytes (i.e., alkaline comet assay). Similarly, when oxidative DNA damage was evaluated (i.e., comet/EndoIII assay) in circulating leukocytes, no statistically significant differences were found when exposed and control nurses were compared. Moreover, to detect low levels of DNA damage, the comet assay protocol was modified by using an inhibitor of DNA repair, such as cytosine arabinoside (i.e., comet/AraC assay). The comet/AraC assay was performed on stimulated lymphocytes, and unexpectedly, nurses exposed to ANPD showed a lower level of DNA migration than the control subjects.

The discrepancy between our previous and the current results might be related to the use of the total leukocyte

**Table 5** Frequency of CA per 100 metaphases in lymphocytes of nurses exposed to ANPD and controls with respect to age and genetic polymorphisms (whole population), and job seniority (exposed subjects)

| | Exposed | | | Controls | | | RoM (95 % CI) |
|---|---|---|---|---|---|---|---|
| | *n* | Chromosome aberrations | | *n* | Chromosome aberrations | | |
| Total | 70 | CA | 3.30 ± 2.05* | 75 | CA | 1.84 ± 1.67 | 1.79 (1.39–2.31) |
| | | CsA | 0.97 ± 1.02 | | CsA | 0.73 ± 0.92 | 1.33 (0.91–1.94) |
| | | CtA | 2.33 ± 1.50* | | CtA | 1.11 ± 1.18 | 2.10 (1.58–2.79) |
| City | | | | | | | |
| Bologna | 16 | CA | 3.13 ± 1.71 | 19 | CA | 2.21 ± 1.23 | 1.42 (0.99–2.03) |
| | | CsA | 0.94 ± 0.68 | | CsA | 0.95 ± 0.85 | 0.99 (0.58–1.68) |
| | | CtA | 2.19 ± 1.33 | | CtA | 1.26 ± 0.81 | 1.74 (1.16–2.62) |
| Brescia | 3 | CA | 2.00 ± 2.00 | 3 | CA | 3.33 ± 3.06 | 0.60 (0.13–2.79) |
| | | CsA | 0.33 ± 0.58 | | CsA | 1.67 ± 1.23 | 0.20 (0.02–1.71) |
| | | CtA | 1.67 ± 2.08 | | CtA | 1.67 ± 1.53 | 1.00 (0.17–5.75) |
| Padova | 16 | CA | 4.56 ± 2.13 | 18 | CA | 1.17 ± 1.47 | 3.90 (2.15–7.08) |
| | | CsA | 1.75 ± 1.29 | | CsA | 0.67 ± 1.03 | 2.61 (1.22–5.61) |
| | | CtA | 2.81 ± 1.60 | | CtA | 0.50 ± 0.71 | 5.62 (2.84–11.12) |
| Parma | 19 | CA | 3.05 ± 2.15 | 18 | CA | 1.50 ± 1.50 | 2.03 (1.16–3.56) |
| | | CsA | 0.84 ± 0.83 | | CsA | 0.56 ± 0.78 | 1.50 (0.69–3.28) |
| | | CtA | 2.21 ± 1.44 | | CtA | 0.94 ± 1.06 | 2.35 (1.29–4.27) |
| Perugia | 16 | CA | 2.75 ± 1.84 | 17 | CA | 2.24 ± 1.99 | 1.23 (0.72–2.10) |
| | | CsA | 0.50 ± 0.89 | | CsA | 0.59 ± 0.87 | 0.85 (0.28–2.59) |
| | | CtA | 2.25 ± 1.61 | | CtA | 1.65 ± 1.69 | 1.36 (0.75–2.48) |
| Diet/alcohol | | | | | | | |
| Low PAH score | 27 | CA | 3.41 ± 2.12# | 38 | CA | 1.61 ± 1.55 | 2.12 (1.44–3.11) |
| | | CsA | 0.93 ± 0.96 | | CsA | 0.74 ± 1.00 | 1.26 (0.70–2.24) |
| | | CtA | 2.48 ± 1.42# | | CtA | 0.87 ± 0.91 | 2.85 (1.92–4.24) |
| High PAH score | 44 | CA | 3.23 ± 2.03# | 39 | CA | 2.08 ± 1.77 | 1.55 (1.12–2.15) |
| | | CsA | 1.00 ± 1.07 | | CsA | 0.73 ± 0.84 | 1.37 (0.85–2.21) |
| | | CtA | 2.23 ± 1.56# | | CtA | 1.35 ± 1.38 | 1.65 (1.13–2.42) |
| ≤4 alcohol units/week | 56 | CA | 3.36 ± 2.06# | | CA | 1.70 ± 1.61 | 1.98 (1.49–2.62) |
| | | CsA | 1.05 ± 1.03 | 63 | CsA | 0.70 ± 0.92 | 1.50 (0.99–2.27) |
| | | CtA | 2.31 ± 1.53# | | CtA | 1.00 ± 1.00 | 2.31 (1.71–3.12) |
| >4 alcohol units/week | 6 | CA | 4.00 ± 2.61 | 14 | CA | 2.43 ± 1.87 | 1.65 (0.85–3.18) |
| | | CsA | 1.00 ± 0.89 | | CsA | 0.86 ± 0.95 | 1.16 (0.46–2.91) |
| | | CtA | 3.00 ± 1.90 | | CtA | 1.57 ± 1.74 | 1.91 (0.88–4.13) |
| Age (years) | | | | | | | |
| <40 | 38 | CA | 3.45 ± 2.04# | 33 | CA | 1.85 ± 1.84 | 1.86 (1.27–2.75) |
| | | CsA | 0.92 ± 0.91 | | CsA | 0.85 ± 1.00 | 1.08 (0.65–1.80) |
| | | CtA | 2.53 ± 1.62# | | CtA | 1.00 ± 1.19 | 2.53 (1.64–3.90) |
| ≥40 | 32 | CA | 3.13 ± 2.09# | 42 | CA | 1.83 ± 1.55 | 1.71 (1.21–2.42) |
| | | CsA | 1.03 ± 1.15 | | CsA | 0.64 ± 0.85 | 1.61 (0.92–2.81) |
| | | CtA | 2.09 ± 1.33# | | CtA | 1.19 ± 1.23 | 1.76 (1.20–2.57) |
| Job seniority (years) | | | | | | | |
| <10 | 38 | CA | 2.87 ± 1.85 | – | – | – | – |
| | | CsA | 0.71 ± 1.01 | | | | |
| | | CtA | 2.16 ± 1.35 | | | | |
| ≥10 | 32 | CA | 3.81 ± 2.19 | – | – | – | – |
| | | CsA | 1.28 ± 0.96$ | | | | |
| | | CtA | 2.53 ± 1.67 | | | | |

**Table 5** continued

| | Exposed | | | Controls | | | RoM (95 % CI) |
|---|---|---|---|---|---|---|---|
| | *n* | Chromosome aberrations | | *n* | Chromosome aberrations | | |
| *GSTM1* | | | | | | | |
| Positive | 36 | CA | 3.53 ± 2.06[#] | 36 | CA | 1.31 ± 1.60 | 2.69 (1.73–4.19) |
| | | CsA | 1.11 ± 1.09[#] | | CsA | 0.47 ± 0.69 | 2.36 (1.33–4.21) |
| | | CtA | 2.42 ± 1.48[#] | | CtA | 0.83 ± 1.36 | 2.92 (1.65–5.16) |
| Null | 30 | CA | 3.30 ± 2.04 | 39 | CA | 2.33 ± 1.59[§] | 1.42 (1.04–1.93) |
| | | CsA | 0.93 ± 0.94 | | CsA | 0.97 ± 1.04 | 0.96 (0.59–1.57) |
| | | CtA | 2.37 ± 1.56[#] | | CtA | 1.36 ± 0.93[§] | 1.74 (1.27–2.40) |
| *GSTT1* | | | | | | | |
| Positive | 58 | CA | 3.52 ± 1.93[#] | 66 | CA | 1.74 ± 1.61 | 2.02 (1.55–2.63) |
| | | CsA | 1.05 ± 1.02 | | CsA | 0.65 ± 0.79 | 1.62 (1.10–2.37) |
| | | CtA | 2.47 ± 1.48[#] | | CtA | 1.09 ± 1.22 | 2.27 (1.66–3.09) |
| Null | 8 | CA | 2.75 ± 2.76 | 9 | CA | 2.56 ± 2.01 | 1.07 (0.45–2.55) |
| | | CsA | 0.88 ± 1.13 | | CsA | 1.33 ± 1.50 | 0.66 (0.21–2.10) |
| | | CtA | 1.88 ± 1.73 | | CtA | 1.22 ± 0.83 | 1.54 (0.71–3.35) |

Data reported as the group mean (±SD) of individual counts

*CA* total chromosome aberrations, *CsA* chromosome-type aberrations, *CtA* chromatid-type aberrations, *RoM* ratio of means, *95 % CI* confidence intervals at the 95 % level

Statistical significance (two-sided $p$ value <0.05, nonparametric Mann–Whitney $U$ test): * whole population, exposed versus controls; [#] subgroups, exposed versus corresponding non-exposed nurses (post hoc analysis, Mann–Whitney $U$ test multiple pairwise comparisons with Bonferroni correction for positive Kruskal–Wallis $H$ tests); [§] subgroups, <40 versus ≥40 years (post hoc analysis with Bonferroni correction)

**Table 6** Multivariate regression analysis[a] of confounding factors[b] and gene polymorphisms[c] on MN or CA frequencies in exposed and control nurses

| Population | Biomarker | Independent variable[b,c] | Regression coefficients | | | *p* |
|---|---|---|---|---|---|---|
| | | | $B$[d] | (95 % CI) | $\beta$[e] | |
| Whole | MN | Exposure | 2.423 | (1.623–3.223) | 0.444 | <0.0001 |
| | | Age | 0.086 | (0.037–0.135) | 0.259 | 0.001 |
| | | *GSTM1* | –0.943 | (−1.737 to −0.149) | −0.174 | 0.020 |
| | CA | Exposure | 1.586 | (0.947–2.226) | 0.390 | <0.0001 |
| | CtA | Exposure | 1.328 | (0.866–1.790) | 0.442 | <0.0001 |
| Exposed | MN | Age | 0.099 | (0.001–0.198) | 0.250 | 0.048 |
| | | *GSTM1* | −1.650 | (−3.143 to −0.157) | −0.274 | 0.031 |
| | CA | Job seniority | 1.061 | (0.007–2.114) | 0.254 | 0.049 |
| Controls | MN | Age | 0.079 | (0.030–0.127) | 0.346 | 0.002 |
| | CA | *GSTM1* | −1.028 | (−1.764 to −0.292) | −0.310 | 0.007 |
| | CsA | *GSTM1* | −0.459 | (−0.865 to −0.054) | −0.251 | 0.027 |

[a] Backwise procedure: $p = 0.05$ for entry into the model

[b] Variables considered: exposure, job seniority, age (years), city, eating habits, alcohol units

[c] Genetic polymorphisms: *GSTM1* and *GSTT1*

[d] Unstandardized $B$ (slope of the regression line)

[e] Standardized $\beta$

population in our earlier study, as that population is composed mainly of cells with short life spans, such as granulocytes. Such as they are, the results of that previous study indicate the absence of short-term events (i.e., primary DNA damage) in a population mostly consisting of short-lived cells. In contrast, in the comet/AraC assay in this study, we used a mitogen-stimulated lymphocyte fraction, which represents the longest surviving white cells in the

blood. Following prolonged exposure to genotoxic xenobiotics (e.g., causing DNA adducts), lesions do accumulate in circulating lymphocytes and such cryptic lesions may be visualized as intermediates of excision repair activity in cells that are treated with an inhibitor of DNA resynthesis (Collins et al. 1993).

In our opinion, the reduced migration extent observed in the exposed subjects as compared to controls might be related to a potential long-term exposure to cross-linking ANPD. As DNA damage induced in circulating lymphocytes is likely to persist and accumulate because of the limited excision repair activity in quiescent ($G_0$) cells (Green et al. 1996), the increased frequency of MN observed in the exposed nurses could be associated with the extent of genome damage (including cross-links) that lymphocytes may have accumulated while circulating within the body in the quiescent phase.

Moreover, analysis of chromosome damage was associated with genotype analysis of genes coding for glutathione *S*-transferases (GST), a family of phase II xenobiotic-metabolizing enzymes involved in catalyzing the conjugation reactions of reactive electrophilic xenobiotics with cytosolic glutathione. Polymorphisms in these genes, possibly by altering their expression and functional activities, may affect carcinogen activation/detoxification and eventually DNA damage. Homozygous deletion, or null genotype, at either the *GSTM1* or the *GSTT1* locus, resulted in enzyme function loss, and this has been hypothesized to account for a higher susceptibility to cancer (Ginsberg et al. 2009). To assess any influence of genetic background on individual susceptibility, we evaluated the potential effect of polymorphisms in *GSTM1* and *GSTT1* on risks from occupational exposures to ANPD. The frequencies obtained for *GSTM1*- and *GSTT1* null genotypes in the whole population (0.50 and 0.13, respectively) were consistent with the data reported in the literature for the European population (Kurose et al. 2012), and the results of this study suggest that *GSTM1* and *GSTT1* null genotypes do not modify MN frequencies in the presence of exposure to ANPD. We cannot compare our findings to published data in the literature because, to the best of our knowledge, ours is the first study in this occupational setting where gene polymorphisms in *GSTM1* and *GSTT1* have been associated with MN frequency. Similarly, *GSTM1* and *GSTT1* null genotypes did not modify CA frequencies in the presence of exposure to ANPD. Our findings are in agreement with the results reported in the published papers with a similar approach (Musak et al. 2009; Testa et al. 2007).

Among the above-cited studies in which cytogenetic end points were evaluated, very few have considered an adequate exposure evaluation in the study design. Environmental and biological monitoring procedures have been considered by Cavallo et al. (2005), which included measurements of surface contamination from the most commonly used ANPD (i.e., CP, cytarabine, 5-fluorouracil, gemcitabine, and ifosfamide,), and measurement of the 5-fluorouracil metabolite α-fluoro-β-alanine in urine samples. Biological monitoring of exposure has been considered only in two studies. Ensslin et al. (1997), in hospital personnel regularly handling ANPD, quantified the incorporation of CP, ifosfamide, and platinum-containing drugs by the determination of urinary concentrations of these drugs. Burgaz et al. (1999) evaluated exposure to ANPD by measuring the urinary concentrations of CP. Environmental monitoring, by the evaluation of contamination of surfaces by 5-fluorouracil (i.e., wipe test), was performed by Ladeira et al. (2014). Overall, the findings in the present and the above-cited studies confirm that ANPD contamination of the work environment in hospital is still possible, and safety measures adopted may not be sufficient to prevent exposure to genotoxic xenobiotics (Hedmer and Wohlfart 2012).

The standard regulation in force regarding this subject in Italy (GURI 1999) incorporates many of the measures included in international warnings and guidelines, as well as rules for safe and appropriate organization of services for ANPD preparations (ASHP 2006; NIOSH 2010; OSHA 2000). On this basis, the nurses exposed to ANPD enrolled in this study had received adequate recommendations for safety handling of ANPD and were recommended to wear appropriate personal protective equipment (i.e., disposable single-use gowns, gloves, and masks) whenever handling ANPD or contaminated materials. However, despite the adoption of methods for preventing exposure to ANPD (e.g., engineering controls, administrative and work practice controls, training, and personal protective equipment), incorporation of trace amounts of these agents still occurs in hospital personnel with a detectable residual genotoxic risks, as revealed by the increased frequency of MN and CA in the exposed subjects.

## Conclusions

In conclusion, our results provide further evidence that handling ANPD, even if under safety controlled conditions, represents a considerable genotoxic risk for healthy subjects occupationally exposed to these chemicals. Moreover, CA (Bonassi et al. 2000, 2004, 2008) and MN (Bonassi et al. 2007) are predictive of increased cancer risk, rather than just reflect the exposure level. This finding clearly indicates the necessity to improve some steps in the administration process of ANPD to appropriately cope with genotoxic risk. It is important to modify the guidelines for the evaluation of mutagenic/carcinogenic hazards in occupationally exposed subjects by considering an integrated chemical/biotoxicological approach. The use of biomarkers

which measure changes in cellular or molecular endpoints (e.g., DNA damage) will allow us to implement a more complete approach according to not only environmental and biological monitoring but also to biological effect monitoring using genotoxicity biomarkers (Villarini et al. 2012).

CA is certainly the most robust biomarker with predictivity related to cancer risk. However, testing of CA has high demands on laboratory training and skills and is very time-consuming. On the other hand, MN is a very promising biomarker with predictivity of cancer and smaller demands on skills (Knudsen and Hansen 2007). The extensive use of CA in future studies is thus limited by the laborious and sensitive procedure of the test. MN testing, because of its ability to detect both clastogenic (e.g., chromosome breakage) and aneugenic (e.g., spindle disruption) effects, could have a role in occupational health surveillance programs for workers occupationally exposed to ANPD to monitor long-term exposure effects (biomarker of early/preclinical biological effects). Finally, genotyping of exposed subjects for *GSTM1* and/or *GSTT1* polymorphisms does not seem to have a role in occupational health surveillance programs in the studied occupational setting.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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