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Research article

Relation between DNA damage measured by comet assay and *OGG1* Ser326Cys polymorphism in antineoplastic drugs biomonitoring

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Abstract: Antineoplastic drugs are hazardous chemical agents used mostly in the treatment of patients with cancer, however health professionals that handle and administer these drugs can become exposed and develop DNA damage. Comet assay is a standard method for assessing DNA damage in human biomonitoring and, combined with formamidopyrimidine DNA glycosylase (FPG) enzyme, it specifically detects DNA oxidative damage.

The aim of this study was to investigate genotoxic effects in workers occupationally exposed to cytostatics (n = 46), as compared to a control group with no exposure (n = 46) at two Portuguese hospitals, by means of the alkaline comet assay. The potential of the *OGG1* Ser326Cys polymorphism as a susceptibility biomarker was also investigated. Exposure was evaluated by investigating the contamination of surfaces and genotoxic assessment was done by alkaline comet assay in peripheral blood lymphocytes. *OGG1* Ser326Cys (rs1052133) polymorphism was studied by Real Time PCR.

As for exposure assessment, there were 121 (37%) positive samples out of a total of 327 samples analysed from both hospitals. No statistically significant differences (Mann-Whitney test, $p > 0.05$) were found between subjects with and without exposure, regarding DNA damage and oxidative DNA damage, nevertheless the exposed group exhibited higher values. Moreover, there

was no consistent trend regarding the variation of both biomarkers as assessed by comet assay with *OGG1* polymorphism.

Our study was not statistically significant regarding occupational exposure to antineoplastic drugs and genetic damage assessed by comet assay. However, health professionals should be monitored for risk behaviour, in order to ensure that safety measures are applied and protection devices are used correctly.

Keywords: DNA damage; Comet assay; *OGG1* polymorphism; antineoplastic drugs; occupational exposure; biomonitoring

1. Introduction

Exposure of patients and health professionals to mixtures of antineoplastic drugs in hospital settings leads to unpredictable and unique effects. This is due to differences in practice at hospital oncology departments, namely regarding the number of patients, the availability of protection devices, and safety procedures employed by the clinical staff. Health care workers who prepare or administer hazardous drugs or work in areas where such drugs are used may be exposed to these agents by direct contact with contaminated workplace surfaces, clothing, medical equipment, patient excreta, and other contaminated materials [1–3].

Exposure may occur by inhalation, resulting from aerosolization of powder or liquid during reconstitution, and by spillage taking place while preparing or administering to patients. It is well known that exposures to even very small concentrations of certain drugs may be hazardous for workers who handle or work near them [1,4,5].

Cytostatic drugs have been proven to be mutagens, carcinogens and teratogens [6–11]. The first chemotherapeutics developed were chemicals that interact directly with DNA by covalent binding or other, or indirectly by interfering with DNA synthesis. Compounds that inhibit the mitotic spindle formation and those that affect endocrine function are also used in cancer chemotherapy [12]. These drugs are often used in combination to achieve synergistic effects on tumour cells resulting from their different modes of action. However, most if not all of such chemical agents are generally nonselective and, along with tumour cells, normal cells may also undergo cytotoxic/genotoxic damage [5,13,14]. According to European Guidelines (Corrigendum to Directive 2004/37/EG), any use of carcinogenic, mutagenic or teratogenic substances, including their application in health care settings, are deemed to be of the highest risk level [8–11,15].

Comet assay has become one of the standard methods for assessing DNA damage, with a wide range of applications, namely in genotoxicity testing, human biomonitoring and molecular epidemiology, as well as in fundamental research on DNA damage and repair [16–20]. To make the assay more specific as well as more sensitive, an extra step is added to the assay using formamidopyrimidine DNA glycosylase (FPG) enzyme that detects the major purine oxidation product 8-oxoguanine (8-OHdG) [16,21–23] providing information about DNA oxidative damage.

The cellular defense system against 8-OHdG mutagenesis involves base excision repair (BER), nucleotide excision repair (NER), and mismatch repair. However, BER via DNA glycosylase (OGG1) represents the main protection mechanism of the integrity of human DNA with respect to 8-OHdG [24]. OGG1 is considered to be the main enzyme responsible for the removal of 8-OHdG in

humans, removing it when it is paired with cytosine [25,26] and the inactivation of *OGG1* appears to play a role in the multistage process of carcinogenesis. The human *OGG1* gene is located on chromosome 3 (3p26), and encodes a bifunctional DNA glycosylase endowed with an AP lyase activity. This is a region frequently lost in various types of cancer, especially in small-cell lung cancers where loss of heterozygosity in nearly 100% of the cases can be observed [27].

Loss of the gene would abrogate OGG1 activity imposing an increased risk of mutagenicity on the cell due to accumulation of 8-OHdG in DNA [27]. A common polymorphism of this gene, Ser326Cys (rs1052133) a C→G polymorphism in codon 326 at exon 7 [28] is associated with an increased risk of cancer [29]. This polymorphism is present in 33–41% of the Caucasian population [30].

The aim of this study is to evaluate genotoxic effects in workers occupationally exposed to cytostatics at two Portuguese hospitals by means of the alkaline comet assay and investigate the potential of the *OGG1* Ser326Cys polymorphism as a susceptibility biomarker.

2. Materials and Method

2.1. Subjects of study

The study is based upon a sample of 46 occupationally exposed workers—pharmacists, pharmacy technicians and nurses and a sample of 46 non-exposed control subjects. The exposed group was formed by workers from two hospitals at the Lisbon region, and the control group included workers from an academic institution, namely teachers and office workers, chosen for having no contact with cytostatic drugs and for being statistically comparable to the exposed group, in terms of sex ratio and age distribution.

This study was performed abiding by high ethical standards and received the necessary approvals. All the participants were informed about the aim and experimental details of the study and gave their informed consent. Each participant completed a standardized questionnaire that covered a detailed medical, family and dietary history, including variables known to influence cytogenetic endpoints (exposure to potential mutagens, oncological therapeutics, and lifestyle activities). The use of individual and collective protection equipment was also assessed by the questionnaire.

2.2. Exposure assessment

Cyclophosphamide (CP), 5-Fluorouracil (5FU) and Paclitaxel (PTX) were used as surrogate markers for surfaces contamination by cytotoxic drugs, since those drugs are among the most used on a daily basis in both hospitals, both in frequency and amount. A similar approach has been used in previous studies [31–35].

Surface areas of 100 cm^2, defined by a stainless frame with an internal size of 10 × 10 cm were wipe sampled with gauze moistened with ethyl-acetate as described in Schmaus et al. [36]. For irregular surfaces such as phones, armrests and handles the wiped area was estimated and the result was used to calculate contamination.

Extraction was performed as described in Schmaus et al. [36]. Briefly, the wipes were extracted with 15 mL of acetonitrile:methanol:water (10:25:65) for 20 minutes at room temperature in a bottle roller homogenizer. Extracts were filtered through a 0.22 μm filter prior to injection. Separation and quantification was performed according to Larson et al. [31] on a Thermo-UNICAM Surveyor

HPLC-DAD; 100 μL sample loop; column Hypersil-GOLD 15 × 5 × 4.6 with a guard column; mobile phase of acetonitrille:methanol:water (19:13:68) at a flow of 0.8 mL min^{-1}. All HPLC grade solvents were purchased from VWR International. CP, 5FU and PTX were purchased from Sigma®. For all the drugs, calibration curves were performed after extraction of spiked wipes. Each sample was injected in triplicate.

2.3. Genotoxicity assessment

Heparinized blood samples were obtained by venipuncture from each subject between 10 a.m. and 12 p.m. and the isolated lymphocytes were cryopreserved following the protocols of Singh and Lai [37] and Duthie et al. [38]. Assessment of genotoxic effects was conducted by applying the alkaline comet assay in peripheral blood lymphocytes, as described in Collins and Azqueta [39]. The FPG [kindly donated by Prof. Andrew Collins (Department of Nutrition, University of Oslo, Norway)] protocol was performed also according to Collins and Azqueta [39]. The slides dried at room temperature, were stained with DAPI (1 μg/mL) and then visualized. All samples were coded and analyzed under blind conditions. Slides were scored by one single observer using Zeiss AxioScope.A1 fluorescence microscope and Comet Assay IV capture system (Perceptive Instruments) and 50 nucleoids were scored per gel, following the parameters described by Collins [40].

2.4. Polymorphism analysis

Whole blood samples were collected and stored at −20 ℃ until total white cell's DNA was extracted by blood spot. Briefly, the whole blood was defrosted and 200 μL were dropped in 3 MM chromatography paper (Watman™) and air dried. Two samples of each biological sample were taken with a perforator and put in a microtube with 500 μL of ultrapure water (Milli-Q®). The perforator was disinfected between samples with ethanol 70%. Each microtube was placed in the vortex and kept at room temperature for 10 min, being centrifuged in the following day at 16.000 g for 2 min. The supernatant was eliminated and 200 μL of Chelex at 6% were added in the microtube and mixed in the vortex. The microtubes were put at 56 ℃ for 10 min and then, after vortex, went for 10 more min at 100 ℃. Finally, the microtubes were centrifuged at 6000 g for 2 min and stored at −20 ℃. All the laboratory procedures were made in the same institution.

The *OGG1*Ser326Cys (rs1052133) genotypes were determined using the TaqMan SNP genotyping assay with Real Time PCR (Applied Biosystems). To perform the genotype analysis of *OGG1* polymorphism the target fragments were amplified in a 20 μL reaction mixture containing 10 μL *TaqManUniversal PCR Master Mix*, 1 μL primers/probe, 5 μL MilliQ water, and 4 μL DNA. Real Time PCR, was then conducted as follows: 10 min at 95 ℃, 50 cycles of 15 sec at 92 ℃ and 1 min at 60 ℃ at *iCycler iQ® Multicolor Real-Time PCR Detection System* (BIO-RAD). All doubtful samples were reanalyzed.

2.5. Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 21.0). Variables were compared with the Normal distribution using the Shapiro-Wilk test ($p > 0.05$). Rejection of the null hypothesis of underlying normality led us to proceed with non-parametric procedures to compare

groups and check associations. The Mann-Whitney, Fisher exact, and Chi-square tests were used to compare groups. The association between each of the genotoxicity biomarkers and *OGG1* genotypes was evaluated by the Kruskal-Wallis test. The analysis of genotype and allele frequency and the Chi-square fit test was made with the *GenePop* on the web software (http://genepop.curtin.edu.au/).

3. Results

Population characteristics such as gender distribution, age, years of exposure and tobacco habits for the control and exposed groups are shown in Table 1.

The exposed group was formed by workers from hospitals A and B (46 = 10 + 36 workers, respectively). There were no significant differences between the two groups in what concerns gender (Fisher exact test, $p > 0.999$), age (Mann-Whitney test, $p = 0.989$), years of exposure (Mann-Whitney test, $p = 0.789$), and tobacco habits (Chi-square test, $p = 0.066$). The statistics of exposure assessment to cystostatic drugs, namely cyclophosphamide, 5-fluorouracil, and paclitaxel are presented in Table 2.

Table 1. Demographics of the study population.

	Control group	**Exposed group**
Number of subjects	46	46
Gender		
Females	34 (73.9%)	40 (87.0%)
Males	12 (26.1%)	6 (13.0%)
***p*-value (Chi-square test)**	0.115	
Age		
(mean ± standarderror of mean, in years)	39.26 ± 1.42	33.85 ± 1.21
Range	20–61	24–58
***p*-value (Mann-Whitney test)**	0.004	
Years of exposure		
(median ± standard error of mean, in years)	n.a.	6.62 ± 0.94
(median ± interquartile range, in years)		5.00 ± 5.00
Range		0.17–30
Tobacco habits		
Non-smokers	34 (77.3%)	42 (91.3%)
Smokers	10 (22.7%)	4 (8.7%)
***p*-value (Chi-square test)**	0.066	

n.a.—non-applicable

There were 121 (37%) positive samples among a total of 327 analysed samples from both hospitals. A sample was considered positive when at least one of the three surrogate markers was detected. At hospital A, 21 (31.3%) out of 67 samples were contaminated. At hospital B, 100 (38.5%) out of 260 samples were positive. Additionally, in Hospital A, 13 samples (19.4%) presented contamination with more than one drug and, in Hospital B, 15 samples (5.8%) showed contamination by more than one drug. The global percentages of contaminated samples in the two hospitals (31.3

and 38.5%) were not statistically different (Chi-square: 1.158, p = 0.28). As for differences in percentages of contamination by drug, they were not different for CP and PTX (respectively, Chi-square: 1.84, 0.66; p = 0.17, p = 0.42), but the difference in percentage of samples contaminated with 5-FU were significantly higher at Hospital A (Chi-square: 18.97, p < 0.01). The median concentrations of CP and PTX were significantly different between hospitals (CP: Mann-Whitney test, p = 0.001 based on LOD; PTX: Mann-Whitney test, p = 0.001).

Table 2. Samples contaminated and total samples per hospital (A and B), median and interquartile range (IR) regarding cyclophosphamide (CP), 5-fluorouracil (5-FU), paclitaxel (PTX), and respective limits of detection (LOD) and quantification (LOQ).

Hospitals	**CP** ($\mu g/cm^2$)	**5-FU** (ng/cm^2)	**PTX** (ng/cm^2)	**Samples with contamination**
A	1/67 (1.5%)	17/67 (25.4%)	17/67 (25.4%)	21/67 (31.3%)
Median	0.18	13.44	12.95	
IR	0.12	2.83	3.12	
B	14/260 (5.4%)	18/260 (6.9%)	54/260 (20.7%)	100/260 (38.5%)
Median	1.00	14.74	21.38	
IR	1.45	11.07	31.45	
Totals	**15/327 (4.6%)**	**35/327 (10.7%)**	**71/327 (21.7%)**	**121/327 (37%)**
LOD ($\mu g/cm^2$)	0.10	3.30	0.167	
LOQ ($\mu g/cm^2$)	0.30	10.00	0.50	

***** Number of contaminated samples is not to be summed across columns, because some samples are multi-contaminated.

There was no statistically significant association between the percentage of contaminated samples and years of exposure (Mann-Whitney test, p > 0.05), meaning that more experience does not necessarily mean less contamination.

Results obtained for DNA damage (% DNA in Tail) and oxidative DNA damage (FPG) are presented in Figure 1. No statistically significant differences (Mann-Whitney test, p > 0.05) were found between subjects with and without exposure, regarding both the mean DNA damage (p = 0.136) and oxidative DNA damage (p = 0.229). However higher values were observed in the exposed group.

DNA damage and oxidative DNA damage in the exposed group showed no differences between hospitals A and B (Mann-Whitney test, p > 0.05)—Figure 2.

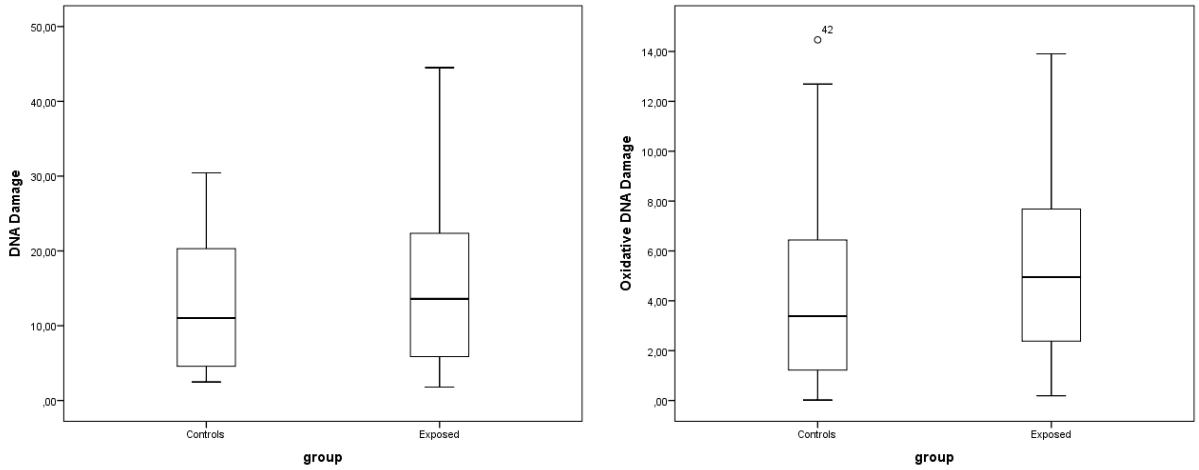

Figure 1. Box plots of DNA damage (left) and oxidative DNA damage (right) in the two groups.

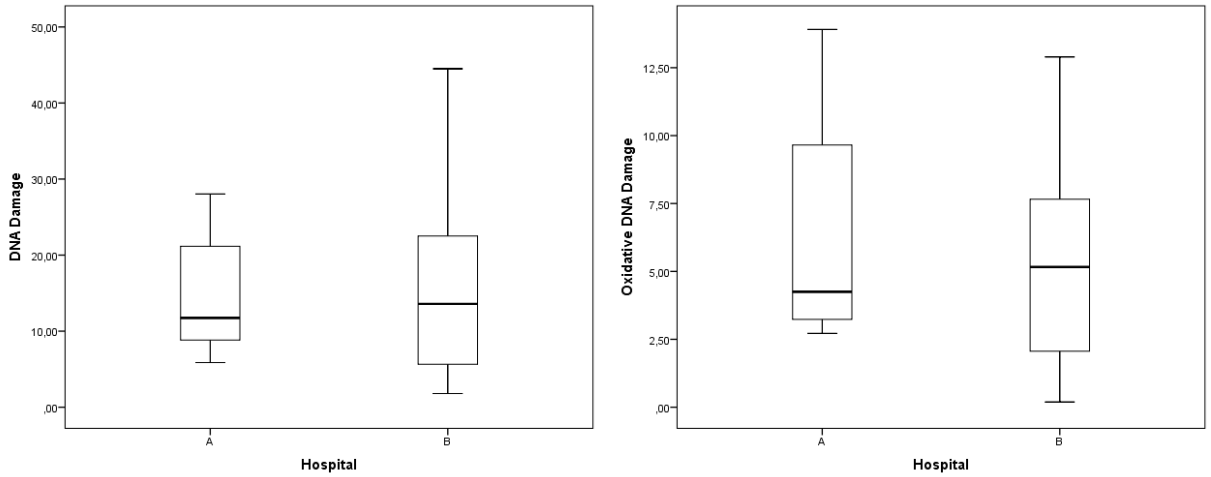

Figure 2. Box plots of DNA damage and oxidative DNA damage between hospitals A and B.

Age, gender, years of exposure and tobacco habits are possible confounding factors that can affect genotoxicity measurement and whose effects can be investigated my multiple regression analysis within the exposed and the control groups. Age and tobacco did not account for significant results ($p < 0.05$). As for gender, the regression coefficients in the model of % DNA damage in the controls indicates that being a woman increases, on average, 6.4% of the % DNA damage in the control group ($p = 0.022$). Genotypic and allelic frequencies of *OGG1* Ser326Cys polymorphism are presented in Table 3. The population was in Hardy-Weinberg equilibrium ($p > 0.05$) and there were no significant differences in genotypic and allelic frequencies between the exposed and control groups (Chi-square fit test, $p > 0.05$) (Table 3).

The descriptive statistics concerning the relationship between genotoxicity biomarkers provided by comet assay and the OGG1 genotypes studied are shown in Table 4.

The results show that the carriers of the Ser/Ser genotype have higher median values of DNA damage and oxidative DNA damage, in comparison with the other two studied genotypes. However,

the Kruskal-Wallis test did not reject the null hypothesis of equality among *OGG1* genotypes regarding the means of the two comet assay parameters ($p > 0.05$).

Table 3. Frequency of genotypes and alleles of OGG1 Ser326Cys polymorphism in the study sample, *p*-value of Fisher's exact test.

Gene	Genotypes	All (%)	Exposed (%)	Controls (%)	*p*-value
	Cys/Cys	9 (9.8)	7 (15.2)	2 (4.4)	
	Ser/Cys	32 (34.8)	14 (30.4)	18 (39.1)	0.446
OGG1	Ser/Ser	51 (55.4)	25 (54.4)	26 (56.5)	
	Cys	50 (27.2)	31 (30.4)	22 (23.9)	0.409
	Ser	134 (72.8)	79 (69.6)	70 (76.1)	

Table 4. **Descriptive statistics of DNA damage and oxidative DNA damage in the studied population (median ± interquartile range, and range) by *OGG1* Ser326Cys genotypes in both groups, *p*-value of Kruskal-Wallis test.**

			Median	Interquartil Range	χ^2_{KW}	d.f.	p
	Cys/Cys	DNA	12.11	16.595	0.059	2	0.971
	Ser/Cys	Damage	13.2	14.987			
OGG1	Ser/Ser		13.4	16.443			
	Cys/Cys	Oxidative	2.78	8.377	0.031	2	0.985
	Ser/Cys	DNA	3.37	5.433			
	Ser/Ser	Damage	4.75	6.139			

4. Discussion

Healthcare workers handling antineoplastic drugs usually have available protection equipment and abide by safety rules to avoid workplace contamination. However, contamination of the working environment is still possible, and the safety measures employed can be insufficient to prevent exposure [13,32,36]. In addition, workers may not apply all safety measures required for handling such substances or some specific working procedures. The antineoplastic drugs handled by pharmacists, pharmacy technicians, and nurses studied in this work can interact with DNA, inducing double and single strand breaks, crosslinks, alkylations, and DNA intercalations, which could account, at least partially, for the genotoxicity observed, even if not yielding statistically significant results.

Therefore, contamination of various surfaces by antineoplastic drugs in workplaces implies an increased risk for health care workers who are dermally exposed [43].

Contaminated surface samples were found for all surrogate markers in both hospitals. These results are a cause for concern, because health effects associated to exposure to carcinogenic, mutagenic, and teratogenic substances usually do not depend on a minimum dose but rather on a prolonged exposure [6,8,9,11]. Therefore, there is no safety dose threshold regarding exposure to these drugs, instead being more appropriate to apply the ALARA principle: keep exposure/contamination levels "As Low As Reasonably Achievable" [41]. Widespread contamination

was also observed in other studies [13,32,36], despite the implementation of safety procedures for handling antineoplastic drugs. Our results showed that the amount of contaminated surfaces varied in both hospitals depending on the drugs considered. This is probably due to daily variability in drugs use and how it relates with the days when sampling was conducted or due to different working procedures and cleaning methods. These two aspects are probably different between hospitals and even between the workers involved on the handling and cleaning within the same hospital.

Some samples were contaminated with more than one drug, bringing up an important point regarding risk assessment: exposure is not occurring to one single drug but rather to combinations of different antineoplastic drugs and the health effects of such mixtures are unpredictable [2,6,42]. Moreover, it was observed that the use of protection devices was frequent in the preparation areas but not in other pharmacy areas and administration services, a concern already brought up before [44].

The comet assay identifies injuries resulting from recent exposure (over the previous few weeks) which are still reparable, such as single and double-strand DNA breaks, alkali labile lesions converted to strand breaks under alkaline conditions, and single-strand breaks associated with incomplete excision repair sites [5,18]. In particular, the comet assay combined with an enzyme which recognizes and cuts specifically oxidized DNA bases, allows for the evaluation of oxidative DNA damage [45]. It is one of the most used methods for biomonitoring genotoxicity in blood lymphocytes [46], and has been widely used to evaluate the genotoxic effects of exposure to specific antineoplastic drugs in several in vitro and in vivo studies [47–53].

In what concerns comet assay, our findings suggest that occupational exposure to antineoplastic drugs in healthcare workers induces DNA damage, as we have found higher mean DNA damage, measured by % DNA in tail and oxidative DNA damage (FPG), in the lymphocytes of exposed subjects as compared to controls, although without reaching statistical significance (Figure 1). Our results are in line with other studies [11,47–52] which used the alkaline comet assay of peripheral blood lymphocytes to monitor subjects exposed to antineoplastic drugs, and also did not find significant differences between exposed and controls, or the weak significant trend reported by Mader et al. [54] who evaluated DNA damage by measuring comet tail moment, and found no significant difference between exposed and control subjects either. A possible explanation for the lack of statistical significance may have to do with comet assay predominantly detecting single-strand breaks and alkali-labile sites induced by antineoplastic drugs [2]. Since both types of DNA damage are continuously and efficiently repaired, the damage level measured results from the balance between the DNA damage inflicted and the speed of repair [55]. Generally, the type, level and persistence of DNA damage in lymphocytes of exposed populations depends on the kind of antineoplastic drugs used as well as on the concentrations of drugs producing the mutagenic response [55]. Also, antineoplastic drugs are well-known cross-linking agents, which can increase the effective molecular weight of DNA, and thereby are known to reduce the ability of DNA with strand breaks to migrate in an electric field. The presence of a cross-linking agent could have hidden an increase in DNA migration associated with the induction of DNA strand breaks by other genotoxic agents, with a higher effect in terms of DNA tail mobility [4].

Contrary to these findings, other researchers evaluating DNA damage in healthcare workers handling antineoplastic drugs were able to show a statistically significant increase in DNA damage on the exposed group as compared to controls, using the comet assay [2,4,51,52,55–61].

The comet assay is recommended to monitor populations chronically exposed to genotoxic agents combined with the cytokinesis-blocked micronucleus assay [46,58]. Our previous published

findings assessing genotoxic effects due to antineoplastic exposure in a nursing group, reported a statistical significant increase of micronucleus in the exposed group when compared with controls [62]. Such results—positive findings with micronucleus and non-significant results by comet assay are similar to those reported by Deng et al. [63] in workers occupationally exposed to another cytotoxic drug: methotrexate.

OGG1 is probably the main enzyme responsible for the removal of 8-OHdG in humans, an oxidation product believed to play an important role in carcinogenesis because of its abundance and high mutagenicity. Epidemiological studies have previously associated the Ser326Cys in *OGG1* genotypes with the risk of different types of cancer, namely esophageal [64], orolaryngeal [65], lung cancer [30], larynx [66], colon cancer [67], and gastric cancer [68]. Our results found that Ser/Ser carriers presented higher levels of DNA damage and oxidative DNA damage but the differences were not statistically significant (Table 4). These results go against studies of Chen et al. [69] that showed *OGG1* Ser/Ser to have higher repair activity toward 8-OHdG than the *OGG1* Cys/Cys, being 8-OHdG levels in lymphocyte DNA significantly higher in the last genotype. Also, Aka et al. [70] and Pawlowska et al. [66] verified that Cys/Cys and Ser/Cys *OGG1* genotypes have less DNA repair capacity compared to the Ser/Ser *OGG1* genotype. Kohno et al. [28] reported that mean 8-OHdG levels were similar in peripheral leukocytes expressing either Ser/Ser or Cys/Cys.

The small size of our sample may have hampered the finding of an association between oxidative damage and this polymorphism. Nevertheless, the effect of Ser326Cys genotypes on DNA repair capacity may differ with the type and strength of DNA-damaging exposures and may be influenced by the interaction between the *OGG1* polymorphism and other genetic polymorphisms [71]. Moreover, this polymorphism may be in linkage disequilibrium with other functional polymorphisms in cancer-related genes [30]. The polymorphism of the *OGG1* gene is worth an investigation, as a population with decreased OGG1 enzyme activity would be at risk of accumulating 8-OHdG in nuclear DNA due to incomplete repair of oxidatively damaged DNA [72].

5. Conclusion

Alkaline comet assay is widely used to detect genotoxic effects induced in vivo by occupational exposure to various mutagens. The relative simplicity and quickness of the method, combined with the fact that few cells are required for the analysis, makes it an attractive technique for biomonitoring purposes in human populations [55]. Our results confirm that it is likely that those continuously handling antineoplastic drugs for occupational reasons are at a greater risk of genotoxic damage, since the results pointed out a slight increase, however not statistically significant, of genetic damage assessed by comet assay. The achieved results can reinforce the importance of keeping and continuously improving safety measures to avoid exposure, and the monitoring of these professionals for risk behaviour, in order to make sure that such hazardous compounds are properly handled. Since genotoxicity may be due to combined effects of all or some of the antineoplastic drugs, it is not possible to attribute damage to any particular agent [56]. We have not found an association between *OGG1* Ser326Cys genotypes and DNA oxidative damage, either because of insufficient sample size or because the effect is modulated by other variables. Nevertheless we suggest that the investigation of this polymorphism is worth pursuing, given its known epidemiological association with cancer.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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