Cytogenetic effects induced by *Prestige* oil on human populations: The role of polymorphisms in genes involved in metabolism and DNA repair

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Abstract:

The spill from the oil tanker *Prestige* (NW Spain, November 2002) was perhaps the biggest ecological disaster that happened worldwide in the last decades. As a consequence of this catastrophe a general concern led to a huge mobilization of human and technical resources. Given that no information was reported in the scientific literature regarding to the chronic repercussions to human health of exposure to oil spills, a pilot study was performed by our group revealing some increased genotoxic effects in the subjects exposed to the oil during cleaning activities. Due to the seriousness of the results, we extended our study comprising a larger population and including an extensive evaluation of the main polymorphic sites in metabolizing and DNA-repair genes. General increases in micronucleus (MN) frequency and decreases in the proliferation index were observed in individuals with longer time of exposure. Age was a significant predictor of MN frequency. *CYP1A1* 3′-UTR, *EPHX1* codons 113 and 139, *GSTP1*, *GSTM1* and *GSTT1* metabolic polymorphisms, and *XRCC3* codon 241 and *XPD* codon 751 repair polymorphisms influenced cytogenetic damage levels. In view of these results, it seems essential to pay more attention to the chronic human health effects of exposure to oil and to focus new studies on such a relevant but overlooked public health field that involves a large number of people all over the world.

Keywords:

Genotoxicity; Genetic polymorphisms; Biomonitoring; *Prestige* oil; Micronucleus test

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1. Introduction

The oil spill from the tanker *Prestige* was perhaps the biggest ecological disaster that happened worldwide in the last decades. Sixty-three thousand tons of oil were spilled from the tanker in November 2002 in front of the Galician coasts and polluted more than 900 km of Spanish and French coasts. The tanker contained fuel oil no. 6, classified as possible human carcinogen (group 2B) by the International Agency for Research on Cancer [1]. The composition of the oil from the *Prestige* was described by the Spanish National Research Council as 50% aromatic hydrocarbons, 22% saturated hydrocarbons and 28% resins and asphalthenes [2]. The aromatic hydrocarbon fraction included high molecular-weight hydrocarbons (complex mixtures of polycyclic aromatic hydrocarbons (PAH) and asphalthenes), light aromatic hydrocarbons (naphthalene and its alkylated derivatives) and volatile aromatic hydrocarbons (primarily benzene, toluene and xylenes).

As a consequence of the high impact that this event had on such a rich and valuable natural environment, a general concern led to a huge mobilization of human and technical resources. Several studies were set-up to ascertain the effects of this disaster especially on the fauna, as had happened after other spills such as those from the Exxon Valdez, the Erika, etc. [3–6]. However, until now no information was reported on the chronic repercussions to human health, which seems paradoxical considering the noxious properties of a large number of compounds present in the oil, many of them classified as human carcinogens. After the sinking of the *Prestige* we started an epidemiological study in which individuals participating in the different kinds of cleaning activity were included, with the aim of evaluating the possible genotoxic and/or endocrine effects induced by the exposure. In a first approximation, the analysis of a part of the whole population revealed some increased genotoxic endpoints [7] and altered endocrine status [8] in the exposed subjects compared with regard the controls, and raised the possibility that some lifestyle and genotypic factors could influence the extent of damage.

In view of the results obtained, given the great relevance of genotoxic events for the outcome of cancer, since they are tightly related to the first stages of carcinogenesis, and taking account of the absence in the international literature of studies focused on this kind of effect in human populations exposed to oil spills, we extended our study by including a larger population, in order to pro-

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vide an analysis with a higher statistical power. As the main health consequences of the exposure seemed to be related to genotoxic phenomena, we selected the micronucleus (MN) test since it is one of the most established cytogenetic assays, it is unlikely to generate false-positive results [9], and there is evidence that the MN frequency in peripheral blood lymphocytes is a predictive biomarker of cancer risk [10]. On the other hand, the cytokinesis-block MN test allows the scoring of nucleoplasmic bridges (NPB), providing a measure of chromosome rearrangement that is otherwise not measured in this assay when only MN are scored [11]. Furthermore, we included an extensive evaluation of the main polymorphic sites in genes encoding for metabolizing enzymes (cytochrome P450 (CYP) *1A1* 3′-UTR *1A > *2A, *CYP1B1* codon 432 *1 > *3, *EPHX1* codon 113 Tyr > His and codon 139 His > Arg, glutathione *S*-transferase (*GST*) P1 codon 105 Ile > Val, and *GSTM1* and *GSTT1* deletion polymorphisms) and for DNA-repair proteins (*XRCC1* codon 194 Arg > Trp and codon 399 Arg > Gln, *XRCC3* codon 241 Thr > Met, and *XPD* codon 751 Lys > Gln) as susceptibility biomarkers. Three of the metabolizing genes analysed (*CYP1A1*, *CYP1B1* and *EPHX1*) have been proposed as possible impact factors on exposures to PAH, one of the major reactive groups of chemicals in the oil, since these genes are directly implicated in the formation of diol-epoxides, responsible for PAH-associated carcinogenicity. In addition, *GSTP1*, *GSTM1* and *GSTT1* participate in the biotransformation of these compounds, their efficacy depending on the species tested. As to DNA-repair proteins, XRCC1 participates in base-excision-repair and recombination-repair pathways [12] and XRCC3 is involved in the homologous recombination pathway [13] for repair of DNA double-strand breaks induced either directly or indirectly following replication of closely spaced single-strand breaks [14]. Lastly, the *XPD* gene encodes a DNA helicase involved in transcription and nucleotide-excision repair [15]. All the pathways mentioned here are somehow involved in the repair of the various DNA lesions induced by the multiple compounds in the oil.

2. Materials and methods

2.1. Study subjects

A total of 219 individuals were included in this study. The performance of different cleaning tasks determined different exposure conditions and allowed the establishment of three exposed groups: voluntary individuals exposed to oil for just five consecutive days by cleaning beaches (V, *N* = 59), workers hired by the Galician Government who developed also manual cleaning tasks during four months (MW, *N* = 53), and hired workers using high-pressure machines for cleaning the rocks during three months (HPW, *N* = 47). These characteristics determined quantitative and qualitative differences as the first group experienced a shorter and more acute exposure, and the employment of high-pressure instrumentation gave rise to a distinctive exposure regarding the direct handling of the oil by the two first groups. Sixty never-exposed individuals constituted the control population. Each subject answered a detailed questionnaire including data on physiological characteristics, consumption habits, professional and medical history, and use of protective devices during the development of the cleaning tasks. The University of A Coruña Research Ethics Committee approved the study, and all participants gave their written informed consent. The characteristics of the study population are listed in Table 1.

2.2. Blood sample collection

Peripheral blood samples were collected between March and May 2003 in heparinized and EDTA tubes before the beginning of the work shift. Samples were immediately transported to the laboratory in a refrigerated container. Every sample was codified in order to ensure a blind study. Heparinized samples were used to immediately establish MN cultures. EDTA samples were aliquoted in 300-μl fractions and frozen at −20 °C until DNA extraction.

2.3. Cytokinesis-block MN test

Heparinized whole blood was cultured in duplicate as described in Laffon et al. [16]. Binucleated cytokinesis-blocked cells, MN and NPB were identified according to the criteria proposed by Fenech et al. [17]. One thousand binucleated cells (500 from each duplicate culture) were scored for each individual to determine the frequencies of MN, binucleated micronucleated cells (BNMN) and NPB. Furthermore, the cytokinesis-blocked proliferation index (CBPI) was evaluated as an estimation of possible cytotoxic effects. Five hundred cells (250 from each replica) were analysed and classified according to the number of nuclei. CBPI was calculated following the formula CBPI = (MI + 2MII + 3(MIII + MIV)/total, where MI–MIV represent the number of cells with 1–4 nuclei.

2.4. Genotype analysis

Frozen whole-blood EDTA samples (300 μl) were thawed at 37 °C and DNA was extracted using the Puregene™ DNA isolation kit (Gentra Systems, Minneapolis, USA). Polymorphisms in metabolizing genes were evaluated according to Laffon et al. [18] for *CYP1A1* 3′-UTR *1A > *2A, Pérez-Cadahía et al. [19] for *CYP1B1* codon 432 *1 > *3 (amino acid substitution Leu > Val), Laffon et al. [20] for *EPHX1* codon 113 Tyr > His and Laffon et al. [21] for codon 139 His > Arg, Saarikoski et al. [22] for *GSTP1* codon 105 Ile > Val and Laffon et al. [20] for simultaneous detection of *GSTM1* and *GSTT1* deletion polymorphisms.

Polymorphisms in DNA-repair genes were analysed as described in Laffon et al. [16] for *XRCC1* codon 194 Arg > Trp and codon 399 Arg > Gln, and Hu et al. [23] for *XRCC3* codon 241 Thr > Met. *XPD* codon 751 Lys > Gln polymorphism was determined by means of melting-curve analysis after a real-time PCR process using resonance energy-transfer probes. A fragment of 338 bp was amplified with a reac-

Table 1
Characteristics of the study population (V: volunteers, MW: hired manual workers, HPW: hired workers using high-pressure machines)

	Controls	Exposed			
		Total	V	MW	HPW
Total no. of individuals (%)	60 (100)	159 (100)	59 (37.1)	53 (33.3)	47 (29.6)
Sex					
Female (%)	37 (61.7)	78 (49.1)	37 (62.7)	19 (35.8)	22 (46.8)
Male (%)	23 (38.3)	81 (50.9)	22 (37.3)	34 (64.2)	25 (53.2)
Age (years)[a]	23.10 ± 4.83	31.84 ± 11.53	22.98 ± 4.61	37.69 ± 11.34	36.30 ± 10.96
Smoking habits					
Non-smokers (%)	41 (68.3)	97 (61.0)	42 (71.2)	33 (62.3)	22 (46.8)
Smokers (%)	19 (31.7)	62 (39.0)	17 (28.8)	20 (37.7)	25 (53.2)
Pack-years[a]	3.57 ± 4.63	9.11 ± 11.36	2.78 ± 2.45	11.50 ± 14.98	10.45 ± 9.79
Use of protection clothes					
New (%)		35 (22.4)	23 (41.1)	5 (9.4)	7 (14.9)
Reused (%)		51 (32.7)	10 (17.8)	1 (1.9)	40 (85.1)
None (%)		70 (44.9)	23 (41.1)	47 (88.7)	0 (0.0)
Use of mask					
New (%)		55 (35.3)	27 (48.2)	20 (37.7)	8 (17.0)
Reused (%)		59 (37.8)	20 (35.7)	3 (5.7)	36 (76.6)
None (%)		42 (26.9)	9 (16.1)	30 (56.6)	3 (6.4)

[a] Mean ± S.D.

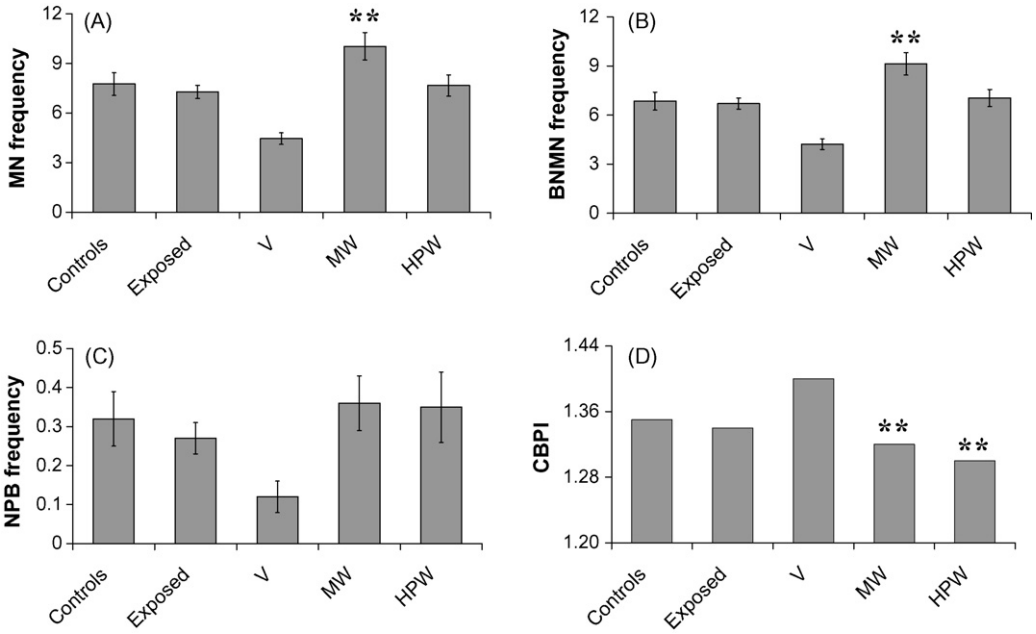

Fig. 1. Results of the cytokinesis-block MN test in the study population: (A) MN frequency, (B) BNMN frequency, (C) NPB frequency, (D) CBPI. $^{**}P \leq 0.01$, significant difference compared with controls.

tion mix composed of 0.4 μM primers (5′-TCT GGA TTA TAC GGA CAT-3′, 5′-GTC ACC TGA CTT CAT AAG A-3′), 0.15 μM hybridization probes (5′-GCC TGG AGC AGC TAG AAT CAG AGG AGA-FL-3′ and 5′-LCR-CTG CAG AGG ATA GAG CAG ATT GC-3′), 30 ng DNA, and 4 μl LightCycler® FastStart Reaction Mix (LightCycler® FastStart DNA Master^{PLUS} HybProbe, Roche, Germany). In every set of reactions a negative control was included. After an initial 10 min 95 °C denaturation step, 45 rounds of amplification were undertaken in a LightCycler® (Roche, Germany) according to the following reaction conditions: 3 s at 95 °C, 12 s at 55 °C and 15 s at 72 °C. Melting curves were recorded from 45 to 80 °C with temperature increase of 0.2 °C/s. PCR and melting procedures were detected online with the LightCycler® instrument. The melting-curve analysis showed a single melting maximum $[-(dF_2/F_1)/dT]$ of 60.5 °C for *XPD*-751^{Lys} allele and of 67 °C for *XPD*-751^{Gln} allele.

2.5. Statistical analysis

Analysis of variance (ANOVA) and Tukey's test were employed to assess the existence of differences among groups. Multifactorial analysis was carried out to evaluate the contribution of potential confounding factors to the response variables considered. The level of statistical significance was set at 0.05, and all analyses were performed using the SPSS for Windows statistical package, version 14.0 (IL, USA).

3. Results

Results obtained for all MN test parameters in the study population classified according to exposure characteristics are shown in Fig. 1. A general increase in the genotoxic damage was observed in the two groups of hired workers but not in the volunteers, although statistical significance compared with controls was only reached for MN and BNMN frequencies in the hired workers who did manual cleaning. Likewise, significant delays in the cell cycle, evaluated by means of CBPI, were detected in both groups of hired workers.

Associations were studied between genotoxic parameters and well-known lifestyle factors that may affect the outcome of cytogenetic assays. When considering sex, age and tobacco consumption separately, a significant effect of the first two was observed on BNMN frequency, after adjusting for exposure (Table 2). Among these, age had the strongest influence, as it was the only factor that remained significant in a further analysis where variables were mutually adjusted, i.e. each one was adjusted for the other two (partial *P*-value 0.010, model *P*-value < 0.001). A significant influence of these factors on NPB frequency was not seen, neither separately nor when mutually adjusted.

Exposed individuals were provided with protective devices (clothes and masks). The effect of the use of these devices was evaluated and, interestingly, no effect was observed for any of them either on BNMN frequency (*P* values 0.726 for reuse of clothes and 0.118 for their non-use, and 0.487 for reuse of masks and 0.326 for their non-use) or on NPB frequency (*P* values 0.277 and 0.836, respectively, for reuse and non-use of protective clothing, and 0.642 and 0.882 for reuse and non-use of masks).

All studied polymorphisms were in Hardy–Weinberg equilibrium, except the *GSTM1* and *GSTT1* deletion polymorphisms, for which no information on allele distribution was available. Analyses

Table 2
Influence of sex, age and smoking habits on the frequencies of binucleated micronucleated cells (BNMN) and nucleoplasmic bridges (NPB)

Model	Unstandardized coefficients β	95% CI	Partial *P* value	R^2	Model *P* value
1. BNMN					
Males vs. females	−1.120	−2.19 to −0.05	0.041	0.172	<0.001
Age (years)	0.085	0.02 to 0.15	0.011	0.179	<0.001
Smoking habits (pack-year)	−0.014	−0.09 to 0.07	0.733	0.155	<0.001
2. NPB					
Males vs. females	−0.038	−0.18 to 0.10	0.592	0.021	0.074
Age (years)	−0.002	−0.01 to 0.01	0.684	0.012	0.168
Smoking habits (pack-year)	−0.008	−0.02 to 0.00	0.142	0.035	0.026

Models are adjusted for exposure.

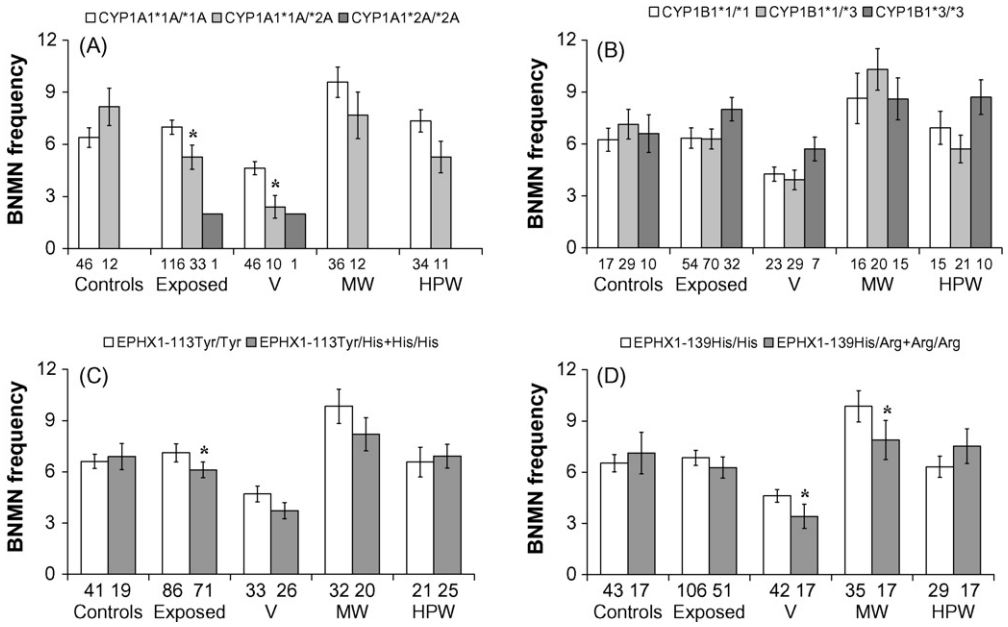

Fig. 2. Effect of genetic polymorphisms in *CYP1A1* 3′-UTR (A), *CYP1B1* codon 432 (B), *EPHX1* codon 113 (C) and *EPHX1* codon 139 (D) on BNMN frequency. The number of individuals in the groups is indicated under each bar. $^{*}P \leq 0.05$, significant difference compared with controls.

of variance were performed to assess the most suitable heredity model for each gene, in order to establish the genotypic level categories, as no defined heredity models were available in the literature, except for *EPHX1*, which is being referred to as dominant [24].

Data on the effect on BNMN frequency of genetic polymorphisms in the main enzymes participating in the metabolism of oil components are summarized in Fig. 2 for cytochrome P450 (*CYP*) and *EPHX1* genes and in Table 3 for glutathione *S*-transferases (*GST*). Due to technical problems, there are some missing values in Fig. 2 and Tables 3 and 4. The decrease in BNMN frequency was related to the presence of the *CYP1A1*^{*2A} variant allele in all exposed groups, although statistical significance was only reached in the volunteer group and in the total exposed population. In contrast, no effect could be observed when considering the allele *CYP1B1*^{*3}. General reduction in damage levels was also associated with the presence of the *EPHX1* variant alleles in both codons 113 and 139 in the exposed populations, although statistical significance was not

Table 3
Influence of *GST* polymorphisms on binucleated micronucleated cells (BNMN) frequency (mean ± S.E.)

Genotype	Controls	Exposed	V	MW	HPW
GSTP1$^{Ala/Ala}$	7.6 ± 0.7 (30)	7.5 ± 0.6 (75)	4.2 ± 0.5 (25)	10.5 ± 1.1 (27)	7.3 ± 0.8 (23)
GSTP1$^{Ala/Val+Val/Val}$	6.0 ± 0.8 (25)	5.8 ± 0.4[a] (74)	4.6 ± 0.5 (32)	7.2 ± 0.8[a] (21)	6.2 ± 0.7 (23)
GSTM1 positive	7.4 ± 0.8 (33)	6.7 ± 0.5 (81)	4.6 ± 0.4 (38)	9.4 ± 1.0 (23)	7.4 ± 0.9 (20)
GSTM1 null	5.8 ± 0.6[a] (27)	6.6 ± 0.6 (76)	3.7 ± 0.6 (21)	9.0 ± 1.0 (29)	6.3 ± 0.7 (26)
GSTT1 positive	6.8 ± 0.5 (58)	6.7 ± 0.4 (142)	4.2 ± 0.3 (53)	9.4 ± 0.8 (49)	6.8 ± 0.6 (40)
GSTT1 null	4.0 ± 3.0 (2)	5.8 ± 1.0 (15)	4.8 ± 1.8 (6)	6.3 ± 1.8 (3)	6.5 ± 1.7 (6)

N is indicated between brackets.
[a] $P \leq 0.01$, significant difference compared with the wild-type homozygous or positive genotype.

Table 4
Influence of polymorphisms in DNA-repair genes on binucleated micronucleated cells (BNMN) frequency (mean ± S.E.)

Genotype	Controls	Exposed	V	MW	HPW
XRCC1-194$^{Arg/Arg}$	7.0 ± 0.6 (49)	6.4 ± 0.4 (115)	4.2 ± 0.4 (47)	8.6 ± 0.8 (35)	6.9 ± 0.6 (36)
XRCC1-194$^{Arg/Trp+Trp/Trp}$	5.2 ± 1.5 (6)	7.4 ± 0.9 (31)	3.8 ± 0.7 (9)	10.5 ± 1.6 (13)	6.4 ± 1.0 (9)
XRCC1-399$^{Arg/Arg}$	6.6 ± 0.7 (34)	6.6 ± 0.5 (80)	4.3 ± 0.5 (31)	8.5 ± 0.9 (29)	7.6 ± 0.9 (20)
XRCC1-399$^{Arg/Gln+Gln/Gln}$	7.1 ± 0.9 (21)	6.5 ± 0.5 (70)	4.0 ± 0.5 (26)	10.1 ± 1.2 (19)	6.3 ± 0.7 (25)
XRCC3-241$^{Thr/Thr}$	7.0 ± 0.9 (23)	5.9 ± 0.4 (74)	4.0 ± 0.4 (25)	7.5 ± 0.9 (24)	6.4 ± 0.7 (25)
XRCC3-241$^{Thr/Met}$	6.9 ± 0.8 (25)	7.3 ± 0.6[a] (67)	4.4 ± 0.6 (27)	9.9 ± 1.1[a] (23)	8.1 ± 1.0 (17)
XRCC3-241$^{Met/Met}$	4.6 ± 1.1 (5)	6.8 ± 1.8 (12)	4.7 ± 1.0 (7)	19.0 ± 4.0[b] (2)	3.7 ± 0.9 (3)
XPD-751$^{Lys/Lys}$	7.1 ± 0.6 (28)	6.4 ± 0.5 (70)	4.7 ± 0.5 (22)	8.2 ± 1.0 (22)	6.4 ± 0.7 (26)
XPD-751$^{Lys/Gln}$	5.7 ± 0.8 (18)	6.6 ± 0.6 (64)	4.0 ± 0.5 (26)	9.4 ± 1.2[b] (20)	7.4 ± 0.9 (18)
XPD-751$^{Gln/Gln}$	8.1 ± 2.2 (9)	7.8 ± 1.3 (21)	4.5 ± 0.9 (10)	11.6 ± 2.1[b] (9)	5.5 ± 2.5 (2)

N is indicated between brackets.
[a] $P \leq 0.05$, significant difference compared with the wild-type homozygous genotype.
[b] $P \leq 0.01$.

always achieved. Lower BNMN frequencies were related to the presence of the *GSTP1*-105^{Val} allele in all groups except the volunteers. Similar reductions in damage levels associated with the absence of *GSTM1* or *GSTT1* activity were detected in all cases, both in controls and in the exposed groups, but the differences were generally not large enough to be statistically significant.

Table 4 shows the results on the effect of polymorphisms in several DNA-repair genes on the genetic damage levels. No significant influence was observed for either of the *XRCC1* polymorphic sites analysed. In contrast, a clear and significant increase in BNMN frequency was obtained related to the presence of *XRCC3*-241^{Met} variant allele in heterozygosis in the exposed population, with a higher impact in the individuals who did manual cleaning. Similarly, the presence of the *XPD*-751^{Gln} variant allele was generally associated with increased BNMN rates, although significant differences were only obtained in the manual cleaner group. The NPB frequency was not influenced by any of these metabolic or DNA-repair polymorphisms.

4. Discussion

Significant association between human exposure to oil during clean-up operations and cytogenetic damage was found in the present study. Higher BNMN frequencies were obtained in both groups of hired workers, although statistical significance was only reached in the manual cleaners. This suggests that a relatively prolonged exposure time was required for this cytogenetic damage to become obvious. Nevertheless, the decrease in cytogenetic frequencies observed in volunteers is rather surprising. It may be somewhat related to the lower average age of the individuals and/or the lower percentage of smokers in this group, since these factors may influence MN test results, as thoroughly discussed below. Similar data were obtained for NPB frequency parameter, although in this case the effect was much weaker and statistical significance in the manual cleaners was not reached.

The MN test reveals both aneugenic and clastogenic types of damage. Thomas et al. [25] proposed the relation NPB/MN as an index to determine the type of damage, in such a way that if the coefficient is close to 0 the effect would be aneugenic, while the index for clastogens would be around 0.77. The values obtained in this study are 0.03, 0.03 and 0.04 for the groups of volunteers, the manual cleaners and the group involved in mechanical cleaning, respectively. This would indicate that the oil from the *Prestige* contained essentially a mainly aneugenic mixture of agents.

The increase of cytogenetic damage in the exposed population was already indicated in our previous study where a pilot population was analysed in terms of genotoxicity and where two different phenomena emerged [7]. On the one hand, although all exposed groups showed an increase in DNA strand breaks (assessed by the comet assay) compared with the controls, this event became much more accentuated in the volunteer group, comprising individuals whose environmental dosimetry of volatile organic compounds (VOC) was highest, and who, therefore, experienced an intense short-term exposure. On the other hand, the cytogenetic biomarkers showed only slight effects in the long-term exposed groups. This could agree with the fact that the comet assay is more related to exposure and does not represent a true biomarker of effect but a biomarker of exposure [26]. It would also be in line with adaptive response theory, nowadays well accepted, by which subjects shortly exposed to oil would be more susceptible in the first instance than longer exposed individuals [27–29]. According to these data, it seems that exposure to oil gave rise to strand breaks almost immediately after it started, but a longer time period was required to induce stable cytogenetic alterations.

Based on current knowledge, it could be anticipated that agents that induce genome damage are also likely to cause cell-cycle delay [30]. This fact could be observed in the response of CBPI to exposure, revealing a decrease in lymphocytes from the hired workers, i.e. the two groups with longer exposures. This suggests that a relatively long period of exposure to the oil is required both for the genotoxic and the cytotoxic effects to become obvious.

A major problem affecting the design of studies in molecular epidemiology is the uncertainty about the impact of confounding factors. Given that some lifestyle factors are widely considered to influence the events evaluated in our study, we have conducted a specific analysis considering three of the most relevant ones: sex, age and tobacco consumption. BNMN frequency alterations were significantly associated with the first two when setting up independent models, but only age remained significant when they were mutually adjusted, agreeing with previous data presented by the HUman MicroNucleus (HUMN) international project [31]. The increase of cytogenetic damage with age is in accordance with the rise in background levels of DNA damage, the loss of efficiency in the repair processes and the subsequent accumulation of errors related to the ageing process. It also fits with the reduction in defence mechanisms associated with generation of reactive oxygen species (ROS) and the reduced levels of agents participating in detoxification pathways, such as glutathione [32].

Although tobacco smoke contains a great number of potent human carcinogens and smoking is a well-established determining factor in cancer development, no influence of this factor was observed on the levels of cytogenetic damage. This supports the findings of our previous study [7], indicating that the absence of effect was not a result of a reduced statistical power. Moreover, results from the HUMN project showed that only heavy smokers (>30 cigarettes/day) exhibited a significant increase in MN frequency [29], and individuals included in our study were all light smokers (mean, 13.04 ± 8.85 cigarettes/day).

Unexpectedly, the use of protective clothing or masks by the exposed population did not influence the genotoxic damage levels. Two possible reasons may explain these surprising results: the unsuitable characteristics of the protective devices or their incorrect use by the individuals.

This is the first study on genotoxicity associated with exposure to spilled oil that comprises a set of genetic polymorphisms, both in metabolizing enzymes and in DNA-repair enzymes. Significant reductions in damage levels were found to be associated with the presence of the variant alleles *CYP1A1*^{*2A} and *EPHX1*-139^{Arg}. This could be related to higher activities of the variant enzymes [33] and, as a consequence, the increased detoxification efficacy of the route. The protective effect was especially observed in the group of volunteers, pointing again to an adaptive response of the wild-type homozygous hired workers, who were exposed for much longer periods. Some further influence was detected for *EPHX1* codon 113 polymorphism, but just when taking the entire exposed population together. Since the effects of all these genetic polymorphisms were detected in the exposed groups but not in the control population, they probably come from exposure–genotype interactions [34].

Regarding the *GST* genes, a slight influence on cytogenetic damage levels was seen from the results of sister-chromatid exchange (SCE) in 120 individuals exposed to *Prestige* oil [8]. In the present study, the enlargement of the population further supported these initial results. Because these effects were observed in all groups, both control and exposed, they are probably due to genotype effects on baseline levels of these cytogenetic biomarkers [34]. Moreover, the results of a previous pooled analysis indicated that *GSTT1*-null subjects had lower micronucleus frequencies than their positive

due to the combined activity of all GST enzymes [36] may help explain the higher damage levels shown by positive individu-als, since it could make it even more difficult for these detoxification pathways to run properly.

Three of the most relevant and widely studied polymorphic genes in DNA repair were included in this study. The possible influ-ence of their polymorphisms in the development of several types of cancer was previously reviewed [37,38]. The two *XRCC1* poly-morphic sites (codons 194 and 399) were not observed to have any significant influence on the level of cytogenetic damage. More pronounced effects could be assigned to *XRCC3* and *XPD* poly-morphisms, both variant alleles led to decreases in the enzyme activity [39]. The XRCC3 protein is involved in the general main-tenance of chromosome stability in mammalian cells [40], and XPD participates in the removal of DNA adducts, a general con-sequence of PAH exposure [41] in the nucleotide-excision-repair pathway. General increases in BNMN were associated with the presence of the variant alleles, reaching statistical significance in the manual cleaning group, indicating that these polymor-phisms must be fundamentally taken into account in chronic exposures. This effect was not significant in individuals who did mechanical cleaning, probably due to the obvious differences in their exposure conditions determined by the use of high-pressure machines.

Data obtained in this work showed cytogenetic effects related to exposure to oil from the tanker *Prestige*, influenced by age and by some genetic polymorphisms in metabolizing and DNA-repair enzymes. In view of these results, it seems essential to pay more attention to the human health effects of exposure to oil after – unfortunately too frequent – accidental spills, and focus new stud-ies on such a relevant but overlooked aspect of public health, which involves a large number of people all over the world. If similar effects would support those reported here, a major urgent topic would be to develop a more optimal international protocol-of-action, to ensure the safety of individuals involved in the recovery and cleaning tasks.

Conflict of interest

All authors disclose any actual or potential conflict of interest.

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References

[1] IARC, Occupational exposures in petroleum refining: crude oil and major petroleum fuels, IARC monographs on the evaluation of carcinogenic risk to humans, vol. 45, International Agency for Research on Cancer, Lyon, 1989.
[2] CSIC (Centro Superior de Investigaciones Científicas), Characterization of the spill and preliminary evolution of in the environment, CSIC technical report No. 01 (in Spanish), 2003, http://csicprestige.iim.csic.es/desarro/informcsic/1/index.htm.
[3] J.E. Hose, M.D. McGurk, G.D. Marty, E.D. Biggs, T.T. Baker, Sublethal effects of the Exxon Valdez oil spill on herring embryos and larvae: morphologic, cytogenetic, and histopathological assessments, 1989–1991, Can. J. Fish. Aquat. Sci. 53 (1996) 2355–2365.
[4] S.A. Steinert, R. Streib-Montee, J.M. Leather, D.B. Chadwick, DNA damage in mussels at sites in San Diego Bay, Mutat. Res. 399 (1998) 65–85.
[5] M. Carls, G. Marty, J. Hose, Synthesis of the toxicological impacts of the Exxon Valdez oil spill on Pacific herring (Clupea pallasi) in Prince William Sound, Alaska, U.S.A, Can. J. Fish. Aquat. Sci. 59 (2002) 153–172.
[6] B. Laffon, T. Rábade, E. Pásaro, J. Méndez, Monitoring of the impact of Prestige oil spill on *Mytilus galloprovincialis* from Galician coast, Environ. Int. 32 (2006) 342–348.
[7] B. Pérez-Cadahía, B. Laffon, E. Pásaro, J. Méndez, Genetic damage induced by accidental environmental pollutants, TheScientificWorldJOURNAL 6 (2006) 1221–1237.
[8] B. Pérez-Cadahía, A. Lafuente, T. Cabaleiro, E. Pásaro, J. Méndez, B. Laffon, Initial study on the effects of Prestige oil on human health, Environ. Int. 33 (2007) 176–185.
[9] M. Fenech, Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death, Mutat. Res. 600 (2006) 58–66.
[10] S. Bonassi, A. Znaor, M. Ceppi, C. Lando, W.P. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, M.P. Bigatti, C. Bolognesi, A. Cebulska-Wasilewska, E. Fabianova, A. Fucic, L. Hagmar, G. Joksic, A. Martelli, L. Migliore, E. Mirkova, M.R. Scarfi, A. Zijno, H. Norppa, M. Fenech, An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans, Carcinogenesis 28 (2007) 625–631.
[11] K. Umegaki, M. Fenech, Cytokinesis-block micronucleus assay in WIL2-NS cells: a sensitive system to detect chromosomal damage induced by reactive oxygen species and activated human neutrophils, Mutagenesis 15 (2000) 261–269.
[12] L.H. Thompson, M.G. West, XRCC1 keeps DNA from getting stranded, Mutat. Res. 459 (2000) 1–18.
[13] M.A. Brenneman, A.E. Weiss, J.A. Nickoloff, D.J. Chen, XRCC3 is required for efficient repair of chromosome breaks by homologous recombination, Mutat. Res. 459 (2000) 89–97.
[14] M. O'Driscoll, P.A. Jeggo, The role of double-strand break repair—insights from human genetics, Nat. Rev. Genet. 7 (2006) 45–54.
[15] E. Evans, J.G. Moggs, J.R. Hwang, J.M. Egly, R.D. Wood, Mechanism of open complex and dual incision formation by human nucleotide excision repair factors, EMBO J. 16 (1997) 6559–6573.
[16] B. Laffon, R. Fraga-Iriso, B. Pérez-Cadahía, J. Méndez, Genotoxicity associated to exposure to Prestige oil during autopsies and cleaning of oil-contaminated birds, Food Chem. Toxicol. 44 (2006) 1714–1723.
[17] M. Fenech, W.P. Chan, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, Mutat. Res. 534 (2003) 65–75.
[18] B. Laffon, B. Pérez-Cadahía, E. Pásaro, J. Méndez, Individual sensitivity to DNA damage induced by styrene in vitro: influence of cytochrome P450, epoxide hydrolase and glutathione S-transferase genotypes, Toxicology 186 (2003) 131–141.
[19] B. Pérez-Cadahía, E. Pásaro, J. Méndez, V. Valdiglesias, B. Laffon, Genetic polymorphism in cytochrome P450 1B1 in a Spanish population, Basic Clin. Pharmacol. Toxicol. 101 (2007) 70–72.
[20] B. Laffon, B. Pérez-Cadahía, E. Pásaro, J. Méndez, Effect of epoxide hydrolase and glutathione S-transferase genotypes on the induction of micronuclei and DNA damage by styrene-7,8-oxide in vitro, Mutat. Res. 536 (2003) 49–59.
[21] B. Laffon, J.P. Teixeira, S. Silva, J. Roma-Torres, B. Pérez-Cadahía, J. Méndez, E. Pásaro, O. Mayan, Assessment of occupational genotoxic risk in the production of rubber tyres, Ann. Occup. Hyg. 50 (2006) 583–592.
[22] S.T. Saarikoski, A. Voho, M. Reinkainen, S. Anttila, A. Karjalainen, C. Malaveille, H. Vainio, K. Husgafvel-Pursiainen, A. Hirvonen, Combined effect of polymorphic GST genes on individual susceptibility to lung cancer, Int. J. Cancer 77 (1998) 516–521.
[23] J.J. Hu, T.R. Smith, M.S. Miller, H.W. Mohrenweiser, A. Golden, L.D. Case, Amino acid substitution variants of APE1 and XRCC1 genes associated with ionizing radiation sensitivity, Carcinogenesis 22 (2001) 917–922.
[24] E.S. Vesell, Pharmacogenetics: multiple interactions between genes and environment as determinants of drug response, Am. J. Med. 66 (1979) 183–187.
[25] P. Thomas, K. Umegaki, M. Fenech, Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay, Mutagenesis 18 (2003) 187–194.
[26] L. Migliore, A. Naccarati, F. Coppedè, E. Bergamaschi, G. De Palma, A. Voho, P. Manini, H. Järventaus, A. Mutti, H. Norppa, A. Hirvonen, Cytogenetic biomarkers, urinary metabolites and metabolic gene polymorphisms in workers exposed to styrene, Pharmacogenet. Genomics 16 (2006) 87–99.
[27] H. Gourabi, H. Mozdarani, A cytokinesis-blocked micronucleus study of the radioadaptive response of lymphocytes of individuals occupationally exposed to chronic doses of radiation, Mutagenesis 13 (1998) 475–480.
[28] A. Rothfuss, C. Dennog, G. Speit, Adaptive protection against the induction of oxidative DNA damage after hyperbaric oxygen treatment, Carcinogenesis 19 (1998) 1913–1917.
[29] S. Bonassi, M. Neri, C. Lando, M. Ceppi, Y. Lin, W.P. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, M. Fenech, Effect of smoking habit on the frequency of micronuclei in human lymphocytes: results from the HUman MicroNucleus project, Mutat. Res. 543 (2003) 155–166.
[30] M. Fenech, A mathematical model of the in vitro micronucleus assay predicts false negative results if micronuclei are not specifically scored in binucleated cells or in cells that have completed one nuclear division, Mutagenesis 15 (2000) 329–336.
[31] S. Bonassi, M. Fenech, C. Lando, Y. Lin, M. Ceppi, W.P. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, L.R. Ferguson, A. Fucic, O.G. Lima,

P. Hrelia, A.P. Krishnaja, T.-K. Lee, L. Migliore, L. Mikhalevich, E. Mirkova, P. Mosesso, W.-U. Müller, Y. Odagiri, M.R. Scarfi, E. Szabova, I. Vorobtsova, A. Vral, A. Zijno, HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei, Environ. Mol. Mutagen. 37 (2001) 31–45.
[32] B. Pesch, R. Düsing, S. Rabstein, V. Harth, D. Grentrup, T. Brüning, O. Landt, H. Vetter, Y.-D. Ko, Polymorphic metabolic susceptibility genes and longevity: a study in octogenarians, Toxicol. Lett. 151 (2004) 283–290.
[33] S. Pavanello, E. Clonfero, Biological indicators of genotoxic risk and metabolic polymorphisms, Mutat. Res. 463 (2000) 285–308.
[34] H. Norppa, Cytogenetic biomarkers and genetic polymorphisms, Toxicol. Lett. 149 (2004) 309–334.
[35] M. Kirsch-Volders, R.A. Mateuca, M. Roelants, A. Tremp, E. Zeiger, S. Bonassi, N. Holland, W.P. Chang, P.V. Aka, M. DeBoeck, L. Godderis, V. Haufroid, H. Ishikawa, B. Laffon, R. Marcos, L. Migliore, H. Norppa, J.P. Teixeira, A. Zijno, M. Fenech, The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo, Cancer Epidemiol. Biomarkers Prev. 15 (2006) 1038–1042.
[36] F.F. Parl, Glutathione S-transferase genotypes and cancer risk, Cancer Lett. 221 (2005) 123–129.
[37] E.L. Goode, C.M. Ulrich, J.D. Potter, Polymorphisms in DNA repair genes and associations with cancer risk, Cancer Epidemiol. Biomarkers Prev. 11 (2002) 1513–1530.
[38] S. Benhamou, A. Sarasin, ERCC2/XPD gene polymorphisms and cancer risk, Mutagenesis 17 (2002) 463–469.
[39] S. Pavanello, Metabolic and DNA repair variations in susceptibility to genotoxins, Polycyclic Aromatic Compounds 23 (2003) 49–107.
[40] X. Cui, M. Brenneman, J. Meyne, B. Oshimura, E.H. Goodwin, D.J. Chen, The XRCC2 and XRCC3 repair genes are required for chromosome stability in mammalian cells, Mutat. Res. 434 (1999) 75–88.
[41] Q. Wei, L. Cheng, C.I. Amos, L.-E. Wang, Z. Guo, W.K. Hong, M.R. Spitz, Repair of tobacco carcinogen-induced DNA adducts and lung cancer risk: a molecular epidemiologic study, J. Natl. Cancer Inst. 92 (2000) 1764–1772.