

Article

# Winter Air Pollution and Genotoxic Effects in Children Living in a Highly Polluted Urban Area

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**Citation:** Zani, C.; Ceretti, E.; Feretti, D.; Villarini, M.; Moretti, M.; Verani, M.; De Donno, A.; Bonetta, S.; Buschini, A.; Bonetti, A.; et al. Winter Air Pollution and Genotoxic Effects in Children Living in a Highly Polluted Urban Area. *Atmosphere* **2021**, *12*, 1191. <https://doi.org/10.3390/atmos12091191>

Academic Editors: Azhar Siddique, Mirza M. Hussain and Haider A. Khwaja

Received: 26 July 2021

Accepted: 13 September 2021

Published: 15 September 2021

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**Abstract:** Air pollutants, especially PM, have been found to determine various effects on human health, including genotoxic effects. The aim of this study was to assess DNA damage with micronuclei (MN) and comet tests on buccal cells of 6–8 years old children living in an area with high air pollution. Both tests were repeated in the same children in two consecutive winters to compare the levels of DNA damage under different pollution conditions. A complete data set including lifestyle, air pollutants levels and biological sampling was available for 180 children in the two winters. A high mean MN frequency was found in both seasons, with higher value in the first ( $0.51 \pm 0.59$ ) than the second winter ( $0.40 \pm 0.52$ ), whereas DNA damage measured with comet test showed higher damage in the second versus the first winter (visual score  $208.8 \pm 67.1$  vs.  $173.2 \pm 50.8$ ). The associations between air pollutant levels (CO, NO<sub>2</sub>, SO<sub>2</sub>, benzene, O<sub>3</sub>, PM<sub>10</sub> and PM<sub>2.5</sub>; PM<sub>0.5</sub> and PAHs) and DNA damage were investigated at different lag times, and mainly, no significant association was found. This study on repeated measure of MN frequency and DNA damage in children's buccal did not show an association with various air pollutants evaluated in an area with high levels of air pollution.

**Keywords:** air pollution; children; early biological effects; micronuclei; DNA damage

## 1. Introduction

Air pollution in urban areas is one of the major environmental problems in both industrialised and developing countries, and the International Agency for Research on Cancer (IARC) has classified it as a human carcinogen [1]. The European Environment

Agency has assessed the impact that air pollution has on the general population, as well as on susceptible population groups and on the environment, both as a whole and as a single pollutant [2,3]. It has also been highlighted that a large percentage of the European population and ecosystems are still exposed to air pollution in exceedance of European standards and WHO Air Quality Guidelines [4].

Urban pollution is mainly caused by traffic, and this situation is common to all cities and in all seasons. Air pollution is rich in particulate matter (PM) of different particle sizes, PAHs, NO<sub>2</sub>, O<sub>3</sub>, SO<sub>2</sub>, and benzene, and it is highly variable and complex mixture of particles and gases emitted directly from sources (primary pollutants) or formed in the atmosphere by gaseous emissions (secondary pollutants) [5–11].

There are numerous studies linking air pollution, and especially PM, to various effects on human health beyond the well-known cardiovascular and respiratory diseases and lung cancer [1,12]. Furthermore, many studies have shown that there is a genotoxic activity of air pollution, found with tests focused on different genetic endpoints in various organisms [13–22]. Genetic tests are also particularly useful for an early assessment of population exposure to substances that are hazardous to health, and they make it possible to identify more sensitive population groups [23].

The main tests for measuring DNA damage in human biomonitoring are the micronucleus and comet assays in human lymphocytes from peripheral blood samples; the micronucleus test can provide information on permanent damage in the cell, while the comet test can provide information on recent exposures to genotoxic substances, and part of the damage can still be repaired [24,25].

Human biomonitoring tests can be conducted on different cells and, as of lately, the use of buccal cells (directly exposed to pollutants) may be a viable alternative to blood cells, as the sampling is less invasive and better accepted by enrolled subjects [26,27].

Most studies have compared DNA damage levels with pollution levels, but few studies assess whether cell damage is maintained in subjects continuously exposed to high levels of pollution, especially in the most susceptible populations. In the last few years, many studies were conducted on genotoxic effects in children, as they are a high-risk group in regards to the early and long-term health effects derived from air pollution exposure. Several studies suggest that early exposure during childhood can play an important role in the development of chronic diseases in adulthood [28].

The aim of this study is to assess, in a group of 6–8 years old children living in an area with high levels of pollution, the DNA damage with MN and comet tests on buccal cells, repeating the tests in two consecutive winters to verify the levels of damage under different pollution conditions.

## 2. Material and Methods

### 2.1. Study Design

This study was part of the MAPEC project (“Monitoring Air Pollution Effects on Children for Supporting Public Health Policy” funded by the European Life+Programme (LIFE12 ENV/IT/000614), a cohort study that aimed to evaluate early biological effects of air pollution on children [29]. The markers of early biological effect were primary DNA damage evaluated with the comet assay and the presence of micronuclei (MN) in buccal cells taken from 6–8 years old children living in Brescia (Northern Italy). This study ran for two consecutive winters and biological and air sampling was repeated in each season in order to evaluate the intra-individual variability of biomarkers of early biological effects.

The choice of the schools was based on the school director’s willingness to participate in the study. The schools 1, 2 and 3 are located near factories in the south of the city, whereas schools 4 and 5 are located in a residential area north of the city. We chose the first, second and third classes (6, 7 and 8 years old, respectively) in the first winter season because the children had to be followed for two consecutive years. For each school class,

children's enrolment was performed after a public meeting with parents and teachers. A total of 283 children were enrolled from the schools.

Children with severe diseases and those who had been exposed to antineoplastic agents, had undergone radiation therapy or X-rays in the previous 12 months, or had a dental brace were excluded.

DNA damage was investigated in saliva leukocytes taken from sputum. To collect buccal cells (leukocytes) for the comet assay, the children rinsed their mouths twice with water and the mouthwashes and sputum were collected in tubes containing 25 mL of saline solution (NaCl 0.9%).

Small-headed toothbrushes were used to collect exfoliated buccal cells for the micronucleus test by gently scraping the inside of both cheeks. The head of the toothbrush was then dipped into tubes containing 15 mL of Saccomanno's fixative (50% ethanol, 2% polyethylene glycol, *v/v*; solution diluted in water and stored).

To characterize air pollution, ultra-fine particulate matter (PM<sub>0.5</sub>) was collected. A high-volume air sampler was located near the schools for 72 h during the days of biological sampling. A chemical analysis of PM<sub>0.5</sub> extracts collected in each season was performed for the determination of polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs using high pressure liquid chromatography (HPLC).

Furthermore, the data on the concentrations of routinely evaluated air pollutants (CO, NO<sub>2</sub>, SO<sub>2</sub>, benzene, O<sub>3</sub>, PM<sub>10</sub>, and PM<sub>2.5</sub>) were retrieved from the website of the Regional Agency for Environmental Protection (ARPA).

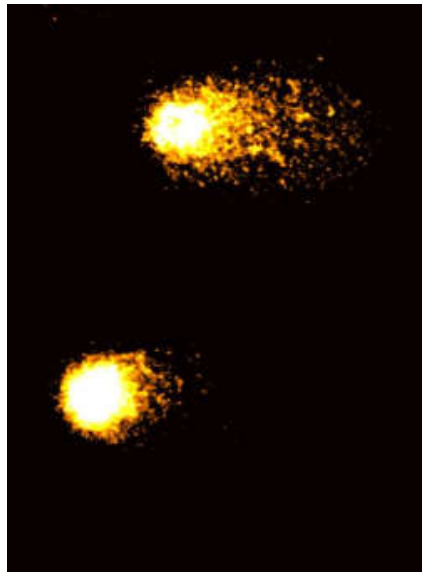
Other indoor and outdoor exposures were investigated using an ad hoc questionnaire administered to the children's parents [30]. The questionnaire included questions on demographic and socioeconomic variables, children's lifestyle, physical activity and diet. On the basis of daily intake of various foods, we also calculated the Italian Mediterranean Index (IMI) score as suggested by Agnoli et al. [31] to compute adherence to the Mediterranean diet (MD), categorized as low ( $\leq 3$ ), medium (4–5) or high ( $\geq 6$ ) [32].

## 2.2. Comet Assay in Salivary Leukocytes

For the evaluation of the primary DNA damage in salivary leukocytes, the comet assay was performed in alkaline conditions (pH > 13) to detect single- and double-strand breaks and alkali labile sites. For the first winter, a high-throughput approach was applied to the comet assay by using 12-gel units. However, based on the analysis made on the first sampling period's slides, in the second winter, the comet assay was performed with little protocol modifications and two-spot slides were used to perform the test. Cell suspension in saline solution was centrifuged for 10 min at 1100 g (at 4 °C), and the pellet was re-suspended in 10 mL of PBS. Cell suspension was gently centrifuged for 10 min at 45 g. Afterward, the supernatant (in which leukocytes were still suspended) was collected in a second tube, centrifuged for 10 min at 1100 g, and the pellet was re-suspended in 1 mL of PBS. Leukocyte viability was determined using the trypan blue exclusion technique. Epithelial buccal cells also present in the mouthwash samples were not considered. The comet assay was performed on salivary leukocytes, according to the methods of Singh et al. [33] on white blood cells, with minor modifications. PBS cell suspension was centrifuged for 4 min at 8700 g, and the pellet was re-suspended in an appropriate volume of LMA (low melting point agarose, 0.7%) and layered onto pretreated (NMA, normal melting point agarose, 1%) slides: with the high-throughput approach, 10  $\mu$ L of cell-LMA suspension were placed into each spot of the 12-gel slide; with the standard approach, two 20  $\mu$ L drops were layered on a single slide. After overnight lysis (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 8 mM TrisHCl, 10% DMSO, 1% Tryton X-100, 4 °C, pH 10) of cellular and nuclear membranes, the slides were placed in a horizontal electrophoresis box and allowed to unwind for 20 min in an electrophoretic alkaline buffer (10 mM Na<sub>4</sub>EDTA, 300 mM NaOH, 10% DMSO, pH > 13) and then subjected to electrophoresis (4 °C) for 20 min in the same buffer by applying an electric field of 1 V/cm and adjusting the current to 300

mA. Lastly, the microgels were neutralized (0.4 M TrisHCl, pH 7.5) and fixed with absolute ethanol ( $-20\text{ }^{\circ}\text{C}$ ). For each electrophoresis, two slides prepared with A549 cells with EMS (2 mM) for 1 h at  $37\text{ }^{\circ}\text{C}$  were added in the tank as internal control.

To evaluate DNA damage, the slides were stained with ethidium bromide ( $10\text{ }\mu\text{g}/\text{mL}$ ) and examined using a fluorescence Leica DMLS microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a high-sensitivity CCD (charge-coupled device) camera connected to a computerized image analysis system (Figure 1) (Comet Assay IV, Perceptive Instruments, Staffordshire, UK).



**Figure 1.** Comet test: image of damaged and undamaged salivary leukocytes observed through fluorescence microscopy.

The analysis of comet slides of the first campaign, made by semi-automatic modality, encountered difficulties: many slides appeared at microscopic analysis without nucleoids, including if the cell count made before preparing the slides showed a correct number of cells. Little modifications were performed to the sampling protocols during the first campaign in order to reduce the problem, but at the end of the sampling period, the analysis of the results was unsatisfactory. The semi-automatic analysis, chosen for the objectivity of the measure, was probably not the best type of analysis for this type of samples. Most of the slides presented fluorescent inclusions that produced confounding effects on the semi-automatic analysis. Based on these results, the reading modality changed for the subsequent evaluations, switching to a manual measure of the DNA migration, and then the visual score, a parameter derived from visual classification of the comets into four different damage classes, was used as a DNA damage measure.

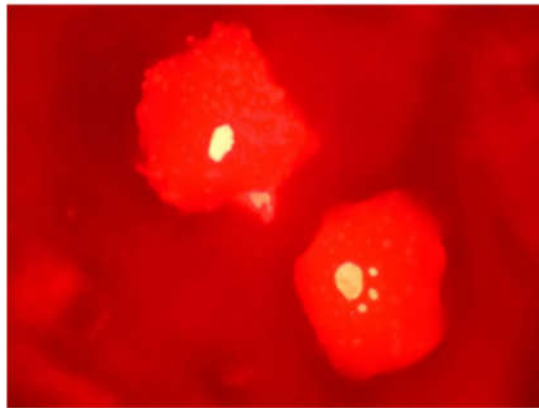
### 2.3. Micronucleus Test in Buccal Cells

The epithelial buccal cells collected were centrifuged in Saccomanno's fixative and washed twice with PBS. Cell suspensions were then drawn into a syringe using an 18 G needle, filtered through a  $100\text{ }\mu\text{m}$  nylon filter, and centrifuged again. Cell pellets were resuspended in ice-cold PBS, aliquots ( $10\text{ }\mu\text{L}$ ) of cell suspensions were diluted 1:1 with a 0.4% Trypan Blue solution, and cell count was performed using a hemocytometer or a Countess automated cell counter (Invitrogen Srl, Milan, Italy). Buccal cells were then fixed with ice-cold Carnoy's fixative (methanol and glacial acetic acid 3:1).

The MN assay was performed according to the procedure described by Thomas and Fenech [34], with minor modifications. For each subject, two slides were prepared by smearing  $100\text{ }\mu\text{L}$  of cell suspension onto precleaned slides (approximately  $1 \times 10^5$

cells/slide). The cells were then stained by applying the Feulgen plus Light Green method. Briefly, the slides were fixed in ethanol, 1 min each in 50% and 20% ethanol, washed with distilled water and treated in 5 M HCl for 30 min. After washing in distilled water, the slides were drained and then stained with Schiff's reagent for 60 min. The slides were further washed with distilled water and then stained with 0.2% Light Green for 20 s. Air dried slides were finally mounted with DePex mounting medium.

The slides were scored initially to determine the frequency of the various cell types in a minimum of 1000 cells. Cell types, anomalies associated with cell death, and nuclear abnormalities indicative of chromosomal instability or DNA damage were classified according to established criteria [35]. The slides were then scored for cells with MN at least 2000 differentiated cells (1000/slide) as measures of DNA damage (Figure 2). The tests were performed in duplicate and the results expressed as the mean of two tests.



**Figure 2.** MN test: image of buccal mucosa cells with and without MN observed through fluorescence microscopy.

#### 2.4. Statistical Analysis

Median, mean and standard deviation (SD) count and percentage were reported for the continuous and categorical variables, respectively.

Due to asymmetric non-normal distribution of the MN count and visual score of comet test, a comparison between the distributions of these variables among groups was made using the non-parametric Kruskal–Wallis test. A comparison between the values observed in the same children in two seasons was assessed using the Wilcoxon test for paired data.

The associations between levels of air pollutants and early effect biomarkers were assessed using two regression models. Univariate and multivariate analyses were performed to assess the associations between MN frequency and air pollutants considering all the variables of interest and possible confounding factors.

The MN count was considered as the response variable in hierarchical mixed-effect Poisson regression models. Demographic, socio-economic and lifestyle and indoor and outdoor exposure variables retrieved from questionnaire were also included in the models. The MN data were structured in two repeated measures for each subject. Incidence rate ratios (IRRs) for each independent variable were estimated with their 95% confidence intervals (95% CIs).

A multiple regression analysis was also performed with DNA damage measured with comet test as the dependent variable and the levels of air pollutants as predictors, adjusting for confounding factors (sex, BMI, parents' smoking and education, and diet).

The associations between MN frequency and DNA damage with comet test as the dependent variables, and the levels of PM<sub>10</sub>, PM<sub>2.5</sub>, benzene, NO<sub>2</sub>, SO<sub>2</sub>, CO, and O<sub>3</sub> were also assessed measuring the air pollutants in various lag times between exposure and biological sampling. Particularly, the mean levels ( $\pm$ SD) on 1, 2, 7, 14, and 21 days before the

biological sampling and the peaks of concentration of PM<sub>10</sub>, PM<sub>2.5</sub>, benzene, NO<sub>2</sub>, SO<sub>2</sub>, CO, and O<sub>3</sub> measured in Brescia during the week before sampling were included in the models.

Two-tailed statistical tests were performed with 0.05 *p* value as the threshold for rejecting the null hypothesis. All the analyses were performed using the Stata TM 12.0 statistical package (Stata Statistical Software Release 12.0, 2012; Stata Corporation, College Station, TX, USA).

### 3. Results

A total of 283 children living in Brescia were enrolled in the first winter, and complete data were available for 257 of them (biological sampling, questionnaire and environmental data). In the second winter, the children were contacted again and 191 (70%) agreed to participate and provided a second biological sample. Overall, a complete dataset with the two seasons data was available for 180 children.

Table 1 reports the socio-demographic and anthropometric characteristics and lifestyle variables of the children. A similar proportion of males and females were enrolled. The majority of children's parents were of Italian nationality, 43.4% of the mothers and 37.2% of fathers had high education level. The majority of children had normal weight; 23.3% of children were overweight or obese. Adherence to Mediterranean diet was satisfactory only in 16%; 11.7% were exposed to second-hand smoke and about one-third practiced outdoor sports. The distribution of the children's characteristics was similar in the two winters (data not reported in the table).

**Table 1.** Characteristics of children enrolled in the study and twice sampled: socio-demographic, anthropometric characteristics and lifestyle.

	N.	%
Gender		
Male	80	45.0
Female	100	55.0
Mother of Italian nationality		
No	31	17.2
Yes	149	82.8
Father of Italian nationality		
No	24	13.3
Yes	156	86.7
Mother degree		
No	102	56.6
Yes	78	43.4
Father degree		
No	113	62.8
Yes	67	37.2
BMI categories		
Normal weight (BMI < 17.4)	138	76.7
Overweight/obese (BMI ≥ 17.4)	42	23.3
Adherence to Mediterranean diet		
No (IMI < 6)	151	83.9
Yes (IMI ≥ 6)	29	16.1
Exposure to second-hand smoke		
No	159	88.3
Yes	21	11.7
Children's outdoor sport		

No	117	65.0
Yes	63	35.0

In Table 2, MN frequency and percentages of children with at least one micronucleus in exfoliated buccal cells in two winters are reported. The MN mean and median were higher in the first than in the second winter (0.51 and 0.5 vs. 0.40 and 0.0 MN frequency), and the statistical test was next to the significance threshold, using the Wilcoxon test ( $p = 0.06$ ). The percentage of children with at least one micronucleus in exfoliated buccal cells was higher in the first (57.8%) than the second winter (47.8%) as well. A total of 51 children had at least one MN in both winter seasons (28.3%) and 41 children never showed MN (22.8%).

**Table 2.** Distribution of MN in children's buccal mucosa cells and visual score in comet test in salivary leukocytes in the two winters.

	Winter Season 1	Winter Season 2	Difference with Wilcoxon Test $p$
MN test			
Number of subjects	180	180	
MN frequency (%)			
Mean $\pm$ SD	0.51 $\pm$ 0.59	0.40 $\pm$ 0.52	0.06
Median	0.5	0.0	
Range	0.0–2.5	0.0–2.0	
N. (%) children with at least one MN	104 (57.8)	86 (47.8)	0.05
N. (%) children with at least one MN in all two seasons	51 (28.3)		
N. and (%) of children without MN in any season	41 (22.8)		
Comet test			
Number of subjects	71	71	
Visual score (arbitrary unit)			
Mean $\pm$ SD	173.2 $\pm$ 50.8	208.8 $\pm$ 67.6	0.009
Median	162.0	193.0	
Range	111.1–327.2	114.0–349.3	

Due to the difficulties reported in Material and Methods for the microscopic analysis of slides, only 71 samples were analysed with the comet test in two winter seasons. The primary DNA damage measured with the comet test and expressed as visual score (arbitrary unit) showed a statistically significant increase from the first to second winter, with means and medians of 173.2 and 162.0 and 208.8 and 193.0, respectively ( $p = 0.009$ ).

Table 3 reported the mean concentrations of PM<sub>0.5</sub> and PAHs measured in ultra-fine particulate matter collected near the schools attended by the children. The mean PM<sub>0.5</sub> was higher in the second than in the first winter, but PAHs (polycyclic aromatic hydrocarbons), nitroPAHs, cPAHs (carcinogenic PAHs), and BaP (benzo(a)pyrene) concentrations measured in PM<sub>0.5</sub> were significantly lower in the second than the first winter; therefore, the chemical characterization of PM<sub>0.5</sub> showed a different composition of ultrafine particulate.

**Table 3.** Mean concentration ( $\pm$ SD) of PM<sub>0.5</sub>, PAHs (polycyclic aromatic hydrocarbons), nitroPAHs, cPAHs (carcinogenic PAHs) and BaP (benzo(a)pyrene) measured in PM<sub>0.5</sub> collected with a high-volume air sampler near the schools attending by the children (five samples for each season).

Pollutants	Winter Season 1 (Mean $\pm$ SD)	Winter Season 2 (Mean $\pm$ SD)	<i>p</i>
PM <sub>0.5</sub> (mg/m <sup>3</sup> )	14.14 $\pm$ 4.68	15.62 $\pm$ 4.80	0.001
PAH (ng/m <sup>3</sup> )	7.96 $\pm$ 4.60	6.52 $\pm$ 1.15	<0.001
NitroPAH (ng/m <sup>3</sup> )	0.09 $\pm$ 0.03	0.04 $\pm$ 0.002	<0.001
cPAH (ng/m <sup>3</sup> )	4.23 $\pm$ 3.47	3.47 $\pm$ 0.54	<0.001
BaP (ng/m <sup>3</sup> )	0.81 $\pm$ 0.48	0.65 $\pm$ 0.14	<0.001

The associations between MN frequency and concentration of PM<sub>0.5</sub>, PAHs, cPAHs and BaP are reported in Table 4. The association with nitroPAH could not be assessed due to small values with a high dispersion. The season, temperature, child BMI, adherence to the Mediterranean diet, exposure to secondhand smoke at home, having Italian parents, and having university-graduated mother and father were included in the model as potential confounders. MN frequency showed an association with temperature (IRR 1.12; 95% CI: 1.07–1.18;  $p < 0.001$ ); the mean temperature was 5.3 and 3.0 °C in the first and second winter, respectively ( $p < 0.001$ ) (Table S1 of Supplementary Material). The regression models for MN frequency did not show statistically significant associations between MN frequency and air pollutants apart from an inverse association with PM<sub>0.5</sub> concentration. No association was found between these pollutants and comet test.

**Table 4.** Analysis of the associations between MN frequency and DNA damage detected with comet test and concentration of PM<sub>0.5</sub>, PAHs (polycyclic aromatic hydrocarbons), cPAHs (carcinogenic PAHs) and BaP (benzo(a)pyrene) using hierarchical mixed effect Poisson regression models. The incidence rate ratio (IRR), and coefficient of regression (coeff.) and 95% confidence intervals (95% CIs) and *p* value are reported.

	IRR	95% CIs	<i>p</i> Value
<b>MN TEST</b>			
PM <sub>0.5</sub>	0.95	0.92; 0.98	0.009
PAH	0.97	0.93; 1.04	0.08
cPAH	0.94	0.88; 1.01	0.07
BaP	0.74	0.52; 1.05	0.09
<b>COMET TEST</b>			
PM <sub>0.5</sub>	−0.003	−0.017; 0.010	0.67
PAH	−0.0004	−0.020; 0.019	0.96
cPAH	−0.0002	−0.38; 0.38	1.0
BaP	−0.00002	−0.18; 0.18	1.0

All the associations were assessed including season, temperature, child BMI and adherence to Mediterranean diet, exposure to secondhand smoke at home, having Italian parents, having graduated mother and father in the model as potential confounders.

The associations between the levels of air pollutants routinely measured in urban air and MN frequency at various lag times between exposure and biological sampling were also assessed: no association was found between these pollutants and MN count, but the peak of concentration of PM<sub>10</sub> in the 7 days before the biological sampling showed a slightly negative association with MN frequency (Table S1). Accordingly, no association was found for DNA damage analyzed by the comet test (Table S2 of Supplementary Material), except a negative association with the mean SO<sub>2</sub> concentration in the 1, 2, and 7 days and a positive association with the mean SO<sub>2</sub> concentration in 21 days before biological sampling.



#### 4. Discussion

In this study, the frequency of micronuclei and the DNA damage were evaluated in buccal cells of 6–8 years old children living in a highly polluted Italian city in two consecutive winters. The same children were twice sampled to evaluate the genotoxic effects in different air pollution conditions in the same subjects and the exclusion criteria (e.g., severe diseases, therapy with antineoplastic agents or radiation therapy, exposure to X-rays, or use of dental braces) allowed to exclude the influence of other factors on genotoxic effects. The MN and comet tests were performed on buccal cells and salivary leukocytes respectively, which are cells directly exposed to airborne compounds and can be collected by non-invasive sampling. The biological sampling was well tolerated by children.

The results showed a higher mean MN frequency in the first (0.51%) than in the second winter (0.40%), with a corresponding proportion of children with at least one MN in exfoliated buccal cells decreasing from 57.8% in the first winter to 47.8% in the second winter.

The air pollution levels in this area (Table S3 of Supplementary Material) are always high, leading to potential chronic exposure of people living here. Concentrations of PM<sub>10</sub> and especially of PM<sub>2.5</sub> are always higher than the UE limits (PM<sub>10</sub> EU daily: 50 µg/m<sup>3</sup>; PM<sub>2.5</sub> EU annual 25 µg/m<sup>3</sup>). NO<sub>2</sub> peak concentrations are also high, up to four times the annual average (NO<sub>2</sub> EU annual: 40 µg/m<sup>3</sup>). We hypothesised that these exposure levels may induce DNA damage in the buccal cells of exposed subjects.

The MN frequency was particularly high in both winters compared with that found in healthy children living in areas with lower air pollution levels and in young working population [36,37]. In the multicentre MAPEC study conducted in five Italian urban areas (including Brescia), the MN frequency was higher in children living in Brescia than in those living in the other cities [38]. A previous study conducted on preschool children (3–6 years old) living in the same town [39] during two consecutive winters, also found a high mean MN frequency in children's buccal mucosa cells, similarly to that found in present study. Other studies evaluated the frequency of MN in exfoliated buccal cells in children [40–49] and found a significant association between MN frequencies and various air pollutants in children exposed to air pollution.

The MN frequency was not influenced by sex, BMI or other demographic or lifestyle characteristics reported in the questionnaire filled in by the children's parents, but it was associated with temperature measured on the environmental air sampling days.

MN are stable DNA damage and the most significant exposure period for observing associations with MN frequency in exfoliated buccal cells corresponded to the 15–21 days before biological sampling, due to the lifetime of these cells, which could differentiate and migrate from basal layer to surface layer between 7–21 days, and thus showing the MN formation [34,36,50]. However, this study did not find any association between the routinely measured pollutants and micronuclei at various lags. Instead, Ceretti et al. [51] found positive and statistically significant associations between MN frequency in children and PM<sub>10</sub>, PM<sub>2.5</sub>, benzene, SO<sub>2</sub> and ozone in a multicentre Italian study; they also found a weak, statistically significant, association of MN with concentration of PM<sub>10</sub>, PM<sub>2.5</sub>, and NO<sub>2</sub> in preschool children living in Brescia in another study [39].

Two possible explanations of the lack of associations between MN frequency and air pollutants in our study may be the presence of unmeasured confounding and the lack of measures of MN frequency at low air pollutants levels. Regarding confounding factors, we assessed various factors, both indoor and outdoor environment, passive smoking, and many others through questionnaires. However, we cannot exclude that questionnaire data did not allow us an adequate control of confounding factors, or that other unmeasured confounders may have played a major role.

The second point deserves attention. To evaluate an association, it is necessary to compare people exposed to high levels to those exposed to low levels of risk factor. When all people are equally exposed, no or weak associations can be found. Our study design allowed us to evaluate repeatability of the MN and comet test by performing them in the

same children at similar air pollution levels. Conversely, this study design did not allow to compare the MN and comet test results at high and low air pollutants levels. Various studies found a positive association between air pollution and DNA damage tests at lower levels of air pollutants than that found in our study [40–49]. However, other studies provided negative or inconsistent results regarding the association between MN frequency in children's cells and air pollutant exposure, highlighting the role of other behavioural factors in the MN occurrence [47,52,53].

Contrary to MN findings, the evaluation of primary DNA damage measured with comet test showed higher damage in second winter than in the first one (mean visual score 208.8 vs. 173.2). The comet assay allows to evaluate the effects of a recent exposure, and our study did not show any association with the level of air pollutants, apart from statistically significant negative relations with the mean SO<sub>2</sub> concentration in 1, 2, and 7 days before biological sampling. Accordingly, negative results were also found in younger children (3–6 years old) living in the same urban area [54].

The results of the comet test were not easy to compare with those of other studies [24,25,36] because we included children instead of adult population and used buccal cells instead of blood lymphocytes and because the different studies used different comet parameters (e.g., tail intensity, tail length, and tail olive moment).

In the same days of biological sampling, an air high-volume sampler was sited near each school attended by the children for 72 h. PM<sub>0.5</sub> was collected and analysed; the mean concentration of PM<sub>0.5</sub> collected was higher in the second winter, but the analysis of PAHs, nitroPAH, carcinogenic PAHs, and BaP showed higher concentrations in the first winter, as reported also in Bonetta et al. [14]. The results confirmed the importance of characterizing the particulate matter, because not only was the size and the concentration important, but the chemical composition was also relevant. Moreover, also the air physical parameters influenced the biological response to air pollution exposure, for instance temperature, which was associated positively with MN frequency in our study and therefore may be a confounding factor.

The results of the MN and the comet tests had opposite trends: the former was not associated with the levels of routinely measured environmental pollutants but correlated with the levels of PM<sub>0.5</sub> and with the levels of PAHs in this ultrafine fraction. The comet test did not show any association with air pollutants, but the DNA damage appeared higher during the second winter in the presence of higher air pollution levels. Similar results have been reported by other authors, highlighting that MN and comet tests detect independent effects induced by different substances not necessarily attached to ultrafine particulate matter [23,36,55], in contrast to other studies showing a concordance among these tests, including if with lower concentration of air pollutants [49].

The PM<sub>0.5</sub> usually showed the greatest toxicity, especially due to organic compounds such as PAHs, as reported by other authors [56–59]. The smallest PM fractions were the most toxic, probably because they are richer in PAHs and had a higher equivalent toxicity, as reported by Feretti et al. [13] in air samples of the same urban area.

The children were enrolled on a voluntary basis and were a selected sample of the population with parents of high socio-economic level [60]. There was no difference among children attending school in different areas as well as the socio-demographic and lifestyle characteristics, which, however, were not confounding factors in our and in other studies [38,51]. For the same characteristics, there was no difference between the children included and excluded in the study. There were no differences in MN frequency and visual score in children's buccal cells according to school and living area. Most children participated in the second winter (about 70%) without differences between those who dropped out and those who decided to continue to participate in the study.

This study has limitations. We did not include a control group of unexposed children. The enrolled children were exposed to high levels of air pollutants in different conditions using the daily levels of air pollutants routinely measured. The environmental sampling (PM<sub>0.5</sub>; PAHs, etc.) performed near the school provided an estimate of the exposure levels

in the period, as it was provided on the days of biological sampling. Indirect measures of exposure to other potential agents causing DNA damage were provided by the questionnaires completed by children's parents, as it is often performed in epidemiological research of health effects of air pollution.

This study also had strengths. The buccal mucosa cells were twice collected in healthy children for a total of 360 MN tests, allowing the comparison between the genotoxic effects in somewhat different air pollution conditions. The number of enrolled children was higher than those recruited in other studies that used biomarkers.

The comparison of MN test between the two winters showed that only 28.3% of children had at least one MN in both seasons, whereas about the half of children showed a positive result in each evaluation. These data suggest that repeatability of the test is low; the probability that a positive MN test in children's buccal cells is confirmed when repeated in the same period of subsequent year is about 50%. This is the first study showing the results of repeated MN tests in the same subjects in a relatively short interval time, to the best of our knowledge.

## 5. Conclusions

This study on repeated measures of MN frequency and DNA damage in children's buccal cells in two consecutive winter seasons, as biomarkers of early DNA damage, did not show an association with various air pollutants evaluated in the area on the same days of biological sampling or with various lag times. However, this study confirms the high level of MN frequency in children living in an area with poor air quality as compared to those living in less polluted areas, suggesting that more attention should be devoted to environmental factors possibly influencing children's health.

**Supplementary Materials:** The following are available online at [www.mdpi.com/2073-4433/12/9/1191/s1](http://www.mdpi.com/2073-4433/12/9/1191/s1), Table S1: Analysis of the associations between MN frequency and PM10, PM2.5, benzene, NO2, SO2, CO and O3 measured in Brescia at various lag time between exposure measure and biological sampling, Table S2: Analysis of the associations between DNA damage with comet test and PM10, PM2.5, benzene, NO2, SO2, CO and O3 measured in Brescia at various lag time between exposure measure and biological sampling, Table S3: Mean ( $\pm$  SD) and peak of concentrations of PM10, PM2.5, benzene, NO2, SO2, CO and O3 measured in Brescia during the weeks before the biological sampling, in two winters (data from the local Environmental Protection Agency). All differences in pollutant concentrations and temperature in two seasons are statistically significant ( $p < 0.001$ ).

**Author Contributions:** Conceptualization: C.Z., E.C., D.F., M.M., M.V. (Marco Verani), A.D.D., S.B. (Sara Bonetta), and U.G.; data curation, C.Z., E.C., M.V. (Milena Villarini), and M.M.; formal analysis, C.Z.; funding acquisition, E.C., S.B. (Silvia Bonizzoni), and U.G.; investigation, C.Z., E.C., D.F., M.V. (Milena Villarini), M.M., M.V. (Marco Verani), A.D.D., S.B. (Sara Bonetta), and A.B. (Annamaria Buschini); methodology, M.V. (Milena Villarini), S.B. (Sara Bonetta), and A.B. (Annamaria Buschini); project administration, E.C., M.M., A.D.D., A.B. (Alberto Bonetti), and U.G.; writing—original draft, C.Z.; writing—review and editing, C.Z., D.F., M.V. (Milena Villarini), M.M., M.V. (Marco Verani), A.D.D., S.B. (Sara Bonetta), A.B. (Annamaria Buschini), A.B. (Alberto Bonetti), S.B. (Silvia Bonizzoni), and U.G. All authors have read and agreed to the published version of the manuscript.

**Funding:** This project was supported by a grant from the European Commission Life+Programme 2012 (grant ID: LIFE12 ENV/IT/000614 MAPEC\_LIFE). The funder had no role in the design and conduct of the study; collection, analysis and interpretation of the data; preparation, review or approval of the manuscript; or decision to submit the manuscript for publication. The researchers were independent of the study funder.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Brescia.

**Informed Consent Statement:** The MAPEC\_LIFE project has been approved by the local Ethics Committees of Brescia. Names of the Ethics Committees and dates of approval: Comitato Etico Provinciale della Provincia di Brescia, 15 January 2014 (approval n. NP1577). Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data is available in the paper and the Supplementary Material.

**Acknowledgments:** The authors are grateful to the children and their parents for their participation in this study. The authors also wish to thank directors, teachers and janitors of the primary schools involved: Istituto Comprensivo Est1 (Schools “Raffaello”, “Giovanni XXIII” and “Calvino”) and Istituto Comprensivo Nord2 (Schools “Arici” and “Quasimodo”) in Brescia.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. IARC. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Outdoor Air Pollution*; IARC Publications: Lion, France, 2016; Volume 109; ISBN 9789283201755.
2. EAA. *Air Quality in Europe 2019*. European Environment Agency, Technical Report No 10/2019; Luxembourg: Publications Office of the European Union: Copenhagen, Denmark, 2019.
3. EAA. *Air Quality in Europe 2020*. European Environment Agency, Technical Report No 9/2020, Luxembourg: Publications Office of the European Union: Copenhagen, Denmark 2020.
4. WHO. *Air Quality Guidelines: Global Update 2005—Particulate Matter, Ozone, Nitrogen Dioxide and Sulphur Dioxide*; World Health Organization, Regional Office for Europe: Copenhagen, Denmark, 2006.
5. Wang, Q.; Li, J.; Yang, J.; Chen, Y.; Li, Y.; Li, S.; Xie, C.; Chen, C.; Wang, L.; Wang, L.; et al. Seasonal characterization of aerosol composition and sources in a polluted city in Central China. *Chemosphere* **2020**, *258*, 127310.
6. Pongpiachan, S.; Hattayanone, M.; Suttinun, O.; Khumsup, C.; Kittikoon, I.; Hiruyatrakul, P.; Cao, J. Assessing human exposure to PM10-bound polycyclic aromatic hydrocarbons during fireworks displays. *Atmos. Pollut. Res.* **2017**, *8*, 816–827.
7. Pongpiachan, S.; Kositanont, C.; Palakun, J.; Liu, S.; Ho, K.F.; Cao, J. Effects of day-of-week trends and vehicle types on PM 2.5-bounded carbonaceous compositions. *Sci. Total Environ.* **2015**, *532*, 484–494.
8. Topinka, J.; Rossner, P., Jr.; Milcova, A.; Schmuczerova, J.; Pencikova, K.; Rossnerova, A.; Ambrož, A.; Štolcpartová, J.; Bendl, J.; Hovorka, J.; et al. Day-to-day variability of toxic events induced by organic compounds bound to size segregated atmospheric aerosol. *Environ. Pollut.* **2015**, *202*, 135–145.
9. Srimuruganandam, B.; Nagendra, S.S. Source characterization of PM10 and PM2.5 mass using a chemical mass balance model at urban roadside. *Sci. Total Environ.* **2012**, *433*, 8–19.
10. Perrone, M.G.; Gualtieri, M.; Ferrero, L.; Lo Porto, C.; Udisti, R.; Bolzacchini, E.; Camatini, M. Seasonal variations in chemical composition and in vitro biological effects of fine PM from Milan. *Chemosphere* **2010**, *78*, 1368–1377.
11. Pey, J.; Querol, X.; Alastuey, A. Discriminating the regional and urban contributions in the North Western Mediterranean: PM levels and composition. *Atmos. Environ.* **2010**, *44*, 1587–1596.
12. Kim, K.H.; Kabir, E.; Kabir, S. A review on the human health impact of airborne particulate matter. *Environ. Int.* **2015**, *74*, 136–143.
13. Feretti, D.; Pedrazzani, R.; Ceretti, E.; Dal Grande, M.; Zerbini, I.; Viola, G.C.V.; Gelatti, U.; Donato, F.; Zani, C. “Risk is in the air”: Polycyclic aromatic hydrocarbons, metals and mutagenicity of atmospheric particulate matter in a town of Northern Italy (Respira study). *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* **2019**, *842*, 35–49.
14. Bonetta, S.; Bonetta, S.; Schilirò, T.; Ceretti, E.; Feretti, D.; Covolo, L.; Vannini, S.; Villarini, M.; Moretti, M.; et al. Mutagenic and genotoxic effects induced by PM0.5 of different Italian towns in human cells and bacteria: The MAPEC\_LIFE study. *Environ. Pollut.* **2019**, *245*, 1124–1135.
15. Palacio, I.C.; Barros, S.B.M.; Roubucek, D.A. Water-soluble and organic extracts of airborne particulate matter induce micronuclei in human lung epithelial A549 cells. *Mutat. Res. Toxicol. Environ. Mutagen.* **2016**, *812*, 1–11.
16. Ceretti, E.; Zani, C.; Zerbini, I.; Viola, G.; Moretti, M.; Villarini, M.; Dominici, L.; Monarca, S.; Feretti, D. Monitoring of volatile and non-volatile urban air genotoxins using bacteria, human cells and plants. *Chemosphere* **2015**, *120*, 221–229.
17. Dumax-Vorzet, A.F.; Tate, M.; Walmsley, R.; Elder, R.H.; Povey, A.C. Cytotoxicity and genotoxicity of urban particulate matter in mammalian cells. *Mutagenesis* **2015**, *30*, 621–633.
18. Traversi, D.; Cervella, P.; Gilli, G. Evaluating the genotoxicity of urban PM2.5 using PCR-based methods in human lung cells and the Salmonella TA98 reverse test. *Environ. Sci. Pollut. Res. Int.* **2015**, *22*, 1279–1289.
19. Lepers, C.; Dergham, M.; Armand, L.; Billet, S.; Verdin, A.; Andre, V. Mutagenicity and clastogenicity of native airborne particulate matter samples collected under industrial, urban or rural influence. *Toxicol. Vitro.* **2014**, *28*, 866–874.
20. De Brito, K.C.; Lemos, C.T.; Rocha, J.A.; Mielli, A.C.; Matzenbacher, C.; Vargas V.M. Comparative genotoxicity of airborne particulate matter (PM2.5) using Salmonella, plants and mammalian cells. *Ecotoxicol. Environ. Saf.* **2013**, *94*, 14–20.
21. Lemos, A.T.; Coronas, M.V.; Rocha, J.A.; Vargas, V.M. Mutagenicity of particulate matter fractions in areas under the impact of urban and industrial activities. *Chemosphere* **2012**, *89*, 1126–1134.

22. Claxton, L.D.; Woodall, G.M. A review of the mutagenicity and rodent carcinogenicity of ambient air. *Mutat Res.* **2007**, *636*, 36–94.
23. Coronas, M.V.; Rocha, J.A.; Salvadori, D.M.; Vargas, V.M. Evaluation of area contaminated by wood treatment activities: Genetic markers in the environment and in the child population. *Chemosphere* **2016**, *144*, 1207–1215.
24. Neri, M.; Bonassi, S.; Knudsen, L.E.; Sram, R.J.; Holland, N.; Ugolini, D.; Merlo, D.F. Children's exposure to environmental pollutants and biomarkers of genetic damage. I. Overview and critical issues. *Mutat. Res.* **2006**, *612*, 1–13.
25. Neri, M.; Ugolini, D.; Bonassi, S.; Fucic, A.; Holland, N.; Knudsen, L.E.; Šrám, R.J.; Ceppi, M.; Bocchini, V.; Merlo, D.F. Children's exposure to environmental pollutants and biomarkers of genetic damage. II. Results of a comprehensive literature search and meta-analysis. *Mutat. Res.* **2006**, *612*, 14–39.
26. Szeto, Y.T.; Benzie, I.F.; Collins, A.R.; Choi, S.W.; Cheng, C.Y.; Yow, C.M.; Tse, M.M. A buccal cell model comet assay: Development and evaluation for human biomonitoring and nutritional studies. *Mutat. Res.* **2005**, *578*, 371–381.
27. Gajski, G.; Gerić, M.; Oreščanin, V.; Garaj-Vrhovac, V. Cytogenetic status of healthy children assessed with the alkaline comet assay and the cytokinesis-block micronucleus cytome assay. *Mutat. Res.* **2013**, *750*, 55–62.
28. Wild, C.P.; Kleinjans, J. Children and increased susceptibility to environmental carcinogens: Evidence or empathy? *Cancer Epidemiol. Biomark. Prev.* **2003**, *2*, 1389–1394.
29. Feretti, D.; Ceretti, E.; De Donno, A.; Moretti, M.; Carducci, A.; Bonetta, S.; Marrese, M.R.; Bonetta, A.; Covolo, L.; Bagordo, F. et al. Monitoring air pollution effects on children for supporting public health policy: The protocol of the prospective cohort MAPEC study. *BMJ Open* **2014**, *4*, e006096.
30. Zani, C.; Donato, F.; Grioni, S.; Viola, G.C.V.; Ceretti, E.; Feretti, D.; Festa, A.; Bonizzoni, S.; Bonnetti, A.; Monarca S.; et al. Feasibility and reliability of a questionnaire for evaluation of the exposure to indoor and outdoor air pollutants, diet and physical activity in 6–8-year-old children. *Ann. Ig.* **2015**, *27*, 646–656.
31. Agnoli, C.; Krogh, V.; Grioni, S.; Sieri, S.; Palli, D.; Masala, G.; Sacerdote, C.; Vineis, P.; Tumino, R.; Frasca, G.; et al. A Priori-Defined Dietary Patterns Are Associated with Reduced Risk of Stroke in a Large Italian Cohort. *J. Nutr.* **2011**, *141*, 1552–1558, <https://doi.org/10.3945/jn.111.140061>.
32. Zani, C.; Ceretti, E.; Grioni, S.; Viola, G.C.V.; Donato, F.; Feretti, D.; Festa, A.; Bonizzoni, S.; Bonnetti, A.; Monarca S.; et al. Are 6–8 years old Italian children moving away from the Mediterranean diet? *Ann. Ig.* **2016**, *28*, 339–348.
33. Singh, N.P.; McCoy, M.T.; Tice, R.R.; Schneider, E.L. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **1988**, *175*, 184–191.
34. Thomas, P.; Fenech, M. Buccal Micronucleus Cytome Assay. In *DNA Damage Detection In Situ, Ex Vivo, and In Vivo. Met Mol Biology (Methods and Protocols)*; Didenko, V., Eds.; Humana Press: Totowa, NJ, USA, 2011; pp. 235–248.
35. Bolognesi, C.; Knasmueller, S.; Nersesyan, A.; Thomas, P.; Fenech, M. The HUMNxl scoring criteria for different cell types and nuclear anomalies in the buccal micronucleus cytome assay—An update and expanded photogallery. *Mutat. Res.* **2013**, *753*, 100–113.
36. Bonassi, S.; Coskun, E.; Ceppi, M.; Lando, C.; Bolognesi, C.; Burgaz, S.; Holland, N.; Kirsh-Volders, M.; Knasmueller, S.; Zeiger, E.; et al. The HUman MicroNucleus project on exfoliated buccal cells (HUMNXL): The role of life-style, host factors, occupational exposures, health status, and assay protocol. *Mutat. Res. Mutat. Res.* **2011**, *728*, 88–97, <https://doi.org/10.1016/j.mrrev.2011.06.005>.
37. Karahalil, B.; Karakaya, A.E.; Burgaz, S. The micronucleus assay in exfoliated buccal cells: Application to occupational exposure to polycyclic aromatic hydrocarbons. *Mutat. Res.* **1999**, *442*, 29–35.
38. Villarini, M.; Levorato, S.; Salvatori, T.; Ceretti, E.; Bonetta, S.; Carducci, A.; Grassi, T.; Vannini, S.; Donato, F.; Bonetta, S.; et al. Buccal micronucleus cytome assay in primary school children: A descriptive analysis of the MAPEC\_LIFE multicenter cohort study. *Int. J. Hyg. Environ. Health* **2018**, *221*, 883–892, <https://doi.org/10.1016/j.ijheh.2018.05.014>.
39. Ceretti, E.; Feretti, D.; Viola, G.C.V.; Zerbini, I.; Limina, R.M.; Zani, C.; Capelli, M.; Lamera, R.; Donato, F.; Gelatti, U. DNA Damage in Buccal Mucosa Cells of Pre-School Children Exposed to High Levels of Urban Air Pollutants. *PLoS ONE* **2014**, *9*, e96524, <https://doi.org/10.1371/journal.pone.0096524>.
40. Lahiri, T.; Roy, S.; Basu, C.; Ganguly, S.; Ray, M.R.; Lahiri, P. Air pollution in Calcutta elicits adverse pulmonary reaction in children. *Indian J. Med. Res.* **2000**; *112*, 21–26.
41. Demircigil, G.Ç.; Erdem, O.; Gaga, E.O.; Altuğ, H.; Demirel, G.; Özden, Ö.; Ari, A.; Örnektekin, S.; Döğeroğlu, T.; et al. Cytogenetic biomonitoring of primary school children exposed to air pollutants: Micronuclei analysis of buccal epithelial cells. *Environ. Sci. Pollut. Res.* **2014**, *21*, 1197–1207.
42. Mergener, M.; Rhoden, C.R.; Amantéa, S.L. Nuclear abnormalities in cells from nasal epithelium: A promising assay to evaluate DNA damage related to air pollution in infants. *Jornal de Pediatria.* **2014**, *90*, 632–636.
43. Silva da Silva, C.; Marzari Rossato, J.; Vaz Rocha, J.A.; Ferrão Vargas, V.M. Characterization of an area of reference for inhalable particulate matter (PM2.5) associated with genetic biomonitoring in children. *Mutat. Res.* **2015**, *778*, 44–55.
44. Cavalcante, D.; Sposito, J.C.V.; Crispim, B.; Nascimento, A.; Barufatti Grisolia, A. Genotoxic and mutagenic effects of passive smoking and urban air pollutants in buccal mucosa cells of children enrolled in public school. *Toxicol. Mech. Meth.* **2017**, *27*, 346–351.
45. Huen, K.; Gunn, L.; Duramad, P.; Jeng, M.; Scalf, R.; Holland, N. Application of a geographic information system to explore associations between air pollution and micronucleus frequencies in African American children and adults. *Environ. Mol. Mutagen.* **2006**, *47*, 236–246, <https://doi.org/10.1002/em.20193>.

46. Sisenando, H.A.; de Batistuzzo, S.R.; Artaxo, P.; Saldiva, P. H.; de Souza Hacon, S. Micronucleus frequency in children exposed to biomass burning in the Brazilian Legal Amazon region: A control case study. *BMC Oral Health* **2012**, *12*, 6.
47. Marcon, A.; Fracasso, M.E.; Marchetti, P.; Doria, D.; Girardi, P.; Guarda, L.; Pesce, G.; Pironi, V.; Ricci, P.; De Marco, R. Outdoor Formaldehyde and NO<sub>2</sub> Exposures and Markers of Genotoxicity in Children Living Near Chipboard Industries. *Environ. Health Perspect.* **2014**, *122*, 639–645, <https://doi.org/10.1289/ehp.1307259>.
48. Panico, A.; Grassi, T.; Bagordo, F.; Idolo, A.; Serio, F.; Tumolo, M.R.; De Giorgi, M.; Guido, M.; Tutino, M.; De Donno, A. Micronucleus Frequency in Exfoliated Buccal Cells of Children Living in an Industrialized Area of Apulia (Italy). *Int. J. Environ. Res. Public Health* **2020**, *17*, 1208, <https://doi.org/10.3390/ijerph17041208>.
49. Lemos, A.T.; Lemos, C.T.; Coronas, M.V.; Rocha, J.R.D.; Vargas, V.M.F. Integrated study of genotoxicity biomarkers in school-children and inhalable particles in areas under petrochemical influence. *Environ. Res.* **2020**, *188*, 109443, doi:10.1016/j.envres.2020.109443.
50. Holland, N.; Bolognesi, C.; Kirsch-Volders, M.; Bonassi, S.; Zeiger, E.; Knasmueller, S.; Fenech, M. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: The HUMN project perspective on current status and knowledge gaps. *Mutat. Res.* **2008**, *659*, 93–108.
51. Ceretti, E.; Donato, F.; Zani, C.; Villarini, M.; Verani, M.; De Donno, A.; Bonetta, S.; Feretti, D.; Carducci, A.; Idolo, A.; et al. Results from the European Union MAPEC\_LIFE cohort study on air pollution and chromosomal damage in children: Are public health policies sufficiently protective? *Environ. Sci. Eur.* **2020**, *32*, 74.
52. Idolo, A.; Grassi, T.; Bagordo, F.; Panico, A.; De Giorgi, M.; Serio, F.; Guido, M.; Piscitelli, P.; De Filippis, G.; Raho, A.; et al. Micronuclei in Exfoliated Buccal Cells of Children Living in a Cluster Area of Salento (Southern Italy) with a High Incidence of Lung Cancer: The IMP.AIR Study. *Int. J. Environ. Res. Public Health* **2018**, *15*, 1659, <https://doi.org/10.3390/ijerph15081659>.
53. O'Callaghan-Gordo, C.; Fthenou, E.; Pedersen, M.; Espinosa, A.; Chatzi, L.; Beelen, R.; Chalkiadaki, G.; Decordier, I.; Hoek, G.; Merlo, M.F.; et al. Outdoor air pollution exposures and micronuclei frequencies in lymphocytes from pregnant women and newborns in Crete, Greece (Rhea cohort). *Environ. Res.* **2015**, *143*, 170–176, <https://doi.org/10.1016/j.envres.2015.10.011>.
54. Zani, C.; Ceretti, E.; Zerbini, I.; Viola, G.C.V.; Donato, F.; Gelatti, U.; Feretti, D. Comet Test in Saliva Leukocytes of Pre-School Children Exposed to Air Pollution in North Italy: The Respira Study. *Int. J. Environ. Res. Public Health* **2020**, *17*, 3276.
55. Zani, C.; Donato, F.; Ceretti, E.; Pedrazzani, R.; Zerbini, I.; Gelatti, U.; Feretti, D. Genotoxic Activity of Particulate Matter and In Vivo Tests in Children Exposed to Air Pollution. *Int. J. Environ. Res. Public Health* **2021**, *18*, 5345, doi:10.3390/ijerph18105345.
56. Billet, S.; Landkocz, Y.; Martin, P.; Verdin, A.; Ledoux, F.; Lepers, C.; André, V.; Cazier, F.; Sichel, F.; Shirali, P.; et al. Chemical characterization of fine and ultrafine PM, direct and indirect genotoxicity of PM and their organic extracts on pulmonary cells. *J. Environ. Sci.* **2018**, *71*, 168–178, <https://doi.org/10.1016/j.jes.2018.04.022>.
57. Valle-Hernández, B.L.; Mugica-Alvarez, V.; Salinas-Talavera, E.; Amador-Muñoz, O.; Murillo-Tovar, M.A.; Villalobos-Pietrini, R.; De Vizcaya-Ruiz, A. Temporal variation of nitro-polycyclic aromatic hydrocarbons in PM<sub>10</sub> and PM<sub>2.5</sub> collected in Northern Mexico City. *Sci. Total Environ.* **2010**, *408*, 5429–5438.
58. Slezakova, K.; Castro, D.; Pereira, M.C.; Morais, S.; Delerue-Matos, C.; Alvim-Ferraz, M.C. Influence of traffic emissions on the carcinogenic polycyclic aromatic hydrocarbons in outdoor breathable particles. *J. Air Waste Manag. Assoc.* **2010**, *60*, 393–401.
59. Oliveira, M.; Slezakova, K.; Delerue-Matos, C.; Pereira, M.C.; Morais, S. Children environmental exposure to particulate matter and polycyclic aromatic hydrocarbons and biomonitoring in school environments: A review on indoor and outdoor exposure levels, major sources and health impacts. *Environ. Int.* **2019**, *124*, 180–204.
60. Bagordo, F.; MAPEC\_LIFE Study Group; De Donno, A.; Grassi, T.; Guido, M.; DeVoti, G.; Ceretti, E.; Zani, C.; Feretti, D.; Villarini, M.; et al. Lifestyles and socio-cultural factors among children aged 6–8 years from five Italian towns: the MAPEC\_LIFE study cohort. *BMC Public Health* **2017**, *17*, 233, <https://doi.org/10.1186/s12889-017-4142-x>.