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

Claudia Zani [1,*], Elisabetta Ceretti [1], Donatella Feretti [1], Milena Villarini [2], Massimo Moretti [2], Marco Verani [3], Antonella De Donno [4], Sara Bonetta [5], Annamaria Buschini [6], Alberto Bonetti [7], Silvia Bonizzoni [8], Umberto Gelatti [1] and on behalf of the MAPEC-LIFE Study Group [†]

1 Department of Medical and Surgical Specialties, Radiological Science and Public Health, University of Brescia, 11 Viale Europa, 25123 Brescia, Italy; elisabetta.ceretti1@unibs.it (E.C.); donatella.feretti@unibs.it (D.F.); umberto.gelatti@unibs.it (U.G.)
2 Unit of Public Health, Department of Pharmaceutical Sciences, University of Perugia, Via del Giochetto, 06122 Perugia, Italy; milena.villarini@unipg.it (M.V.); massimo.moretti@unipg.it (M.M.)
3 Department of Biology, University of Pisa, 35/39 Via S. Zeno, 56127 Pisa, Italy; marco.verani@unipi.it
4 Department of Biological and Environmental Sciences and Technologies, University of Salento, 165 Via Monteroni, 73100 Lecce, Italy; antonella.dedonno@unisalento.it
5 Department of Public Health and Pediatrics, University of Torino, 94 Piazza Polonia, 10126 Turin, Italy; sara.bonetta@unito.it
6 Department of Chemistry, Life Sciences and Environmental Sustainability, University of Parma, Parco Area delle Scienze 11/A, 43124 Parma, Italy; annamaria.buschini@unipr.it
7 Multi-Sector and Technological Service Centre—CSMT Gestione S.c.a.r.l, 45 Via Branze, 25123 Brescia, Italy; a.bonetti@csmt.it
8 Brescia Municipality, 1 Piazza Repubblica, 25100 Brescia, Italy; sbonizzoni@comune.brescia.it
* Correspondence: claudia.zani@unibs.it
† The MAPEC_LIFE Study Group: Francesco Donato, Andrea Festa, Gaia C.V. Viola, Ilaria Zerbini, Cristina Fatigoni, Annalaura Carducci, Gabriele Donzelli, Giacomo Palomba, Francesco Bagordo, Tiziana Grassi, Marcello Guido, Adele Idolo, Alessandra Panico, Francesca Serio, Elisabetta Carraro, Tiziana Schilirò, Silvia Bonetta, Cristina Pignata, Marta Gea, Valeria Romanazzi, Giorgio Gilli, Alessio Perotti, Serena Montalbano, Beatrice Casini, Beatrice Bruni, Giovanna Mazzoleni.

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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 ± 0.59) than the second winter (0.40 ± 0.52), whereas DNA damage measured with comet test showed higher damage in the second versus the first winter (visual score 208.8 ± 67.1 vs. 173.2 ± 50.8). The associations between air pollutant levels (CO, NO_2, SO_2, benzene, O_3, $PM_{10,}$ and $PM_{2.5}$; $PM_{0.5}$ 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_2, O_3, SO_2, 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 ($PM_{0.5}$) 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 $PM_{0.5}$ 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_2, SO_2, benzene, O_3, $PM_{10,}$ and $PM_{2.5}$) 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 µL of cell-LMA suspension were placed into each spot of the 12-gel slide; with the standard approach, two 20 µL drops were layered on a single slide. After overnight lysis (2.5 M NaCl, 100 mM Na_2EDTA, 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_4EDTA, 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 °C). For each electrophoresis, two slides prepared with A549 cells with EMS (2 mM) for 1 h at 37 °C were added in the tank as internal control.

To evaluate DNA damage, the slides were stained with ethidium bromide (10 µg/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).

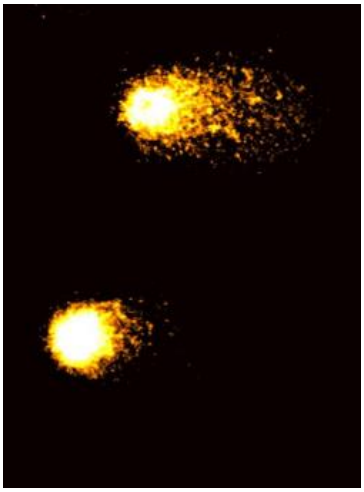

Figure 1. Comet test: image of damaged and undamaged salivary leukocytes observed through fluorescence microscopy.

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 florescent 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 µm nylon filter, and centrifuged again. Cell pellets were resuspended in ice-cold PBS, aliquots (10 µL) 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 µL of cell suspension onto precleaned slides (approximately 1×10^5 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.

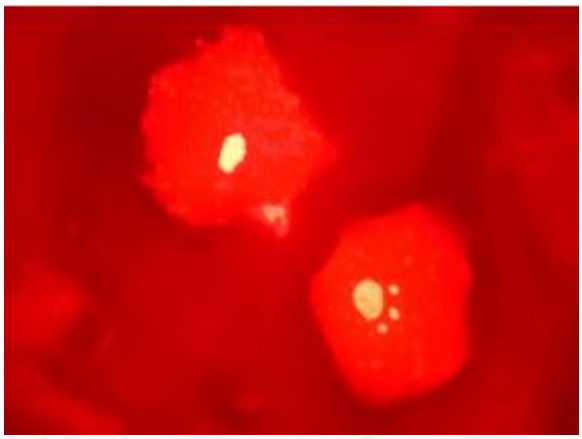

Figure 2. MN test: image of buccal mucosa cells with and without MN observed through fluorescence microscopy.

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_{10}, $PM_{2.5}$, benzene, NO_2, SO_2, CO, and O_3 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_{10}, $PM_{2.5}$, benzene, NO_2, SO_2, CO, and O_3 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 $\geq$ 17.4)	42	23.3
Adherence to Mediterranean diet		
No (IMI < 6)	151	83.9
Yes (IMI $\geq$ 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 ± SD	0.51 ± 0.59	0.40 ± 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 ± SD	173.2 ± 50.8	208.8 ± 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 $PM_{0.5}$ and PAHs measured in ultra-fine particulate matter collected near the schools attended by the children. The mean $PM_{0.5}$ 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 $PM_{0.5}$ were significantly lower in the second than the first winter; therefore, the chemical characterization of $PM_{0.5}$ showed a different composition of ultrafine particulate.

The associations between MN frequency and concentration of $PM_{0.5}$, 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 $PM_{0.5}$ concentration. No association was found between these pollutants and comet test.

Table 3. Mean concentration (±SD) of $PM_{0.5}$, PAHs (polycyclic aromatic hydrocarbons), nitroPAHs, cPAHs (carcinogenic PAHs) and BaP (benzo(a)pyrene) measured in $PM_{0.5}$ collected with a high-volume air sampler near the schools attending by the children (five samples for each season).

Pollutants	Winter Season 1 (Mean ± SD)	Winter Season 2 (Mean ± SD)	*p*
$PM_{0.5}$ (mg/m^3)	14.14 ± 4.68	15.62 ± 4.80	0.001
PAH (ng/m^3)	7.96 ± 4.60	6.52 ± 1.15	<0.001
NitroPAH (ng/m^3)	0.09 ± 0.03	0.04 ± 0.002	<0.001
cPAH (ng/m^3)	4.23 ± 3.47	3.47 ± 0.54	<0.001
BaP (ng/m^3)	0.81 ± 0.48	0.65 ± 0.14	<0.001

Table 4. Analysis of the associations between MN frequency and DNA damage detected with comet test and concentration of $PM_{0.5}$, 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	*p* Value
MN TEST			
$PM_{0.5}$	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
COMET TEST	**Coeff.**	**95% CIs**	***p* Value**
$PM_{0.5}$	−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_{10} 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_2 concentration in the 1, 2, and 7 days and a positive association with the mean SO_2 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_{10} and especially of $PM_{2.5}$ are always higher than the UE limits (PM_{10} EU daily: 50 $\mu g/m^3$; $PM_{2.5}$ EU annual 25 $\mu g/m^3$). NO_2 peak concentrations are also high, up to four times the annual average (NO_2 EU annual: 40 $\mu g/m^3$). 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_{10}, $PM_{2.5}$, benzene, SO_2, and ozone in a multicentre Italian study; they also found a weak, statistically significant, association of MN with concentration of PM_{10}, $PM_{2.5}$, and NO_2 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_2 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. $PM_{0.5}$ was collected and analysed; the mean concentration of $PM_{0.5}$ 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 $PM_{0.5}$ 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 $PM_{0.5}$ 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' 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 ($PM_{0.5}$; 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 in 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 https://www.mdpi.com/article/10.3390/atmos12091191/s1, Table S1: Analysis of the associations between MN frequency and PM_{10}, $PM_{2.5}$, benzene, NO_2, SO_2, CO and O_3 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 PM_{10}, $PM_{2.5}$, benzene, NO_2, SO_2, CO and O_3 measured in Brescia at various lag time between exposure measure and biological sampling, Table S3: Mean ($\pm$ SD) and peak of concentrations of PM_{10}, $PM_{2.5}$, benzene, NO_2, SO_2, CO and O_3 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 ($p < 0.001$).

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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.

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