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Monitoring air pollution effects on children (MAPEC) living near cement factories: The MAPEC_Gubbio Study

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ABSTRACT

The present study examined the association between airborne pollutants and early biological effects in children, a group particularly vulnerable to immediate and long-term effects, underscoring the importance of early detection. Researchers have performed the buccal micronucleucytome (BMCyt) assay focusing particularly on the assessment of two biomarkers of early genotoxic damage – micronuclei (MN) and nuclear buds – in exfoliated buccal mucosa cells of kids of 6–8 years from Gubbio (exposed area with two cement factories) and Città di Castello (control area). Overall, 164 children were enrolled, of whom 161 underwent cytome analysis. Air quality data were obtained from the publicly available database of the Regional Agency for Environmental Protection (ARPA Umbria). Information on indoor exposure, sociodemographic characteristics, diet, and health, were collected via questionnaires. Sampling was conducted in late spring and winter, seasons with differing pollutant concentrations. Analysis explored correlations between MN frequency and pollution levels. Results showed a significantly ($p < 0.001$) doubled MN frequency in exposed children during late spring, with a median of 2.0 (IQR 1.0–2.5), compared with 1.0 (IQR 0.5–1.5) of the control group, likely due to unmonitored summer-specific pollutants, despite measured pollutants being below legal limits. The findings indicate a residual genotoxic risk for children in exposed areas, underscoring the need to enhance air pollutant monitoring to better address potential genetic damage.

1. Introduction

Air pollution is defined as the presence of one or more compounds in the atmosphere at concentrations or for periods above their natural levels, with the potential to adversely affect the environment and/or human health (Seinfeld and Pandis, 2016). Outdoor air pollution comprises complex mixtures of thousands of distinct compounds, including gaseous pollutants such as carbon monoxide (CO), nitrogen dioxide (NO_2), sulphur oxides (SO_X), ozone (O_3), volatile organic compounds (VOCs) like benzene, heavy metals such as lead (Pb), and particulate matter (PM). PM is usually categorised into three size classes based on its median aerodynamic diameter (AD): coarse particles with an AD of 2.5–10 μm (PM_{10}), fine particles with ADs less than 2.5 μm ($PM_{2.5}$), and ultrafine particles with ADs less than 0.1 μm ($PM_{0.1}$) (WHO, 2006).

It is widely acknowledged that exposure to airborne pollutants significantly impacts human health (WHO, 2019; Shazia et al., 2025): short-term exposure to ambient air pollution correlates with aggravated asthma, leading to increased hospital admissions (Zheng et al., 2015; Ivan et al., 2025), while long-term exposure is linked to a higher prevalence of cardiovascular and respiratory diseases (Pope et al., 2011; Pelucchi et al., 2009), birth defects (Padula et al., 2013), and neurodegenerative disorders (Moulton and Yang, 2012). The health impacts are not caused solely by exposure to individual air pollutants or single chemical compounds, but rather by complex mixtures that exert

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synergistic, harmful effects on multiple targets (Vuong et al., 2020; Constantinescu et al., 2025; Zhang et al., 2020; Jarvis et al., 2013; Valavanidis et al., 2009; Lagunas-Rangel et al., 2022).

Among all the chemical species of pollution mixture, PM, which encompasses a complex mixture of solid and liquid particles suspended in the air, has gained increasing attention due to its strong association with various noxious effects, from in vitro mutagenicity for bacteria, plants and mammalian cells (Massolo et al., 2002) to cancer induction in mice (Aoki et al., 2018). The size of PM particles is directly linked to their potential to cause health problems, and fine particles ($PM_{2.5}$) pose the most significant health risk. These fine particles can penetrate deeply into tissues, and some may even enter the bloodstream, thereby contributing significantly to the development of chronic degenerative diseases (WHO Europe, 2013). Many epidemiological studies have highlighted a relevant association between PM exposure and various human diseases incidence and mortality: natural cause mortality (So et al., 2023), cardiovascular diseases (Abdul-Rahman et al., 2024), diabetes (Marchini, 2023), and lung cancer (Holme et al., 2023). Based on sufficient evidence of carcinogenicity in humans and experimental animals and strong support by mechanistic studies, the International Agency for Research on Cancer (IARC) has classified outdoor air pollution and fine PM in outdoor air pollution as carcinogenic to humans (Group 1) (Loomis et al., 2013; IARC, Outdoor Air Pollution, International Agency for Research on Cancer, Lyon, France, 2015). Recent European Union official reports have highlighted that air pollution is not only a significant problem for urban inhabitants' health but also Europe's most considerable environmental health risk (EEA, 2024, 2022).

One of the critical anthropogenic sources of PM and other hazardous air pollutants is the cement manufacturing industry (Marcon et al., 2014; Rovira et al., 2018). PM and other pollutants are generated at almost every stage of the cement manufacturing process, from quarrying raw materials to packaging (Abdul-Wahab, 2006; Adeyanju and Okeke, 2019). Cement factories release significant amounts of dust and gaseous byproducts, including SO_X, NO_X, CO, and VOCs, which contribute to the formation of environmental PM and atmospheric contamination (Rovira et al., 2018; Ciobanu et al., 2021). These pollutants are of particular concern when emitted close to residential areas, as they can significantly increase the local population's exposure to genotoxic agents.

Children are particularly vulnerable to the health impacts of air pollution due to their distinct exposure pathways, developmental physiology, and longer life expectancy (ERS, 2010; WHO, 2008, 2007). Specifically, children may be more susceptible to the harmful effects of air pollution than adults due to their smaller airways and higher minute ventilation relative to body weight, as well as underdeveloped detoxifying and metabolic systems, and behaviours that increase inhalation exposure, such as time spent outdoors and proximity to ground-level pollution (Kurt et al., 2016; Sly and Carpenter, 2012). Moreover, epidemiological research indicates that children are more vulnerable to genotoxic chemicals than adults, and that genetic damage occurring at a young age may influence the lifetime risk of unfavourable health outcomes (Neri et al., 2006; Acito et al., 2022), including an increased risk of cancer later in life (Acito et al., 2022). In general, data from the literature suggest that early childhood exposure to ambient air pollution may significantly affect the development of chronic diseases in adulthood (Bateson and Schwartz, 2008; Grigg, 2009; Landrigan, 2004; WHO, 2005; Wild and Kleinjans, 2003).

The long-term health effects, such as chronic obstructive pulmonary disease, cardiovascular disease, or cancer, of moderate or low air pollution levels may not be clearly highlighted by classic epidemiology approaches, particularly in small-scale studies (Brunekreef and Holgate, 2002). For that reason, there is an increasing number of molecular epidemiology studies that use biological markers to examine the genotoxic effects of exposure to environmental pollutants (Coronas et al., 2009; Hrelia et al., 2004; Kyrtopoulos et al., 2001). In this field, genotoxic hazard biomonitoring is typically evaluated using outcomes such as primary DNA damage (*e.g.*, by the comet assay) or cytogenetic effects (*e.g.*, micronuclei or sister chromatid exchanges) (Acito et al., 2022; Ladeira and Smajdova, 2017).

Micronucleus (MN) frequency is among the most widely used biomarkers in studies assessing environmental or occupational risks associated with potential exposure to genotoxic xenobiotics (Knudsen and Hansen, 2007). MN testing can detect both clastogenic (*e.g.*, chromosomal breakage) and aneugenic (*e.g.*, spindle disruption) effects, making it a biomarker of early biological effects (Kirsch-Volders et al., 2011; NRC, 2006). MN occur in the cytoplasm of interphasic cells as little extra nuclei that are smaller than the primary nucleus. During anaphase, MN can form from acentric chromosomal fragments (resulting from clastogen-induced breakage) or complete chromosomes (resulting from aneuploidy or malsegregation). Acentric or entire chromosomes are left behind during mitotic cellular division and so excluded from both daughter nuclei (Fenech et al., 2011a; Bolognesi and Fenech, 2019; Thomas and Fenech, 2011a; Fenech, 2002; Thomas and Fenech, 2011b). MN are regarded as biomarkers of early biological effects due to their capacity to detect both clastogenic (*e.g.*, chromosomal breakage) and aneugenic (*e.g.*, spindle alterations) effects (Kirsch-Volders et al., 2011; NRC, 2006).

The buccal micronucleus cytome (BMCyt) assay is a non-invasive technique for evaluating genomic damage and cell death indicators in exfoliated buccal cells in human population studies (Bonassi et al., 2011; Fenech et al., 2011b; Bolognesi et al., 2013). The BMCyt assay in buccal exfoliated cells allows the assessment not only of chromosomal and DNA damage markers (MN and nuclear buds), but also cell proliferation markers (basal and binucleated cells), and cell death/apoptosis markers (cells with condensed chromatin, and karyorrhectic, pyknotic, and karyolytic cells) (Thomas and Fenech, 2011a; Thomas et al., 2009). Of no less importance, given the minimally invasive nature of the sampling procedure, the BMCyt is particularly useful in biomonitoring studies involving children, thereby avoiding traumatic and painful sampling and minimising children's discomfort.

The present molecular epidemiological approach (*i.e.*, MAPEC_Gubbio Study) aimed to investigate the relationship between air pollutant concentrations and cytogenetic alterations in school-aged children from Gubbio, a town located in Umbria, Italy (Fig. 1), where environmental pollutant monitoring is particularly rigorous due to the presence of two cement factories.

2. Materials and methods

2.1. Chemicals

Analytical grade reagents were used throughout the study. Polyethene glycol, DPX mounting medium, and Light Green were purchased from VWR International Srl (Milan, Italy). Methanol, Schiff's reagent, and phosphate-buffered saline pH 7.4 (PBS) were obtained from Sigma Aldrich Srl (Milan, Italy). Acridine orange (AO), 4,6-diamidino-2-phenylindole (DAPI), and NC-Slide A8 were bought from ChemoMetec (Allerød, Denmark). Ethanol was from ITW Reagents Srl (Monza, Italy). 18 G needles were from PIC Spa (Como, Italy). Nylon filters (100 μm) were from Merck Spa (Milan, Italy). Acetic acid, conventional microscope slides, and coverslips were supplied by Thermo Fisher Inc. (Monza, Italy). Deionised milli-Q water from Carlo Erba Srl (Milan, Italy) was used throughout the experiments.

2.2. Study design

The study design proposed in the MAPEC_Gubbio Study is based on the MAPEC_LIFE (Monitoring Air Pollution Effects on Children for Supporting Public Health Policy) multicentre study (LIFE+; #LIFE12 ENV/IT/000614) (Ceretti et al., 2020; Feretti et al., 2014; Villarini et al., 2018). The MAPEC_Gubbio Study is a cross-sectional study designed to evaluate the potential association between air pollutant concentrations

Fig. 1. Geographical location of Umbria region (Italy) and the two sampling cities (*i.e.*, Gubbio, exposed area, and Città di Castello, control area).

and children's early biological effects, as indicated by the presence of cytogenetic biomarkers detected by the BMCyt assay.

Before the children's enrolment, public meetings with parents and teachers were held in Gubbio (the exposure zone, with two cement factories located north-west and south-east of the town) and in Città di Castello (the control town, 30 km from Gubbio). The schools were then selected based on the willingness of the children's parents and school directors to participate in the study. A total of 164 children aged 6–8 years were enrolled from participating schools. Biological samples were requested from children whose parents had duly filled out the informed consent form.

Before sampling buccal mucosa (BM) epithelial cells, informed consent to participate in the study was also obtained from the children using a structured form resembling a comic strip (Supplementary Figure S1). The inclusion/exclusion criteria for study participants are reported in Table 1. Participation in the study was voluntary, and no incentives were offered.

BM cell sampling was performed twice on the same children, covering two seasonal periods: late spring/summer 2023 (from the last week of May to the first week of June) and winter 2024 (specifically in February). For the BMCyt assay, biomarkers indicative of DNA damage (*i.e.*, micronuclei and/or nuclear buds), cellular proliferation potential (*i.e.*, basal and/or binucleated cells), and/or cell death (*i.e.*, condensed chromatin, karyorrhectic, pyknotic, and karyolytic cells) were evaluated in BM cells taken from the enrolled children.

The analysis of individual coded data was conducted in accordance with the Declaration of Helsinki and in compliance with the measures outlined in the General Data Protection Regulation (EU) 2016/679 (GDPR). The MAPEC_Gubbio Study has received ethical approval from the University of Perugia's Bioethics Committee (January 18, 2023; Protocol No. 1/2023).

Table 1
Inclusion/exclusion criteria for MAPEC_Gubbio participant children.

Inclusion criteria	Exclusion criteria
• 6–8 years old children. • Children living in Gubbio or Città di Castello.	• Affected by serious illnesses. • Orthodontic appliance. • Treatment with antineoplastic drugs in the last 12 months. • Radiation therapy or X-ray exposure in the last 12 months.

2.3. Sampling of buccal mucosa cells

Before cell sampling, children were asked to rinse their mouths twice with natural mineral water. To harvest BM epithelial cells, small-headed, soft-bristled toothbrushes featuring children's favourite comic-book characters were used to gently scrape the inner surface of both cheeks (10 times in a circular motion). The toothbrush head was dipped and quickly rotated inside tubes containing 15 mL of Saccomanno's fixative (50% ethanol, 2% polyethylene glycol, *vol./vol.*; the solution was diluted in deionised water and stored at +4 °C) to dislodge and release the collected cells. Each tube was univocally labelled with an alphanumeric code that reported the date, the sampling zone, and the children's progressive number. The samples were then transferred to the laboratory and stored at + 4°C until further processing.

The BM cells preserved in Saccomanno's fixative were centrifuged and rinsed twice with Dulbecco's PBS (pH 7.4) buffer. The resulting cell suspensions were aspirated into a syringe fitted with an 18-gauge needle, filtered through a 100 μm nylon mesh, and centrifuged once more. The final cell pellets were then resuspended in chilled PBS and treated with the AO/DAPI dye solution, after which they were loaded onto NC-Slides A8. The slides were then inserted into the fluorescence-based image cytometer, NucleoCounter NC-3000 (Chemometec, Allerød, Denmark), for cell counting (di Vito et al., 2022). The cells were then fixed with ice-cold Carnoy's fixative (methanol and glacial acetic acid 3:1) and stored at + 4°C until slide preparation.

2.4. Buccal micronucleus cytome (BMCyt) assay

The BMCyt assay was performed according to the protocol described by Thomas and Fenech (Thomas and Fenech, 2011a), with minor modifications (Villarini et al., 2018). For each child, two independent slides were prepared and analysed by two trained operators in a blinded manner (one slide per operator). Each slide was prepared by smearing 100 μL of cell suspension onto pre-cleaned slides (about 1×10^5 cells/slide). The cells were stained using the Feulgen plus Light Green technique. The slides were first fixed in absolute ethanol, then rinsed with deionised water and treated with 5 M HCl. After washing with deionised water, the slides were drained and stained with Schiff's reagent. The slides were rewashed with deionised water and counter-stained with 0.2% Light Green. Air-dried slides were finally mounted with DPX mounting medium.

Adhering to the scoring criteria established by Thomas and Fenech (Thomas and Fenech, 2011a), cell proliferation was determined by the

number of basal cells (BC), and other cellular anomalies indicating cytokinesis defect (*i.e.*, binucleated cells, BNC), and early-to-late stages of apoptosis and cell death (*i.e.*, condensed chromatin cells, CCC; karyorrhectic cells, KHC; pyknotic cells, PYK; karyolitic cells, KYL) were detected (Tolbert et al., 1992). On each slide, cytotoxicity biomarkers were evaluated in the first 500 differentiated epithelial cells. Micronuclei (MN) and nuclear buds (NBUD) were scored in 1000 differentiated epithelial cells per slide (total number of scored cells per child: 2000). MN frequency was then expressed as the number of micronuclei per 1000 differentiated cells. For each child, the two operator-specific MN/1000 values were averaged to obtain a single individual value, which was then used for statistical analyses. The median of each group was calculated from the individual means obtained from independent slide readings performed by different operators.

2.5. Collection of urban air chemical data

Children's exposure to air pollution was evaluated by gathering data on the air pollutants regulated by the EU Ambient Air Quality Directives (EU, 2008) (*i.e.*, PM_{10}, $PM_{2.5}$, NO_2, SO_2, benzene, and O_3), which are measured daily by the Air Quality Monitoring Network of the Regional Agency for Environmental Protection (ARPA Umbria). Data on pollutant concentrations were obtained from the publicly available ARPA Umbria database (ARPA Umbria).

The epithelial turnover time for BM epithelial cells is approximately 14–21 days (Brizuela and Histology, 2025); therefore, we assessed the associations between the frequency of cytogenetic biomarkers and air pollutant concentrations in the 3 weeks preceding cell sampling (considering the sampling day as "day 0", week I means 1–7 days before, week II stays for 8–14 days before, and week III 15–21 days before the sampling). We considered the average daily concentrations of the 7, 14, and 21 days preceding BM cell collection, as well as the peak hourly concentrations (*i.e.*, maximum hourly concentrations) in the 7, 14, and 21 days preceding biological sampling. For PM_{10}, the number of exceedance days of the daily EU law limit (*i.e.*, 50 $\mu g/m^3$) occurring in the 7, 14, and 21 days preceding biological sampling was also considered (Ceretti et al., 2020).

2.6. Questionnaires

Demographic, socioeconomic, and anthropometric variables were collected using a validated questionnaire (Altavilla and Caballero-Pérez, 2019) previously employed in the MAPEC_LIFE Study. The questionnaire presents 148 items linked to personal information (*e.g.*, sex, age, height and weight), children's health, physical activity and parents' characteristics (*e.g.*, birthplace, education, work and smoking habits), indoor pollutant sources such as home heating systems, exposure to second-hand smoke at home, traffic intensity perception near children's home and school. In addition, the adherence to the Mediterranean Diet was evaluated by analysing children's eating habits using the validated KIDMED questionnaire (Altavilla and Caballero-Pérez, 2019). The eating habits questionnaire also included items about foods (e.g., fried and grilled foods, toasted bread, wood-oven pizza, and smoked foods) commonly associated with polycyclic aromatic hydrocarbon (PAH) exposure (Bansal and Kim, 2015).

2.7. Statistical analysis

Approximation to the normal distribution of continuous variables was evaluated using the Kolmogorov-Smirnov test. All considered cell types, as well as anomalies associated with DNA damage, cell death or chromosomal instability (*i.e.*, cells with MN or NBUD, and CCC, KHC, PYK, and KYL cells), showed significant departures from the normal distribution, even after logarithmic transformation. Cytogenetic parameters were then summarised as median and interquartile range. Means, standard deviations (SD), counts, and percentages were reported for other continuous and categorical variables. For cytome analysis, statistically significant differences in values within the same children across two seasons were assessed using the Wilcoxon test for paired data, and differences between towns were evaluated using the non-parametric Mann-Whitney *U*-test.

Regarding parametric variables (*e.g.*, air pollutants, ages), differences among groups were investigated using the Student's t-test. Comparisons among proportions were performed using Pearson's χ^2 test.

Multivariate regression analysis of atmospheric pollutants and children's BMI on MN frequencies was conducted using backward stepwise regression analysis. All statistical tests were two-sided with an α level of 0.05. The data analyses were performed using SPSS 20 statistical package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Study population

In the present cross-sectional study, 164 school-aged children were enrolled (n = 123 in the exposed group from Gubbio and n = 41 in the control group from Città di Castello). In total, slides appropriate for cytome analysis were successfully prepared from n = 161 children. In particular, n = 34 (85.0%) and n = 37 (92.5%) for Città di Castello, and n = 112 (98.6%) and n = 108 (89.3%) for Gubbio, during the first and second seasons, respectively. Moreover, 122 (74.4%) questionnaires from the entire cohort were obtained and analysed.

Table 2 summarises the main characteristics of the study population according to town of residence. Children aged 6–8 years, and 86 (52.4%) were girls. Most of the parents were of Italian origin; only 39.2% of mothers and 19.5% of fathers attained a high level of education. Significant variations were observed across the different towns.

Furthermore, 16.0% of mothers and 27.6% of fathers were active smokers, with significant variation in these proportions across cities.

Considering health status and eating habits (Table 3), no significant differences were observed between the two groups. Body mass index (BMI) was similar between the two cohorts, with most children (68.2%) in the normal-weight category and 11.8% classified as obese. According to the KIDMED Score, most children (60.3%) require improvement in

Table 2
Socio-demographic profile of the children enrolled in the study and their parents.

Characteristics	Gubbio	Città di Castello	*p*-value
Children[a]	123 (75.0)	41 (25.0)	
Boys / Girls[b]	59 / 64 (0.92)	19 / 22 (0.86)	
Children's age[c]	7.2 ± 1.0	6.6 ± 0.8	*n.s.*[$]
Parents of Italian nationality[d,e]			
Mother	92.6	85.7	*n.s.*[a#]
Father	93.6	78.6	*0.019*[#]
Parents' percentage high education[d,e]			
Mother	45.4	17.9	*0.033*[#]
Father	23.2	7.1	*n.s.*[#]
Parents' smoking habits[d,e]			
Mother	11.3	32.1	*n.s.*[#]
Father	22.1	46.4	*0.011*[#]

Statistical significance: [#]comparisons ($p < 0.05$) among proportions were performed using the Pearson's χ^2 test; [$]differences between children's ages were assessed using the Student's *t*-test; *n.s.*, not significant.

[a] Number of subjects and % (in parentheses), respectively.
[b] Number of subjects and masculinity ratio (in parentheses), respectively.
[c] Group mean ± standard deviation (age expressed in years).
[d] Percentage of subjects.
[e] Data relating to the 122 correctly completed questionnaires.

Table 3
Health status and eating habits characteristics of children included in the study.

Characteristics[a]	Gubbio	Città di Castello	*p*-value
Children's BMI[b,c]	16.5 ± 2.8	16.3 ± 2.5	*n.s.*[#]
Underweight[d]	9.3	8.3	*n.s.*[$]
Normal weight	66.3	75.0	
Overweight	11.6	8.3	
Obese	12.8	8.3	
Children's KIDMED Score[c,e]	6.8 ± 2.2	6.4 ± 2.2	*n.s.*[#]
Very low-quality diet[d]	6.5	0.0	*n.s.*[$]
Need to improve the food pattern	58.1	67.9	
Optimal Mediterranean diet	35.5	32.1	

Statistical significance: [#]differences between the towns were investigated using the Student's *t*-test; [$]comparisons among proportions were performed using the Pearson's χ^2 test with the Bonferroni correction for multiple comparisons; *n.s.*, not significant.

[a] Data related to the 122 correctly completed questionnaires.

[b] BMI = body mass index, calculated as weight (kg) divided by the square of the height (m^2). Children were categorised as underweight, normal weight, overweight, or obese according to their BMI percentiles.

[c] Group mean ± standard deviation.

[d] Percentage of subjects.

[e] The KIDMED Score was calculated using the proper validated questionnaire (see Materials and Methods section). Based on this score, children's eating habits were grouped into four categories: very-low-quality diet (< 3), need to improve the food pattern by adjusting to a Mediterranean diet (4–7), and optimal Mediterranean diet (> 8).

their dietary pattern, with only 5.0% following a very low-quality diet.

PAH food-related exposure assessment (Table 4) indicated that more than half of the children (54.5%) had low exposure levels. A similar percentage of children from Gubbio and Città di Castello (24.7% and 25.0%, respectively) exhibited respiratory problems other than the common cold in the last 12 months (Table 4).

When it comes to traffic density (Supplementary Table S1), parents of children affirmed that both in their children's place of residence and the school area, car and truck density were generally low.

Atmospheric pollutants (*i.e.*, NO_2, PM_{10}, and $PM_{2.5}$) 24-hour averages were also considered, with concentrations differentiated by one, two, or three weeks before sampling, for both Season I (late spring) and Season II (winter) (Fig. 2). Significant differences ($p < 0.05$) were observed in the concentrations of each atmospheric pollutant between the two cities. Overall, ARPA monitoring stations consistently recorded higher pollutant levels during winter (Season II).

NO_2 was significantly more concentrated in Gubbio than in Città di Castello, except for the week before sampling in both seasons, reaching a maximum concentration of 53.79 ± 11.00 μg/m^3 in the exposed group during winter. Conversely, the two other airborne contaminants were consistently more prevalent in the control group.

Nevertheless, within the exposed group, PM_{10} exhibited the highest concentration (35.54 ± 16.79 μg/m^3) two weeks before sampling (Season II), rather than the lowest concentration (9.60 ± 7.61 μg/m^3) a week before sampling (Season I). Regarding $PM_{2.5}$, the two weeks before the children's buccal cells collection in winter displayed the peak concentration of 22.83 ± 9.55 μg/m^3, while the lowest one (17.36 ± 6.08 μg/m^3) was obtained at one week before sampling in the late spring season.

Table 4
Children's PAHs exposure through foods commonly associated with their presence, and children with respiratory problems other than a common cold.

Characteristics[a]	Gubbio	Città di Castello	*p*-value
Children's PAH Score[b,c]	9.0 ± 5.1	10.2 ± 6.0	*n.s.*[#]
Low exposure[d]	59.1	39.3	*n.s.*[$]
Moderate exposure	35.5	50.0	
High exposure	5.4	10.7	
Children with respiratory problems[d,e]	24.7	25.0	*n.s.*[$]

Statistical significance: [#]differences between the towns were investigated using the Student's *t*-test; [$]comparisons among proportions were performed using the Pearson's χ^2 test with the Bonferroni correction for multiple comparisons; *n.s.*, not significant.

[a] Data relating to the 122 correctly completed questionnaires.

[b] The children's PAHs Score was calculated considering the consumption frequencies (*i.e.*, never, once a month, twice a month, 3,4, and 5 or more times a month) of some foods commonly associated with the presence of PAHs (*i.e.*, fried and grilled foods, food cooked on a griddle, toasted bread, wood oven pizza and smoked food). It ranges from 0 to 30, classifying exposure as low (≤ 9), medium (≤ 19), and high (≥ 20).

[c] Group mean ± standard deviation.

[d] Percentage of subjects.

[e] Children with respiratory problems other than a common cold (*e.g.* asthma, bronchial wheeze, cough, mucus, allergic symptoms, and persistent nasal and sinus disorders) in the last 12 months.

3.2. BMCyt assay

The results of cytome analyses are summarised in Fig. 3 and Table 5. MN (chromosomal damage marker) frequencies are displayed in Fig. 3. Whereas DNA damage markers (NBUD), cell proliferation markers (BC and BNC), and cell death/apoptosis markers (CCC, KHC, PYK, and KYL) are reported in Table 5.

Overall, the MN frequency decreased slightly from late spring (Season I) to winter (Season II), moving from a median value of 1.5 (IQR 1.0–2.5) to 1.0 (IQR 0.5–1.5). During Season I, MN frequency was significantly doubled ($p < 0.001$) in Gubbio compared to Città di Castello, with a median of 2.0 (IQR 1.0–2.5) to 1.0 (IQR 0.5–1.5), respectively. Subsequently, the frequency of MN realigned during Season II to 1.0 (IQR 0.5–1.5). Moreover, comparing the two sampling seasons in Gubbio, the first showed a significantly higher MN frequency ($p < 0.05$).

Considering cell proliferation markers, BNC frequency was slightly higher in Season I than in II, each displaying a median frequency of 4.5 (IQR 3.5–5.5) and 4.0 (IQR 2.5–5.0). Season II showed a significantly higher BNC frequency in the case area ($p = 0.045$), with a median of 4.0 (IQR 2.8–5.5), compared to 3.5 (IQR 2.5–4.5) in the control group. Basal cells were not found. All other markers of death cells followed the same trend, with higher values in Season I than in Season II, except for KYL cells. Specifically, the CCC frequency was significantly higher ($p < 0.001$) in Gubbio than in Città di Castello during the first sampling, with medians of 7.0 (IQR 5.0–10.5) and 4.8 (IQR 3.5–6.0), respectively. Subsequently, the same frequency was observed during Season II, with scores of 2.0 (IQR 1.0–3.0) for Gubbio and 2.0 (IQR 1.5–2.5) for the control city. A similar pattern was observed for KHC, where Gubbio exhibited the highest frequency in Season I with a median of 6.0 (IQR 4.0–9.8) compared to a significantly lower ($p < 0.001$) value of 1.5 (IQR 0.5–3.0) for Città di Castello. The gap was then closed for Season II. Finally, KYL cells appeared significantly higher ($p = 0.005$) in Gubbio for the first sampling, reaching a median of 4.0 (IQR 3.0–6.0), compared with the control group. No significant differences were observed for PYK cell frequencies.

From the multivariate regression analyses (back wise procedure; $p < 0.05$ for entry into the model) of MN frequencies and questionnaire items, no statistically significant correlation was observed neither for nutritional behaviour nor lifestyle (*e.g.*, physical activity) (data not shown), except for children's BMI and MN frequencies in Season I, where a significant positive correlation ($p = 0.044$) was obtained in the exposed group (Gubbio) and the entire population (Table 6).

When it comes to atmospheric pollutant concentrations, statistically significant positive correlations were observed with MN frequency for NO_2 and $PM_{2.5}$ ($p = 0.003$ and $p = 0.020$, respectively) in Season I (late spring) and three weeks before sampling, in both the exposed children and the whole population (Table 6). No statistically significant correlation was found for Città di Castello when considered separately.

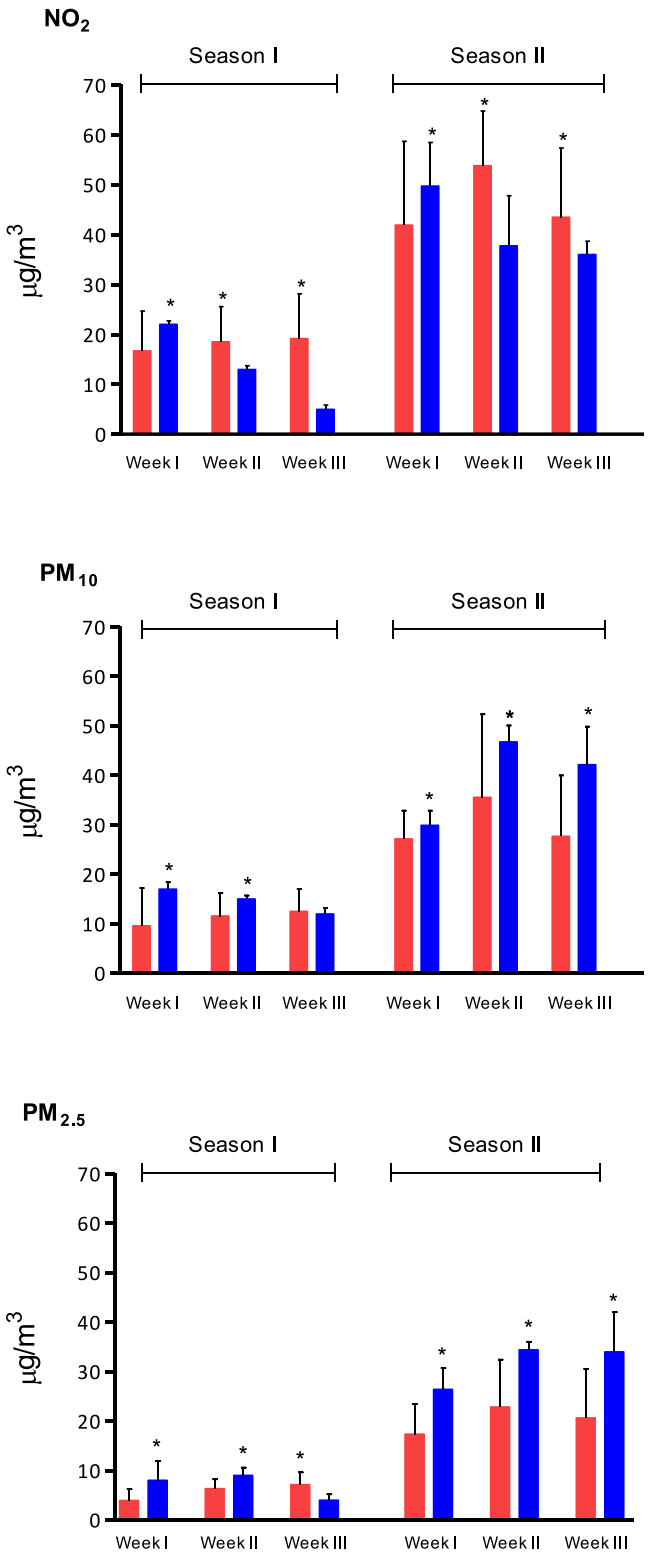

Fig. 2. Atmospheric pollutants (*i.e.*, NO_2, PM_{10}, and $PM_{2.5}$, 24-hour averages) concentrations (µg/m3), one, two, or three weeks before sampling, for both Season I (late spring) and Season II (winter). Gubbio (exposed group) in red and Città di Castello (control group) in blue. Results are expressed as the mean ± SD. *$p < 0.05$.

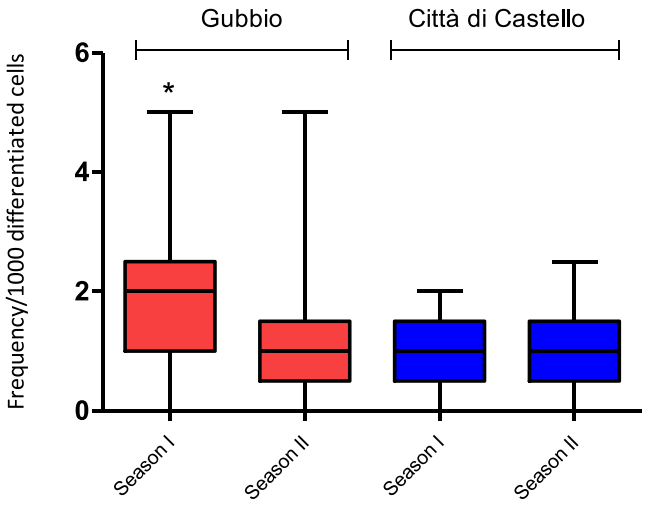

Fig. 3. MN frequencies of sampled children from Gubbio (exposed group) and Città di Castello (control group), in the two sampling seasons (Season I = late spring; Season II = winter). Results are expressed as the median and interquartile range (IQR). *$p < 0.05$.

Table 5
Frequencies of chromosome damage markers (NBUD), cell proliferation markers (BC and BNC), and cell death markers (CCC, KHC, PYK, and KYL), as well as the replication index (RI), were measured in exfoliated buccal cells of children by town of residence, with results presented as group medians and interquartile ranges (IQR). The median of each group was calculated from the individual means obtained from independent slide readings performed by different operators.

Biomarker[a]		Gubbio	Città di Castello	*p*-value[#]
NBUD (‰)	Season I[b]	0.0 (0.0–0.5)	0.0	
	Season II	0.0	0.0 (0.0–0.5)	
BNC (‰)	Season I	4.0 (3.5–5.5)	5.0 (3.6–6.4)[$]	
	Season II	4.0 (2.8–5.5)	3.5 (2.5–4.5)	*0.045*
CCC (‰)	Season I	7.0 (5.0–10.5)[b]	4.8 (3.5–6.0)[$]	*< 0.001*
	Season II	2.0 (1.0–3.0)	2.0 (1.5–2.5)	.
KHC (‰)	Season I	6.0 (4.0–9.8)[b]	1.5 (0.5–3.0)	*< 0.001*
	Season II	2.5 (1.5–5.0)	2.0 (1.0–3.0)	
PYK (‰)	Season I	0.0 (0.0–0.5)	0.0 (0.0–0.5)	
	Season II	0.0	0.0	
KYL (‰)	Season I	4.0 (3.0–6.0)	2.5 (1.5–5.0)[$]	*0.005*
	Season II	3.8 (2.3–6.0)	4.0 (2.5–6.0)	

Statistical significance: [#]differences between the two towns were investigated using the non-parametric Mann-Whitney *U*-test; [$]Statistically significant differences ($p < 0.05$) were observed when comparing the same children in the two seasons (non-parametric Wilcoxon test for paired data).

[a] NBUD, nuclear buds; BNC, binucleated cells; CCC, condensed chromatin cells; KHC, karyorrhectic cells; PYK, pyknotic cells; KYL, karyolytic cells.

[b] Season I = late spring; Season II = winter.

4. Discussion

In the present cross-sectional study, 164 school-aged children were enrolled from Gubbio, a town in Umbria, Italy, where environmental pollutant monitoring is particularly rigorous due to the presence of two cement factories, and from Città di Castello (control population), a town 30 km from Gubbio. Of these, 161 were children from whom the frequency of markers of chromosomal and DNA damage, cell proliferation, and cell death/apoptosis (cytome) was evaluated in exfoliated BC. Sample collection was performed during two seasons: winter and late spring.

Children's breathing habits differ from those of adults, which could influence the way toxins are deposited in their lungs. Adults usually breathe primarily through their noses at rest, whereas children are more likely to breathe through their mouths (Bateson and Schwartz, 2008).

Table 6
Multivariate regression analysis of atmospheric pollutants and children's BMI, on MN frequencies in the entire population and the exposed group.

	Population	Independent variable[a]	Regression coefficients			*p*-value
			B[b]	S.E.	β[c]	
Health	**Whole**	BMI	0.275	0.135	0.201	0.044
	Gubbio	BMI	0.310	0.145	0.234	0.036
Pollutants	**Whole**	NO_2 *3 weeks before sampling*	0.035	0.011	0.352	0.003
		$PM_{2.5}$ *3 weeks before sampling*	0.088	0.037	0.272	0.020
	Gubbio	NO_2 *3 weeks before sampling*	0.400	0.015	0.361	0.008
		$PM_{2.5}$ 3 *weeks before sampling*	0.089	0.041	0.287	0.032

Backward procedure: $p < 0.05$ for entry into the model.
[a] Season I.
[b] Unstandardised B (slope of the regression line).
[c] Standardised β.

Therefore, exfoliated BCs are a suitable option for investigating how air contaminants affect children.

Except for data observed in Gubbio during Season I (late spring-summer), the MN frequencies reported in this study align with the estimated average MN values — deriving from a comprehensive database compiled from studies involving both children and adults — in exfoliated buccal cells of healthy controls, which are 0.74‰, ranging from 0.3 to 1.7 MN per 1000 cells (Bonassi et al., 2011).

In fact, the results of this study showed a higher median MN frequency in late spring (2.0‰) than in winter (1.0‰) among the exposed children, despite pollutant levels being generally higher in Season II. A high BMI in children was associated with increased MN frequency, suggesting a potential relationship between dietary habits and MN levels, a topic that remains under discussion in both adults and children (Bonassi et al., 2011).

Contrary to our initial assumptions, the concentration of the monitored pollutants (*i.e.*, NO_2, PM_{10}, and $PM_{2.5}$) was generally higher in Città di Castello (the control area) than in Gubbio (the exposed area) during winter. In some cases, values were also higher in late spring. Regarding cytome analysis, no differences were observed in the frequency of MN retrieved from the exfoliated BC of school-age children residing in the two towns under consideration during the winter season. Nevertheless, in late spring, with the concentrations of the monitored pollutants significantly lower than in winter, the frequency of MN in Gubbio almost doubled relative to Città di Castello. The results do not seem to be affected by parental tobacco smoking habits. These pieces of evidence suggest the role of an unmonitored summer-specific factor (or factors).

Similar results have been reported in a study conducted in Eskişehir (Turkey) (Demircigil et al., 2014). The authors conducted winter and summer campaigns to assess genotoxicity among children at two schools in suburban and urban traffic areas in Eskişehir. At both sites, MN frequencies were significantly higher in the summer than in the winter. Similarly, in another study conducted in Oakland, California, USA, the authors observed a peak in MN frequency in children during summer and associated cytogenetic damage with exposure to high ozone concentrations (Huen et al., 2006). Analogously, Giovannelli and co-workers demonstrated a seasonal effect on DNA damage – measured via the Comet assay – in human lymphocytes from healthy donors, with the highest levels observed in summer, and further reported a positive correlation between DNA damage and air temperature, alongside weaker associations with global solar radiation and air ozone concentrations (Giovannelli et al., 2006). Moreover, another study showed that Comet assay parameters in blood cells were elevated when samples were collected in warmer seasons, peaking in summer, and were positively correlated with certain climatic variables (*i.e.*, air temperature, ozone, solar radiation) (Geric et al., 2018).

Our findings can be interpreted in the context of the large multicentre MAPEC_LIFE study, which investigated micronucleus (MN) frequency in buccal epithelial cells of children living in several Italian urban areas characterised by different levels of air pollution. In that study, MN frequencies generally ranged from approximately 0.2–0.4 MN per 1000 differentiated cells, with higher values typically observed during the winter season and in cities with higher levels of airborne pollutants (Villarini et al., 2018). When comparing our results with those reported in the MAPEC_LIFE study, some differences emerge. In our study, slightly higher MN frequencies were observed for Città di Castello in both seasons and for Gubbio during the second sampling period. Whereas, a more evident difference was observed for Gubbio in the first season, where the median value of 2 MN per 1000 differentiated cells appears higher than the range reported in the MAPEC_LIFE cohort.

However, this comparison should be interpreted with caution, since the two studies refer to different urban contexts and sampling periods. Variability in MN frequency across populations may reflect differences in local sources of air pollution, meteorological conditions, seasonal dynamics of pollutants, and other environmental or lifestyle-related factors influencing exposure patterns (Ceretti et al., 2020). Overall, despite these differences, the values observed in our study remain within the range reported in the literature for paediatric populations assessed using the buccal micronucleus cytome (BMCyt) assay, which has been widely applied in biomonitoring studies investigating early genotoxic effects associated with environmental exposures (Thomas et al., 2009; Aykanat et al., 2016).

Returning to the present study, in Gubbio and Città di Castello, pollution levels, relative to the monitored parameters (*i.e.*, NO_2, PM_{10}, $PM_{2.5}$), are of the same order of magnitude during the late-spring sampling period. ARPA Umbria air sampling units do not monitor ozone in Gubbio and Città di Castello. When focusing specifically on environmental pollutants, unmonitored factor(s) may be represented by photochemical smog—commonly referred to as "summer smog"—or by one or more of its major components. Photochemical smog is strongly linked to automobile traffic. It forms when solar ultraviolet radiation (acting as a catalyst) triggers chemical reactions between nitrogen oxides (NO, NO_2) and volatile organic compounds, producing harmful secondary pollutants such as ground-level ozone and other dangerous contaminants, such as peroxyacetyl nitrates and aldehydes. This process is particularly prevalent in urban areas with high traffic density and warm, sunny weather.

The present study has some weaknesses. For instance, heavy metals and other toxic trace compounds have not been included within the monitored parameters. In future studies, combining chemometric methods with conventional biomonitoring approaches could help assess environmental pollution status and related health risks more effectively and accurately (Onjia et al., 2022). Moreover, the number of participants was relatively low. Hence, to increase the sample size, future research would benefit from integrating additional recruitment methods (*e.g.*, raising public awareness through the enhanced dissemination of educational materials in both print and digital formats, as well as through collaboration with local healthcare providers).

In conclusion, this study showed that in late spring, an increased MN frequency in buccal cells of children residing in Gubbio was associated with an unmonitored factor(s), plausibly a summer-specific pollutant (or

pollutants). The levels of the monitored pollutants were essentially below the limit values specified in the applicable legislation. However, a residual genotoxic risk was evidenced in school-age children with residential exposure. These findings strongly suggest the need to improve the monitoring process of airborne pollutants to manage genotoxic risk effectively. ARPA Umbria air sampling units, at least in Gubbio, should also monitor O_3, in addition to NO_2, PM_{10}, and $PM_{2.5}$. Pollutant monitoring should not focus solely on cement plant activity but also on the resulting heavy-vehicle traffic.

Finally, our data confirm the importance of adopting an integrated chemical/biotoxicological approach to evaluate genotoxic/carcinogenic hazards in individuals exposed to residential environments. The use of biomarkers that assess changes in cellular or molecular endpoints, combined with chemometric methods, will enable us to adopt a more comprehensive approach that incorporates not only environmental and biological monitoring but also biological effect monitoring using genotoxicity biomarkers (Villarini et al., 2012; Moretti et al., 2015). Our study provides a starting point for further research that may consider other exposure scenarios.

CRediT authorship contribution statement

Cristina Fatigoni: Investigation. **Tommaso Rondini:** Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation. **Massimo Moretti:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Milena Villarini:** Validation, Supervision, Software, Formal analysis, Data curation, Conceptualization. **Mattia Acito:** Writing – original draft, Software, Investigation. **Edoardo Franceschini:** Writing – original draft, Software, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxlet.2026.111884.

Data availability

The research data are provided within the article or the supplementary material.

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Glossary

AD: Aerodynamic diameter
AO: Acridine orange
ARPA: Agenzia Regionale per la Protezione dell'Ambiente
BC: Buccal cells
BM: Buccal mucosa
BMCyt: Buccal micronucleus cytome assay
BNC: Binucleated cells
CCC: Condensed chromatin cells
DAPI: 4′,6-diamidino-2-phenylindole
DPX: Distyrene Plasticizer Xylene
EU: European Union
GDPR: General Data Protection Regulation
IARC: International agency for research on cancer
KHC: Karyorrhectic cells
KIDMED: Mediterranean Diet Quality Index for Children and Adolescents
KYL: Karyolytic cells
MAPEC: Monitoring Air Pollution Effect on Children
MN: Micronuclei
NBUD: Nuclear buds
PAHs: Polycyclic aromatic hydrocarbons
PBS: Phosphate buffer saline
PM: Particulate matter
PYK: Pyknotic cells